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Full Length Research Paper

Phenotypic and genetic characterization of selected Kenyan groundnut (*Arachis hypongaea* L.) varieties

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Groundnuts are among the principle economic oilseed largely cultivated in warm regions of Kenya and contribute to domestic food security and cash revenue. The study aimed at evaluating genetic diversity and phenotypic characterization of groundnut varieties grown in some parts of Kenya. Six varieties (ICG83708*, ICGV99568, CG7, ICGV12991, RV* and Chalimbana) obtained from KALRO (Kakamega) based on quality traits. Seeds were planted in cylindrical cans with 0.2 m^3 of soil and allowed to germinate for 3 weeks. Leaf trait measurement was done using 15 cm ruler and the data was recorded in excel spreadsheet and exported to MINITAB v17 software. Extracted DNA was amplified using 11 SSR primers with manual scoring of bands. A dendrogram of cluster analysis was constructed based on simple matching coefficient of 11 microsatellite markers. Principle components showed Eigen values with 67.2% variability of all the traits studied. A total of 35 alleles were detected across the loci of 11 primers used. Major allele frequency ranged from 0.9333 in IPAHM 165 to the least frequency of 0.6333 in IPAHM 176 with an average of 0.7610 across all the markers. The expected heterozygosity had a mean of 0.3178 with highest value of 0.4511 in IPAHM 176. Phylogenetic tree had three major clusters and with high bootstrap values in genotype icgv99568 replicates. Primer IPAHM176 was the most informative marker and should be utilized in selection of parent plants with good quality traits. Breeding programs on groundnuts should utilize grain and seed traits since they are informative and discriminatory.

Key words: Groundnut varieties, phenotypic and genetic characterization, gene diversity.

INTRODUCTION

Groundnut (*Arachis hypongaea* L.) is an essential plant both nutritionally and economically for oil and protein source (Ingale and Shrivastava, 2011). The study aimed at phenotypic and genetic characterisation of selected six groundnut varieties grown in Kenya using physical trait measurement and SSR markers. Phenotypic characterization is based on visually accessible traits such as length, width, ratio of length to width of the leaves and seeds. A detailed account on genotypes ought to help in resolving documentation challenges that arise while keeping records on varieties (Suprapto et al., 2013). Genetic markers linked with morphological

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characteristics to determine plant type are beneficial to choose a variety for groundnut breeder or farmer. These DNA fragments include isozyme, RFLP, AFLP, RAPD, SSR, ISSR and SNPs which gets a lot of utility in plant inheritance series. These markers have different benefits and flaws in their use (Guo et al., 2014). The SSR markers are chosen due to use simplicity, scoring ease, higher allele range and their definite distribution genome-wise (Wang et al., 2015). It is important to gain insights into the genetic base of the *A. hypongaea* L. genetic resources currently being used by the farmers in Kenya. There is scanty information on Kenyan groundnuts phenotypic characterization and hence the need to analyze the varieties for prerequisite information in molecular breeding and variety advancement.

MATERIALS AND METHODS

Collection of samples and preparation

Six Kenyan groundnuts varieties were collected from the Rift valley, Coast and Western Regions agro-ecological zones of Kenya. The varieties include *RV, CG7, Chalimbana, ICGV83708, ICGV12991*, and *ICGV99568.* The varieties were selected based on high farmer preference to seed oil quality, nutritional quality, pest resistance and disease resistance, environmental stress and yield. The groundnut samples were designated into populations based on the variety, with each population comprising of 15 seeds. The seeds were then taken to Kenyatta University Plant Transformation Laboratory photographed and prepared for measurements of weight in grams, seed length, seed breadth, seed length/breadth in millimeters. Five seeds of each variety were planted in pots containing soil and were watered daily for two weeks. Mature leaves were harvested in five replicates per variety, frozen then stored at -20°C in preparation for DNA extraction.

Phenotypic characterization

Morpho-agronomic traits developed by Biodiversity International were targeted on all the varieties. Five seeds from each variety were selected at random and the measurements in centimeters (cm). The mass of each groundnut seed for each variety was determined. Traits targeted were grain length (GL), grain breadth (GB), grain length/breadth (G-L/B) and grain weight (GW), leaf length (LL), and leaf breadth (LB), leaf length/breadth (L-L/B). The data was recorded in excel and exported to Minitab software v17 for further analysis.

Genetic characterization

DNA extraction and PCR assay

Plant genomic DNA was extracted using Doyle and Doyle (1990) protocol from each sample. Fifteen SSR markers set (Table 1) were obtained and selected from published data for groundnut simple sequence repeats primers (Moretzsohn et al., 2004). PCR was conducted as per the Taq polymerase conditions. The PCR products from the different markers were resolved in 2% agarose gel. Scoring of bands was done manually with presence of a band being recounted as one (1) while absence was reported as zero (0).

Data management and statistical analysis

The data obtained was both qualitative and quantitative and was subjected to descriptive statistics and presented in tables and figures. Phenotypic data was exported from excel worksheet to Minitab statistical software v17 (Pennsylvania, U.S.A). Mean were computed. One-way analysis of variance (ANOVA) followed by Tukey's Post Hoc test with confidence level at 95% ($p > 0.05$) was computed. Euclidian distance tool was used before forming complete linkage distance among the different varieties. The source of variation was determined using principal coordinates analysis (PCA) based on the seven morphological traits. PowerMarker Version 3.3 (Tamura et al., 2007) software was used to analyze polymorphisms of the markers. Phylogenetic tree, dissimilarity matrix with 1000 bootstraps was done using Darwin version 7. Principle coordinate analysis and AMOVA was calculated using GenALEx 6.5 software (Peakall and Smouse, 2012).

RESULTS

Phenotypic characterization

Measurements of grain and leaf characteristics

Measurements of leaf and seed characteristics for the 6 varieties and their mean values are shown in Table 2. From the results, genotype *ICG83708* had the longest grain at 15.37 mm while RV had the shortest at 10.06 mm. There was significant difference between genotype *ICG83708*, *Chalimbana*, *CG7* and *ICGV99568*. No statistical difference was observed between genotype *RV* and *ICGV12991* ($p > 0.05$). Significant statistical difference was observed between *RV* and *ICGV12991* against the grain lengths of genotypes *ICG83708, Chalimbana, CG7* and *ICGV99568* ($P > 0.05$). In terms of grain breadth, the highest breadth was observed in *Chalimbana* at 9.16 mm while the lowest 6.09 mm was observed in *RV* genotype. Significant difference was observed between *Chalimbana and ICG83708* against *ICGV12991, CG7*, and *RV* varieties.

The grain length/breadth ratio ranged from 1.47 in *ICGV12991* to 2.02 mm in genotype *ICG83708*. No significant difference was observed amongst genotypes *CG7, ICG83708* and *RV*. In addition, *Chalimbana*, *ICGV12991* and *ICGV99568* also showed no statistically significant difference amongst their ratio (Table 2). Significant statistical difference was observed between *Chalimbana, ICGV12991* and *ICGV99568* against *CG7, ICG83708* and *RV* (Table 2). Grain weight ranged from 0.78 g in *ICGV83708* and the lowest was in RV genotype at 0.32 g. Significant difference was observed between *ICGV83708* against *RV, ICGV12991*, *Chalimbana*, *CG7* and *ICGV99568* genotypes (Table 2). No significant statistical difference was observed between genotypes CG7, *Chalimbana*, *ICGV12991*and *ICGV99568*. Moreover, I*CGV12991* and *RV* showed no significant difference amongst its mean grain weights (Table 2). Leaf length ranged from 2.06 cm in *ICGV99568* to 2.80 cm in *ICGV12991*. This value was significantly different with

Table 1. Groundnut 11 SSR markers used in PCR amplification.

Marker ID	Gene bank number	Repeat motif	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
IPAHM 229	ER974475	(CA)14TA(CA)3	TCAGCCTGCGAAACTAAGGT	TGGAGAACTAGGATCTCTTTTGTG
IPAHM 103	ER974437	(CA)3(GA)17	GCATTCACCACCATAGTCCA	TCCTCTGACTTTCCTCCATCA
IPAHM 123	ER974446	(GA)18	CGGAGACAGAACACAAACCA	TACCCTGAGCCTCTCTCTCG
IPAHM 165	IPAHM 165	(GA)13	CAACACGTTCGCTTCCAGAT	TCACTCTCATTTCCGCCATT
IPAHM 166	ER974463	(CAA)9(TAA)3	GGACAATTATGCCCCTCAGC	TCCTTCCTCTGAGCTTTTCG
IPAHM 108	ER974439	(TC)18	CTTGTCAAACTCTGTGACTTAGCA	CATGAACAATTACACCCAGTCA
IPAHM 23	ER974415	(CA)17(TA)3	GTGTCTTTTCGTTCGCGATT	CGACTCTTAGGGTGGATTATAGTAA
IPAHM 105	ER974438	(CT)18	CAGAGTTTGGGAATTGATGCT	GCCAGATCTGAGCAAGAACC
IPAHM 229	ER974475	(CA)14TA(CA)3	TCAGCCTGCGAAACTAAGGT	TGGAGAACTAGGATCTCTTTTGTG
IPAHM 176	ER974468	(GA)16	CAACACAAGCCCACAACAAA	TCCATCATCACCCTCATCAA
IPAHM 171a	ER974466	(TC)7TGTT(TC)9	TTGGTTGTTCGTAGCTCTGC	AGCACGGCAAACACTAACACT

Table 2. Analysis of variance of the phenotypic traits with mean ± standard Error.

Genotype	N	Grain length (mm)	Grain breadth (mm)	Grain length/breadth	Grain weight (g)	Leaf length (cm)	Leaf width (cm)	Leaf length/width
CG7	5	13.98±0.20^{a}	7.53±0.16^{b}	1.87±0.05^{a}	0.56±0.04^{b}	2.28±0.08^{cd}	1.36±0.04^{b}	1.55±0.02^{a}
Chalimbana	5	13.77±0.49^{a}	9.16±0.18^{a}	1.54±0.03^{b}	0.56±0.06^{b}	2.68±0.07^{ab}	1.82±0.04^{a}	1.46±0.01^{ab}
ICG83708	5	15.37±0.36^{a}	8.78±0.30^{a}	2.02±0.11^{a}	0.78±0.03^{a}	2.60±0.05^{ab}	1.92±0.07^{a}	1.35±0.02^{b}
ICGV12991	5	11.16±0.86^{b}	6.18±0.29^{c}	1.47±0.03^{b}	0.40±0.03^{bc}	2.80±0.03^{a}	1.92±0.03^{a}	1.53±0.02^{a}
ICGV99568	5	13.65±0.43^{a}	8.36±0.09^{ab}	1.58±0.06^{b}	0.52±0.03^{b}	2.06±0.06^{d}	1.54±0.02^{b}	1.34±0.03^{b}
RV	5	10.06±0.15^{b}	6.09±0.14^{c}	1.91±0.06^{a}	0.32±0.03^{c}	2.46±0.05^{bc}	1.76±0.05^{a}	1.54±0.04^{a}

values of genotypes *CG7, ICGV99568* and *RV*. Also, significant difference was observed between *CG7* and *Chalimbana*, *ICG83708*, and *ICGV12991* (Table 2).

Leaf width results showed that *CG7* had the lowest value of 1.36 cm while the highest value of 1.92 cm observed in two genotypes *ICGV12991* and *ICG83708*. Significant difference was observed between *CG7* and *ICGV99568* against *Chalimbana, RV, ICGV83708* and *ICGV12991* (Table 2). No significant difference was observed between Chalimbana, RV, *ICGV83708* and *ICGV12991* and also between *CG7* and *ICGV99568* (Table 2 and Figure 1). In terms of the leaf length/width ratio, the highest value was observed in CG7 (1.55) while the least was observed in *ICGV99568* (1.34). Insignificant difference at $p > 0.05$ was observed between *CG7, Chalimbana*, and *ICGV12991* and *RV* genotypes. Genotypes *ICGV99568, ICG83708* and *Chalimbana* showed no significant difference (Table 2).

Euclidian distance based dendrogram on 7 phenotypic traits

The mean values of the 7-phenotypic traits of the seed and leaves phenotypic characteristics were used to generate a dendrogram and it indicated major clusters I and II (Figure 2). Cluster I had two sub clusters; IA and IB. Cluster II had two sub clusters; IIA and IIB. Subcluster IA had two subclusters; IA1 and IA2. Sub-cluster IB had two sub-clusters; IB1 and IB2. Sub-cluster IA1 had two minor subclusters IIIA and IIIB. Sub-cluster IIIA contained first replicates of genotype *Chalimbana and ICG83708*. Minor subcluster IIIB had four *Chalimbana* genotypes from the coastal region and *ICGV12991-1* a genotype from Western region. Sub-cluster IA2 consisted of *ICG83708* genotypes. Subclusters IB1 and IB2 consisted of genotypes mainly from Rift Valley with *CG7* in cluster IB1 and *ICGV99568* in cluster IB2. The Western region of Kenyan genotypes clustered in subcluster IIA as *RV* genotypes and *ICGV12991* genotypes in subluster IIB. Based on the observed classification, it is clear that the major cluster I was the most diverse relative to the major cluster II (Figure 2).

Principal component analysis

Figure 3 shows the PCA was generated from 7

Figure 1. Photographs of *Arachis hypongaea.l* variety seeds corrected from KARLO Kakamega (a, b, c, d, e, f); planted groundnut genotypes in pots according to variety for phenotypic and genetic characterisation (g and h).

Table 3. Eigen values and vectors based on the 7 phenotypic traits.

	PC 1	PC 2	PC 3
Eigen value	2.90	1.79	1.06
% Total variance	41.6	25.7	15.1
% Cumulative	41.6	67.2	82.4
Traits		**Eigen vectors**	
Grain length	**0.533**	0.013	0.012
Grain breadth	0.512	-0.081	**-0.299**
Grain length/breadth	0.141	0.054	**0.923**
Grain weight	0.491	-0.184	0.180
Leaf length	-0.150	-0.681	0.045
Leaf width	-0.053	**-0.700**	0.027
Leaf length/width	**-0.409**	**0.055**	0.156

phenotypic characteristics. In terms of principal components generated, the first two components showed more Eigen values exhibiting 67.2% variability of all the traits studied. The PC1 had 41.6%, PC2 showed 25.7% and PC3 had 15.1% of the total variability observed. Eigen values were observed, where it decreased from PC1 to PC3. Based on Table 3, PC1 had 2.90, PC2 (1.79) and PC3 exhibited an Eigen value of 1.06. Principal component 1 had high and positive correlation with seed length, seed breadth and weight and negative correlation with leaf width, leaf length and leaf length/width ratio. The second principal coordinate, exhibited high and negative correlation with leaf width, leaf length, grain weight, and grain breadth. Positive correlation was observed in leaf length/width ratio. The third principal coordinate showed high and positive correlation with grain length/breadth ratio and grain weight. Negative correlation was observed in grain breadth (Table 3). The six genotypes with five replicates clustered into four quadrants (Figure 4). Principal component 1 reported 41.6% while the principal component two reported 25.7% of the total variation observed. The first quadrant comprised of three *RV* genotypes and one *CG7* genotype (Figure 5). The second quadrant comprised of two genotypes; *CG7* replicates clustered closely, a similar observation exhibited by the groundnut genotypes from Rift Valley, *ICGV99568*. The third quadrant consisted of replicates of *RV* and *ICGV12991* clustering together. Moreover, *Chalimbana-5* and *ICGV12991*-1 ordinated on the same point in the quadrant (Figure 5). The fourth quadrant had the most scattered distribution of genotypes (Figure 5). Genotypes *ICG83708-1* and *ICG83708-5* ordinated on the same point as shown in (Figure 5). Moreover, genotype *ICG83708* and *Chalimbana* were grouped in this quadrant (Figure 5).

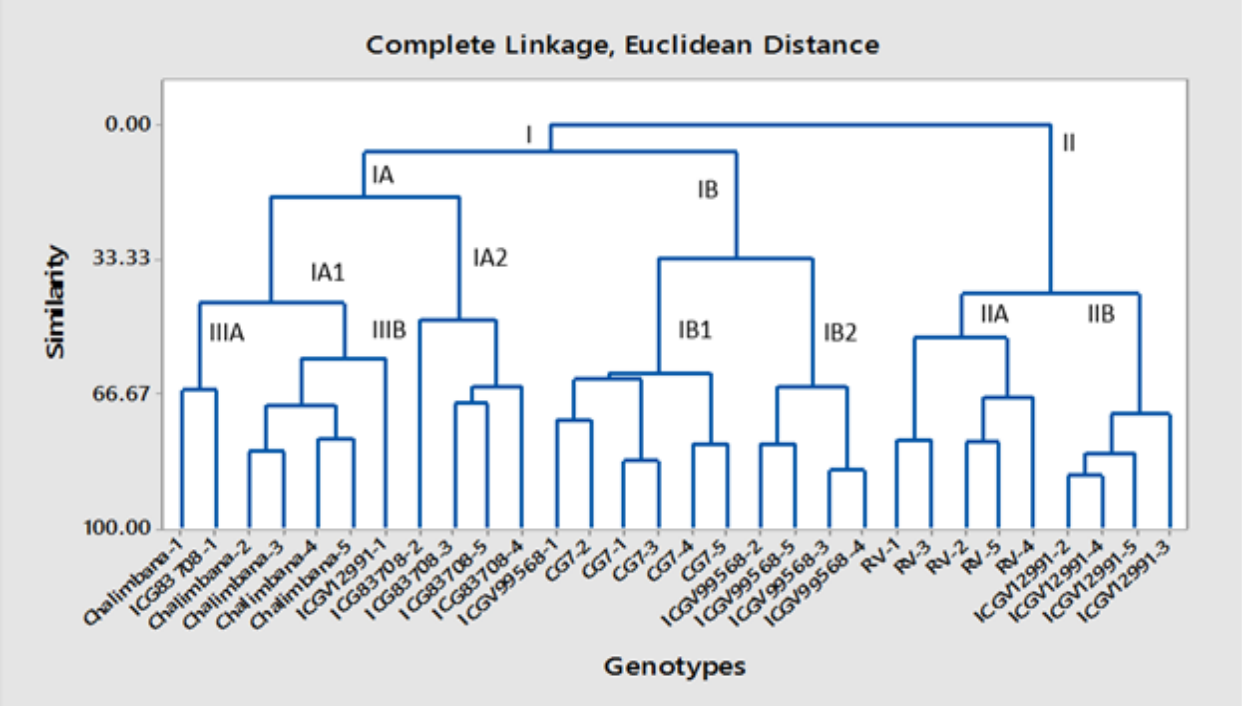

Figure 2. Complete linkage Euclidian distance tree of the 7 phenotypic traits studied on the groundnut varieties.

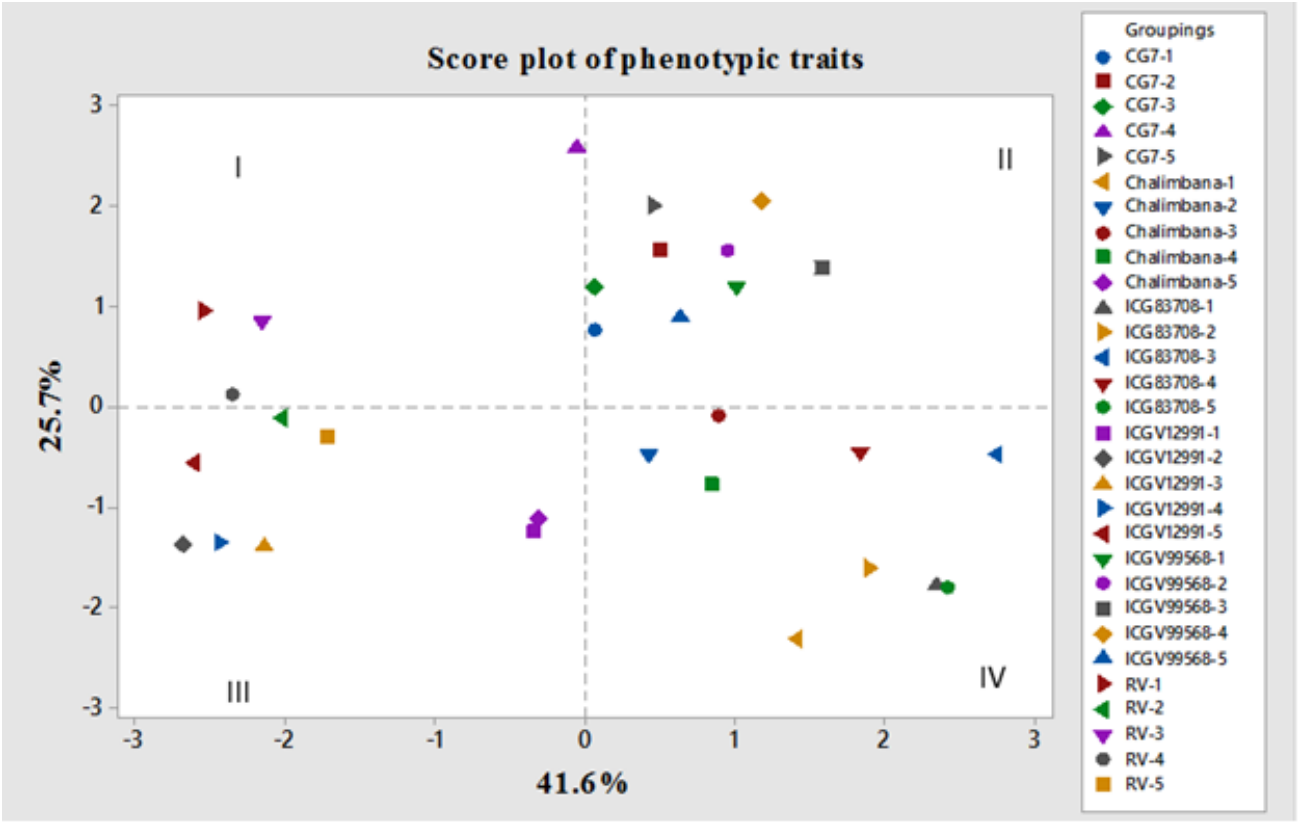

Figure 3. Cluster analysis showing major principle components of groundnuts varieties on the phenotypic traits.

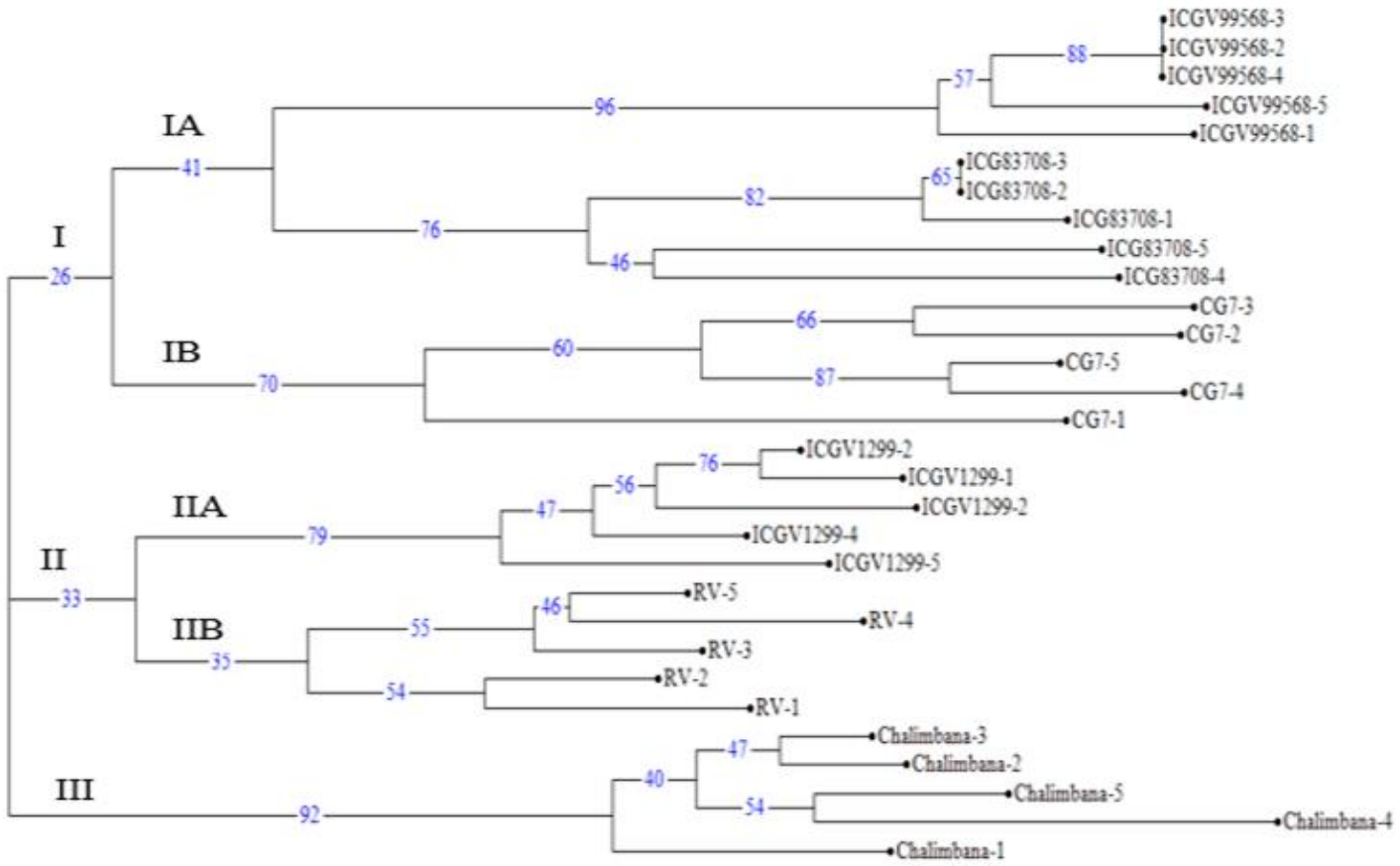

Figure 4. Dendrogram of the 6 groundnut varieties with 5 replicates on the basis of 11 SSR markers using the neighbor joining tool from PowerMarker software.

Gene diversity

SSR markers polymorphism patterns

Among the 15 primers used in the study, 11 markers were polymorphic and formed unambiguous and distinct bands and hence were included in calculation of genetic parameters. All the monomorphic primers were excluded since they were not informative (Supplementary material Figure 1). 35 alleles were detected across the loci of the 11 markers used. The major allele frequency ranged from 0.9333 in primer IPAHM 165 to the least frequency of 0.6333 in primer IPAHM 176 with an average of 0.7610 across all the polymorphic markers. The expected heterozygosity had a mean of 0.3178 where the highest was observed value of 0.4511 in IPAHM 176 while the least diversity of 0.1222 was observed in IPAHM 165. Heterozygosity also referred to as the observed Heterozygosity showed a common value of 0.0000 across the polymorphic markers used. Polymorphism information content (PIC) showed the levels of polymorphism in groundnut varieties for a marker locus which is used in linkage studies. Based on this study the highest PIC was obtained in IPAHM 176 with a PIC value of 0.3484 while the least was in IPAHM 165 with a PIC of 0.1131 with a mean PIC value of 0.2528 across the markers used (Table 4) Occurrence of rare alleles was observed in Marker IPAHM 219 at 400bp in genotype *ICG83708* where an allele present in <5% of the study population was considered exceptional (Table 4).

Phylogenetic tree

Unweighted neighbour joining tree generated in this study showed the genetic relatedness of the genotypes (Figure 4). The phylogenetic tree had three major clusters, I, II and III. Subsequently, there was also other sub clusters IA, IB, IIA, and IIB (Figure 4). Bootstrap values ranged from the highest at 96% in IAI to the lowest in major cluster II. Cluster I consisted two sub clusters IA and IB where in IA there was genotype *ICGV99568* which had the highest bootstrap value of 96%. Replicate *ICGV99568-3*, *ICGV99568-2* and *ICGV99568-4* clustered

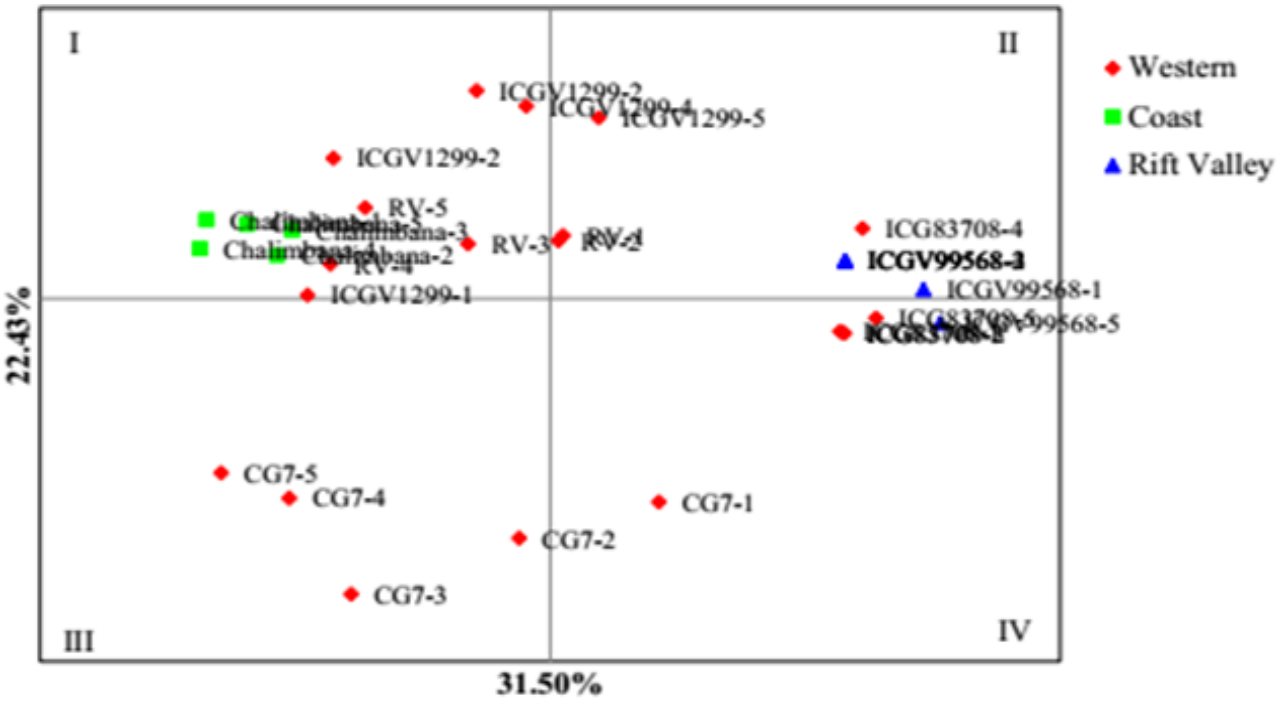

Figure 5. Scatter plot on groundnut genotypes based on Principle coordinate analysis.

Table 4. Genetic parameters of the SSR markers used.

Marker	Major allele frequency	Allele numbers	Gene diversity	Heterozygosity	PIC	f
IPAHM 219	0.7733	2.0000	0.3333	0.0000	0.2726	1.0000
IPAHM 103	0.7267	2.0000	0.3516	0.0000	0.2765	1.0000
IPAHM 123	0.8667	2.0000	0.2207	0.0000	0.1901	1.0000
IPAHM 165	0.9333	2.0000	0.1222	0.0000	0.1131	1.0000
IPAHM 166	0.8000	2.0000	0.2400	0.0000	0.1824	1.0000
IPAHM 108	0.6917	2.0000	0.4017	0.0000	0.3171	1.0000
IPAHM 23	0.6667	2.0000	0.4163	0.0000	0.3248	1.0000
IPAHM 105	0.6792	2.0000	0.4090	0.0000	0.3210	1.0000
IPAHM 229	0.7889	2.0000	0.2881	0.0000	0.2258	1.0000
IPAHM 176	0.6333	2.0000	0.4511	0.0000	0.3484	1.0000
IPAHM 171a	0.8111	2.0000	0.2615	0.0000	0.2085	1.0000
Mean	0.7610	2.0000	0.3178	0.0000	0.2528	1.0000

at the same node with 88% confidence limits. Similarly, *ICG83708-3* and *ICG83708-2* clustered in the same node with 65% confidence limits. Sub cluster IB only consisted of *CG7* replicates with high confidence limits of 70%. Cluster II consisted of two varieties all from Western region, sub cluster IIA had the highest bootstrap value compared to IIB which consisted of all the *RV* genotypes. Cluster III had the second highest bootstrap value of 92% and consisted of all the replicates of *Chalimbana* coastal groundnut genotype.

Principal coordinate analysis

The principal coordinate analysis (PCA) was done to show the relationship among the studied groundnut genotypes. This complements the phylogenetic tree in elaborating genetic relationships amongst genotypes. The 2-Dimentional plot obtained had coordinate one accounting for 31.5% of the total variation while the coordinate two had 22.43% of the total variation observed. Quadrant I comprised of three varieties;

Table 5. Analysis of molecular variance.

Source	DF	SS	MS	Est. Var.	% Variation	P-value
Among Pops	2	41.517	20.758	2.136	31	
Within Pops	27	127.950	4.739	4.739	69	
Total	29	169.467		6.875	100	<0.001

Chalimbana; *RV* and *ICGV12991*. Quadrant II comprised of three genotypes, *ICGV99568* from Rift valley and three genotypes *RV*, *ICGV12991* and *ICG83708* all from Kenya's western region. Quadrant III has sparse distribution of *CG7* replicates. Four replicates of *ICG83708* ordinated in two positions in quadrant IV as in Figure 4.

Analysis of molecular variance

This illustrates the distribution of variation amongst and within populations. Based on Table 5, out of the total variation, 69% variation was distributed within populations while 31% was attributed to among population variation ($p<0.001$; Table 5).

DISCUSSION

The findings from this study show that there is both phenotypic and genetic variations that exist within and among the varieties. These differences substantiate observations by local farmers on leaf length, leaf width, leaf breadth, seed length, seed width, seed breadth and seed weight. Leaf lengths variations between genotypes under study were observed. The relatively low leaf lengths observed in genotype *ICGV99568* could be attributed to known adaptation that the genotype have undergone to suit saline and drier environmental conditions observed in the Rift Valley region that this genotype is best adapted to xerophytic conditions to reduce water loss by evaporation. Matheri et al. (2016) have observed similar findings in Kenyan *Passiflora edulis* genotypes. High leaf lengths could be due to genotypes being hybrids and having adapted to the Kenyan agronomic and environmental influence (Wu and Campell, 2007). The means leaf lengths are lower than the leaf length value of 5.42 cm attained by Upadhyaya et al. (2009) in cold-tolerant Indian peanuts and this could be due to environmental influence. Leaf width had significant difference amongst genotypes under study; this could be due to agronomic adaptation to the different agronomic and environmental conditions since these genotypes are widely grown in Western, Rift valley and Coastal regions. These values are relatively lower than an average of 2.3 cm in cold-tolerant Indian peanuts (Upadhyaya et al., 2009). Leaf length/width ratio is used to give estimates on if the leaf is narrow or wide. Leaf with a ratio less than 2 are broad, since the leaf width is higher while those with a ratio more than 3 are narrow due to the higher leaf length. Based on this, all the varieties under study had broad leaves. Similar findings were found in Jerusalem artichoke (Diederichsen, 2010).

Based on the grain length/breadth ratio, groundnuts that have length 4-5times longer than the width are considered as long grain while those with a ratio of length 2 to three times longer than the breadth are considered as medium-grained. Grain weight showed significant difference amongst the genotypes of study. Findings in Indian groundnuts showed a mean grain weight of 0.539 g (Upadhyaya et al., 2009).

Based on Euclidian distance of the 7 grain and leaf traits the genotypes clustering showed that *Chalimbana* a coastal genotype had close relation with *ICG83708* a genotype majorly grown in the western region of Kenya. This shows that these genotypes have similar phenotypic traits resulting from environmental adaptability. *CG7* and *ICGV99568* clustered together implying that these genotypes have similar phenotypic traits. It is, notable that these clustering contained genotypes that originated from Rift valley region and one from Western region, this means that despite the different geographical origin these genotypes possess similar leaf and grain traits. Western Kenya genotypes *RV* and *ICGV12991* clustered into one major cluster implying that they have similar phenotypic traits. These could be due to similar environmental conditions and also existence of similar genes that causes expression of similar phenotypic characteristics (Lobo and Shaw, 2008).

Eigen values generated showed that the first principal component accounted for higher contributions to the total variation observed compared to other principal components. The principal component techniques is an important factor in phenotypic diversity studies since it allows the evaluation of the importance of a trait to the total variation observed (Hamid et al., 2009). The total variation In PC1 was 41.6%, phenotypic traits such as grain weight, grain length and grain breadth accounted the most to the diversity observed. Leaf length/width ratio and grain length/width ratio are important traits that contributed to the variation patterns observed in PC2.

Determination of genetic diversity amongst the groundnut genotypes showed considerable levels of genetic diversity. Finding from a study by Khera et al. (2013) utilizing SNPs to carry out genetic diversity, showed a major allele frequency ranging from 0.5 to 0.99 with a mean of 0.67 on a reference set of groundnuts. The difference in values attained can be attributed to different genotypes of groundnuts use and the type of molecular marker used. According to Goddard et al. (2000) markers that have a major allele frequency that ranges from 0.5 to 0.8 can be used in mapping of QTLs since they contribute approximately equally to information in the linkage equilibrium studies. The low mean allele number per locus observed can be as a result of founder effects, bottlenecks or genetic isolation that the genotypes under study have experienced. The 0.3178 mean gene diversity value observed was lower than 0.683 observed in genetic diversity studies on Brazilian cultivated peanut genotypes using SSR markers (Moretzsohn et al., 2004). Observed heterozygosity was lower than 0.6974 observed in genetic diversity of rhizome peanuts using microsatellite markers (Pandey et al., 2012). The difference between observed and expected heterozygosity seen in this study is attributed to the high inbreeding coefficient observed in this study. Similar findings have been observed in genetic diversity of selected rice genotypes using microsatellite markers (Chemutai et al., 2016).

Polymorphic information content shows the informativeness of molecular marker (Hildebrand et al., 1994). Based on PIC classification standards most of the markers are reasonably informative markers. The observed PIC is lower than 0.49 observed by Ncube Kanyika et al. (2015) in African peanut germplasm in a study to identify SSR markers to be used in QTL mapping studies. In a study by Shoba et al. (2010), 11 groundnut genotypes were studied using 17SSR markers and only six were polymorphic (24%) and PIC values ranged from 0.17 to 0.63 with an average of 0.41. The neighbour joining tree used in this study was most suitable since it does not assume a rigid molecular clock as observed in the Un-weighted pair group method using arithmetic average (UPGMA) (Ngari et al., 2018). The observed replicates in sub cluster IA implies that these genotypes are identical genotypically and have no genetic divergence amongst them Each genotype and its replicates clustered into a single sub cluster with no admixture implying that low genetic variation was prevalent within and among the genotypes under study (Matheri et al., 2016).

The PCoA attained in this study supports the clustering observed in the phylogenetic tree. Genotypes that were located closer to each other confirm the neighbour joining tree findings on existence of a similar ancestry (Matheri et al., 2016). Genotypes that ordinated on one spot within the quadrant, for instance, in quadrant IV shows the existence of replicates. Also, *RV* replicates were scattered across quadrant I and II implying that these replicates harbour a large genetic variation amongst the genotypes which could be attributed to chromosomal mutations amongst these genotypes. The principal coordinates obtained were lower than 88.64% molecular variance (coordinate 1) and 3.74 (coordinate 2) obtained by Ren et al. (2014) in a study on genetic diversity of major peanuts grown in China using SSR markers. Low bootstrap values observed in sub clusters of major clusters III and IIA could be attributed to the high within population variation, which is observed in the AMOVA table. High within population variation attained in AMOVA implies that within the Kenyan peanut genotypes there exists a significantly high within population variation.

Conclusions

The phenotypes diversity indicated variation based on the seed and leaf traits. Cluster analysis grouped the groundnut genotypes intro three major groups, with cluster III containing genotypes from Western region contrary to the other with an admixture.PIC values showed that IPAHM 176 is most informative marker for diversity studies using SSR markers. Phylogenetic neighbour joining tree showed that coastal variety, *Chalimbana* was distinct from other genotypes under study.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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SUPPLEMENTARY MATERIAL

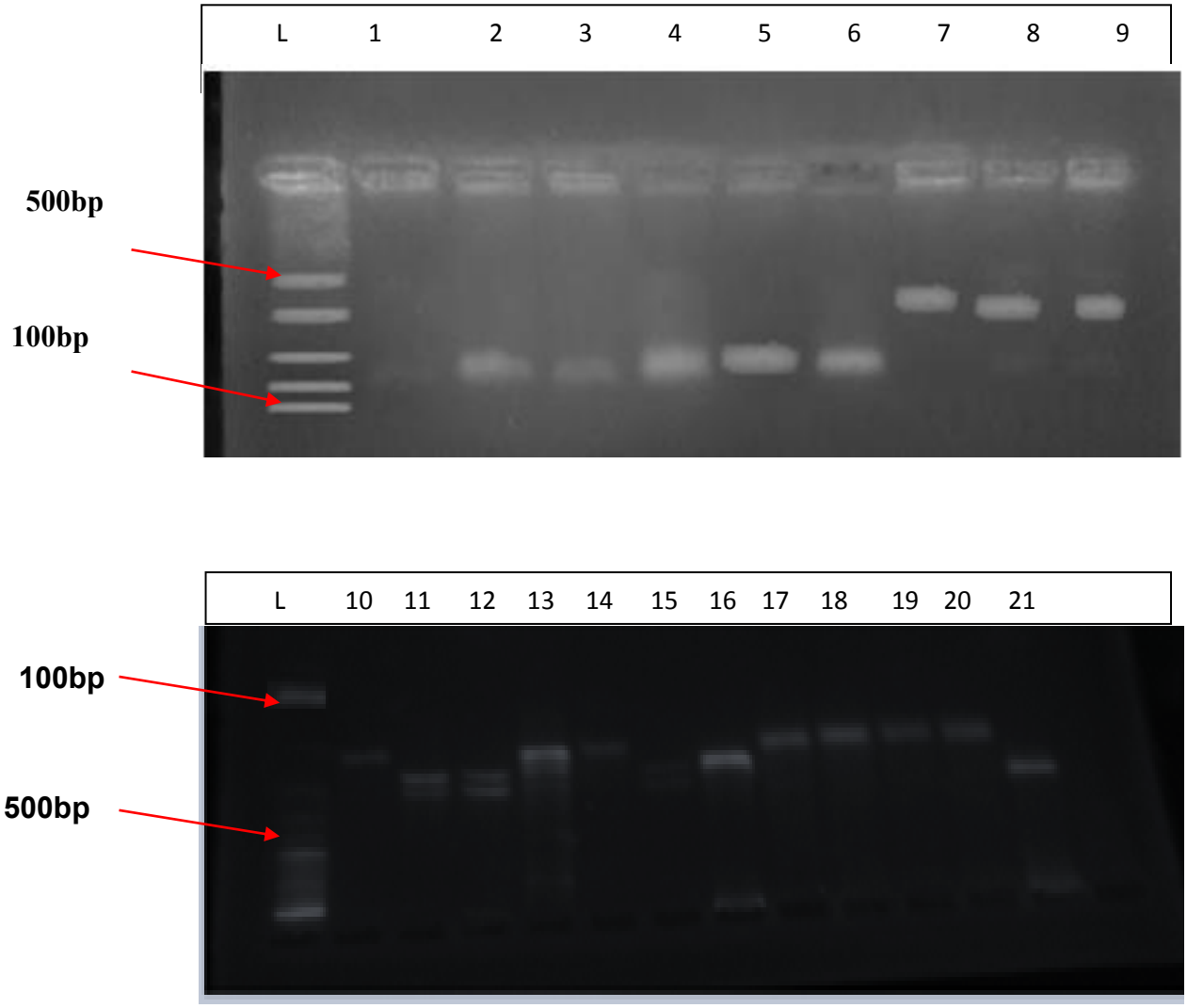

Figure 1. Gel photo for primer IPAHM 229: L-100BP DNA Ladder.1-Control, 2, 3, 4, 5-*ICGV83708. 6, 7, 8, 9, 10-ICGV99568*.11, 12, 13, -CG7.14, 15, 16, -*ICGV12991*.17, 18, 19 RV.20, 21 *C* Tables.

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Evaluation of sugar beet monogerm O-type lines for salinity tolerance at vegetative stage

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Increased production of sugar beet under rainfed conditions on saline–sodic soils in the Iranian areas highlights the importance of salt tolerant varieties. Screening of genotypes for salinity tolerance is difficult in field due to heterogeneity of physical and chemical properties of soil. In order to evaluate the salinity tolerance of 21 sugar beet monogerm O-types lines, a pot experiment was conducted using a split plot design. The evaluation of plants was performed using 11 morphological and physiological traits at vegetative growth stage under severe salt stress (~16 dS m^{-1}) and control (0.3 dS m^{-1}) for 8 weeks. Salinity stress significantly reduced weight related traits. The response of genotypes for total weights and stem weights was very similar under both conditions. But, ranking of O-type lines for root weights under normal and stress condition was different. Indeed, there was high significant genotype × treat interaction for two these traits. Cluster analysis by using STI index of all traits allowed the identification of tolerant, moderate tolerant and sensitive genotypes toward salinity. The four salt-tolerant genotypes, O-type 9669, O-type 1609, O-type 463-2, and O-type 463-5 identified in this study, could be used in the development of salt-tolerant sugar beet varieties. In the second part of this study in order to assess a simple, rapid, and nondestructive method to estimate chlorophyll content, the chlorophyll meter (SPAD 502) readings were recorded and the relation was determined. Regression analysis indicated that there was a significant linear regression between chlorophyll content and chlorophyll meter and about 74% of changes in chlorophyll meter based on chlorophyll content were predicted.

Key words: Sugar beet (*beta vulgaris* L.), salt tolerant index, screening, hybrid.

INTRODUCTION

Threats to the 21st Century include depletion of water resources, environmental contamination, and excessive salinity of soil and water. It has been estimated that 20% of the world's lands and almost twice as much of the irrigated lands are affected by salinity. By 2050, the worldwide 50% of total cultivated land will be salinized (Rozema and Flowers, 2008; Jamil et al., 2011; Zhang et al., 2014). The increased production of sugar beet under

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rainfed conditions on saline–sodic soils highlights the importance of salt tolerant varieties. Fortunately, compared to other crops, sugar beet is comparatively tolerant to abiotic stress conditions, owing to *Beta vulgaris* sp. *maritima* as the wild progenitor of sugar beet, which prospered insuch harsh conditions (Ober and Rajabi, 2010). In sugar beet, the selection for improving stress tolerance in seedling stage ameliorates plant establishment in the field (McGrath et al., 2008). Thus, it is necessary for the majority of sugar beet breeding programs to focus on increasing germination and establishment in saline environments in order to maintain crop productivity. So far, in many researches, different agronomy and physiological traits have been used to evaluate salinity tolerance in crop species, but due to the complexity of the tolerance mechanism and the lack of suitable technique, limited improvement has been made (Munns and James, 2003). Most studies screening genetic sources under salt conditions have been accomplished in controlled environments with a single level of salt stress and no validation of the results under field conditions.

Sugar beet is a salt tolerant plant that shows a great potential for cultivation in salt-affected areas (Wang et al., 2017; Tahjib-UI-Arif et al., 2019) so that it has exhibited better growth status under 3mMNaCl than 0mMNaCl (Peng et al., 2014). The previous report revealed the ability of sugar beet growth at low to moderate (75–100 mM NaCl) salinity in soil culture test (Tahjib-UI-Arif et al., 2019) and to a higher degree in soil containing 85–140 mM salt (Li et al., 2007). The salt tolerance of sugar beet is a complex trait determined by many physiological and metabolic response mechanisms, including: accumulation of the Na^+ and Cl^- in old leaves and petioles, increased accumulation of compatible solutes such as betaine and free amino acids, increased activity of antioxidant enzymes and enhanced activity of photosynthesis relate enzymes under moderate salt stress (Wang et al., 2017).

The most effects of abiotic stress, such as drought and salinity, on the chlorophyll content leads to reduction in growth and photosynthesis (Dadkhah and Rassam, 2017). The measurement of the chlorophyll content is expensive, laborious and time consuming. Thus, a quick and straightway approach, as alternative, can be very effective for estimating leaf chlorophyll concentration. A Chlorophyll Meter SPAD-502 is used for measuring the absorbance of the leaf in two regions, a red 650 nm and an infrared 940 nm (Minolta, 1989). The SPAD Chlorophyll Meter Reading (SCMR) has been positively correlated with chlorophyll content in rice (Turner and Jund, 1991), wheat (Uddling et al., 2007) and sugarcane (Jangpromma et al., 2010). The growth stage, genotype and environmental conditions affects the regression equations of chlorophyll content on the chlorophyll meter (Campbell et al., 1990; Peng et al., 1993; Smeal and Zhang, 1994; Balasubramanian et al., 2000; Esfahani et al., 2008). Due to the involvement of nitrogen in chlorophyll-producing enzymes in plants (Chapman and Barreto, 1997), the researchers have also used chlorophyll reading to predict leaf nitrogen concentration (Peng et al., 1995b; Esfahani et al., 2008).

The detection of cytoplasmic-gene male-sterility (CMS) system has contributed to the practical production of hybrid seed in sugar beet. Propagation and maintenance of CMS plants is feasible with near isogenetic pollen-fertile lines that has normal cytoplasm (N) and two recessive loci ([N]xxzz) in nucleolus (Moritani et al., 2013). This system of genetic fertility restoration was first identified by Owen (1945) and Owen-type (O-type) source was known as maintainer line for CMS line. Therefore, hybrid cultivars in sugar beet are produced by male sterile lines, O-type lines and pollinator (Bosemak, 2006). Studies have been carried out on tolerance to salinity of pollinators; but, there was no information on salinity tolerance of O-type lines in sugar beet. Recently, one study on resistance against rhizoctonia crown and root rot (Rcrr) disease in these lines has been reported (Hassani et al., 2019). Thus, the objectives of the present study were: 1) the evaluation of salt tolerance in sugar beet monogerm O-type lines from Iran at vegetative growth stage based on morphological and physiological parameters in order to select tolerant and sensitive genotypes for use in breeding programs. As regards this, male sterile lines equivalent to salt tolerant O-type lines derived from this study were used in factorial design for genetic study of sugar beet salinity (Abbasi et al., 2019). 2) The determination of the best relationship between SPAD readings with Net CO_2 assimilation rate (A) and Transpiration rate (T) in sugar beet plant for prediction of chlorophyll content using SPAD.

MATERIALS AND METHODS

Plant materials

Twenty one sugar beet monogerm O-type lines provided at Sugar Beet Seed Institute (SBSI) of Iran, were assessed for salinity stress at germination and early seedling growth stages (Table 1) by eleven traits (Table 2). O-type 231 and 7233.P.29, were used as susceptible and tolerant controls, respectively, in greenhouse experiment. The population of 7233-P.29 as a broad open pollinated population, was improved after some cycles of simple recurrent selections using selected roots for salinity tolerance under saline field conditions.

Greenhouse experiment

Due to drip irrigation system, the split plot experiment with two factors of genotypes (nineteen O-type lines along with two controls) and salinity with two levels (0.3 dS m^{-1} and 16 dS m^{-1} (~175 mMNaCl)) were used. Salt water for experiment was prepared from the Agricultural Research Experiment Station located at Rodasht (65 km east of Isfahan, 328290 N and 528100 E, 1560 m asl).in a natural manner. In a previous experiment, EC= 16 dS m^{-1} was identified as critical electrical conductivity to differentiate between sugar beet genotypes (Khayamim et al., 2014). The experiment was

Table 1. Sugar beet O-types lines evaluated in greenhouse.

No.	Pedigree
1	O-type 9621
2	O-type 9669
3	O-type 445
4	O-type 9590
5	O-type 1609
6	O-type 7173
7	O-type 8090
8	O-type 7617
9	O-type 463-1
10	O-type 463-2
11	O-type 463-3
12	O-type 463-4
13	O-type 463-5
14	O-type 419
15	O-type 463-6
16	O-type 474
17	O-type 452
18	O-type 419
19	O-type 428
20	O-type 231- susceptible control
21	7233-P.29 – tolerant control

Table 2. Abbreviations and units of measurement for the measured traits of sugar beet in greenhouse.

Trait	Abbreviation	Unit of measurement
Germination percentage	GP	%
Mean daily germination	MDG	day
Mean time to germination	MTG	day
Establishment percentage	EP	%
Relative water content	RWC	-
Total fresh weight	TFW	g
Shoot fresh weight	SFW	g
Root fresh weight	RFW	g
Total dry weight	TDW	g
Shoot dry weight	SDW	g
Root dry weight	RDW	g
SPAD chlorophyll meter reading	SCMR	-
Chlorophyll content	ChlC	µmol m^{-2}
Net CO_2 assimilation rate	A	µmol CO_2 m^{-2} s^{-1}
Transpiration rate	E	mmol H_2O m^{-2} s^{-1}
Stress tolerance index	STI	-
Field emergence potential	FEP	-

conducted at Isfahan Agriculture and Natural Resources Research Center, Iran in October 2012. The electrical conductivity (EC) of the NaCl solutions was measured directly using a conductivity meter (Model 1481-50, Cole-Parmer Instrument Company, Chicago). The treatment combinations were replicated three times and arranged in a completely randomized design (CRD). Each experimental unit consisted of 24 seeds/pot planted in a circular pattern (at a depth of 1.5 cm) in plastic pots (18cm diameter and 20 cm depth) filled with perlite. Salt stress was imposed from planting time and lasted for two months. The control and saline irrigation solutions were separatelypreparedintwo100-Lreservoirscontainingahalfstrength Hoagland's solution (Table 1S) (Hoagland and Arnon, 1959), and

drip irrigation system was applied. Overflow irrigation was returned through drainage to the reservoirs. The drip irrigation was performed once a day for 30 min. Some control (not planted) pots were placed among the pots to control the EC in perlite. The experiment was conducted under day/night temperatures of 23-34°C/15-20°C, day length of 13–13.5 h and humidity range from 40 to 85%. The number of germinated seeds was recorded daily. Germination percentage (GP) was recorded 24 days after sowing. Plants were harvested after two months. Seedling establishment percentage (EP) was recorded at the end of experiment. Mean daily germination (MDG) that is 'the average number of seeds germinated per day of the actual test period' was calculated as follow (Gidner et al., 2005) (Equation 1):

$$MDG = \frac{FGP}{D}$$

where FGP is the final germination percentage and D is the number of days to the end of the test.

Mean time to germination (MTG) is the index of germination rate calculated as follow (Lein et al., 2008) (Equation 2):

$$MTG = \frac{\sum(nd)}{\sum n}$$

where*n* is the number of germinated seeds in *d*th day and $\sum n$ is the total number of germinated seeds.

Indexes

Field emergence potential (FEP) (McGrath et al., 2000) for all traits was determined as:the ratio of stress to non-stress seedling characteristics represents the salt tolerance during vegetative growth.

Stress tolerance index (STI) was calculated for seedling characteristics using the following equation as example (Fernandez, 1991) (Equation 3):

$$\text{STI (GP)} = \frac{GP_S \times GP_N}{\overline{GP}_N}$$

where GP_S and GP_N represent germination percentage under stress and non-stress conditions, respectively for each genotype and $\overline{GP}_N$ represents the mean of germination percentage in non-stress conditions for all genotypes.

Field emergence potential (FEP) (McGrath et al., 2000) for germination was determined as follow: number of germinated seeds in stress treatment/number of germinated seeds in control treatment. Similarly, this index was calculated for other traits.

Physiological measurements

Biomass

Biomass was determined from control and salt stressed plants. At harvest times, the roots and shoots of plants from each replication were separated. The fresh weight was measured for shoot (SFW), root (RFW) and total fresh weight plant (TFW). After being dried at 70°C in an oven until the samples reached a constant weight, the dry weight of roots (RDW) and shoots (SDW) per plant were measured.

Leaf relative water content (RWC)

Leaf relative water content (RWC) was determined by using the method described by Ghoulam et al. (2002) in fully expanded leaves. Leaf discs were excised from the interveinal areas of each plant. For each plot, discs were pooled and their fresh weight (FW) determined. They were floated on distilled water in Petri dishes for 4 h to regain turgidity, then thawed and re-weighed as turgid weight (TW). The leaf samples were dried at 80°C for 24 h to determine dry weight (DW). RWC was defined as follows:

RWC (%)=[(FW−DW)/(TW−DW)]×100

Percentage variation (increase/decrease) in comparison to control for each trait was calculated as below:

Percentage variation (%)=[(Control - Stress)/(Control)]×100

Photosynthetic parameters

Leaf gas exchange parameters) net CO_2 assimilation rate (A) and transpiration rate (E)) were measured using a Li-Cor 6400 gas-exchange portable photosynthesis system (Li-Cor, Lincoln, Nebraska, USA). The chlorophyll content were measured using the method mentioned in Jamil et al. (2007).

A chlorophyll meter [SPAD-502, Soil and plant analysis development (SPAD), Minolta Camera Co. Osaka, Japan] was used for chlorophyll measurement on fully expanded leaves.Three SPAD readings were taken around the midpoint of each leaf blade averaged to represent the mean SPAD readings of each plot.

Data analysis

Data were assessed by SAS software version 9.2 (SAS Inc., Cary, NC, USA) as the split plot experiment. The comparison of means was determined using LSD test among genotypes for each measurement, under stress and normal condition as separately (Steel and Torrie, 1984). In order to discriminate 21 sugar beet O-type lines for salt tolerance, cluster analysis was performed using STI of traits by Ward's method. Linear regression was used to determine the relationship between SCMR with chlorophyll content, photosynthesis and transpiration.

RESULTS AND DISCUSSION

In this study, the response of 21 sugar beet O-type lines under salinity and normal conditions were assessed by eleven morphological and physiological traits and two index (Table 2).

Morpho-physiological response under stress and normal conditions

The variance analysis revealed significant ($P \leq 0.01$) effects of genotype, treatment and their interaction for germination and establishment percentage, relative water content and weight related traits (data not shown). Salinity showed the negative effect by reducing the value of all traits except MGT and MTG. The percentage variation (decrease/increase) of traits under salinity

Table 3. Mean comparison, mean, percentage decrease and relation between STI and EFP indices for eleven different traits of sugar beet investigated at seedling growth stage.

No.	Genotype	Germination percentage (GP)		Mean daily germination (MGT)		Mean time to germination (MTG)		Establishment percentage (EP)		Relative water content (RWC)		Total fresh weight (TFW)	
		Normal	Saline	Normal	Saline	Normal	Saline	Normal	Saline	Normal	Saline	Normal	Saline
1	Otype 9621	75.00	80.55	5.36	6.71	7.65	8.78	75.00	61.11	90.77	83.81	32.41	10.95
2	Otype 9669	95.83	80.56	6.85	6.71	7.72	8.22	93.75	73.61	88.67	85.12	33.60	10.98
3	Otype 445	81.25	80.55	5.80	6.71	8.01	8.15	81.25	52.78	89.89	86.75	28.93	6.69
4	Otype 9590	70.83	83.33	5.06	6.94	6.58	7.85	77.83	76.39	91.28	85.67	34.17	12.31
5	Otype 1609	91.67	90.28	6.55	7.52	7.40	8.49	91.67	63.89	89.22	86.46	32.34	10.24
6	Otype 7173	83.33	76.39	5.95	6.36	8.18	8.49	81.25	61.11	88.01	86.48	32.45	8.72
7	Otype 8090	77.08	72.22	5.51	6.02	7.78	8.75	77.08	61.11	90.47	86.08	31.07	7.64
8	Otype 7617	72.92	62.50	5.21	5.21	6.87	8.71	72.92	47.22	90.39	85.55	34.09	11.24
9	Otype 463-1	91.67	65.28	6.55	5.44	7.39	7.92	91.67	50.00	87.94	86.27	38.38	7.27
10	Otype 463-2	85.42	88.89	6.10	7.41	7.09	8.79	85.42	75.00	89.49	87.04	29.02	10.48
11	Otype 463-3	97.92	79.17	6.99	6.60	7.58	8.68	97.92	51.39	86.47	86.60	20.08	6.52
12	Otype 463-4	77.08	84.72	5.51	7.06	6.47	8.59	77.08	65.28	90.46	89.93	23.11	8.10
13	Otype 463-5	91.67	84.72	6.55	7.06	7.59	8.26	91.67	61.11	90.74	86.42	30.36	10.51
14	Otype 419	81.25	87.50	5.80	7.29	7.02	8.22	81.25	52.78	89.56	87.04	32.33	7.40
15	Otype 463-6	93.75	73.61	6.70	6.13	7.56	8.13	93.75	52.78	91.50	86.66	35.35	7.73
16	Otype 474	89.58	84.72	6.40	7.06	7.62	8.60	89.58	48.61	89.83	86.05	30.13	6.19
17	Otype 452	81.25	81.94	5.80	6.83	6.85	8.56	79.17	51.39	90.24	85.37	26.27	5.57
18	Otype 419 bulk	72.92	75.00	5.21	6.25	7.11	9.15	72.92	55.55	89.20	86.82	31.52	8.34
19	Otype 428	89.58	80.56	6.40	6.71	7.52	8.49	89.58	54.17	91.40	85.35	33.22	7.41
20	Otype 231	66.67	56.94	4.76	4.74	7.38	7.01	60.42	27.78	88.64	86.90	22.21	3.90
21	7233-P.29	93.75	98.61	6.70	8.22	6.43	8.07	93.75	90.28	89.40	86.72	33.75	18.88
LSD (5%)		15.296	15.377	1.0936	1.2811	1.7811	2.2126	15.038	18.593	1.5052	2.919	1.1211	0.6019
Mean		83.83	79.43	5.98	6.61	8.22	8.34	83.23	58.73	89.69	86.34	30.7	8.91
% decrease		5.25		10.54		1.95		29.44*		3.74		70.99**	
R^2 (STI, EFP) (%)		25.2		21.7		22.34		58.4*		31.2		53.8*	

No.	Genotype	Shoot fresh weight (SFW)		Root fresh weight (RFW)		Total dry weight (TDW)		Shoot dry weight (SDW)		Root dry weight (RDW)		
		Normal	Saline	Normal	Saline	Normal	Saline	Normal	Saline	Normal	Saline	
1	Otype 9621	28.45	9.42	3.96	1.53	3.74	1.99	2.63	1.53	1.11	0.45	
2	Otype 9669	30.83	9.36	2.77	1.62	4.13	1.82	3.43	1.39	0.70	0.43	
3	Otype 445	25.62	5.94	3.31	0.75	3.49	0.97	2.60	0.79	0.89	0.18	
4	Otype 9590	30.00	10.79	4.17	1.51	3.85	1.92	2.69	1.55	1.17	0.37	

Table 3. Contd.

5	Otype 1609	29.74	9.10	2.60	1.14	3.96	1.51	3.22	1.24	0.74	0.27
6	Otype 7173	28.28	7.55	4.17	1.17	5.22	1.28	3.40	1.02	1.82	0.26
7	Otype 8090	27.86	6.64	3.21	1.00	3.64	1.21	2.66	0.92	0.99	0.28
8	Otype 7617	31.55	9.65	2.54	1.59	3.62	1.79	3.03	1.38	0.59	0.41
9	Otype 463-1	35.86	6.40	2.52	0.87	5.04	1.10	4.33	0.87	0.71	0.23
10	Otype 463-2	26.96	8.99	2.06	1.49	3.42	1.51	2.85	1.16	0.58	0.35
11	Otype 463-3	17.30	5.90	2.78	0.62	3.62	0.93	2.34	0.79	1.28	0.14
12	Otype 463-4	20.57	7.41	2.54	0.68	2.64	0.97	1.89	0.79	0.75	0.18
13	Otype 463-5	27.21	8.94	3.15	1.56	3.42	1.53	2.52	1.23	0.90	0.30
14	Otype 419	29.81	6.56	2.52	0.84	3.80	1.04	3.09	0.87	0.71	0.17
15	Otype 463-6	33.09	6.73	2.27	1.00	3.38	1.14	2.80	0.90	0.59	0.23
16	Otype 474	26.36	5.23	3.77	0.95	3.59	0.97	2.69	0.74	0.90	0.23
17	Otype 452	24.42	4.82	1.85	0.75	2.94	0.90	2.42	0.70	0.52	0.19
18	Otype 419 bulk	26.81	7.15	4.71	1.19	4.22	1.20	2.86	0.94	1.37	0.26
19	Otype 428	31.39	6.49	1.83	0.92	3.21	1.15	2.70	0.95	0.51	0.20
20	Otype 231	20.04	3.51	2.17	0.40	2.83	0.54	2.27	0.45	0.56	0.10
21	7233-P.29	30.23	16.34	3.52	2.54	4.85	2.42	3.20	1.91	1.65	0.52
LSD (5%)		1.1211	0.6019	1.1211	0.6019	1.1211	0.6019	0.7052	0.4723	1.7310	0.5711
Mean		27.73	7.76	2.97	1.15	3.74	1.33	2.84	1.05	0.90	0.27
% decrease		72.02**		61.34**		64.44**		63.03**		69.65**	
R^2 (STI, EFP) (%)		52.88*		50.9*		53.3*		53.12*		51.8*	

stress ranged from the increase of 10.54% for mean daily germination (MGT) to the decrease of 72.02% for shoot fresh weight (SFW) (Table 3). Indeed, the most percentage decreases were owned to weight related traits with the difference between genotypes. The genotypes showing the highest percentage decrease are considered as the most sensitive to salt stress.

According to LSD test, significant differences were detected between the analyzed genotype that shows the effect of salinity varied among genotypes (Table 3). For instance, germination percentage ranged from 66.67% “Otype 231“genotype to 97.92 “Otype 463-3“ genotype under normal condition and ranged from 56.94% “Otype 231“genotype to 98.61 “7233-P.29“ genotype under stress condition. The establishment percentage for normal conditions was approximately equal to the germination percentage under the same conditions, but the establishment percentage under salinity stress ranged between 27.78 and 90.28 with 58.73 mean. Seed establishment appears to be more important than seed germination, meaning that the salt tolerant genotypes are those that have survival potential after germination. The present data shows that genotypes#5 and 10 were good for two traits and genotypes#8 and 9 were bad for two traits, but genotype#14 with high germination was not able to overcome salt stress and survive. On the contrary, genotypes#2 and 4 with germination percent about 80% show high survival. These results corroborate those obtained by Chikha et al. (2016).

For weight related traits, significant differences were observed between genotypes (Table 3). The genotypes showed almost the same ranking for TFW and SFW and also for TDW and SDW under both conditions. This result showed that salt

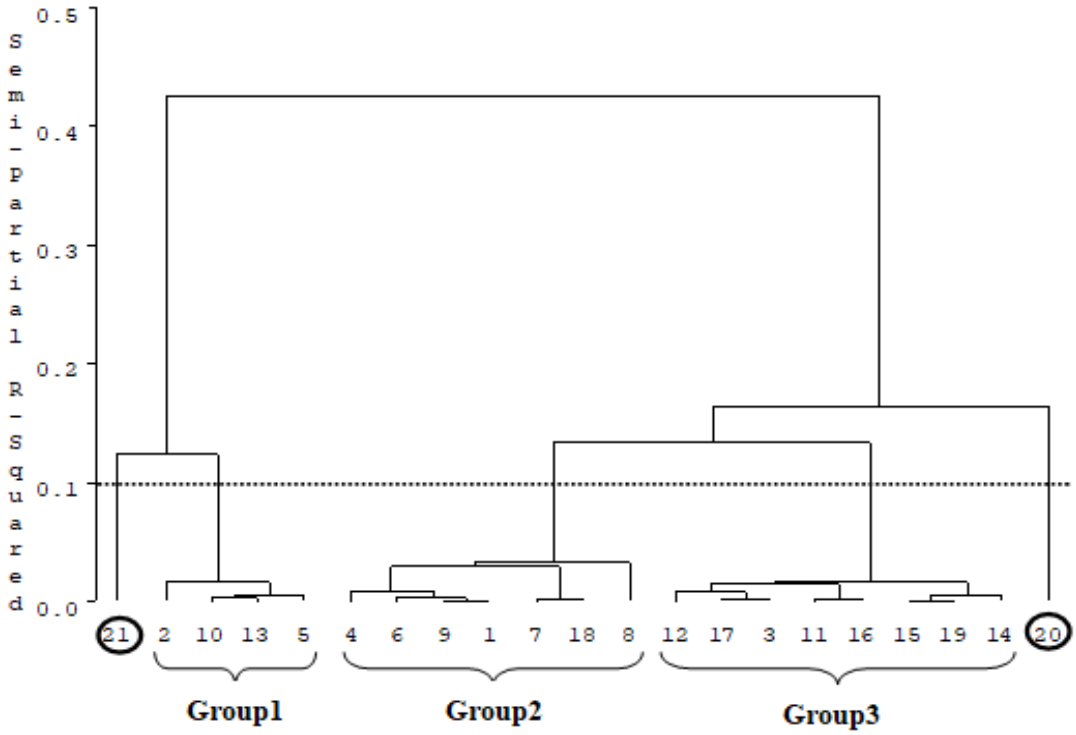

Figure 1. Cluster analysis of 21 sugar beet O-types lines investigated at seedling growth stage using STI of traits by Ward's method.

stress causes more damage to plant aerial part than plant roots that was confirmed by previous studies (Eschie et al., 2002; Wang et al., 2017). Ranking of O-type lines for SFW and SDW under normal and stress condition was different. Indeed, there was high significant genotype×treat interaction for two these traits. Weight loss under stress is a surefire occurrence in all plants. Under salt stress, the phenomenon of necrotic appeared in plant leaves, but only salt tolerant genotypes were able to maintain their biomass and photosynthesis and hence able to overcome salt stress.

These results indicated the existence of genetic potential for salt tolerance among this sugar beet O-type lines that could maintain a good growth status in plant aerial part under salt stress and also show that stress intensity (16 dS/m) used in our study, was appropriate which was able to differentiate between susceptible and tolerant controls, and to differentiate genotypes for different traits. This goes in pair with many other studies (Khayamim et al., 2014; Chikha et al., 2016; Abbasi et al., 2018), which illustrate that severe saline stress, could be used as a rapid method to identify visible phenotypic differences among salt tolerant and sensitive genotypes.

This study documented that the vegetative stage as a very important stage in sugar beet (McGrath et al., 2000) was well able to evaluate genotypes response towards salinity. Several findings in sugar beet indicated that screening at vegetative stage in controlled conditions was accompanied with improving field emergence of sugar beet (Durrant and Gummerson, 1990; McGrath et al., 2000; De los Reyes and McGrath, 2003; McGrath et al., 2008).

Cluster analysis based on the STI values for salt tolerance

The ward's cluster analysis (Figure1) showed that the most sensitive (#20) and resistant (#21) controls were completely separated, indicating that the experiment was performed carefully. According to the dendrogram (Figure 1) and based on the STI of traits, the studied genotypes exhibited different responses toward salt treatment and three distinct groups were identified. The first group with four genotypes #2, 5, 10 and 13 was defined as salt-tolerant genotypes due to high STI value for the traits related to germination and establishment under stress and normal conditions. The second group consisting of seven genotypes were dedicated to moderately tolerant to salinity and the remaining eight O-type lines with low amount of weight related traits, were classified as sensitive to salinity. In many researches, Ward's clustering technique based on STI values was able to distinguish genotypes with contrasting demeanor toward salinity (tolerant/sensitive) (Win et al., 2011; Mini et al., 2015; Kim et al., 2016; Sakina et al., 2016; Abbasi et al.,2018).

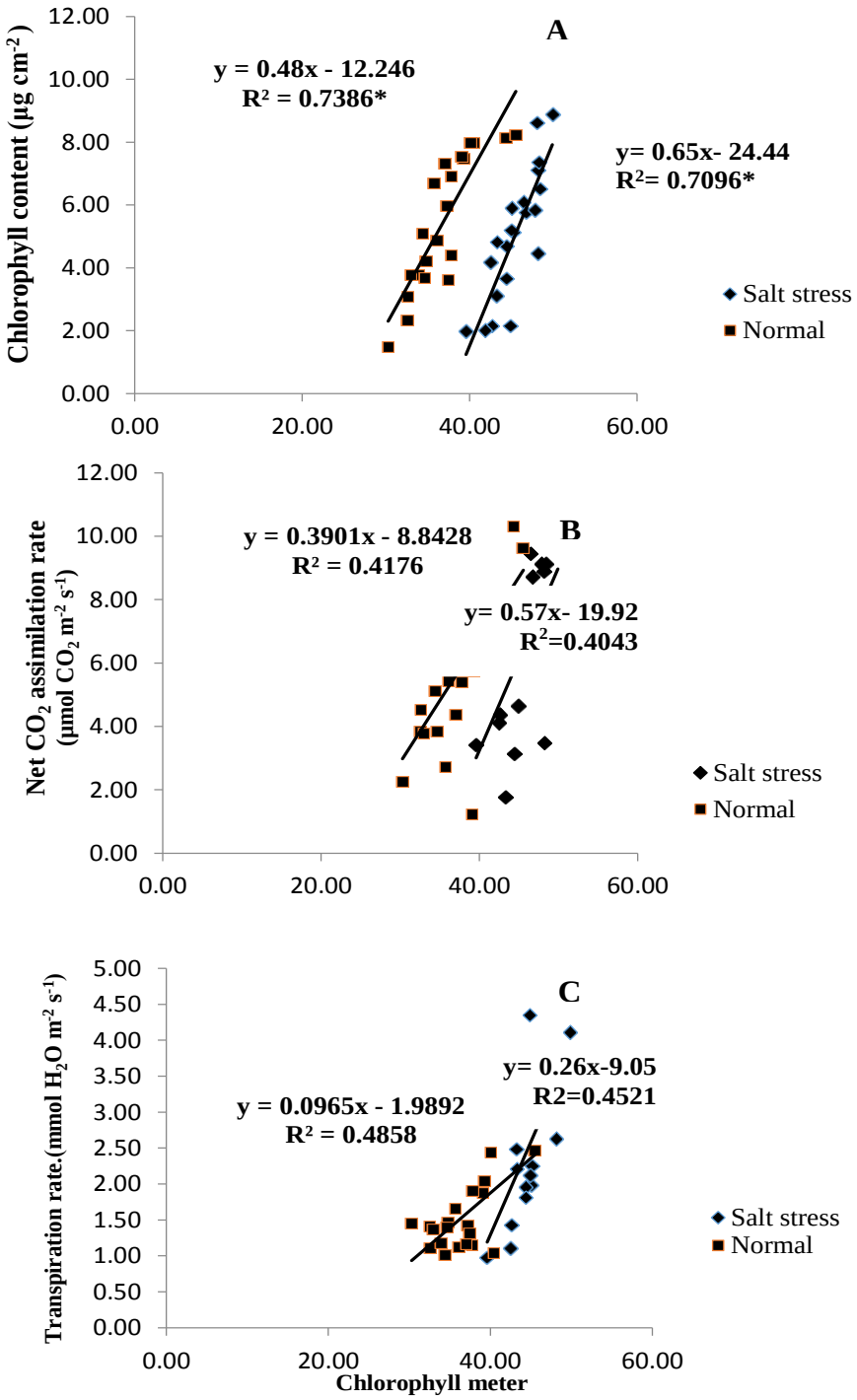

Figure 2. Relationship between (A) total chlorophyll content (µg cm^{-2}), (B) Net CO_2 assimilation rate (µmol CO_2 m^{-2} s^{-1}) and (C) Transpiration rate) mmol H_2O m^{-2} s^{-1} (with chlorophyll meter reading at final establishment of sugar beet (n=21) under salt stress and normal condition. * Significant at p≤0.05.

Relation between SCMR with chlorophyll content, photosynthesis and transpiration

Relationships between total chlorophyll content, net CO_2 assimilation rate and transpiration rate with chlorophyll meter reading (SCMR) at final establishment of sugar beet were shown in Figure 2 (A, B, C). Regression analysis indicated that there was a significant linear regression between chlorophyll content and SCMR and about 74% of changes in SCMR based on chlorophyll

content were predicted (Figure 2A). These results showed that chlorophyll content affected the chlorophyll meter readings; in fact, the accuracy of chlorophyll content prediction is related to SCMR.

Regression analysis showed that there was no significant correlation between net CO_2 assimilation rate (A) and SPAD readings and only about 42% of variation in A was explained by chlorophyll meter reading(Figure 2B). Relationship between transpiration rate (E) and SPAD readings was poor and non-significant (R^2 = 48%) and showed only about half of the changes in E was justified by SCMR (Figure 2C). So, the SPAD chlorophyll meter reading as a simple, low-cost, fast and non-destructive method for prediction of chlorophyll content in salinity research could be used. Since, this relationship is influenced by on growth stage, genotype and environmental conditions, an individual calibration for different cultivars grown under specific growth conditions can increase the accurate prediction. In a study, the relationship between SPAD readings and nitrogen concentration for different rice cultivars increased by an individual calibration (Peng et al., 1995b). Esfahani et al. (2008) presented that adjusting the SPAD readings for specific leaf weight (SLW) improved the estimation of N concentration from 23 up to 88%.

Conclusion

The STI index used in this research could classify sugar beet O-type lines into different categories of sensitive, moderately tolerant and tolerant to salinity; so that the four salt-tolerant genotypes #2, 5, 10 and 13 obtained, were well incorporated in the breeding program after evaluation in the field (in another study). The association between total chlorophyll content with chlorophyll meter reading showed that chlorophyll content of sugar beet leaves can be achieved without cost and time, only by using the chlorophyll meter reading. For different plant species and different growth conditions, the process of testing and calibration may be required.

CONFLICT OF INTERESTS

The author has not declared any conflict of interests.

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Table 1. Supplement. Compounds and amount of ingredients used in Hoagland nutrient solution

No	Name	Amount in stock solution (g/lit)	Amount in 100 liters (ml)
			Solution A
1	H_3BO_3	2.8	100
2	$ZnSO_4$	0.22	
3	$MnSO_4$	4.3	
4	$CuSO_4$	0.1	
5	$(NH_4)_6Mo_7O_{24}$	0.01	
			Solution B
6	H2SO4	5 CC	
			Solution C
7	Na2-EDTA	6.72	
8	Fe- SO4	5.58	
			Solution D
	$NH_4H_2PO_4$	1.2	100
	KNO_3	6.6	
	$Ca(No_3)_2$	9.4	
	$MgSO_4$	5.2	

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Diversity and genetic structure in natural populations of *Croton linearifolius* (Euphorbiaceae) based on molecular markers

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***Croton linearifolius* Mull. Arg, an endemic species of Brazil, has insecticidal activity proven. To the detriment of its importance as a natural resource, the studies of this species are incipient, as well as the strategies applied to its management and conservation. The diversity and genetic structure in 61 individuals of *C. linearifolius* collected in the National Forest Contendas do Sincorá (NFCS) were estimated. Estimates were based on analysis of the amplification profile of nine combinations of pairs of resistance gene analogs (RGA) primers and eight inter simple sequence repeat (ISSR) primers. A total of 134 markers (81.3% polymorphic) were generated. Bayesian analysis indicated as most likely a structuring into two groups. Based on the molecular analysis of variance (AMOVA) it was possible to verify that 64% (p rand <0.01) of the variation occurs within the regions, and a significant amount (36%) (p rand <0.01) was attributed to variations between regions, indicating genetic structure between them. The AMOVA results were corroborated with the Principal Coordinate Analysis (PCoA), indicating an association between the distribution of variability and the geographical distribution of the collection regions. The probable mechanisms pollination and dispersion would justify, at least in part, the genetic structuring observed for *C. linearifolius*.**

Key words: Conservation, genetic variability, genetic polymorphism, resistance gene analogs (RGA) marker, inter simple sequence repeat (ISSR) marker.

INTRODUCTION

The genus *Croton* L. (Euprobiaceae), with about 1,200 species, stands out the second largest in the Euphorbiaceae family, with most of its species dispersed in the tropical regions of the world (Govaerts et al., 2000). Brazil is considered one of the main centers of diversity of the genus *Croton*, with at least 350 species distributed

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Figure 1. General appearance of the species *Croton linearifolius* (A) male flower, fruit (B) and seed (C). Source: Personal collection.

in different regions of the country (Berry et al., 2005). The highest concentration of species of this genus occurs in the Southeast and Northeast regions, with approximately 160 and 120 species, respectively (Cordeiro et al., 2015).

Species of the genus *Croton* are an important natural resource in the Northeast region of Brazil, being used for several purposes. Among the species listed popularly by having biological activity, some have tests that prove its medicinal potential, such as *Croton regelianus* (Antitumoral activity) (Bezerra et al., 2009), *Croton zehntneri* (Antimalarial activity) (Mota et al., 2012), *Croton campestris* (Gastric antiulcerogenic activity) (Júnior et al., 2014), *Croton cordiifolius* (Antinociceptive activity) (Nogueira et al., 2015), and/or insecticide, such as *Croton argyrophylloides, Croton nepetaefolius, Croton sonderianus* and *C. zehntneri* (Lima et al., 2006).

This study highlights the species *Croton linearifolius*, popularly known as "velame pimenta", with proven insecticidal potential against *Aedes aegypti* (Silva et al., 2014) and *Cochliomyia macellaria* (Silva et al., 2010), which corroborates the traditional use indicated in the semi-arid region of Brazil (Silva et al., 2010, 2014). This species also presents diverse chemical composition, being rich in alkaloids, steroids and flavonoids, among other potentially promising compounds (Silva et al., 2010).

C. linearifolius is endemic in Brazil, with occurrence records in the states of Minas Gerais, Piauí, Tocantins and greater representation in Bahia (Cordeiro et al., 2015) (Figure 1), it has shrubby habit and can measure up to 2 m high. The plants of this species are monoecious, with lanceolate leaves and discolores, bisexual inflorescences, the fruits are dehiscent, the seeds measure about 4.0 mm long and 2.5 mm wide, with brown coloration (Lima and Pirani, 2008).

The exploitation of native species by the population is generally based on exploratory extraction, which can lead to a reduction of biodiversity (Mondini et al., 2009). Among the different components of biodiversity, genetic diversity is the basis for the evolutionary potential of a

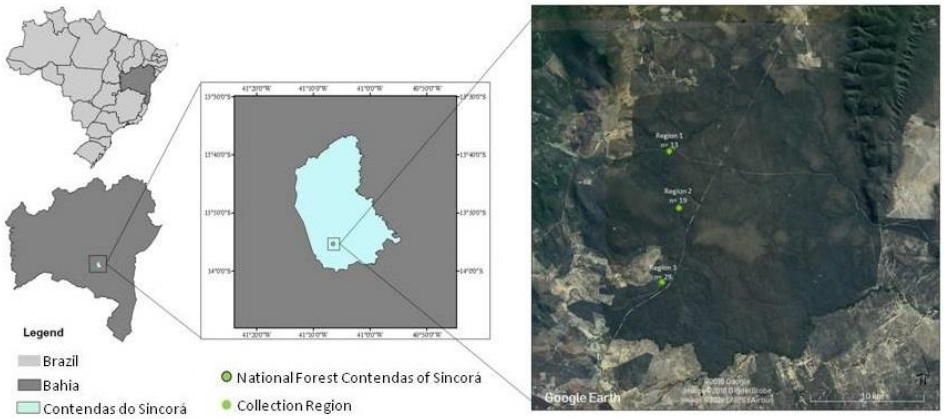

Figure 2. Geographical areas map (Brazil, Bahia State, Contendas do Sincorá city) were from collected samples of *Croton linearifolius*.

species and determines its chances of survival, reproduction and adaptation to possible environmental changes (Fleishman et al., 2001). Therefore, knowledge of the diversity and genetic structure of species that present potential as a natural resource is a prerogative for the elaboration of strategies for sustainable management and conservation of genetic resources. In this context, genetic-molecular markers are an important tool for population studies and have been used in the study of a wide range of organisms (Ibrahim et al., 2010).

Resistance gene analogs (RGA) and inter simple sequence repeat (ISSR) markers are examples of markers that do not require prior information on the genome of the species to be evaluated and enable a rapid and low cost characterization, especially useful for species not yet studied and with commercial interest still little explored.

In detriment of the ecological importance and economic potential of *Croton* species, population genetic studies are limited to a few species (approximately 1%), which hampers the development of management and conservation strategies that may help in the conservation and sustainable use of this biodiversity. Specifically for *C. linearifolius* the genetic-molecular studies are limited to the works of Scaldaferri et al. (2013, 2014) and Silva et al. (2018).

In this study, the genetic diversity and structure of natural populations of *C. linearifolius* were characterized and discussed, and present in the National Forest Contendas do Sincorá (NFCS), based on the application of RGA and ISSR molecular markers.

MATERIALS AND METHODS

Characterization of the biological material

The study was conducted from DNA samples from 61 individuals of *C. linearifolius* collected in three regions understood here as populations, present in the National Forest Contendas do Sincorá (NFCS), in the municipality of Contendas do Sincorá, Bahia, Brazil (Figure 2 and Supplemental Material 1). The exsicatas were deposited in the herbarium of the Universidade Estadual de Feira de Santana, under registration HUEFS 146620. The DNA samples were previously extracted from fresh leaves using the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1990) with modifications previously tested for *Croton* by Scaldaferri et al. (2013). The samples were deposited in the genomic DNA bank of the Laboratory of Applied Molecular Genetics (LAMG), at the Universidade Estadual do Sudoeste da Bahia (UESB), Itapetinga, Bahia, Brazil.

The integrity of the DNA samples was evaluated in 1% agarose gel (m/v) by electrophoresis (2 h in 90 V electric current) and visualized from loading dyes (bromophenol blue and xylene cyanol) and GelRed (Invitrogen Co., Carlsbad, CA, USA) according to manufacturer's specifications, in Kodak photodocumentation system, with incidence of ultraviolet (UV) illumination. To estimate the DNA concentration (ng/µL), the lambda molecular weight marker (Invitrogen Lambda DNA) was adopted as standard.

Molecular genotyping

Genotyping was performed based on 10 ISSR primers (Table 1) and 10 combinations of RGA primers pairs (Table 2) previously selected (Silva et al., 2018). PCR reactions were performed with a final volume of 16 µL containing 12 ng of DNA, 1.7 µL of 10X PCR buffer (LGC Biotechnology, São Paulo, Brazil), 1.0 µL of 50 mM $MgCl_2$ (LGC Biotechnology, São Paulo, Brazil), 1 µL of 2.5 mM dNTP mix (LGC biotechnology), 1 unit of Taq DNA polymerase (LGC Biotecnologia, São Paulo, Brazil), Milli-Q water and 1 µL primer.

In the amplification reactions, the following amplification programs were adopted: for primers RGAs; 5 min at 95°C; followed by 34 cycles (30 s at 95°C, 1 min at 37°C, 1 min and 20 s at 72°C); and 10 min at 72°C. For ISSR primers: 95°C for 5 min; followed by 34 cycles (94°C for 50 s, 48°C for 50 s, 72°C for 1 min); and 5 min at 72°C. All amplifications were performed in the LGMA of the UESB.

Aliquots (5 µL) of the amplification products were visualized from electrophoretic run, on 2% agarose gel (m/v) and 1x TBE solution, for approximately 2 h at 110 V. The visualization of the result of the electrophoretic run was performed with a Kodak photodocumentator, under the influence of ultraviolet light.

Table 1. Description of the 24 combinations of Resistance Gene Analogs (RGA) primer pairs (obtained from 23 primers) used in amplification tests in *Croton linearifolius.*

Combination	Code	Sequence	Reference
1	S1	GGT GGG GTT GGG AAG ACA ACG	
	AS1	CAA CGC TAG TGG CAA TCC	
2	S1	GGT GGG GTT GGG AAG ACA ACG	
	AS2	IAA IGC IAG IGG IAA ICC	
3	S1	GGT GGG GTT GGG AAG ACA ACG	Leister et al. (1996)
	AS3	IAG IGC IAG IGG IAG ICC	
4	AS1	CAA CGC TAG TGG CAA TCC	
	AS2	IAA IGC IAG IGG IAA ICC	
5	AS1	CAA CGC TAG TGG CAA TCC	
	AS3	IAG IGC IAG IGG IAG ICC	
6	NBSF1	GGA ATG GGI GGI GIF GGI AAR AC	Yu et al. (1996)
	AS1	CAA CGC TAG TGG CAA TCC	Leister et al. (1996)
7	NBSF1	GGA ATG GGI GGI GIF GGI AAR AC	Yu et al. (1996)
	AS2	IAA IGC IAG IGG IAA ICC	Leister et al. (1996)
8	NBSF1	GGA ATG GGI GGI GIF GGI AAR AC	Yu et al. (1996)
	AS3	IAG IGC IAG IGG IAG ICC	Leister et al. (1996)
9	S1	GGT GGG GTT GGG AAG ACA ACG	Leister et al. (1996)
	NBSR1	YCT ACT TGT RAY DAT DAY YYT RC	Yu et al. (1996)
10	RGA1F	AGT TTA TAA TTY EAT TGC T	Kanazin et al. (1996)
	RGA8R	AGC CAC TTT TGA CAA CTG C	Kanazin et al. (1996)
11	NBSF1	GGA ATG GGI GGI GIF GGI AAR AC	Yu et al. (1996)
	NBSR1	YCT ACT TGT RAY DAT DAY YYT RC	Yu et al. (1996)
12	RGA8F	AGC GAC GAG AGT TGT ATT TAA G	
	RGA8R	AGC CAC TTT TGA CAA CTG C	
13	RGA1R	ACT ACG ATT CAA GAC GTC CT	
	RGA1F	AGT TTA TAA TTY EAT TGC T	
14	RGA2R	CAC ACG GTT TAA AAT TCT CA	
	RGA1F	AGT TTA TAA TTY EAT TGC T	Kanazin et al. (1996)
15	RGA7R	CCG AAG CAT AAG TTG GTG	
	RGA1F	AGT TTA TAA TTY EAT TGC T	
16	RGA4R	TAC ATC ATG TGT TAC CTC T	
	RGA4F	TGT TAC TGC TTT GTT TGG TA	
17	RGA5R	TCA ATC ATT TCT TTG CAC AA	
	RGA5F	TGC TAG AAA AGT CTA TGA AG	

Table 1. Contd.

18	RGA6R	AAC TAC ATT TCT TGC AAG T
	RGA 6F	AGC CAA AGC CAT CTA CAG T
19	RGA1R	ACT ACG ATT CAA GAC GTC CT
	RGA 4F	TGT TAC TGC TTT GTT TGG TA
20	RGA4R	TAC ATC ATG TGT TAC CTC T
	RGA 1F	AGT TTA TAA TTY EAT TGC T
21	RGA2R	CAC ACG GTT TAA AAT TCT CA
	RGA 4F	TGT TAC TGC TTT GTT TGG TA
22	RGA2R	CAC ACG GTT TAA AAT TCT CA
	RGA 5F	TGC TAG AAA AGT CTA TGA AG
23	RGA2R	CAC ACG GTT TAA AAT TCT CA
	RGA 6F	AGC CAA AGC CAT CTA CAG T
24	RGA6R	AAC TAC ATT TCT TGC AAG T
	RGA 5F	TGC TAG AAA AGT CTA TGA AG

I= A/T/G/C; D= A/G/T; E=C/G; R= A/G; Y= C/T.

Table 2. Description of the 23 Inter Simple Sequence Repeat (ISSR) primers tested in *C. linearifolius.*

Primer Code	Sequence
DiCA3´G	5´- CAC ACA CAC ACA CAC AG- 3´
DiCA3`RG	5´- CAC ACA CAC ACA CAC ARG- 3´
DiCA3`YG	5´- CAC ACA CAC ACA CAC AYG-3`
DiGA3`C	5`- GAG AGA GAG AGA GAG AC- 3`
DiGA3` RC	5`- GAG AGA GAG AGA GAG ARC- 3`
DiGA3`T	5`- GAG AGA GAG AGA GAG AT- 3`
TriCAC3`RC	5`- CAC CAC CAC CAC CAC RC-3`
TriCAC3`YC	5`- CAC CAC CAC CAC CAC YC-3`
TriCAC5`CY	5`- CAC CAC CAC CAC CAC CY-3`
TriCAG3`RC	5`- CAC CAC CAC CAC CAC RC-3`
TriGTG3`YC	5`- GTG GTG GTG GTG GTG YC-3`
TriTGT3`YC	5`- TGT TGT TGT TGT TGT YC-3`
TriAAC3`RC	5`- AAC AAC AAC AAC AAC RC-3`
TriAAG3`RC	5`-AAG AAG AAG AAG AAG RC3`
TriACG3`RC	5`- ACG ACG ACG ACG ACG RC-3`
TriAGA3`RC	5`-AGA AGA AGA AGA AGA RC-3`
TriTGG3`RC	5`- TGG TGG TGG TGG TGG RC-3`
TriCGA3`RC	5`- CGA CGA CGA CGA CGA RC-3`
TriCGC3`RC	5`- CGC CGC CGC CGC CGC RC-3`
TriGAC3`RC	5`- GAC GAC GAC GAC GAC RC-3`
TriGCA3`RC	5`- GCA GCA GCA GCA GCA RC-3`
TriGCC3`RC	5`- GCC GCC GCC GCC GCC RC-3`
TriGGA3`RC	5`- GGA GGA GGA GGA GGA RC-3`

I= I=A/T/G/C; D= A/G/T; E=C/G; R= A/G; Y= C/T.

Table 3. Polymorphism obtained from nine combinations of Resistance Gene Analogs (RGA) primer pairs in the characterization of the 61 individuals of *Croton linearifolius.*

Combination	Code	Number of markers		Polymorphism (%)
		Total	Polymorphic	
2	S1+AS1	9	5	55.5
3	S1+AS3	5	2	40
6	NBSF1+AS1	6	1	16.6
14	RGA2R+ RGA1F	9	9	100
15	RGA7R+ RGA1F	4	4	100
17	RGA5R+ RGA5F	12	12	100
18	RGA6R+ RGA6F	3	3	100
19	RGA1R+ RGA4F	13	13	100
24	RGA6R+ RGA5F	8	8	100
Total	-	69	57	83%

Data analysis and genetic diversity estimation

The obtained band standards, with analysis of the gels by two evaluators, were considered for the construction of a binary data table, where zero (0) was assigned for the absence of bands and one (1) for presence. Based on the table, the percentage of polymorphism was calculated. In order to maintain only the markers that presented a percentage less than 20% of missing data (that is, data whose identification of presence or absence of bands cannot be determined with certainty).

Analyses of population structure were performed with the Bayesian method using the structure software, version 2.3.4 (Pritchard et al., 2000). Considering that the present study was conducted using natural populations, we used an admixture model with independent allele frequencies in each population. The burn-in period and replication numbers were set to 100,000 and 1,000,000, respectively, for each run. The number of groups (K) was systematically varied from 1 to 10 and 20 simulations were performed to estimate each K. The ΔK ad hoc method described by Evanno et al. (2005) and implemented in the online tool Structure Harvester (Earl and vonHoldt, 2012) was used to estimate the most likely K in each set. After estimating the most likely K, the greedy algorithm implemented in CLUMPP v.1.1.1 (Jakobsson and Rosenberg, 2007) was used with a random input order and 1000 permutations to align the runs. Based on the posterior probability of membership (q) of a given accession belonging to a given group compared to the total number of groups (K), individuals were classified with $q > 0.60$ as a member of a given cluster, whereas for clusters with membership (q) values ≤ 0.60, the accession was classified as admixed.

Estimation of genetic similarity was performed based on the Jaccard coefficient, with the aid of the GENES program (Cruz, 2006). The genotype grouping was performed by the Neighbor Joining method, through the DARwin program (Perrier and Jacquemoud-Collet, 2009). Principal coordinate analysis (PCoA) and Molecular Variance (AMOVA), with 999 permutations were performed using the GenALEX v.6.5 program (Peakall and Smouse, 2012) in order to observe the genetic variation between and within the regions collection.

RESULTS AND DISCUSSION

A total of 134 markers were generated after the amplification reactions in 61 individuals of *C. linearifolius*. After sorting the data, nine and eight RGA and ISSR primers were maintained in the analyses, respectively. The combinations of selected RGA primers produced a total of 69 markers (82.6% polymorphic) with an average number of 7.6 markers per primer pair. The combinations 18 (RGA6R + RGA6F) and 19 (RGA1R + RGA4F) generated the lowest (three) and the highest (13) number of brands, respectively (Table 3). The amplification reactions with the ISSR primers produced a total of 65 markers (80% polymorphic), with an average number of 8.1 marks per primer. The primers DiCA3`RG and TriAAC3`RC produced the lowest (6) and highest (10) number of marks, respectively (Table 4).

Although there are no studies with RGA markers for the genus *Croton*, results similar to the polymorphism observed with such markers for *C. linearifolius* were observed in different plant species, such as sugarcane (86.5%) (Jayashree et al., 2010) and rice (96%) (Ren et al., 2013). These results reinforce the potential of RGA markers to access genetic polymorphism. The polymorphism results obtained with the use of ISSR primers in *C. linearifolius* were similar to the results presented for other species of the genus *Croton*, such as *Croton tetradenius* (94.8%) (Almeida-Pereira et al., 2017) and *Croton heliotropiifolius* (94%) (Rocha et al., 2016).

Considering Bayesian analysis (based on Delta K values), the main structuring was observed in two gene pools (Delta K score = 1400) and possible sub-structuring of three or four gene pools (Delta K-400 and K ~200, respectively), as the most probable compositions for the 61 individuals of *C. linearifolius* (Figure 3). The histogram showing the level of structuring in two gene pools (Figure 4A) reveals a predominance of the gene pool represented by blue color in regions 1 (q = 0.78) and 2 (points 2, 3, 4 and 5) (Mean of q = 0.73), whereas in region 3 (points 6, 7, 8, 9 and 10) there is a predominance of the gene pool represented by green color (mean of q = 0.89). Considering a sub-structuring in three gene pools (Figure 4B), although there are no

Table 4. Polymorphism obtained from eight Inter Simple Sequence Repeat (ISSR) primer in the characterization of the 61 individuals of *Croton linearifolius.*

Primer code	Number of markers		Total polymorphism (%)
	Total	Polymorphic	
DiCA3`RG	6	4	66.6
DiGA3` RC	9	7	77.7
TriCAC3`RC	9	9	100
TriCAC3`YC	7	4	57.1
TriGTG3`YC	8	5	25
TriTGT3`YC	9	9	100
TriAAC3`RC	10	8	80
TriACG3`RC	7	6	85.7
Total	65	52	80

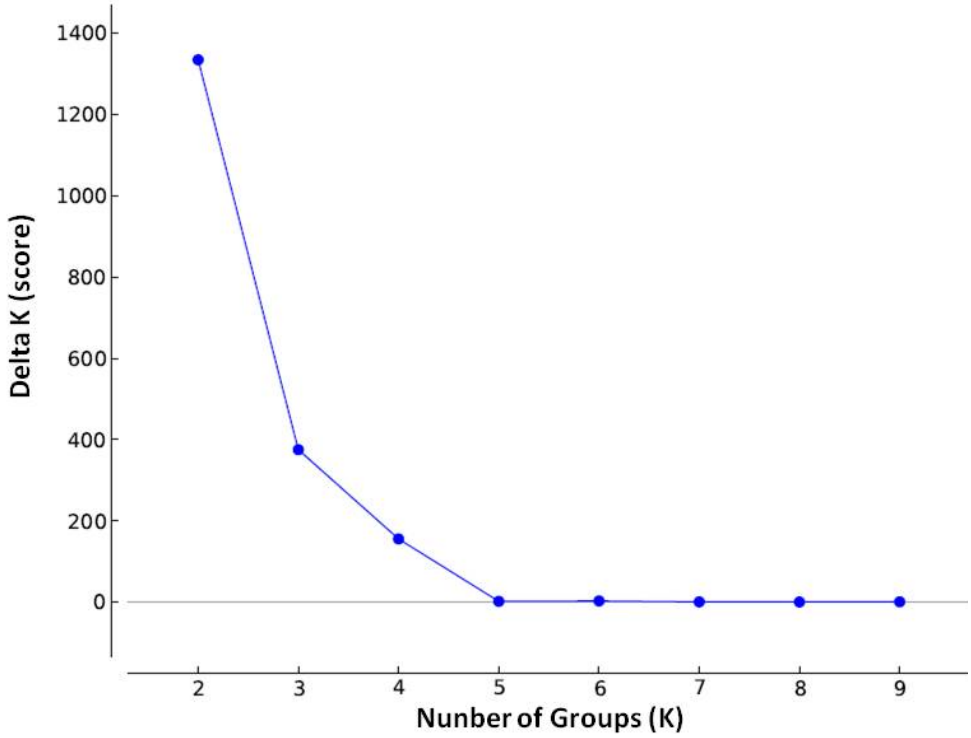

Figure 3. Numbers of genetic pool (clusters) inferred based on Bayesian analyses considering the most probable number of groups (K) estimated with the method described by Evanno et al. (2005).

significant changes in the composition of regions 1 (q = 0.82) and 3 (mean of q = 0.89), the change in composition of region 2 that presents the predominant presence of a distinct gene pool represented by red color (mean of q = 0.62).

The structure observed with Bayesian analysis was corroborated by the results obtained from the molecular analysis of variance (AMOVA), where it was possible to verify that 64% (p rand <0.01) of the variation occurs within the regions, and a significant amount (36%) (p rand <0.01) was attributed to differences between regions, which indicates genetic structuring between them. The AMOVA decomposition presented in the evaluation matrix pair to pair among the regions (Table 5) indicates a greater genetic distance between regions 1 and 3 (45.8%) and lower between regions 2 and 3 (29.7%). A similar result was observed for the species *Croton antisyphiliticus* which according to Oliveira et al.,

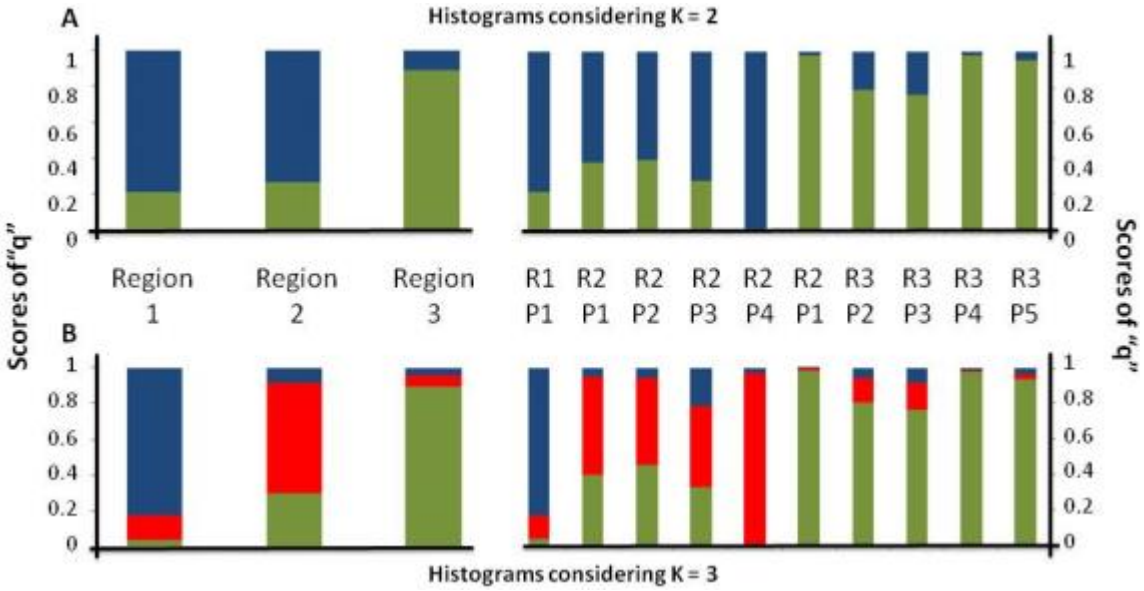

Figure 4. Histograms (based on Delta K values) representing the distribution of probable gene pools in the three regions (R) (upper and lower left) and collection points (P) (upper and lower right) of *Croton linearifolius*. Principal structure in two gene pools (Delta K score = 1400) (A) and sub-structuring in three gene pools (Delta K ~ 400 scores) (B). The colors used in the histograms represent the most probable ancestry of the group from which the individuals were derived.

Table 5. Matrix representative of the values of Φ (lower diagonal) and probability of occurrence (upper diagonal) estimated from the Molecular Analysis of Variance among the different collection regions of *Croton linearifolius*.

Correlation	Region 1	Region 2	Region 3
Region 1		0.001	0.001
Region 2	0.335		0.001
Region 3	0.458	0.297	

(2016) presented approximately 40% variation among the studied localities.

The genetic structure among plant populations is influenced, among other factors, by the effects caused by the dispersion of pollen and seeds, which are directly related to the connectivity among the populations, determining the gene flow rates (Zanella et al., 2012). Pollinators and seed dispersers that have effective dispersion over long distances decrease the likelihood of differentiation between populations, while dispersion at restricted distances has the opposite effect, promoting population genetic structuring (Loveless and Hamrick, 1984).

Although there are no records in the literature of studies on the reproductive biology of *C. linearifolius*, studies carried out with species of the same genus show that there is predominance of pollination by the wind and/or by different insects [*Croton suberosus* (Domínguez and Bullock, 1989), *Croton floribundus* and *Croton priscus* (Passos, 1995), *Croton sarcopetalus* (Freitas et al., 2001), and *Croton urucurana* (Pires et al., 2004)]. Considering that insect-pollinated species tend to have lower gene flow rates and consequently greater genetic differentiation, since the distances traveled are relatively small (Loveless and Hamrick, 1984) it is expected that genus *Croton* will occur between populations.

The primary autochoric dispersion due to fruit dehiscence and secondary zoocoric dispersion due to associations with several ant species is a common strategy among species of the genus *Croton*. The presence of the elaiosomes is an attraction for the ants that act as secondary dispersers, thus reducing predation and competition between the seedlings (Webster, 1994; Passos and Ferreira, 1996; Leal et al., 2007; Lobo et al., 2011). The autochoric species tend to exhibit spatially limited seed dispersion, at maximum distances ranging from 3.4 (Passos and Ferreira, 1996) to 8.0 m (Narbona

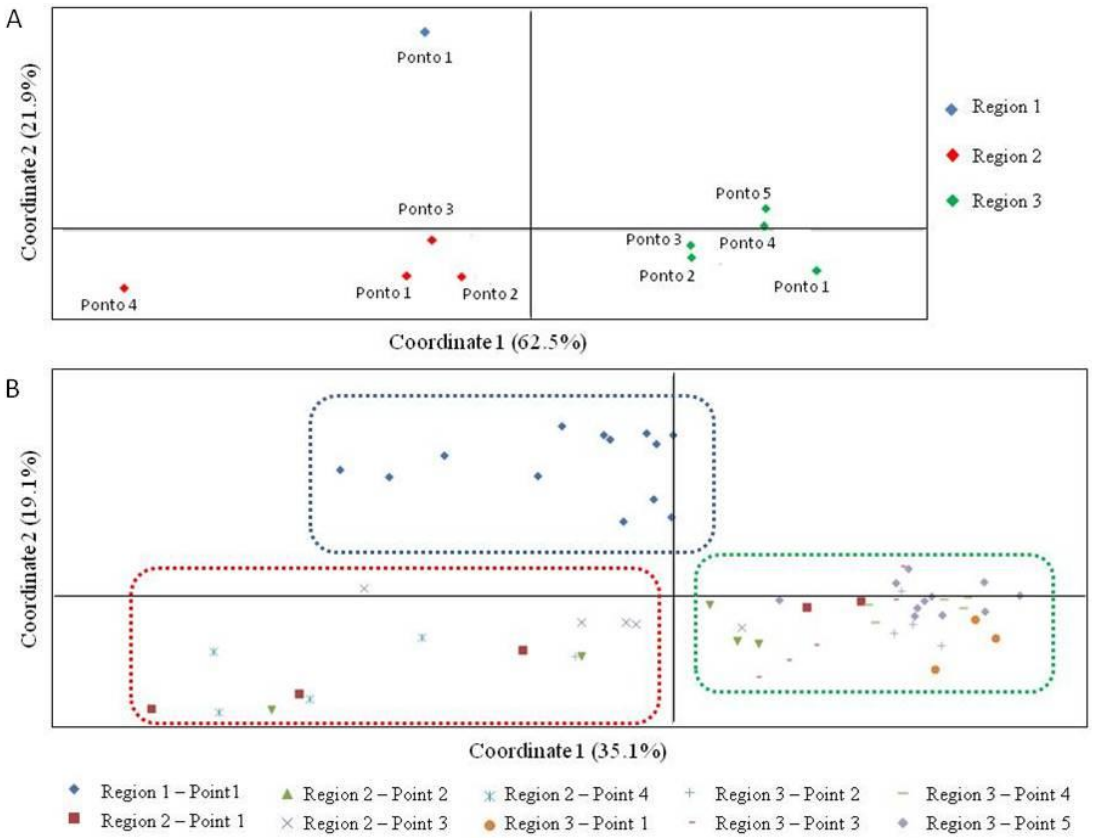

Figure 5. Dispersion graphic based on the main coordinate analysis (PCoA), represented by collection regions (A) and 61 individuals of *Croton linearifolius* (B). The colors of dashed lines correspond to those used to represent the predominant gene pools in the histograms obtained in structure (Figure 3).

et al., 2005). In addition, ants can travel up to 2.5 m away (Passos and Ferreira, 1996), which can also result in greater genetic differentiation between populations.

Therefore, the pollen and dispersion strategies observed for the genus and probable for *C. linearifolius* may be related to the low inferred gene flow between regions 1 and 3, based on STRUCTURE and AMOVA results, as well as the lower structure between these and region 2, which is located in the intermediate region. Similar structure, with existence of three gene pools and presence of greater differentiation between extreme collection points, was observed by Rocha et al. (2016) for *C. heliotropiifolius* from estimates made with ISSR and RAPD markers.

In addition to the mentioned factors, *C. linearifolius* is a monoic species, characterized by inflorescences with staminate (male) flowers in the terminal part and pistil (female) flowers in the lower part (Lima and Pirani, 2008). This arrangement of male and female flowers on an inflorescence may facilitate the occurrence of self-fertilization, a fact that has already been demonstrated in *C. floribundus* and *C. priscus* (Passos, 1995); *C. suberosus* (Domínguez and Bullock, 1989) and *C. sarcopetalus* (Freitas et al., 2001) that present flowers of similar morphology to *C. linearifolius*, which contributes to the increase of inbreeding.

The results obtained through the PCoA analysis (Figure 5) corroborate the hypothesis of sub-structuring in three gene pools obtained by Bayesian analysis (Figure 4B), corresponding to the geographical distribution of the three collection regions. Figure 5B shows the groupings of individuals delimited by rectangles of color corresponding to those used to represent the gene pools in the histogram. Several individuals collected in region 2 presented high q values for the characteristic gene pool of this region, represented by the red color in the histogram ($q \leq 0.60$) (Figure 4B and Supplementary Material 1). These results are in agreement with the collection regions and corroborate with the dispersion of individuals from that region on the PCoA plot (Figure 5B).

Based on the PCoA plot (Figure 5B) it is possible to observe the occurrence of individuals characteristic of region 2 for the gene pool characteristic of the individuals of region 3 (Supplemental Material 1), a fact that was corroborated by the molecular analysis of variance presented in the matrix of pair to pair evaluation among regions (Table 5), which indicated genetic structuring between individuals in regions 2 and 3 (approximately

30%). The observed result may be a reflection of the connectivity between the regions having the region 2 as intermediate, due to its geographical location between points 1 and 3.

In spite of the complementarity observed between the Bayesian analysis, PCoA Analysis and AMOVA results, there was no correspondence with the distance data projection (both by the Neighbor Joining grouping method and by the UPGMA) estimated by the Jaccard coefficient (data not presented). Considering that the choice of coefficients and clustering methods can directly influence the results of the projections (both in scatter plots and in dendrograms) (Scaldaferri et al., 2014; Cerqueira-Silva et al., 2009), it is probable that this absence of association is associated with the influence of the statistical method intrinsic to the procedures of distance estimation and/or clustering method.

Conclusion

The RGA and ISSR molecular markers were efficient in accessing polymorphic markers in *C. linearifolius*. The populations of *C. linearifolius* are structured in at least two gene pools and sub-structured in three and four gene pools, with a correspondence between the genetic structuring and the geographical distribution of the populations. The probable mechanisms of insect pollination and autochoric primary dispersion with secondary dispersion by ants would justify the genetic structuring observed for *C. linearifolius*, since these strategies would not be enough to promote the genetic flow among the collection regions. The results obtained contribute to the genetic-molecular knowledge of *C. linearifolius*, which may help in the strategies applied to the management and conservation of the species.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Supplementary Material 1. Geographic coordinates and number of *Croton linearifolius* individuals sampled by regions and collection points in the Contendas do Sincorá National Forest, Bahia, Brazil.

Collection region	Collection points	Latitude	Longitude	Plant code	Individuals number
Region 1	1	-13.922	-41.117	UESB-Cl-1-13	13
Region 2	1	-13.943	-41.113	UESB-Cl-14-32	5
	2	-13.942	-41.112		5
	3	-13.942	-41.112		5
	4	-13.941	-41.112		4
Region 3	1	-13.97	-41.119	UESB-Cl-33-61	3
	2	-13.97	-41.119		5
	3	-13.97	-41.119		5
	4	-13.969	-41.119		5
	5	-13.97	-41.119		11

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Evaluation of different fungi and bacteria strains for production of cellulases by submerged fermentation using sugarcane bagasse as carbon source: Effect of substrate concentration and cultivation temperature

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Cellulases (enzymes of great potential in biotechnology) are currently of interest due to their applicability in the hydrolysis of cellulose from lignocellulosic materials, producing fermentable sugars for alcohol production. The great demand for efficient enzymes is the driving force to prospect new cellulases-producing microorganisms, as well as to optimize the enzyme production step. Many variables can be optimized in microorganism cultivation, such as pH, type of microorganism, induction, temperature, type and concentration of substrate, among others. This work aimed to evaluate the production of cellulases by submerged fermentation from three strains of filamentous fungi (*Trichoderma koningii*, *Penicillium* species, *Rhizomucor* species) and two strains of bacteria (*Bacillus megaterium* and *Bacillus subtilis)*, using sugarcane bagasse as substrate. Variations in substrate concentrations (0.5, 1.6 and 2.7%, w/v) and temperature (28, 33 and 38°C) were evaluated on volumetric activity. The best fungus was *T. koningii* (3130.4 IU/L) using 2.7% natural sugarcane bagasse at 28°C. Among the bacteria, *B. megaterium* stood out with an enzyme production in range of 130 to 156.7 IU/L (at 28-33°C using natural and acid-alkaline pretreated bagasses), although up to around 20 times lower than the production by the *T. Koningii*.

Key words: Microorganisms, lignocellulosic biomass, cellulolytic enzymes, pretreated biomass.

INTRODUCTION

Cellulases are among the most important hydrolytic enzymes, being the third largest industrial enzyme in the world market. This is due to the wide application of these enzymes, such as starch processing, animal food

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production, extraction of fruit and vegetable juices, pulp and paper industry, and textile industry (Singhania et al., 2010; Akinyele et al., 2014; Jasani et al., 2016). Currently, there is a great interest in cellulases for use in ethanol production from lignocellulosic biomass, and some enzyme companies have launched cellulases specific to the lignocellulosic biomass processing industries (Singhania et al., 2010). Cellulases hydrolyze the β-1,4 glucosidic linkages of cellulose resulting in glucose, which can be fermented to produce second-generation ethanol (2G ethanol).

Cellulase is industrially produced mainly by submerged fermentation (SmF) (Lin et al., 2017), although in the last two decades solid-state fermentation (SSF) has awakening interest due to the high enzyme productivity, reduced energy requirements, etc. (Acharya et al., 2010; Singhania et al., 2010). The scientific literature has reported studies concerning both solid-state and submerged fermentation (Hernández-Domínguez et al., 2014; Zanirun et al., 2014; Jasani et al., 2016; Bentil et al., 2018; Wang et al., 2018). Bentil et al. (2018) reported that submerged fermentation is more efficient than solid state fermentation to obtain higher cellulase production rates when using white-rot basidiomycetous fungi. Submerged fermentation provides a homogeneous environment, continuous oxygen supply and better control over several parameters, such as pH, temperature, dissolved oxygen (DO), and froth formation. Moreover, there is no problem of mass transfer and heat removal (Singhania et al., 2015; Wang et al., 2018).

Regarding microorganisms, there is a wide range of cellulase-producing species, which may be fungi, yeast, aerobic bacteria and actinomycetes (Mmango-kaseke et al., 2016; Behera et al., 2017). Among them, the filamentous fungi are the best producers of cellulases, mainly of the genus *Trichoderma* and *Aspergillus* (Baraldo-Junior et al., 2014; Borges et al., 2014; Juturu and Wu, 2014). The screening and isolation of microbes from nature is one of the important ways to get cellulases with diverse properties and high activity (Juturu and Wu, 2014); thus, researchers are continually searching for new microorganisms. Cellulase is produced as a primary metabolite associated with microorganism growth (Zanirun et al., 2014).

The production of cellulase also depends on the growth parameters, which include pH, temperature, carbon source, nitrogen source, agitation rate, and others (Nagar and Kumar, 2010; Jasani et al., 2016). Agro-industrial residues are generally used as carbon sources, as they are a low-cost raw material and also act as inducers of cellulase production (Zanirun et al., 2014; Catelan and Pinotti, 2019). Different types of lignocellulosic substrate may contribute to different cellulase production (Pandey et al., 2016). Some of the substrates significantly stimulate enzyme production without supplementation of the culture medium with specific inducers (Singhania et al., 2010). Moreover, the use of pretreated biomass can promote the growth of microorganisms since the pretreatment reduces the recalcitrance of the material and facilitates the access to the cellulose (Rodríguez-Zúñiga et al., 2011). Therefore, the selection of suitable substrate capable to induce high yield of cellulase is a very important subject.

In this work, we investigated the production of cellulases by submerged fermentation using different filamentous fungi (*Trichoderma koningii*, *Penicillium* species, *Rhizomucor* species) and two bacterial species (*Bacillus megaterium* and *Bacillus subtilis*). For each microorganism, three types of sugarcane bagasses were evaluated (natural and pretreated with acid-alkaline and with hydrogen peroxide solutions) and two cultivation variables, temperature (28, 33 and 38°C) and concentration of sugarcane bagasse (0. 5, 1.6 and 2.7% w/v). The experiments were performed according to a 3^2 factorial design, in which the temperature and concentration of bagasse were varied for each microorganism and for each type of sugarcane bagasse.

MATERIALS AND METHODS

Microorganisms and substrate

T. koningii INCQS 4031 (CFAM 422) and *B. subtilis* (ATCC 6633) were obtained from Oswaldo Cruz Foundation (Rio de Janeiro, RJ, Brazil). *Penicillium* and *Rhizomucor* spp. were isolated and supplied by the Department of Environmental Engineering of the Federal University of Espírito Santo (Vitória, ES, Brazil). *B. megaterium* ATCC 14945 was donated by the Department of Chemical Engineering of the Federal University of São Carlos (São Carlos, SP, Brazil). The fungi and bacteria were maintained on potato dextrose agar (42 g/L) and nutrient agar slant (23 g/L), respectively, and kept at 4°C prior to use.

Sugarcane bagasse was supplied by Destilaria Itaúnas S/A (DISA, Espírito Santo, Brazil) and the procedure performed to obtain the natural bagasse and pretreated with acid-alkaline and hydrogen peroxide solution was as described in previous work (Salomão et al., 2019).

Inoculum preparations

Bacteria

The inoculum medium was composed of 1% (w/v) peptone, 0.5% (w/v) yeast extract and 1% (w/v) sodium chloride and incubated for 12 h at 30°C and 200 rpm (adapted from Fernandes, 2007).

Fungi

To obtain the precultures, the fungi strains were grown on an agar plate containing 3.9% (w/v) potato dextrose agar at 28°C for 5 days. After this period, spores were harvested by adding 10 mL of 0.1% (v/v) Tween-80 and counted in a Neubauer chamber (Menoncin et al., 2009).

Enzyme production by SmF

For enzyme production, sugarcane bagasse was used with particle

Table 1. Cellulase production by SmF of *T. koningii; Penicillium* spp. and *Rhizomucor* spp. using sugarcane bagasse natural (NB), pretreated with acid-alkaline solution (AAB) and hydrogen peroxide solution (HPB) in different conditions of temperature (T°C) and concentration of sugarcane bagasse ([B]).

Run	Variable		Endoglucanase Activity (IU/L)								
			Trichoderma koningii			*Penicillium* sp.			*Rhizomucor* sp.		
	T ºC	[B] % w/v	NB	AAB	HPB	NB	AAB	HPB	NB	AAB	HPB
1	28	0.5	1112.5	1524.9	2171.4	0.0	0.00	17.1	16.0	7.7	8.6
2	33	0.5	1049.0	*	1947.8	14.6	28.7	15.2	3.9	4.4	0.3
3	38	0.5	174.5	78.2	0.0	7.5	0.0	8.0	5.8	17.7	0.0
4	28	1.6	2241.5	1167.7	1253.8	3.3	62.1	49.1	**63.5**	27.9	24.3
5	33	1.6	1837.4	78.5	1219.0	18.5	**111.8**	31.8	4.4	0.3	9.7
6	38	1.6	554.9	68.9	0.0	9.4	15.5	22.9	8.8	7.4	38.8
7	28	2.7	**3130.4**	237.4	271.6	53.3	107.7	83.6	13.5	29.5	28.2
8	33	2.7	1993.9	57.0	103.8	60.5	54.7	57.4	3.0	3.9	13.0
9	38	2.7	654.2	0.0	0.0	37.8	21.3	22.8	17.1	7.7	0.0
10	33	1.6	1804.3	82.2	1160.0	15.2	111.4	34.5	4.7	0.8	8.8
11	33	1.6	1879.4	74.5	1141.7	17.4	111.0	38.6	4.7	0.3	7.7

*Omitted.

diameter sizes between 0.85 and 2.36 mm (Fernandes, 2007). The bagasse was added to 250 mL Erlenmeyer flasks containing 100 mL mineral salt solution of Mandels and Weber (1969) at pH 5.3. The flasks were autoclaved at 121°C for 20 min and inoculated with concentration of 10^6 spores/mL. The flasks were incubated (28, 33 and 38°C) on a shaker (100 rpm) during 48 h for bacteria and 72 h for fungi. At the end of fermentation, the medium was filtered and the filtrate was used for determination of enzyme activity.

For this study the following conditions were evaluated: cultivation temperature (28, 33 and 38°C) and sugarcane bagasse concentration (0.5, 1.6 and 2.7% w/v) for each microorganism (*B. megaterium*, *B. subtilis*, *T. koningii*, *Penicillium* spp. and *Rhizomucor* spp.) and for each type of sugarcane bagasse (natural and pre-treated with acid-alkaline and hydrogen peroxide solutions, as described in Salomão et al. (2019)). The values of concentration and temperatures selected here were based on works found in the literature, which used agro-industrial residues to produce cellulases (Akinyele et al., 2014; Mesa et al., 2016; Jasani et al., 2016; Irfan et al., 2017; Fernandes et al., 2018). For these experiments, a 3^2 factorial design with two central points was used. Statistical analysis of the data was performed using Statistic v. 13.0, and the values were considered significant for p-values < 0.05. The model obtained had the assumption of normal distribution of errors (difference between the values estimated by the model and the "correct value") admitted as valid and the model equations that correlate the activity of cellulases with the temperature and the concentration of sugarcane bagasse of sugar were obtained using the uncoded variables.

Enzyme activity

The endoglucanase (EG) activity was determined according to Ghose (1987) using 2% (w/v) carboxymethyl cellulose (CMC) in 0.05 M citrate buffer, pH 4.8 as substrate. The mixture containing 0.5 mL of CMC and 0.5 mL of enzyme sample was incubated at 50°C for 30 min. The reaction was stopped by adding 2.0 mL of dinitrosalicylic acid (DNS) reagent and boiled for 5 min. The released reducing sugar was measured according to the DNS curve with glucose as standard (Miller, 1959). One International Unit (IU) of endoglucanase activity was defined as the amount of enzyme that produced 1 µmol of glucose equivalent per milliliter per minute under the assay conditions.

RESULTS AND DISCUSSION

Production of cellulases by fungi

The SmF results with the different fungi (*T. koningii*, *Penicillium* spp. and *Rhizomucor* spp.) and with the different substrates (natural sugarcane bagasse and pretreated with acid-alkaline and with hydrogen peroxide solutions) are shown in Table 1. The best enzyme producers followed the order: *T. Koningii* (3130.4 IU/L at 28°C using 2.7% w/v natural bagasse), *Penicillium* spp. (111.8 IU/L at 33°C using 1.6% w/v acid-alkaline pretreated bagasse) and *Rhizomucor* spp. (63.5 IU/L at 28°C using 1.6% w/v natural bagasse). Salomão et al. (2019) reported similar findings for solid-state fermentations of the same fungi. Under the conditions evaluated in our study reported here, *T. Koningii* was capable of producing 28 and 49 times more cellulose activity than *Penicillium* and *Rhizomucor* spp., respectively. The fact that we observe the best production with the genus *Trichoderma* is important for perspectives from an industrial point of view, since the genus *Trichoderma* is the most culturable and industrially exploited for the production of several multipurpose enzymes. This excellent characteristic of synthesizing a multitude of enzymes, for numerous applications, makes this genus a magnificent industrial cell factory of enzymes (Gautam and Naraian, 2020).

There are few studies in the scientific literature on the production of cellulases with the species *T. koningii* by SmF. Besides, enzymatic activities are measured with

different substrates, making a deeper comparison difficult to achieve. In terms of CMCase (endoglucanase activity), Wang et al. (2012) reported a production of 40,300 IU/L by *T. koningii* D-64 isolated from soil samples from Singapore. This production was obtained after the optimization of the production medium, with the addition of 1% cellulose and 2% wheat bran; however, initially the volumetric activities were between 200 and 7000 IU/L using different carbon sources.

Liu et al. (2012) compared the endoglucanase activity of 4 species of fungi (*T. koningii, Trichoderma reesei, Trichoderma viride* and *Aspergillus niger*) and found that *T. koningii* was the best cellulose producer (31,300 IU/L), followed by *T. viride* (22,000 IU/L), in a medium containing 20 g/L of microcrystalline cellulose. This work corroborates our results, that is, *T. koningii* is the best producer of cellulases by SmF, at least among the evaluated fungi. Also, Wang et al. (2013) found enzyme activities of 28,300 IU/L by a mutant *T. koningii* hyperproducer of cellulase in cultures induced by bran and corncob powder.

With regard to *Penicillium*, it is possible to find very different values of cellulase production in the scientific literature, which depends on the species, culture medium and physical conditions used. Mesa et al. (2016), in a study of culture medium optimization, obtained a production of 13 IU/L (CMCase) by a wild strain of *Penicillium* spp., with the addition of 1.5 g/L of sugarcane bagasse pretreated with acid-alkaline solution. Santa-Rosa et al. (2018) found an activity of 600 IU/L by a strain of *Penicillium* spp. isolated from the Amazon region in a medium containing 7.5 g/L of carboxymethylcellulose as a carbon source. Fernandes et al. (2018) used natural Soybean hulls as well as fractions obtained from pretreatment as carbon sources on the production of cellulases by *Penicillium* spp. Results showed production of 130 IU/L (CMCase) using 1% of the *in natura* residue and 200 UI/L (Avicelase) with pretreated residue. Vázquez-Montoya et al. (2020) found an activity of 1683 UI/L using *Moringa* straw as carbon source in a submerged fermentation of *Penicillium funiculosum*.

In a search of the scientific literature, no reports were found for studies of production of cellulases by *Rhizomucor* spp. As we found in our work, this genus is not a good cellulase producer.

Regarding the solid substrate used for fermentation, we found that the microorganisms can produce enzymes using different sugarcane bagasses (natural and pretreated with acid-alkaline and hydrogen peroxide solutions), but the enzyme production by *T. koningii* is more expressive using natural sugarcane bagasse. For both pretreated sugarcane bagasses, the higher the substrate concentration (1.6 and 2.7%, w/v) and the temperature (33 and 38°C), the lower the enzyme production by this fungus. Probably, the generation of toxic substances in the pretreatment (Vasconcellos et al., 2015) negatively affected the production of cellulases by *T. koningii* when a high pretreated bagasse concentration was used. On the contrary, *Penicillium* spp. seems to be more tolerant to temperature and toxic compounds, because the highest CMCase activities were obtained at 33°C and using 1.6% (w/v) acid-alkaline pretreated bagasse.

The effects of temperature and concentration of sugarcane bagasse on the enzyme production by SmF of *T. koningii* (the best enzyme producer in our work) were statistically analyzed and the results (ANOVA) are shown in Table 2. The variables that were not statistically significantly ($p < 0.05$) were removed, and thus the new values of the effects of the variables on the enzymatic production were obtained (Table 3), as well as the three equations (Equations 1 to 3) that describe the behavior of the variables in the enzyme activity and the response surfaces (Figure 1a, b and c) for natural sugarcane bagasse, pretreated with base-acid and hydrogen peroxide solution, respectively. Both concentration of sugarcane bagasse ([B]) and temperature (T) influenced the production of cellulases (A.E.). When natural sugarcane bagasse was used, higher bagasse concentrations and lower temperatures led to better results in the enzyme production, with the temperature having an effect 1.5 times higher. However, for pretreated bagasse, both the increase in bagasse concentration and temperature interferes negatively in the enzyme production. As pointed out earlier, chemically pretreated bagasse can contain toxic substances (Vasconcellos et al., 2015) that inhibit the growth of the microorganisms; therefore, higher concentrations of bagasse lower the enzyme production. The effect of the concentration of bagasse pretreated with hydrogen peroxide solution was greater when compared with the bagasse pretreated with acid-alkaline solution.

$$A.E\ (IU/L) = -12515.9 + 3409.8 \times [B] - 181.5 \times [B]^2 + 847.1 \times T - 13.7 \times T^2 - 69.9 \times T \times [B] \quad (1)$$

$$A.E\ (IU/L) = 22522.2 - 2078.4 \times [B] - 1149.8 \times T + 14.7 \times T^2 + 55.0 \times [B] \times T \quad (2)$$

$$A.E\ (IU/L) = -11059.1 - 3416.9 \times [B] + 1054.2 \times T - 19.9 \times T^2 + 86.4 \times T \times [B] \quad (3)$$

Production of cellulases by bacteria

The results of enzymatic production with the bacteria *B. megaterium* and *B. subtilis* are shown in Table 4. Among the bacteria, *B. megaterium* showed better results (CMCase activity of 156.7 IU/L at 33°C using acid-alkaline pretreated sugarcane bagasse at 0.5%, w/v), even better than the yields found by SmF of the fungi *Penicillium* and *Rhizomucor*.

Shahid et al. (2016) reported a production of 710 IU/L (CMCase activity) by *B. megaterium* BM 05 using wheat straw as substrate. Al-Gheethi (2015) investigated the potential of different sewage sludge as medium of

Table 2. Results of analysis of variance (ANOVA) for production of cellulases by SmF of *Trichoderma koningii*, using natural sugarcane bagasse (NB) and bagasses pretreated with acid-alkaline solution (AAB) and hydrogen peroxide solution (HPB).

NB	Sum of square	d*f*	Mean square	*F* value	*p* value
(1) [B] (L)	1975134	1	1975134	175.6375	0.000044
[B] (Q)	122218	1	122218	10.8681	0.021554
(2) T (L)	4336360	1	4336360	385.6080	0.000006
T (Q)	297861	1	297861	26.4871	0.003625
1L by 2L	591515	1	591515	52.6001	0.000778
Error	56228	5	11246		
Total SS	7521024	10			
AAB					
(1) [B] (%, w/v) (L)	377260	1	377260	12.57462	0.023883
[B] (Q)	39	1	39	0.00131	0.972889
(2) T (L)	1290755	1	1290755	43.02272	0.002794
T (Q)	254325	1	254325	8.47700	0.043611
1L by 2L	365602	1	365602	12.18602	0.025104
Error	120007	4	30002		
Total SS	2642565	9			
HPB					
(1) [B] (%, w/v) (L)	2336006	1	2336006	41.56241	0.001335
[B] (Q)	15609	1	15609	0.27772	0.620738
(2) T (L)	2277722	1	2277722	40.52541	0.001414
T (Q)	577361	1	577361	10.27246	0.023860
1L by 2L	902310	1	902310	16.05397	0.010255
Error	281024	5	56205		
Total SS	6489933	10			

[B] is bagasse concentration (% w/v), T is temperature in Celsius degree; red font indicates statistically significant results.

Table 3. Effect of concentration of bagasse sugarcane and temperature on the enzyme production by submerged fermentation of *T. koningii* using natural sugarcane bagasse (NB) and bagasses pretreated with acid-alkaline solution (AAB) and hydrogen peroxide (HPB).

Substrate	Variable	Effect
NB	Mean/Interc.	1425.63
	[B] (L)	1147.50
	[B] (Q)	219.64
	T (L)	-1700.27
	T (Q)	342.89
	1 L by 2 L	-769.10
AAB	Mean/Interc.	390.491
	[B] (L)	-581.789
	[B] (Q)	n.s
	T (L)	-927.633
	T (Q)	-367.076
	1 L by 2 L	604.650
HPB	Mean/Interc.	782.24
	[B] (L)	-1247.93

Table 3. Contd.

[B] (Q)	n.s
T (L)	-1232.27
T (Q)	498.33
1 L by 2 L	949.90

* (L) =linear factor. ** (Q) =quadratic factor; n.s. =Not statistically significant; [B] = concentration of sugarcane bagasse; T = temperature (°C).

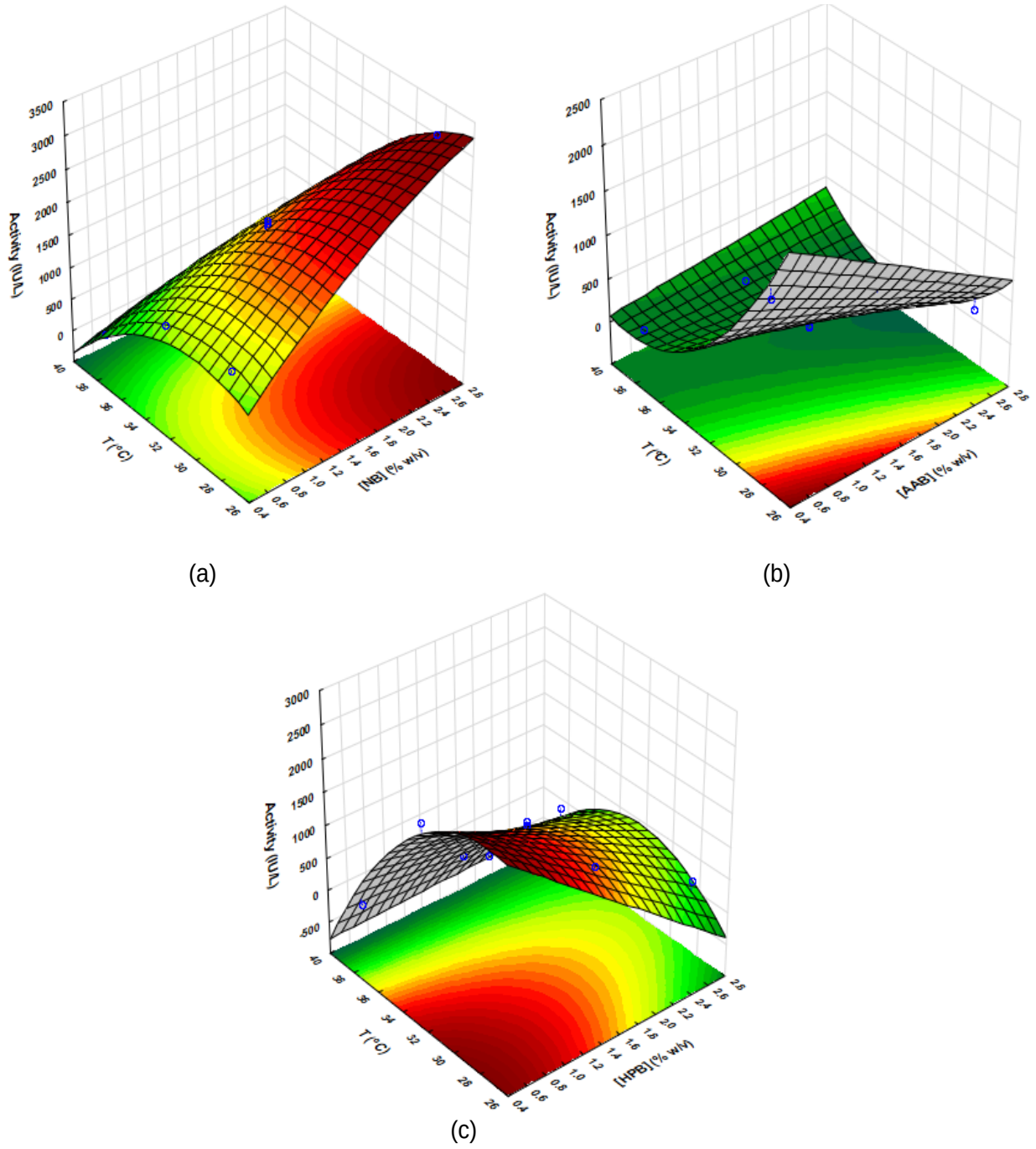

Figure 1. Response surfaces for enzyme production by SmF of *Trichoderma koningii* using NB (a), AAB (b) and HPB (c).

Table 4. Cellulase production by *B. megaterium* and *B. subtilis* using sugarcane bagasse natural (NB), pretreated with acid-alkaline solution (AAB) and hydrogen peroxide solution (HPB) in different conditions of temperature and concentration of sugarcane bagasse.

Run	Variable		Endoglucanase Activity (IU/L)					
			Bacillus megaterium			*Bacillus subtilis*		
	T (ºC)	[B] (% m/v)	NB	AAB	HPB	NB	AAB	HPB
1	28	0.5	79.0	152.9	78	8.3	6.1	1.1
2	33	0.5	111.0	**156.7**	60	15.2	10.2	7.8
3	38	0.5	28.0	63.2	0	19.9	8.6	5.3
4	28	1.6	113.8	130.1	60	7.2	9.1	1.3
5	33	1.6	114.0	127.7	50	11.9	11.0	7.0
6	38	1.6	43.0	57.4	0	**24.0**	9.7	7.1
7	28	2.7	130.8	130.0	52	10.5	11.3	7.9
8	33	2.7	127.0	105.2	37	10.2	10.2	8.0
9	38	2.7	57.0	52.7	0	15.7	15.2	9.0
10	33	1.6	118.2	111.0	56	13.5	11.6	7.1
11	33	1.6	119.0	107.3	59	13.0	12.4	7.1

production of cellulases by *B. megaterium*. The highest and lowest activities were 14,300 and 1400 IU/L, respectively, which did depend on the studied sewage. In addition, the authors studied the use of bagasse as a carbon source and the achieved enzyme activity was 9800 IU/L. The enzyme activities reported by the authors were much higher than those found in this work, which corroborates with the statement that the species of microorganism and the working conditions result in very different enzyme productions.

Regarding the production of cellulases by *B. subtilis*, very different values of enzymatic activity are found in the scientific literature, depending on the species used and the working conditions. Jiménez-Leyva et al. (2017) evaluated three different strains of *B. subtilis* in the production of cellulases using carboxymethylcellulose and microcrystalline cellulose as carbon sources. The authors found CMCase activities between 150 and 450 IU/L. Vaid and Bajaj (2017) studied different sources of carbon and nitrogen in the production of cellulases by the strain *B. subtilis* G2 and the highest activity was 2179 IU/L using banana peel, which was very close to the activity using sugarcane bagasse (2000 IU/L).

Regarding to the substrates used in this work, we can see that the sugarcane bagasse treated with hydrogen peroxide led to the lowest values of enzyme activity, both for *B. megaterium* and *B. subtilis*. For *B. megaterium,* a slightly higher enzyme production was observed when bagasse pretreated with acid-alkaline solution was used. For *B. subtilis*, the enzyme productions were close to the natural bagasse and pretreated with acid-alkaline solution.

The effects of the temperature and the concentration of sugarcane bagasse on the enzyme production were statistically analyzed and the results of ANOVA are shown in Table 5. Statistical analysis was performed only for *B. megaterium*, which showed the best enzyme production. After removing the variables that did not significantly affect the enzyme activities ($p > 0.05$), the values of the effects of the variables on the enzyme production were obtained (Table 6), as well as the equations (Equations 4 to 6) that describe the behavior of the variables in the enzyme activity, and the surface response (Figure 2a, b, and c), when using natural bagasse and bagasses pretreated with acid-base and peroxide solutions, respectively. It can be seen that both variables influenced the enzyme production, similarly to the results obtained with *T. koningii*. When natural sugarcane bagasse was used, higher concentrations produced higher enzymatic activities; however, an increase in the temperature resulted in a decrease in the activity. The effect of the temperature was twice greater than that of the bagasse concentration. For the other two types of pretreated bagasse, there is a decrease in the enzyme production by increasing both substrate concentration and temperature, where temperature is the variable that most influenced the enzyme production.

$$A.E.\ (IU/L) = -1545.0 + 14.7 \times [B] + 105.9 \times T - 1.7 \times T^2 \quad (4)$$

$$A.E\ (IU/L) = - 633.6 - 12.9 \times [B] + 55.0 \times T - 0.9 \times T^2 \quad (5)$$

$$A.E\ (IU/L) = - 567.5 - 46.4 \times [B] + 46.5 \times T - 0.8 \times T^2 + 1.2 \times [B] \times T \quad (6)$$

Conclusion

This study showed that *T. koningii* is a good choice to produce cellulases by SmF using natural sugarcane bagasse as carbon source and relatively low temperature of cultivation. These findings could be attractive from an economic point of view, because pretreatment of the bagasse increases its final cost, as well as higher

Table 5. Results of analysis of variance (ANOVA) for production of cellulases by SmF of *Bacillus megaterium,* using natural sugarcane bagasse (NB) and bagasses pretreated with acid-alkaline solution (AAB) and hidrogen peroxide solution (HPB).

NB	Sum of square	d*f*	Mean square	*F* value	*p* value
(1) [B] (L)	1561.71	1	1561.707	29.5565	0.002857
[B] (Q)	6.15	1	6.148	0.1164	0.746878
(2) T (L)	6376.56	1	6376.560	120.6812	0.000109
T (Q)	4502.46	1	4502.463	85.2125	0.000251
1 L by 2 L	129.96	1	129.960	2.4596	0.177598
Error	264.19	5	52.838		
Total SS	13281.72	10			
AAB					
(1) [B] (L)	1201.34	1	1201.335	8.14107	0.035687
[B] (Q)	280.84	1	280.842	1.90318	0.226230
(2) T (L)	9576.02	1	9576.015	64.89361	0.000477
T (Q)	1802.07	1	1802.074	12.21208	0.017385
1L by 2L	38.44	1	38.440	0.26050	0.631507
Error	737.82	5	147.565		
Total SS	13387.53	10			
HPB					
(1) [B] (L)	400.167	1	400.167	14.8403	0.011973
[B] (Q)	7.875	1	7.875	0.2921	0.612096
(2) T (L)	6016.667	1	6016.667	223.1295	0.000024
T (Q)	1040.175	1	1040.175	38.5751	0.001581
1L by 2L	169.000	1	169.000	6.2674	0.054259
Error	134.825	5	26.965		
Total SS	7900.909	10			

[B] = Sugarcane bagasse concentration (%, w/v); T = temperature (°C).

Table 6. Effect of concentration of bagasse sugarcane and temperature on the enzyme production by SmF of *B. megaterium* using natural sugarcane bagasse (NB) and bagasses pretreated with acid-alkaline solution (AAB) and hydrogen peroxide (HPB).

Substrate	Variable	Effect
NB	Mean/Interc.	89,4578
	[B] (L)	32,2667
	[B] (Q)	s.n
	T (L)	-65,2000
	T (Q)	42,5733
	1 L by 2 L	s.n
AAB	Mean/Interc.	105,6711
	[B] (L)	-28,3000
	[B] (Q)	s.n
	T (L)	-79,9000
	T (Q)	23,8633
	1 L by 2 L	s.n
HPB	Mean/Interc.	52,4000
	[B] (L)	-16,3333

Table 6. Contd.

[B] (Q)	s.n.
T (L)	-63,3333
T (Q)	-41,4667
1 L by 2 L	13,0000

*(L) = Linear factor. ** (Q) =quadratic factor, n.s. =Not statistically significant. [B] = sugarcane bagasse concentration (%, w/v); T = temperature (°C).

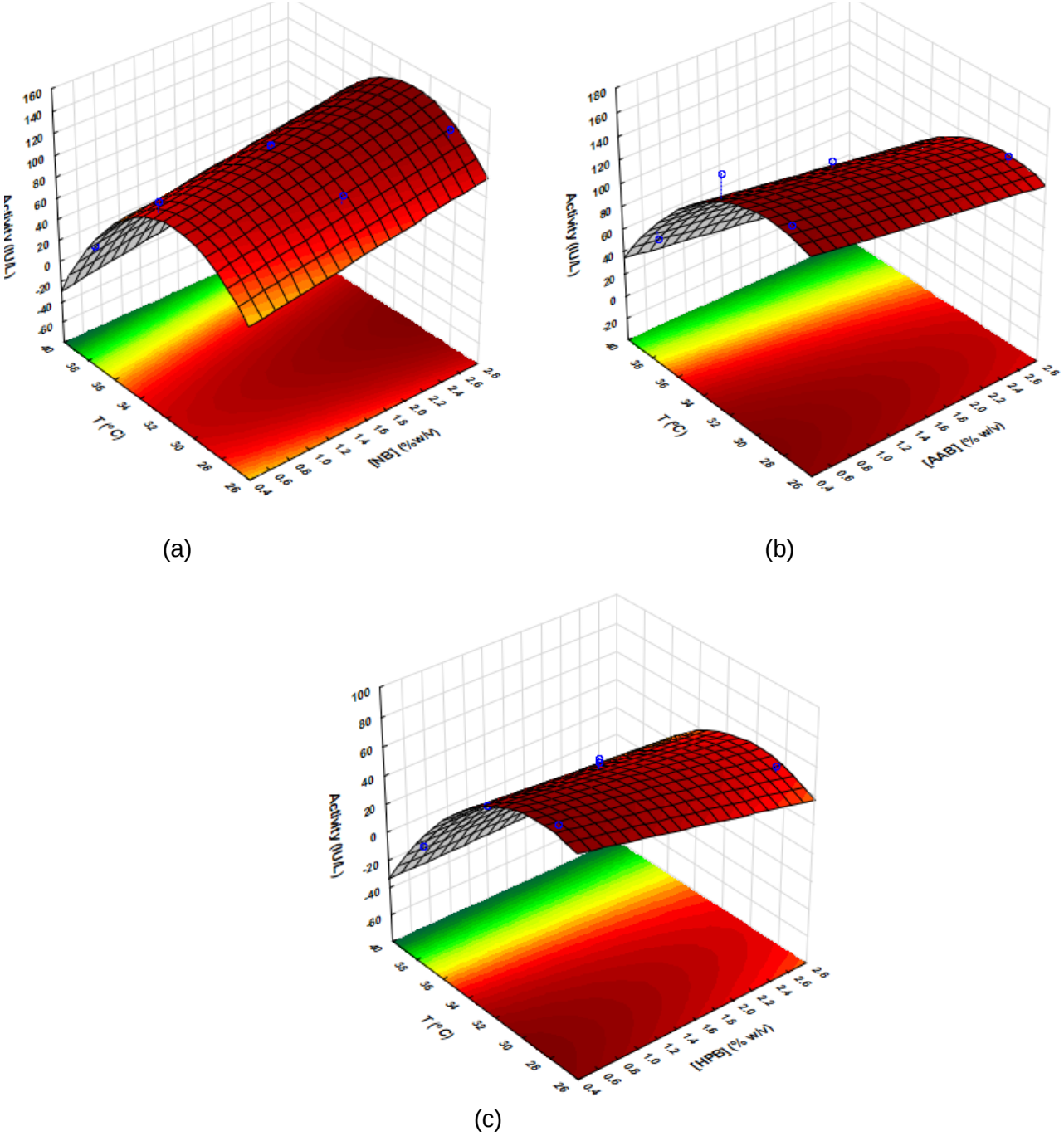

Figure 2. Response surfaces for enzyme production by SmF of *Bacillus megaterium* using NB (a), AAB (b) and HPB (c).

temperature is energy consuming and therefore not environmentally friendly.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Spectroscopic and chemical analysis of a Nigerian hybrid *Hevea Brasiliensis* (Rubber) seed oil for product quality assessment in technical applications

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Rubber seeds from a Nigerian hybrid rubber tree were dried in an oven at 110°C for 2 h to 7 wt% moisture content and cold-screw pressed at 30°C to obtain an oil yield of 28 wt%. Chemical compositional, nuclear magnetic resonance (NMR) and Fourier transform infra-red spectroscopy (FTIR) analyses were conducted on the oil sample extracts. Chemical analysis of the oil indicated iodine value of 136.07 g I_2/100 g oil, peroxide value of 9.45 O_2/kg oil, saponification value of 189.5 mg KOH/g oil, and confirms its suitability for applications, such as biodiesel production, oleochemicals synthesis, polyurethane composites and water-reducible alkyd resins, pharmaceutical products, plasticizers, adhesives, and surfactants. Rubber seed oil is a promising substitute to linseed oil as semi-drying oil for oily paint formulation. ^{1}H NMR analysis revealed that the fatty acid compositions consist of linoleic acid (34.22 wt%), oleic acid (28.6 wt%), linolenic acid (18.6 wt%), and saturated fatty acids (18.57 wt%). FTIR analysis indicated fingerprint regions of 1461 to 585 cm^{-1} which can be used to check adulteration of the oil. The NMR spectra (^{1}H and ^{13}C) of the oil are similar to those of other vegetable oils with well-identified peaks and regions that can be used to authenticate the quality of the oil.

Key words: Rubber seed oil, characterization, nuclear magnetic resonance (NMR), Fourier transform infra-red spectroscopy (FTIR), quality assessment, technical applications.

INTRODUCTION

Edible vegetable oils have several food applications and they are increasingly being used for other purposes such as resinous pigments and drying oils in paints and coatings production (Aigbodion and Bakare, 2005). To reduce costs associated with the use of edible vegetable oils in the food industry, non-edible oils of good industrial applications are highly sought to replace edible oils (Roschat et al., 2017). In recent times, with the development of new technologies and research future areas expansion on non-edible oils applications to replace edible oils, detailed characteristic property assessment of oils quality are imperative to the entrepreneurs (Reshad et al., 2015). Therefore, novel oils characterization to determine their possible applications

and to forestall adulteration because of price differentials with low quality oils is paramount for high quality derivable products (Barison et al., 2010).

Recently, several researchers studied rubber seed oil (RSO) usefulness to authenticate its industrial application as a renewable resource material to complement the fast depleting non-renewable mineral oils of fossil origins (Onoji et al., 2019). Rubber tree accounts for 99% of world's natural rubber (NR) latex used for the production of several rubber products (Atabani et al., 2013). Currently, the seeds are underutilized and allowed to rot away in the plantations, apart from a minimal use in subsequent tree plant breeding process in Nigeria (Onoji et al., 2017). The rubber seeds used in this study were collected from the 40-hectare (ha) NIG800 series plantations of Rubber Research Institute of Nigeria (RRIN), Iyanomo, Benin-City (Aigbodion and Bakare, 2005). The foreign (Malaysia, Sri Lanka, etc.) rubber seeds (RRIM 600, RRIM 501, PB 28/59, PB 5/63, RRIM 628, RRIC 45, RRIM 614, AVROS 1581, RRIM 605, PB 5/51, GT1, RRIM 62, RRIM707, PR107 and PB217) used as parentage stock in these plantations were pre-germinated in pre-nursery beds (Umar et al., 2010). Ground and polythene bags nursery techniques were used for seedlings production. Certified rubber seedlings obtained through budding techniques were used for planting in these plantations. Specie of Nigerian hybrid is a high-yield latex (3000 to 3500 dry NR/hectare/year) tree, resistant to wind and with a capacity to produce about 1200 seeds/tree/year (Onoji et al., 2020). The specie has an average weight of 4 g/seed (Onoji et al., 2019), and a non-edible oil content yield of about 43 wt% (Onoji et al., 2016). Non-edible seed oil from rubber tree has been identified as a potentially promising material for several industrial applications such as, the production of biodiesel (Yang et al., 2011), oleochemicals (Hosamani and Katagi, 2008), polyurethane composites and water-reducible alkyd resins (Bakare et al., 2010), pharmaceutical products, plasticizers, adhesives, and surfactants (Onoji et al., 2016). De-oiled cake, after pretreatment to remove toxic material such as hydrogen cyanide, is suitable as a valuable source of proteins for farm animals and poultry feeds (Eka et al., 2010). RSO has been characterized as a semi-drying oil (Aravind et al., 2015), and of a high quality that makes it a promising substitute for linseed oil in paint formulation (Ebewele et al., 2010). In Nigeria, linseed oil is imported for use in the paint industry and other applications (Okiemen et al., 2005). However, available report shows that RSO was yet to have any commercial value in Nigeria, which has the capacity to produce 13,000 tons RSO/year (Okiemen et al., 2005). The oil can be processed into a semi-drying oil for the paint industry to reduce importation of linseed oil which depletes the Nigerian foreign exchange reserves. There is therefore a need to characterize the Nigerian hybrid RSO in order to authenticate its quality against any adulteration with respect to the aforementioned usage.

Vegetable oils authentication for product quality via spectroscopic methods use is well reported in literature (Sadowska et al., 2008). Gas chromatography coupled with mass spectrometry (GC-MS) has been in use for several decades (Skooge et al., 2007). However GC-MS, a destructive method, involves oil sample chemical modification (oxidation) with the tendency to produce unreliable results. In addition, GC-MS is cumbersome, cost in-effective, time consuming and may pose problems in result interpretations, especially the fatty acid compositions (Barison et al., 2010). Fourier transform infra-red spectroscopy (FTIR) is a non-destructive technique reported for use in the analysis of free fatty acids and to monitor oil quality to check adulteration (Valente et al., 2016) through the identification of the functional groups present in the sample (Bohre, 2013). Proton (^{1}H) and carbon-13 (^{13}C) nuclear magnetic resonance (NMR) spectroscopy is a non-destructive technique used to determine the proportion of different acyl groups present in oils and fats, and other liquids in a very short time for sample preparation and experimental spectrum acquisition compared to GC-MS (Scano et al., 2008; Guillén and Ruiz, 2003a). ^{1}H NMR spectra area signals are proportional to the number of hydrogen atoms that produce the signals (Guillén and Ruiz, 2003a). This method has many advantages because it does not involve chemical modification of sample like the GC-MS (Yeung et al., 2008).

In this study, seed oil was extracted by mechanical means notable for high-quality oil (Ebewele et al., 2010). This method is cost effective and can be adapted to small- to medium-scale entrepreneurship due to its low operating and maintenance costs. For instance, a demonstration-scale rubber seed oil mechanical extractor designed and fabricated by RRIN located in Benin City at the cost of US$1,050 has a capacity to extract 500 L of oil/day. The extracted oil physico-chemical properties were determined by using standard methods described elsewhere (Onoji et al., 2016), while FTIR and NMR spectroscopy were deployed to determine oil functional groups and fatty acids, respectively.

EXPERIMENTAL

Materials and reagents

Fresh and glossy rubber seeds used for this study were handpicked

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from the hybrid rubber estate plantation of RRIN. All reagents from BDH Chemicals Ltd., Poole England, and GFS Chemicals, Inc., 867 McKinley Ave., Columbus, OH 43223 and used in the analyses were of analytical grades.

Seed oil extraction

Seed kernels were separated from seed shells by gentle cracking using a laboratory mortar and pestle. The kernels were weighed, and dried in an oven at 110°C for 2 h to attain constant weight for moisture content determination. About 2 kg of fresh seed kernel was dried at 110°C to 7 wt% moisture content for a maximum oil yield (Igeleke and Omorusi, 2007). Seed oil was extracted at 30°C from the dried kernel using a mechanical oilseed screw press. Extracted oil was stored in screwed airtight plastic containers for physico-chemical characterization, and spectroscopic analysis.

Physico-chemical analysis of rubber seed oil

The standard methods adopted for the determination of physico-chemical properties of the rubber seed oil extracted using a 99.9% n-hexane solvent in a soxhlet extractor were obtained elsewhere (Onoji et al., 2016). The experiments were carried out in duplicate, and the average values recorded for accuracy of data.

Spectroscopic analysis of rubber seed oil

Oil infrared spectra were recorded using an FTIR spectrometer (Model: TENSOR 27, Bruker Optics Inc., USA) equipped with a detector, and interfaced to a personal computer operating under OPUS spectroscopy software supplied together with the instrument. A film of 2 µL of the RSO sample was deposited between two disks of KBr, in the absence of air (Guillén and Cabo, 2002). Sinclair et al. (1952) reported that the degree of unsaturation in vegetable oils correlates with some absorbance bands in the FTIR spectrum, hence the frequencies of such bands are closely related to the proportions of saturated, monounsaturated, and polyunsaturated acyl groups (Guillén and Ruiz, 2003a). Consequently all spectra were recorded between 4000 and 500 cm^{-1}.

Extracted oil ^{1}H NMR spectrum was recorded on *Bruke*r ultra-shield TM 500-MHz NMR spectrophotometer. Approximately 0.2 g of oil sample was dissolved in 500 µL of deuterated chloroform ($CDCl_3$) as solvent (δ = 7.26 ppm) containing a small amount of tetramethylsilane (TMS) as an internal standard (δ = 0 ppm). This was then placed in 5 mm diameter NMR test tubes to commence the analysis (Guillén and Ruiz, 2003a). The acquisition data were: spectral width 8.1 kHz, relaxation delay 3s, 15 scans, and pulse width 30°.

^{13}C NMR experiments were also carried out with *Bruke*r ultra-shield TM 500-MHz NMR equipment. About 25 mg of RSO was dissolved in 1 mL of $CDCl_3$. Samples were placed in 5 mm NMR tubes at 20°C and analyzed within 2 days as described by Scano et al. (2008). The experiments were conducted at room temperature (28°C). ^{13}C spectra were recorded at 235.2 ppm spectral width, relaxation delays 3s, and a total of 350 scans. The carbon atom in $CDCl_3$ was observed at 77.42 ppm. All other peaks were assigned with respect to it.

Fatty acid compositions of rubber seed oil using NMR spectroscopy

Unsaturation degree in oils and fats can also be determined from the proportion of olefinic hydrogen atoms obtained from ^{1}H NMR data, besides different acyl groups present in oil sample (Guillén and Ruiz, 2003b). The proportions of Linolenic (Ln), Linoleic (L), Oleic (O), and saturated (S) fatty acids present in the RSO were calculated from the areas of the identified peaks (1 – 10) in ^{1}H NMR spectra (Figure 1) using parameters shown in Equations 1 to 4 for oils containing similar acyl groups as that mentioned previously (Reshad et al., 2015; Guillén and Ruiz, 2003b).

$$Ln\ (\%) = 100\left(\frac{B}{A+B}\right) \quad (1)$$

$$L(\%) = 100\left[\frac{E}{D} - 2\left(\frac{B}{A+B}\right)\right] \quad (2)$$

$$O(\%) = 100\left[\frac{C}{2D} - \frac{E}{D} + \left(\frac{B}{A+B}\right)\right] \quad (3)$$

$$S(\%) = 100\left[1 - \frac{C}{2D}\right] \quad (4)$$

where A, is the area signal 1, corresponding to methyl hydrogen atoms of saturated, oleic (omega-9), and linoleic acyl groups; B is area signal 2, corresponding to methyl hydrogen atoms of linolenic acyl groups; C is area signal 5, corresponding to methylenic hydrogen atoms in position α, in relation to one double bond (allylic protons); D is area signal 6, corresponding to methylenic hydrogen atoms in position α, in relation to the carboxyl group; and E is area signal 7, corresponding to methylenic hydrogen atoms in α position with two double bonds (bis-allylic protons).

RESULTS AND DISCUSSION

Physico-chemical properties of rubber seed oil

Mechanically extracted hybrid oil properties are presented in Table 1, with oil yield of 28 wt%. This yield closely agrees with the yield of 23 wt% reported by Aigbodion and Bakare (2005) on similar experiment with RSO. The oil parameters such as iodine value of 136.07 g I_2/100 g oil, peroxide value of 9.45 O_2/kg oil, saponification value of 189.5 mg KOH/g oil, and refractive index of 1.472 are within the range reported by other researchers on RSO (Reshad et al., 2015; Aravind et al., 2015).

FTIR and NMR spectroscopic analysis of rubber seed oil

Results obtained from oil FTIR and NMR analysis are geared towards providing an insight into the fatty acid structural configuration and, compositions that enables easy evaluation of its potential applications by entrepreneurs.

FTIR analysis of rubber seed oil

Figure 2 shows the FTIR spectrum of the extracted oil. The main peaks and their assignment to functional groups are presented in Table 2. The O-H stretching vibrations bands (for alcohols, carboxylic acids, and hydroperoxides) usually in the absorption range of 3200 to 3600 cm^{-1} for vegetable oils (Ogbu and Ajiwe, 2016)

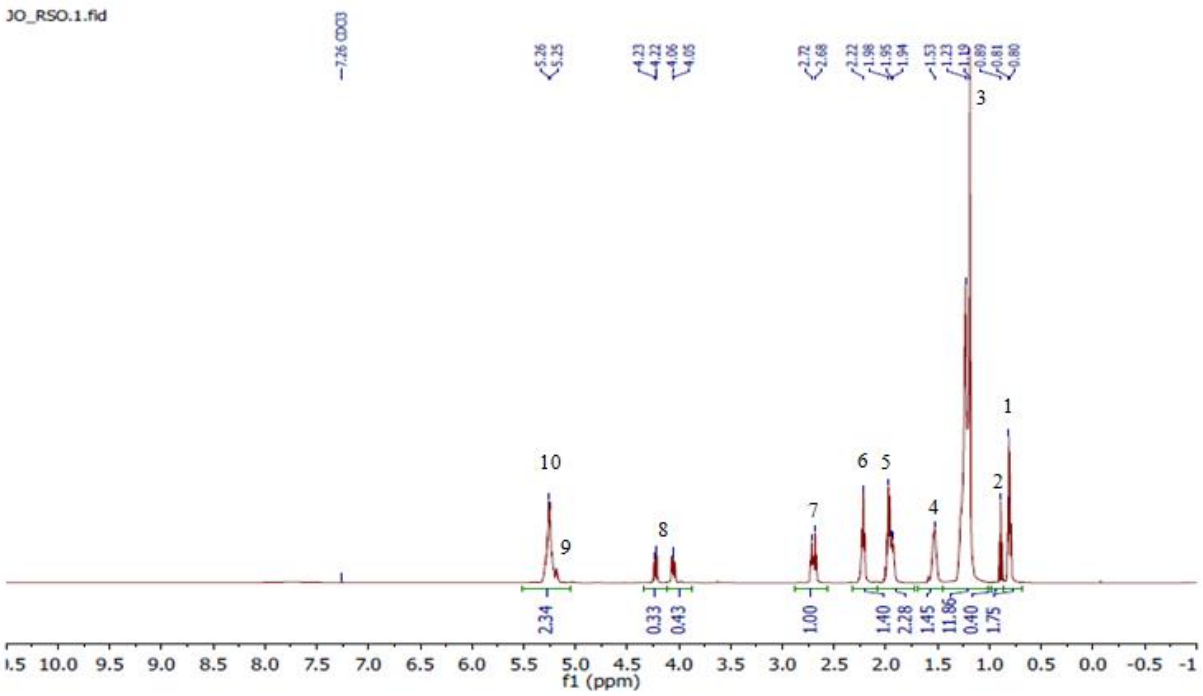

Figure 1. ^{1}H NMR spectra of rubber seed oil.

Table 1. Physico-chemical properties of rubber seed oil.

Parameter	Reshad et al. (2015)	This study
Colour	Golden yellow	Dark brown
Density, g/cm^3 at 25^{o}C	0.910	0.892 ± 0.01
Specific gravity at 15^{o}C	-	0.91 ± 0.02
Oil content (wt%)	40–50	28 ± 0.2
Oil water content (wt%)	-	0.075 ± 0.01
Initial kernel water content (wt%)	-	9.85 ± 0.03
Iodine value, g I_2/100 g oil	113	136.07 ± 0.04
Peroxide value, meq. O_2/kg oil	-	9.45 ± 0.06
Saponification value, mg KOH/g oil	235.28	189.5 ± 0.13
Acid value, mg KOH/g oil	24	16.5 ± 0.1
Free fatty acid (%FFA as oleic acid)	12.12	8.25 ± 0.05
Kinematic viscosity, mm^2/s at 40^{o}C	30	38.5 ± 0.04
Refractive index at 20^{o}C	1.47	1.472 ± 0.001
Pour point, ^{o}C	–1.5	–5
Cloud point, ^{o}C	3	4.8
Cold filter plugging point, ^{o}C	-	–0.62
Flash point, ^{o}C	-	243
Fire point, ^{o}C	-	254
Aniline point, ^{o}C (^{o}F)	-	21 (69.8)
Boiling point, ^{o}C	-	122
Freezing point, ^{o}C	-	–18
Higher heating value (HHV), MJ/kg	39.34	39.81
Mean mol. weight of fatty acids (g/mol)	-	295.5
Average mol. weight of RSO (g/mol)	-	924.56

Values are mean ± standard deviation of duplicate data.

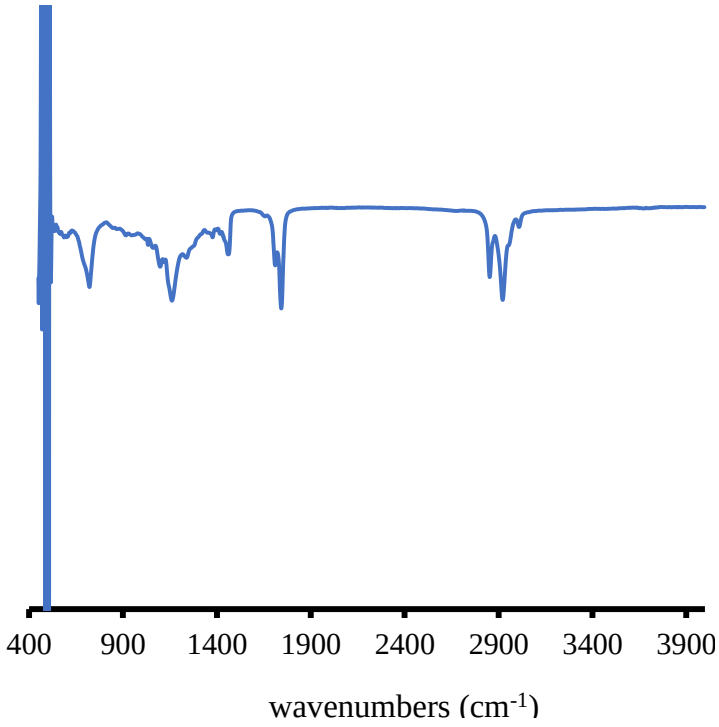

Figure 2. FTIR spectrum of rubber seed oil.

Table 2. Description of FTIR analysis of rubber seed oil.

Wavenumber (cm^{-1})	Functional group	Description of vibration
3009	Olefinic	=C–H stretching
2921-2853	Methylene	Strong peaks stretching vibration of C–H
1742	Carbonyl	–C=O stretching vibration of esters
1710	Carboxylic acids	Stretching vibration of C=O
Fingerprint region (1461-585 cm^{-1})		
1461	Methyl, methylene	Medium signal due to bending vibration of alkyl C–H bonds
1377	Methyl	Alkyl C–H bending vibrations
1238	Carboxylic acids, Esters	Stretching vibration of C–O
1160	Esters	C–O stretching vibration of ester groups
1118-1033	Esters	=C–O–C stretching vibration of ester groups
721	Methylene	Bending (rocking) vibration of C–H
585	Alkanes	Weak signal due to C–H vibration

are absent in the RSO used for this study. The observed spectrum implies that triglyceride is the main component of the oil as a result of the strong presence of ester carbonyl group (C=O) at 1742 cm^{-1}. Ester group presence could be attributed to the stretching vibrations of C–O observed at 1160 cm^{-1}. Results on fingerprint obtained are within the range (1461 to 585 cm^{-1}) which provides useful information to detect oil adulteration. The results obtained are within the range reported by other researchers on RSO (Reshad et al., 2015).

NMR spectra analysis of rubber seed oil

Figure 1 depicts the ^{1}H NMR spectrum of RSO. The assignment of signals is listed in Table 3. Signal 1 is the overlap of doublet signals of methyl group protons that appears between 0.8 and 0.81 ppm. Signal 2 is a singlet methyl group proton, and appears at 0.89 ppm. Signal 3 appears between 1.19 and 1.23 ppm corresponding to linolenyl chains protons, and saturated methylene group of acyl chains, respectively. Signal 4 appears at 1.53 ppm,

Table 3. ^{1}H NMR and ^{13}C NMR analysis of rubber seed oil.

^{1}H (ppm)	^{13}C (ppm)	Functional groups	Assignments
0.80 - 0.89	13.91	CH_3 (saturated, oleic, and linoleic acids)	All acyl chain except linolenic
1.19	33.97	$CH=CHCH_2CH_3$	Linolenic chains
1.23 - 1.4	29 - 31	(CH_2)n saturated aliphatic	All acyl chains
1.53	21.9	$CH_2–CH_2COOH$	All acyl chains
1.94 - 1.98	26.1	$CH_2CH=CH$	All unsaturated acyl chains
2.22	33.97	$CH_2–COOH$	All acyl chains
2.68; 2.72	24.9	$CH=CHCH_2CH=CH$	Linolenic and linoleic chains
4.05, 4.06; 4.22, 4.23	61.93 - 77.16	$CH_2–OCOR$	Triglycerides (glyceryl group)
5.25	-	CH–OCOR	Triglycerides (glyceryl group)
5.26	126.99 - 131.99	CH=CH (olefinic group)	All unsaturated fatty acids
-	172.41 - 172.82	C=O (carbonyl group)	-
-	178.64	COOH (carboxylic acids)	-

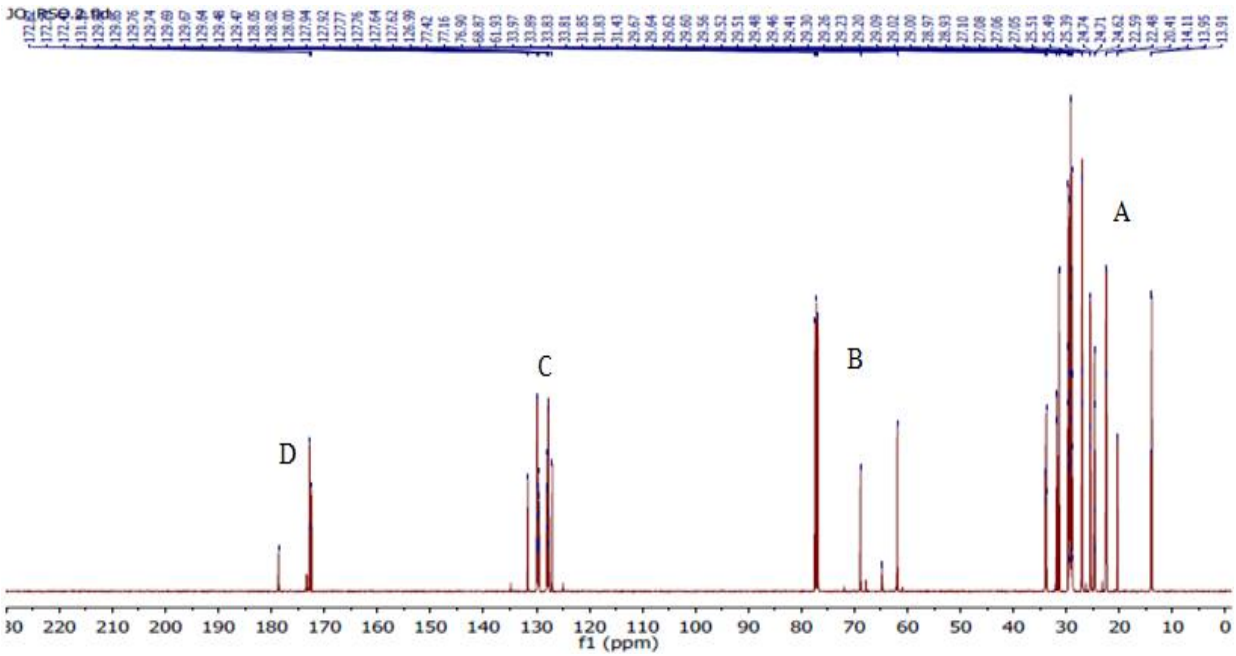

Figure 3. ^{13}C NMR spectra of rubber seed oil.

representing the methylene protons in the β position. Signal 5 appears between 1.94 and 1.98 ppm, due to α-methylene proton related to a protons in α position in relation to a single double bond (allylic protons). Signal 6 occurred due to the methylene protons in α position, and it appears at 2.22 ppm. Signal 7 is a signal overlap between 2.68 and 2.72 ppm, due to responses from α-methylene protons related to the double bonds (bi-allylic protons). Signal 8 appears at 4.05, 4.06 and 4.22, 4.23 ppm, due to the protons on carbon atoms 1 and 3 of the glyceryl group. Signal 9 appears at 5.25 ppm, corresponding to the proton on carbon 2 atom of the glyceryl group, and it overlaps with Signal 10 at 5.26 ppm that represents olefinic protons of different acyl groups.

^{13}C NMR spectra analysis of rubber seed oil

Figure 3 shows the ^{13}C NMR spectrum of the oil under analysis with well-defined four distinct regions (A, B, C and D): A (13.91 - 33.97 ppm) due to methyl or alkyl functional groups for saturated acids, oleic, and linoleic acids; B (61.93 - 77.16 ppm) due to glyceryl C-atoms; C (126.99 - 131.99) due to the presence of unsaturated alkenes; and D (172.41 - 178.64) due to the presence of carbonyl and carboxylic acid groups. The assignment of

Table 4. Fatty acid compositions of rubber seed oil via ^{1}H NMR spectra.

Fatty acid/systemic name	Chemical formulae	Composition (%)	
		Reshad et al. (2015)	This study
Saturated ($C_{XX:0}$)		19.91	18.57
Oleic ($C_{18:1}$)/ *cis*-9-Octadecenoic	$C_{18}H_{34}O_2$	27.06	28.6
Linoleic($C_{18:2}$)/ *cis*-9-*cis*-12-Octadecadienoic	$C_{18}H_{32}O_2$	39.86	34.22
α-Linolenic ($C_{18:3}$)/ *cis*-9-*cis*-12-*cis*-15-Octadecatrienoic	$C_{18}H_{30}O_2$	13.17	18.6
Total saturated		19.91	18.57
Total monounsaturated		27.06	28.6
Total polyunsaturated (linoleic, linolenic)		53.03	53.82
Total unsaturated (oleic, linoleic, linolenic)		80.09	81.42

XX means 14, 16 or 18.

signals is listed along with those from ^{1}H NMR spectrum in Table 3. Both ^{1}H and ^{13}C NMR spectra of the oil are similar to those of known vegetable oils (Sadowska et al., 2008; Guillén and Ruiz, 2003b).

Fatty acid compositions of rubber seed oil through ^{1}H NMR spectra

The fatty acid compositions of vegetable oils depend to a large extent on the seed storage and processing time, extraction technique employed, and method of oil analysis etc. Based on Equations 1 to 4 and the identified peak areas of the ^{1}H NMR spectrum (Figure 1), the fatty acids composition was quantified and tabulated in Table 4. The spectra analysis showed that the fatty acid compositions of the extracted RSO consist of linoleic acid (34.22 wt%), oleic acid (28.6 wt%), linolenic acid (18.6 wt%), and saturated fatty acids (18.57 wt%), and it compared favorably with results on RSO from other researchers (Reshad et al., 2015). The results show that linoleic acid is the predominant fatty acid present in this hybrid RSO and over 80% of the fatty acids are unsaturated.

Conversely, the oil may be categorized among the less nutritional quality due to the low profile of oleic acid (28.6 wt%) compared to olive oil (64.6 - 84.4 wt%) and canola (60 - 75 wt%) (Reshad et al., 2015). In addition, due to the high content of unsaturated fatty acids coupled with the low temperature properties, the oil is a promising material for biodiesel production, and as a substitute for linseed oil suitable for making drying oil for paint and varnish formulations.

Conclusions

In this study, rubber seed oil was mechanically extracted from the seeds of Nigerian hybrid rubber trees with oil yield of 28 wt%. An Adulteration of high-value RSO with low-grade oils often constitutes economic and commercial loss. Consequently chemical analysis to check for adulteration revealed that the RSO is non-edible and possesses significant potentials as substitute to the edible oils on different industrial applications. For instance, the oil can be used as a replacement for linseed oil as a semi-drying oil in the paint industry. The spectroscopic analytical methods (FTIR and NMR spectroscopy) employed in the study are non-destructive, simple, fast, reliable, cost-effective, and with no sample pre-treatment required compared to the GC-MS analytical tool which involves chemical modification of oil samples. The ^{1}H NMR spectra analysis of the oil shows high degree of unsaturation which implies susceptibility to low temperature, hence suitable for low climatic regions. The observed FTIR fingerprint regions of the oil are adequate for adulteration detection of low quality RSO that may impair derived products. The –C=O, =C–O–C, and C–O vibrations in the FTIR bands of 1742, 1118-1033, and 1160 cm^{-1} indicate the presence of strong ester groups supportive of biodiesel production. The findings of this study are useful parametric data for rubber seed oil identification, quantification, and authentication purposes.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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In vitro efficacy of two microbial strains and physicochemical effects on their aflatoxin decontamination in poultry feeds

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The efficacy of two microbial isolates, *Bacillus* spp. (B285) and *Saccharomyces* spp. yeast strain (Y833), in reducing aflatoxin concentration in poultry feeds in comparison with the commonly used commercial chemical binder, bentonite, was investigated using the VICAM ® fluorometer. The influence of the poultry feed matrix, pH (4.5 and 6.5), and temperature (room temperature, 37 and 42ºC) on the aflatoxin reducing activity by the two microorganisms was also explored. All microorganisms and bentonite reduced aflatoxins by over 74% of the original concentration. Bentonite registered the highest reduction at 93.4%; followed by Y833 (83.6%), then the combination of Y833 and B285 (77.9%); and lastly B285 (74.9%). Temperature and pH did not have significant effect on the performance of the biological agents and bentonite. The aflatoxin reducing activity was lower in presence of feeds compared to that in phosphate buffered saline except for Y833. The yeast strain was more effective than the bacterial strain in reducing the aflatoxin levels; however, both are promising strategies for countering the aflatoxin challenges in animal feeds. In response to the advocacy for use of biological control agents, there is need for more investigations to establish the safety of the microorganisms and the mechanism of aflatoxin decontamination.

Key words: Aflatoxin decontamination, *Saccharomyces*, *Bacillus*, commercial binder, animal feeds, pH, temperature.

INTRODUCTION

Consumption of contaminated foods of animal origin has been linked to increasing incidences of human illnesses and deaths affecting about 1 in 10 people worldwide (FAO et al., 2019). The safety of food and feed is compromised by contamination with different materials including chemically active fungal metabolites, especially

aflatoxins. Aflatoxins are a large group of secondary metabolites that are highly toxic and carcinogenic. The toxins are produced by specific species of fungi, that is, *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius*; which are ubiquitous in soil, decaying vegetation, hay and grains (Coppock et al., 2018). Aflatoxins associated with major detrimental effects are B_1, B_2, G_1, and G_2; with aflatoxin B_1 being the most potent hepatocarcinogen known (Rajarajan et al., 2013). Aflatoxin B_1 is also reported to be mutagenic, teratogenic and estrogenic (Benkerroum, 2020).

Aflatoxins occur in most of the staple foods produced in Africa such as maize, sorghum, groundnuts, peanuts and cotton seed (Osuret et al., 2016). The contamination is associated with high moisture content, ranging from 18 to 18.5% in the grains. Unfortunately, subsequent processing steps, such as drying and boiling, do not reduce the aflatoxin levels (Benkerroum, 2020; Khaneghah et al., 2018). The European Economic Community (EEC) as well as the United States Food and Drug Authority (FDA) set the maximum permitted levels of total aflatoxins in poultry feed as 20 µg/kg (Lubna et al., 2018), while the Uganda National Bureau for Standards (UNBS) set a limit of 10 µg/kg for all foods and feeds but only those intended for export (Grace et al., 2015).

In Uganda, reports of persistent occurrence of aflatoxin contamination, beyond acceptable levels, in selected food and feedstuffs exist (Lukwago et al., 2019; Omara et al., 2020). Indeed, a previous study in Uganda revealed poultry feed contamination with aflatoxins at levels between 7.5± 0.71 and 393.5 ± 19.09 µg/kg; with only 17.9% passing the FDA limits (Nakavuma et al., 2020). Unfortunately, there is limited monitoring and enforcement of the regulations; in addition to unsuccessful strategies for management of mycotoxin contamination in the animal feed industry in Uganda.

Management of aflatoxin contamination in the food/feed chain calls for improvement in the farming practices, pre- and post-harvest handling; with the latter emphasizing appropriate storage of the grains and processed feeds (Pankaj et al., 2018). These strategies require additional treatments to effectively eliminate aflatoxin contamination. Therefore, further efforts in development of safe, effective, affordable and environmentally safe aflatoxin decontamination methods are required (Mwakinyali et al., 2019). The most widely used approach in the detoxification of feed and food is the application of sorbents for selective removal of toxins by adsorption before and during passage through the gastrointestinal tract (Ismail et al., 2018). Aflatoxin binders adsorb the toxin while *in-situ,* in the feed and in the gut resulting in the excretion of the toxin-binder complex in the faeces (Boudergue et al., 2009). Inorganic binders, such as hydrated sodium calcium Aluminosilicates (HSCAS), bentonites, phyllosilicates, smectites, kaolinites, zeolites and activated charcoal are used especially in developed countries (Womack et al., 2014). Bentonite is among the most widely used sequestering agents and has been reported to adsorb over 85% of aflatoxins present in the feed (Gan et al., 2019; Mgbeahuruike et al., 2018; Wang et al., 2020) and was therefore included in the current analyses for comparison purposes. The effectiveness of inorganic substances varies with the toxin type; and in some cases, there is need to eliminate them from the treated feeds before presenting to the animals (Kim et al., 2017). Hence, investigations into the effectiveness and feasibility of applications of biological materials are recommended as alternative strategies for aflatoxin decontamination in feeds (Ismail et al., 2018).

Several microorganisms including bacteria, yeast, and non-toxigenic strains of *A. flavus* and *A. parasiticus* have exhibited aflatoxin detoxification capabilities and are thus promising as alternatives to chemical binders (Ismail et al., 2018). Biological detoxification, which is due to biotransformation or degradation of the toxin by the microorganism or its enzymes, yield metabolites that are either non-toxic when ingested by animals or less toxic than the parent toxin molecule (Boudergue et al., 2009). Despite considerable research on microbial detoxification and their premise on utilization in feeds and foods (Kim et al., 2017), they are not widely available, especially in developing countries, such as Uganda. Besides, given the regulatory rigour on introduction of microbes into a country and the biosecurity issues, there is need to develop products from sources obtained from the local environment. This study aimed at evaluating the efficacy of two locally isolated microbial strains in reducing aflatoxin concentration in poultry feeds in comparison to the commercial chemical binder, bentonite. The influence of the feed matrix, pH, and temperature on the aflatoxin reducing activity of the microbes and bentonite was also determined.

MATERIALS AND METHODS

Study design and source of microorganisms

An experimental laboratory-based study that followed a factorial design was carried out to establish the efficacy of two microbial strains in reducing aflatoxin concentrations in poultry feeds. The microbes were obtained from previous studies that involved *in vitro* screening for aflatoxin reduction activity of isolates from various sources in Uganda. A commercial binder, bentonite, was included as the positive control. Bentonite, as well as the specifications for its use, was obtained from local traders in Kampala, Uganda. The microbes included a bacterial strain (*Bacillus* spp B285) and a yeast *Saccharomyces* spp strain (Y833) that were isolated from poultry

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droppings and brewer's waste, respectively.

Preparation of the aflatoxin-reducing microbial test materials

Stock cultures of the microbial isolates, *Bacillus* spp (B285) and a *Saccharomyces* spp strain (Y833) were resuscitated in Trypticase Soy Broth (TSB) and Czapek Dox Broth (CDB), respectively. Purity of the isolates was confirmed by sub-culturing on Trypticase Soy Agar (TSA) and Potato dextrose agar (PDA) for the bacteria and yeast, respectively. All media were manufactured by Laboratorios Conda S.A, Spain. Working cultures were prepared by inoculation into the respective broth medium and incubated at 37ºC for 18 h for bacteria and at room temperature for 5 days for yeast.

From the overnight microbial broth culture, 2.0 ml were centrifuged at 3000 rpm (Zentrifugen, Germany) for 15 min at 10ºC. The cells were washed twice with PBS (pH 7.2) and once with double distilled water; and the cell pellet was suspended in 1.0 ml of sterile double distilled water. The microbial preparation was standardized to about 10^6 CFU/ml for yeast and 10^8 CFU/ml for bacteria by comparing with McFarland standard No.5. The viable bacterial and yeast cell concentrations were confirmed by surface spread method and expressed as colony-forming units per millilitres (CFU/ml).

Compounding of the poultry feed

Freshly supplied raw materials for feed preparation were purchased from produce dealers in Kampala. The ingredients were used in compounding of about 2 kg of broiler starter feed following an in-house formulation at the Nutrition Laboratory, College of Veterinary Medicine, Animal Resources and Biosecurity (COVAB), Makerere University. The ingredients and proportions included: cotton seed cake – 300 g, silver fish meal (*Rastrineobola argentea*, locally known as "mukene") – 240 g, bones – 120 g, maize – 1230 g, salt – 10 g and soya – 100 g. The ingredients were ground separately and mixed to obtain the required compounded feed. From the compounded feed, 250 g were ground further using a laboratory mill to pass No.20 and then No.14 sieve. The feed was mixed thoroughly for homogeneity and placed in paper bags and stored at refrigeration temperature. The compounded feed was used for the aflatoxin -reducing experiments.

Source of aflatoxins for feed sample spiking

In-house production of aflatoxins was carried out following the methods described by Shotwell et al. (1966) with modifications. Coarsely ground rice was used as the substrate instead of the kernels. The inoculated sterile rice slurry was incubated for 7 days at 25°C in the dark. An aflatoxigenic *A. flavus* strain Y20, was obtained from the National Agricultural Research Laboratory, Kawanda.

Aflatoxin extraction was made following the procedure by Kana et al. (2013); with modifications; and all procedures were carried out in a fume hood. Briefly, 50 g samples of the rice culture was blended with 250 ml of methanol:water mixture (60:40 w/v) for 5 min at a high speed. The solution was filtered through Whatman No. 1 filter paper (Sigma-Aldrich, Germany). To the filtrate, 30 ml of 2% sodium chloride solution and 500 ml n-hexane were added, placed in a separating funnel, and shaken vigorously for 5 min. The n-hexane layer was discarded and the lower methanolic layer was placed into another separating funnel to which 40 ml of chloroform was added; shaken and allowed to rest undisturbed for separation of layers. The chloroform layer was collected in a flask containing 5 g of cupric carbonate, agitated, and allowed to settle down. The extract was filtered through a bed of anhydrous sodium sulphate over a filter paper; the various portions were pooled and then evaporated to dryness on a heat block. The residue was dissolved or reconstituted in 1 ml acetonitrile, to have a crude extract that was kept in a refrigerator until further analysis or use. Presence of aflatoxins in the crude extract was confirmed using thin layer chromatography (TLC) plates (Sigma- Aldrich, Germany) alongside 0.04 µg/ml aflatoxin B1, 0.024 µg/ml aflatoxin B2, 0.03 µg/ml aflatoxin G1 and 0.04 µg/ml aflatoxin G2 (Romer Labs, UK) as the aflatoxin standards. The bands were visualised under UV light at 365 nm wavelength.

Further purification of the aflatoxin extract was achieved by passing it through an Aflatest Immunoaffinity column (VICAM, Milford-Massachusetts, USA) following the manufacturer's instructions. The column was washed with PBS and the toxins eluted with 100% High Performance Liquid Chromatography (HPLC) grade methanol. Quantification of aflatoxin content was achieved using VICAM®, a fluorometric method following the manufacturer's instructions.

Preparation of the aflatoxin working solution involved evaporation of the methanol by keeping the loosely closed vials at 50°C for overnight, after which the concentration was adjusted to 1 µg/ml using PBS. The working solution was kept in an amber bottle in the refrigerator.

Preparation of aflatoxin spiked feeds and non-spiked feeds

Preparation of the feeds was as described by Manafi et al. (2009), with modifications, where 10 g of compounded feed instead of 25 g were employed and pH of the feeds was not modified. Briefly, to 10 g of the compounded poultry feed, 0.8 g of sodium chloride were added and mixed properly. A slurry was prepared by adding 10 ml PBS (pH 7.2) and then autoclaved. To 10 ml of the feed slurry, the aflatoxin working solution was added to obtain a concentration of about 40 µg/L (Gul et al., 2017), that is, 400 µL of 1 µg/ml aflatoxin working solution were added. To ensure proper distribution, small amounts of the feed slurry were continuously mixed with the aflatoxin working solution and homogenised. For non-spiked feeds, instead of aflatoxin solution, 400 µL of PBS was added.

Determination of the effect of feed matrix, pH and temperature on aflatoxin reduction

A factorial study design was used to evaluate the aflatoxin reducing efficacy of the two selected microorganisms and the control (bentonite) in aflatoxin-contaminated feed *in vitro* and under simulated *in situ* gastrointestinal tract environment (pH and temperature) of chickens according to Manafi et al. (2009). However, in this case, evaluation of the effects of pH and temperature were carried out in PBS not the feed. For each treatment, the toxin reduction was tested in triplicates at pH levels of 4.5 and 6.5; and at 37, 42ºC and at room temperature; after which the residual aflatoxin concentrations were determined.

To the aflatoxin-spiked and non-spiked feed samples, the appropriate test reducing agents were added. For bentonite, 0.3% w/v was added according to the instructions given by the local agrochemical supplier and Magnoli et al. (2008). For the microbial agents, 5 mL of the standardized yeast and bacterial cell suspensions with concentration of 1.0×10^6 and 1.0×10^8 CFU/mL, respectively, were used. In order to determine the effect of the feed matrix, PBS instead of feeds was used. The tests were carried out in triplicates and incubated at the appropriate temperatures for four hours. The experimental set-up is detailed below and summarized in Table 1.

For comparison of the activity of the test materials, the treatment groups included aflatoxin spiked feeds with yeast (Group 1); non-spiked feed with yeast (Group 2); spiked feed with bacteria

Table 1. The experimental set up for determination of aflatoxin reduction in poultry feeds, and the effect of feed matrix, pH and temperature**.**

Reducing agent	Aflatoxin (40 µg/L) status			
	Feeds		PBS*	
	Spiked	Non-spiked	Spiked	Non-spiked
5 ml 1×10^6cfu/ml of yeast	1	2	11	12
5 ml 1×10^8 cfu/ml of *Bacillus* spp	3	4	13	14
2.5 ml 1×10^6 cfu/ml yeast + 2.5 ml 1×10^8 cfu/ml *Bacillus* spp	5	6	15	16
Bentonite (Positive control)	7	8	17	18
None (Negative control)	9	10	19	20

PBS – Phosphate Buffered Saline. Arabic numerals 1 – 20 indicate the treatment groups. *The same set-up for PBS was followed for evaluating the effect of pH and temperature on Aflatoxin reduction.

(Group 3); non-spiked feeds with bacteria (Group 4); spiked feeds with 50% yeast and 50% bacteria (Group 5); and non-spiked feeds with 50% yeast and 50% bacteria (Group 6). In order to determine the effect of the feed matrix, the same set up was used, however, PBS instead of feeds was used. For positive control, bentonite was added, while for the negative control, nothing was added to the spiked or non-spiked feeds.

For determination of the effect of pH on aflatoxin reduction, the pH of PBS was adjusted to 4.5 and to 6.5; one portion was spiked with aflatoxins, while the other was not. The test materials were then added following the same set-up as for the feed experiments. For the effect of temperature, PBS experimental set up as described above was employed. Two sets, one for incubation at 37°C and the other at 42°C were put-up. Positive and negative controls involved addition of bentonite (positive) or nothing (negative) to the spiked and non-spiked preparations (Groups 7, 8, 9, 10; and Groups 17, 18, 19 and 20, respectively).

Residual aflatoxin concentration was determined after 4 h of exposure. For each treatment, aflatoxin extraction and quantification followed a procedure described by Kana et al. (2013), using the VICAM ® fluorometric method according to the manufacturer's instructions. Absolute values of the aflatoxin concentrations for all the treatments were recorded.

For safety precautions, the cultures for aflatoxin production were autoclaved and later treated with 2% sodium hypochlorite before incineration. Likewise, all the plastic ware and glassware were soaked overnight in sodium hypochlorite before disposal or washing for reuse. All preparations were carried out in a Class II Biological Safety Cabinet (Faster sil, Ferrari, Italy).

Data analysis

Using the absolute concentration values, percent aflatoxin reduction was calculated from the amount of unbound (residual) aflatoxins extracted after the experiments compared to negative control (without reducing agent). The formula below was employed.

$$A = 100\left[1 - {}^{A_1}/_{A_0}\right]$$

$A = \%\ of\ AF\ bound$
$A_1 = Amount\ of\ AF\ in\ supernatant\ solution\ after\ binding\ assay$
$A_0 = Amount\ of\ AF\ in\ negative\ control$

Data were analyzed by STATA v12 and the differences between the treatments were determined using ANOVA.

RESULTS

Reduction of aflatoxin concentration in poultry feeds subjected to the different agents

Results of aflatoxin reducing activities in poultry feeds are presented in Table 2. It was impossible to locally obtain aflatoxin-free ingredients for feed formulation; thus, the non-spiked feed contained 1.55±0.001 µg/L total aflatoxins. Addition of 0.3% (w/v) bentonite (positive control) to aflatoxin-spiked feed yielded a 93.43% reduction of total aflatoxin concentration, while the addition of the same to a non-spiked feed sample yielded a 100% removal of the toxins present. Mean aflatoxin concentration in presence of bentonite was significantly ($p<0.00001$) lower compared to that in absence of the binder.

Yeast was the most effective biological agent with a mean aflatoxin reduction from 41.53 ± 0.062 to 6.80 ± 0.002 µg/L giving a percentage reduction of 83.7%. The combination of yeast and *Bacillus* spp produced a higher percentage reduction (77.8%) compared to use of *Bacillus* spp (74.9%) only. The aflatoxin reduction by biological agents followed the trend of Yeast > Yeast + *Bacillus* spp > *Bacillus* spp. In non-spiked feeds, the biological agents reduced the aflatoxins to undetectable levels and hence produced 100% reduction from 1.549±0.001 to 0.00 (undetectable levels).

All test materials reduced aflatoxin concentration in feeds by more than 74%. However, the commercial binder, bentonite, had the highest reduction percentage (93.4%) followed by the yeast isolate with 83.7%; and lower values were recorded where bacteria were added.

Effect of feed matrix on aflatoxin reduction capacities of the test agents

Results indicating the effects of feed matrix on aflatoxin reduction activity of the different test agents are presented in Table 3. Aflatoxin reducing activity was

Table 2. Mean aflatoxin concentrations in poultry feed exposed to different treatments.

Treatment	*Mean AFT (µg/L)		Reduction (%)	P-value
	Start	End		
Non-AF spiked feed + No binder	1.55±0.001	1.55±0.001		
Aflatoxin spiked feed + No binder	41.60 ± 0.007	41.60±0.007		
Aflatoxin spiked feed + Bacteria	41.60 ± 0.007	10.41±0.004	75.0±0.01	0.000
Aflatoxin spiked feed + Yeast	41.60 ± 0.007	6.80±0.004	83.7±0.03	
Aflatoxin spiked feed + Bacteria and Yeast	41.60 ± 0.007	9.20±0.001	77.9±0.01	
Aflatoxin spiked feed + 0.3% Bentonite	41.58 ± 0.03	2.73±0.003	93.4±0.03	

All treatments were carried out in triplicates (n=3); *Total Aflatoxin concentration at start and end of experiment (after 4 h of incubation).

Table 3. The effect of feed matrix on the aflatoxin-reducing activity of two microbial strains (bacteria and yeast) and bentonite.

Treatment	Feed matrix		Phosphate buffered saline	
	Aflatoxin content (µg/L)	% Aflatoxin reduction	Aflatoxin content (µg/L)	% Aflatoxin reduction
AF without binder	41.57 ± 0.017	-	40.00 ± 0.000	-
AF with Bentonite	2.73±0.003	93	3.57 ±0.001	91
AF with *Bacillus*	10.41 ± 0.002	75	8.80 ± 0.005	78
AF with Yeast	6.79±0.002	84	6.23±0.000	84
AF with *Bacillus* and Yeast	9.19±0.001	78	5.58±0.001	86
P-value	0.0001		0.00001	

AF: Aflatoxin spiked, all treatments were carried out in triplicates (n=3)

lower by 3 and 8% in the presence of feeds as compared to PBS, for bacteria and bacteria plus yeast test materials, respectively. No differences were observed in case of the yeast isolate while bentonite performed better in presence of feeds than in PBS. The bacterial strain had lower activity compared to the yeast strain, when used singly; however, higher performance was observed in PBS, when combined with the yeast. Performance of bentonite was higher than the biological materials by 9-18% and by 5-13% in presence of feeds and in PBS, respectively. There were significant differences in aflatoxin reducing activity in the presence of feed ($p < 0.001$) and in PBS ($p < 0.0001$).

Effect of pH and temperature on aflatoxin reduction capacities of the agents

Aflatoxin reduction activities of the test agents at various temperatures and pH are presented in Figure 1. The percentage reduction ranged from 74 to 96%. Generally, the aflatoxin reduction activity for all the materials at different physicochemical conditions was above 70% with the commercial binder having > 90% in all cases. The yeast strain performed second best, with 83 – 86% reduction; and the *Bacillus* spp showed the least activity (74 – 78%) except at 42°C, where 96% reduction was observed. The bacteria and yeast combination exhibited 77 - 80% reduction. Aflatoxin reduction increased with rise in temperature, although it was not significant ($p = 0.0940$).

There was no significant effect ($p = 0.9817$) of pH on aflatoxin reduction by the various test materials. However, bentonite had the highest activity (95%) at pH 6.5. For the other treatments, there was a 1% reduction in activity at pH 6.5 compared to that at pH 4.5.

DISCUSSION

The continuous search for effective and appropriate mycotoxin control strategies has biological control as a promising strategy due to the associated efficiency, minimal effect on feed and/or food quality and safety; and environmental friendliness. This study screened two microbial agents for *in vitro* aflatoxin reduction activity at a single concentration. The selected strains, one of each of bacteria (*Bacillus*) and yeast reduced aflatoxin concentrations in contaminated poultry feed by 75 - 83.7%. The duration of exposure used was informed by the transit time of feed on ingestion, while the total aflatoxin concentration was twice the FDA recommended levels.

The yeast strain Y833 exhibited similar reduction activity in both feed and PBS, which fits in aflatoxin decontamination activities of 40 - 99.3% reported by

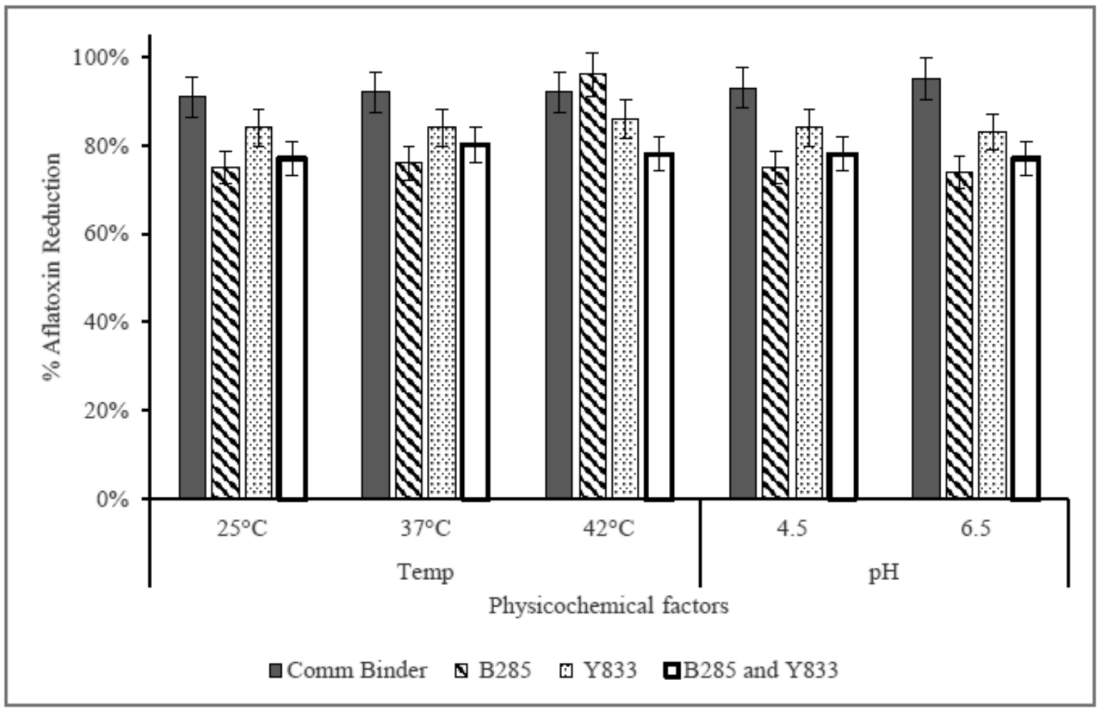

Figure 1. The effect of temperature and pH on the aflatoxin reducing activity of *Bacillus* spp. strain B285 and Yeast strain Y833 in comparison with a commercial aflatoxin binder, bentonite.

previous researchers (Kana et al., 2013; Manafi et al., 2009; Shotwell et al., 1966). Various species including *Saccharomyces cerevisiae* and *Candida krusei* were shown to bind > 60% of aflatoxins in a study that determined the binding efficacy of the yeast isolates (Gul et al., 2017). The reduction activity of Y833 was comparable to what was reported for the yeast cell wall and brewery dehydrated residues; but lower than the autolysed and the dried cane sugar yeasts at almost similar conditions as the current study (Manafi et al., 2009). Lower reduction activity was reported by some researchers, but the measurements were made at or beyond six hours post exposure, such as 60 - 72.8% AFB1 reduction by selected *Saccharomyces* spp. (Assaf et al., 2019; Chlebicz and Śliżewska, 2020; Magnoli et al., 2008). The decontamination activity depends on the yeast strains, pH and temperature conditions, duration of exposure and initial aflatoxin concentration (Gonçalves et al., 2014; Mukandungutse et al., 2019; Shetty and Jespersen, 2006). The reversible binding of aflatoxins by the yeast influences the detoxification levels, which have been reported to reduce with time (Manafi et al., 2009). An alternative mode of decontamination, through both intracellular and extracellular biodegradation by yeasts, such as *Candida versatilis* was previously reported (Li et al., 2018). The current study did not evaluate the activity over a period of time, which could have offered a clue on the mechanism of decontamination; hence, there is need for determining the mode of action of the selected isolates.

The aflatoxin reducing activity of the *Bacillus* spp. strain coded B 285 from this study is in agreement with findings by previous researchers who reported activities up to 97.3%, depending on the bacterial strain as well as the conditions and duration of the experiment (Petchkongkaew et al., 2008; Siahmoshteh et al., 2017; Wang et al., 2018). Our findings are similar to those of Petchkongkaew et al. (2008); who reported *Bacillus lichenformis* exhibiting 74% while *Bacillus subtilis* had 85% reduction of aflatoxin B1 at 37°C after 48 h of exposure, although the current exposure lasted 4 h only. The mode of activity by the *Bacillus* spp, is more of biodegradation than adsorption, which despite the different duration of exposure, results in comparable level of activities. However, like for the yeast studies, the analyses did not explore the effect of time on the decontamination activity.

In this study, the combined microbial activity (77.8%) was lower than that exhibited by the yeast strain (83.7%) but higher than that for bacteria (75%). The lower activity of the combined microbes than yeast is likely to have been due to the reduced amount of yeast cells in the preparation, however, this contributed to a higher activity than what was experienced by the bacterial strain. A higher effect compared to that by each strain was expected but the different mode of aflatoxin decontamination, and reduced concentration of each microbe probably affected their activity. These current findings contrast those from a previous study where degradation rates of 38.38 and 21.08% when *B. subtilis*

and *Candida utilis* were used singly, respectively; and a much higher rate of 45.49% by a combination of the two microbial species (Karaman et al., 2005). The lower activity compared to yeast alone, was probably due to some growth-inhibitory activity of *Bacillus* spp against the yeast in addition to the lower concentration, thus affecting the aflatoxins decontamination. Determining the mode of decontamination, and the level of aflatoxins against time and microbial cell population can elucidate the combined microbial activity to obtain conclusive explanations.

Bentonite exhibited highest reduction, followed by yeast, then a combination of yeast and *Bacillus spp*, while the latter was the least active. Compared to bentonite, the microbes exhibited lower reduction activity. Vekiru et al. (2015) reported similar findings where sodium bentonite showed the strongest binding capacity for aflatoxin B1. Bentonite is one of the inorganic toxin binders that are more effective in binding aflatoxin B1 than others, which is due to the purification of the inorganic clays (EFSA et al., 2017). The surface area and chemical affinities between adsorbent and mycotoxins have been reported to increase the process of binding (Romoser et al., 2013). The latter explains why bentonite had higher aflatoxin reduction activity than the microbes. The performance of yeast was next to that of bentonite, probably because of the similar mode of action, that is, adsorption of the aflatoxins. The lower performance of bacteria is most likely due to the differences in mode of action; the biodegradation effect, where enzyme induction and production needs more time than what was used in this study. The experimental feed contained some aflatoxins because it was impossible to obtain toxin-free ingredients. Removal of the basal aflatoxins in the non-spiked feed sample to undetectable levels by both the bentonite and the microbes; is probably an indication that the efficiency depends on the levels of contamination. However, the decontamination activity at different aflatoxin levels calls for further evaluation. Previous researchers used aflatoxin B1 for the decontamination experiments, while the current study did not purify the aflatoxin by type. Similar reduction activities probably point to presence of the aflatoxin B1 as the major type in the in-house aflatoxin extract used during the current study.

Generally, in-feed aflatoxin decontamination was lower than *in vitro* activity in PBS for the bacteria; and for the yeast and bacteria combination. This could have been influenced by the substrate, the feed that did not offer a conducive environment, which probably affects the growth and therefore aflatoxin biodegradation process. The current finding contrasts with that by Siahmoshteh et al. (2017) where *B. subtilis* had 85.66% reduction in aflatoxin B1 toxin in broth culture compared to 95% in the pistachio nuts. The latter was probably because the bacteria can grow luxuriantly in presence of the nuts, as the substrate. Bentonite exhibited higher activity in feed than in PBS and there was no difference in case of yeast, which was probably influenced by the mode of action. Shetty and Jespersen (2006) reported that live yeast strains and heat-treated preparations were efficient at binding aflatoxin B1 on mixing as feed additives, thus emphasizing that adsorption was not affected by the media where the toxin was suspended. Significant differences on the effect of feed matrix between the test agents were observed, but not on the decontamination activity of each test item, this attribute needs to be evaluated further before availing the product to the industrial processors.

There were no significant differences in the aflatoxin decontamination at various pH. The same trend of reduction of bentonite being the highest, followed by yeast, then a combination of yeast and bacteria; and lastly bacteria, was observed. Bentonite performed better at pH 6.5 than at pH 4.5; which contrasted to the microbial agents' activity. Yeast, just like other fungi, prefer acidic pH for growth, but the adsorption mode of action, which depends on the cell wall components does and not on cell viability, is probably reason for the observed activity. Similar findings were reported by Gonçalves et al. (2014). Dogi et al. (2011) demonstrated three yeast strains that survived under gastrointestinal conditions and effectively adsorbed aflatoxin B1 at different pH levels, which is similar to the findings of the current study. The bacterial agent was also not affected by pH since *Bacillus* spp have a wide range of pH at which they survive or grow. It is likely that the enzyme that is involved in biodegradation is resistant to the low pH, hence the comparable activity observed in this study. The 1% decrease in aflatoxin reducing activity at pH 6.5 compared to that at pH 4.5 contrasts Jouany et al. (2005) findings of greatest degradation at pH 5 – 6.5, probably due to differences in the microorganisms analyzed, since the previous researcher evaluated *Lactobacillus* spp.

The trends of decontamination by the tested microbial agents were similar at the same temperature, except at 42°C where B285 performed better than the others, including bentonite. Alteration of temperatures in the present study did not have any significant effect ($p>0.05$) on the efficacy of bentonite on aflatoxin reduction; similar to findings by Ramos and Hernandez (1996). However, our findings contrast some earlier reports, where a significant decrease in the adsorbed aflatoxin B1 by bentonite was recorded when the temperature was elevated from 25 to 45°C (Wongtangtintan et al., 2016). The ability of the *Bacillus* spp to grow at high temperatures could have resulted in increased cell populations and/or their enzymatic activity; hence exhibition of the highest aflatoxin reduction activity was at 42°C. Having been isolated from chicken droppings and given the body temperature of the birds, the *Bacillus* spp strain B285 was already adapted to such thermal conditions. The current findings agreed with Farzaneh et al. (2012) who previously reported, an optimum temperature for *B. subtilis* cell free supernatant was 35 to 40°C. Besides, an earlier study indicated that bacterial

adsorption of aflatoxins was both temperature and concentration dependent (Haskard et al., 1998). Since yeast and other eukaryotic cells' optimum temperature for growth is 37°C, lower activity would be expected at higher temperatures. However, the yeast activity was highest at 42°C because non-viable cells can also adsorb the toxins. The high activity at 42°C, pH 6.5 and at pH 4.5 implies that these microbial strains can impart their effect *in vivo*, since these conditions simulated those of chicken intestinal tract. Indeed, the crop and the proventriculus have a pH of about 4.5 whereas the small and large intestines have a pH of about 6.5 in chickens (Mabelebele et al., 2014).

Identification of potential mycotoxin-detoxifying agents is based on the ability to adsorb more than 80% aflatoxins available in solution; and determination of the mechanisms and conditions favorable for adsorption (Ahlberg et al., 2015; Pereyra et al., 2013). These are considered the minimum for passing the *in vivo* tests. However, the selected yeast candidate strain should be analyzed further to establish the mechanism of action and the appropriate concentrations for *in vivo* application in feed.

Conclusions

This study demonstrated the *in vitro* aflatoxin reducing activity of two selected microbial strains. From the results, it can be concluded that bentonite is more effective in adsorbing aflatoxins from poultry feed compared to their biological counterparts. Yeast strain Y833 was more effective than the bacterial strain B285 (*Bacillus* spp.) in decreasing the aflatoxin concentration in the poultry feeds. It can also be concluded that alternation of temperatures between 25°C through 37 to 42°C has no significant effect on the aflatoxin reduction capacity of the tested agents. Likewise, changing pH from 4.5 to 6.5 has insignificant influence on the aflatoxin reducing capacity of both biological and commercial chemical/mineral binders. In determining the mode of decontamination, reduction activity in relation to aflatoxin concentration, the duration of exposure and microbial cell population can elucidate the combined microbial activity to obtain conclusive explanations before recommendation for use by the feed processors. In addition, safety studies of the selected microbial candidates need to be performed before *in vivo* evaluation and large-scale production is embarked upon.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Characterization of Kenyan French Bean genotypes into gene pool affiliations using allele specific markers

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French bean (*Phaseolus vulgaris* L.) is a major export crop in Kenya where it serves as a cash crop for smallholder farmers and a source of employment. However, there is limited information on characterization of available germplasm which is essential in genetic improvement of this crop. The present study was therefore aimed to identify the gene pool affiliations of Kenyan French bean germplasm using specific molecular markers in order to understand the available germplasm for future use in breeding programs. The germplasm panel consisted of 46 accessions, comprising 40 French bean and six dry bean genotypes. The accessions were characterized using four gel-based molecular markers: SHP1-A, SHP1-B, SHP1-C and phaseolin protein marker. The most informative marker was the phaseolin protein marker which indicated that 82% of the French bean genotypes are of Andean origin, while 18% are of the Mesoamerican origin. Low polymorphism was observed for the SHP1 markers and the data from the three SHP1 markers did not correspond to the phaseolin protein marker for the French bean germplasm although SHP1-A and SHP1-B were able to differentiate the Andean from the Mesoamerican differential cultivars. The information on the gene pool affiliations of Kenyan French bean accessions is important for breeders to harness the divergence between the two gene pools in order to broaden the genetic base of the crop.

Key words: Gene pool, phaseolin protein, shatterproof, molecular markers, French beans.

INTRODUCTION

French beans designate common beans (*Phaseolus vulgaris* L.) with fleshier immature pods that are consumed as a vegetable (Singh and Singh, 2015; Myers and Kmiecik, 2017). The crop is a major export vegetable in Kenya although production has not matched its yield potential due to institutional challenges and a number of biotic and abiotic constraints. French bean breeding efforts in eastern Africa have mainly focused on pod yield improvement, local adaptation and incorporating disease resistance into contemporary cultivars (Wasonga et al., 2010; Wahome et al., 2011). Despite the breeding efforts in Kenya, cultivar release has been slow partly due to the limited understanding of the available germplasm. Few studies have attempted to characterize French bean

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germplasm in Kenya (Arunga et al., 2015). There is therefore a need for more studies on French bean germplasm because successful plant-breeding programs depend on characterizing genetic diversity in order to identify parents to be used as sources of breeding stocks.

The domestication of the common bean has the Mesoamerican and Andean gene pools (Beebe et al., 2001). The classification of these gene pools was according to phaseolin seed protein (Gepts and Bliss, 1988), different allozymes (Singh et al., 1991), nucleotide sequences (McClean and Lee, 2007; Nanni et al., 2011) and a number of molecular markers (Acosta-Gallegos et al., 2007; Papa et al., 2006). Worldwide, genetic diversity among French bean germplasm has been conducted based on multilocus molecular markers (Kwak and Gepts, 2009; Wallace et al., 2018). Previously, French beans were grouped as of the Andean origin (Singh et al., 1991), but later reports have grouped them in both gene pools of common bean depending on the region of collection (Blair et al., 2010; Wallace et al., 2018). Hybridization between the two gene pools have also been reported (Cunha et al., 2004).

The earliest studies of variations in common bean were based on the phaseolin protein (Gepts et al., 1986). This protein plays an essential role as a molecular marker in the understanding of common bean gene pools and genetic diversity analyses (Gepts and Bliss, 1988; Singh, 2001). These phaseolin diversity studies have been achieved under denaturing conditions through some procedures such as sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis or mass spectrometry (De La Fuente et al., 2012). Kami et al. (1995) developed a gel-based co-dominant SCAR marker that was able to discriminate between the two gene pools of common beans in an easy, fast and reliable process. The marker has been useful in understanding the common bean germplasm and also for marker-assisted selection for quantitative trait loci (QTL) conferring partial resistance to white mold in dry bean (Miklas, 2007; De la Fuente et al., 2012). In addition, Nanni et al. (2011) developed sequence tagged site (STS) markers that can be used to distinguish Mesoamerican and Andean gene pools. The three polymerase chain reaction (PCR) -based indel spanning markers (SHP1-A, SHP1-B, and SHP1-C) were designed based on *PvSHP1* gene sequences in common bean, a genomic sequence that is homologous to SHATTERPROOF-1 (SHP1) of *A. thaliana*. The *PvSHP1* in common bean is linked to the *V* locus for flower color and is a genomic region where QTLs for days to flowering and common bacterial blight resistance have been mapped (Blair et al., 2006; Miklas et al., 2006). The *PvSHP1* sequence is highly polymorphic and it has been particularly useful in distinguishing Mesoamerican and Andean gene pools (Angioi et al., 2010; Nanni et al., 2011; Pipan and Meglic, 2019). The present study was therefore aimed at characterizing Kenyan French bean germplasm into affiliated gene pools in order to understand the material for future use in breeding programs.

MATERIALS AND METHODS

Plant material

The studied plant material was made up of 46 accessions, comprising 40 French bean accessions (designated FBK1-40) and six dry bean cultivars: Pan 72, G5686, BAT 332, G11796, Mex 54 and G2333 (Table 1). The French bean accessions comprised of 32 commercial French bean varieties (obsolete and current), five breeding lines and three landraces. The French bean seeds were obtained from farmers, various research organizations and seed distributors in Kenya, while the landraces were obtained from the National gene bank of Kenya. Seed for the differentials was sourced from the International Center for Tropical Agriculture, Uganda. The gene pools of the differential cultivars are known (Liebenberg and Pretorius, 1997) and included in the study as controls.

DNA analysis

Plants used for DNA extraction were planted in a greenhouse maintained at 25±5°C at the University of Embu, Kenya. Leaves were collected from three-week old plants and DNA was extracted using the Mahuku DNA extraction protocol (Mahuku, 2004). PCR amplification used three STS markers: SHP1-A, SHP1-B, SHP1-C (Nanni et al., 2011) and the Phaseolin protein SCAR (Kami et al., 1995) (Table 2). The PCR reactions were composed of a total reaction volume of 10 µl in FrameStar® Break-A-Way PCR tubes containing 1X Dream Taq buffer (containing 2 mM $MgCl_2$), 0.2 mM dNTPs, 0.5 µM of each reverse and forward primers, 0.1U *Taq* Polymerase (Thermo Fisher Scientific) and 5 ηg/µl of genomic DNA. The DNA was amplified using the following thermocycler regime: an initial denaturation step at 94°C for 3 min; followed by 35 cycles of the following three steps: denaturation at 94°C for 10 s, annealing for 40 s, an extension at 72°C for 2 min; and a final extension step at 72°C for 5 min. The annealing temperatures for each marker are shown in Table 2. After amplification, a volume of 2 µl of 6x DNA loading dye (NEB) was added to each PCR reaction. The contents were loaded in 1.5% agarose gel pre-stained with 5 µM Ethidium bromide in 1x Sodium borate buffer and run at 100 volts for 3 h. For confirmation, a repeat of electrophoresis was done using 6% non-denaturing polyacrylamide gel, run horizontally at 100 V for 3 h, before staining with ethidium bromide. The DNA bands were viewed under ultraviolet light (UVP® GelDoc-it system). The major allele frequency, number of alleles per locus, gene diversity and polymorphic information content (PIC) were determined using Power Marker version 3.25 (Liu and Muse, 2005). Genetic distances among the 46 accessions were calculated using the Jaccard distance measure and the unweighted paired group method with arithmetic mean (UPGMA) tree was constructed using DARwin version 6.0.021 (Perrier and Jacquemoud-Collet, 2006).

RESULTS

All the four markers successfully amplified the 46 accessions revealing a mean of 2.25 alleles (Table 3). The most informative marker was the phaseolin protein SCAR marker with the polymorphism information content (PIC) value of 0.33. The phaseolin protein SCAR marker

Table 1. Description of germplasm under study.

S/N	Accession name	Cultivar class	Gene pool
1	FBK1	Obsolete commercial	Undesignated
2	FBK2	Current commercial	Undesignated
3	FBK3	Breeding line	Undesignated
4	FBK4	Breeding line	Undesignated
5	FBK5	Obsolete commercial	Undesignated
6	FBK6	Breeding line	Undesignated
7	FBK7	Breeding line	Undesignated
8	FBK8	Current commercial	Undesignated
9	FBK9	Current commercial	Undesignated
10	FBK10	Breeding line	Undesignated
11	FBK11	Current commercial	Undesignated
12	FBK12	Current commercial	Undesignated
13	FBK13	Current commercial	Undesignated
14	FBK14	Current commercial	Undesignated
15	FBK15	Gene bank landrace	Undesignated
16	FBK16	Current commercial	Undesignated
17	FBK17	Gene bank landrace	Undesignated
18	FBK18	Current commercial	Undesignated
19	FBK19	Current commercial	Undesignated
20	FBK20	Current commercial	Undesignated
21	FBK21	Obsolete commercial	Undesignated
22	FBK22	Gene bank landrace	Undesignated
23	FBK23	Current commercial	Undesignated
24	FBK24	Current commercial	Undesignated
25	FBK25	Current commercial	Undesignated
26	FBK26	Obsolete commercial	Undesignated
27	FBK27	Current commercial	Undesignated
28	FBK28	Current commercial	Undesignated
29	FBK29	Current commercial	Undesignated
30	FBK30	Current commercial	Undesignated
31	FBK31	Current commercial	Undesignated
32	FBK32	Current commercial	Undesignated
33	FBK33	Current commercial	Undesignated
34	FBK34	Current commercial	Undesignated
35	FBK35	Current commercial	Undesignated
36	FBK36	Current commercial	Undesignated
37	FBK37	Current commercial	Undesignated
38	FBK38	Current commercial	Undesignated
39	FBK39	Current commercial	Undesignated
40	FBK40	Current commercial	Undesignated
41	Pan 72	ALS differential	Mesoamerican
42	G05686	ALS differential	Andean
43	BAT 332	ALS differential	Mesoamerican
44	G11796	ALS differential	Andean
45	Mex 54	ALS differential	Mesoamerican
46	G2333	Anthracnose differential	Mesoamerican

(*Phs*) amplification produced two major profiles across the 46 entries. The first one consisting of two fragments of 249 and 270 bp and was considered of the Mesoamerican gene pool, and the second one contained

Table 2. Primer sequences, annealing temperatures and product size of molecular markers used for DNA amplification.

S/N	Molecular marker	Primer sequence (5' - 3')	Annealing temperature (°C)	Product size
1	SHP1 - A	F- TTGAGGGTAGATTGGAGAAAGG R-GGAAAATTTCATCAAAACATATCCA	57	198-206
2	SHP1 - B	F- GGAAATTGAGCTGCAAAACC R-CACAGTGTTCCCTGCATCAT	57	119-127
3	SHP1 - C	F- TTGAGGGTAGATTGGAGAAAGG R- TTGGGTTTATAAGAAAACCTTCCA	57	211-221
4	*Phs*	F- ACGATATTCTAGAGGCCTCC R- GCTCAGTTCCTCAATCTGTTC	55	249-285

Source: Nanni et al. (2011); Kami et al. (1995).

Table 3. Major allele frequency, number of alleles identified, gene diversity and polymorphism information content (PIC) of four gene pool specific markers analyzed on 46 common bean accessions.

Marker	Major allele frequency	Number of alleles	Gene diversity	Polymorphism information content (PIC)
SHP1-A	0.905	2.000	0.172	0.158
SHP1-B	0.889	2.000	0.198	0.178
SHP1-C	0.930	2.000	0.130	0.121
Phs	0.739	3.000	0.396	0.333
Mean	**0.866**	**2.250**	**0.224**	**0.198**

three fragments of 249, 264 and 285 bp, and was considered of the Andean gene pool (Figure 1). Based on the phaseolin protein markers, 82% of the French bean genotypes are of Andean origin while 18% are of the Mesoamerican origin. Further, the Mesoamerican differentials produced two fragments of 249 and 270 bp while the Andean differential cultivars contained three fragments of 249, 264 and 285 bp.

The PIC values for SHP1 markers were very low with SHP1-B having the highest value (0.17). The data from the three SHP1 markers did not correspond to the phaseolin protein marker results for the French bean germplasm. However, SHP1-A and SHP1-B were able to differentiate the Andean differential cultivars (G5686 and G11796) from the Mesoamerican (G2333, Mex 54, Pan 72 and BAT 332) differential cultivars. For SHP1-A, the Andean differential cultivars had the 206 bp allele while the Mesoamerican differentials had the 198 bp, while for SHP1-B the Andean differential cultivars had the 211 bp allele while the Mesoamerican differentials had the 221 bp (Figure 2). Only three French bean accessions (FBK20, 26 and 38) were different from the rest when amplified with SHP1-A and SHP1-B. Furthermore, SHP1-C was monomorphic for all the accessions except for three French bean accessions. Majority of the accessions possessed the 127 bp band while FBK 3, 7 and 38 possessed the 119 bp band.

Cluster analysis grouped the 46 accessions into three major clusters (Figure 3). Cluster I was made up of 29 French bean accessions which showed no variability for the three markers. These accessions were grouped as Andean genotypes using the phaseolin protein marker. Cluster II was made up of 5 breeding lines (1, 3, 4, 7, 10) one obsolete commercial cultivar (21) and a landrace (22). These group was classified as of Mesoamerican gene pool based on the phaseolin protein marker. However, they showed no polymorphism for the SHP markers. Cluster III was made up of the differential cultivars (42, 44) and three French bean accessions (20, 26, 38) that were polymorphic for SHP-A and SHP-B markers. The members of the third cluster were classified to be of Andean origin based on the phaseolin protein marker.

DISCUSSION

The phaseolin seed storage protein is an important molecular marker in common bean (Carović-Stanko et al. 2017). This marker has been used to characterize common bean genotypes in East Africa (Tanzania and Uganda) recording the predominance of Andean genotypes as compared with the Mesoamerican genotypes (Okii et al., 2014; Chilagane et al., 2016). In Europe the phaseolin protein marker, together with SHP1 markers, were able to successfully indicate the gene pool

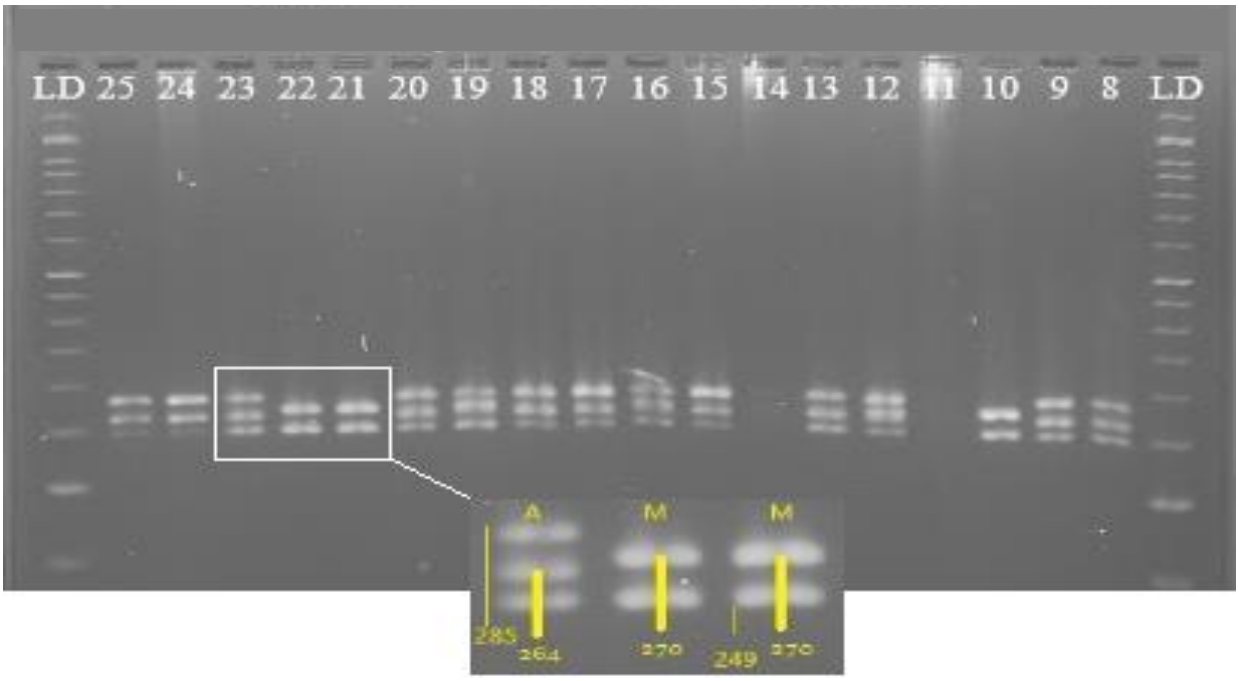

Figure 1. PCR amplification products of genomic DNA from *P. vulgaris* L. genotypes amplified with Phs SCAR marker electrophoresed on 1.5% agarose gels. Ld = 50 bp ladder, 8 = FBK8 9 = FBK9, 10 = FBK10, 11 = FBK11 12 = FBK12, 13 = FBK13, 14 = FBK14, 15 = GBK FBK15, 16 = FBK16, 17 = FBK17, 18 = FBK18, 19 = FBK19, 20 = FBK20, 21 = FBK21, 22 = FBK22, 23 = FBK23, 24 = FBK24, 25 = FBK25.

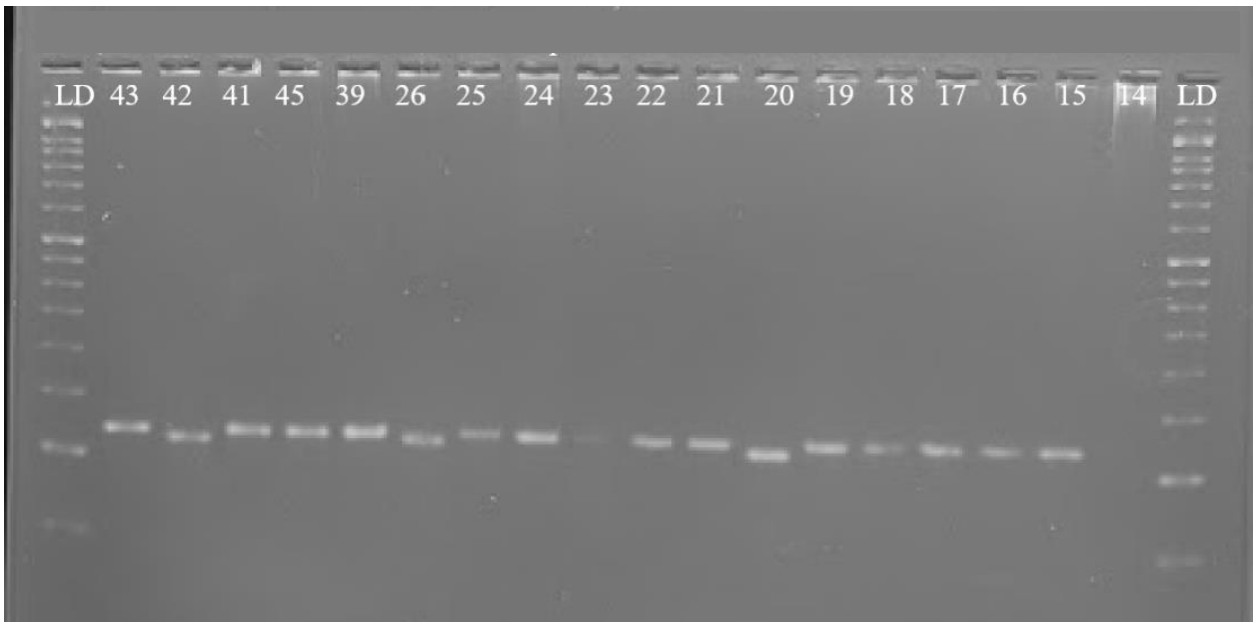

Figure 2. PCR amplification products of genomic DNA from *P. vulgaris* L. genotypes amplified with SHP1-B electrophoresed on 1.5% agarose gels. Ld = 100 bp ladder, 14 = FBK14, 15 = GBK FBK15, 16 = FBK16, 17 = FBK17, 18 = FBK18, 19 = FBK19, 20 = FBK20, 21 = FBK21, 22 = FBK22, 23 = FBK23, 24 = FBK24, 25 = FBK25, 26=FBK26, 39 = FBK39, 45= Mex 54, 41 = PAN 72, 42 = G05686, 43 = BAT 332.

origins of common bean germplasm from various parts of Europe (Pipan and Meglic, 2019). In contrast, the information obtained from the three SHP1 markers in this study was not enough to categorize the French bean

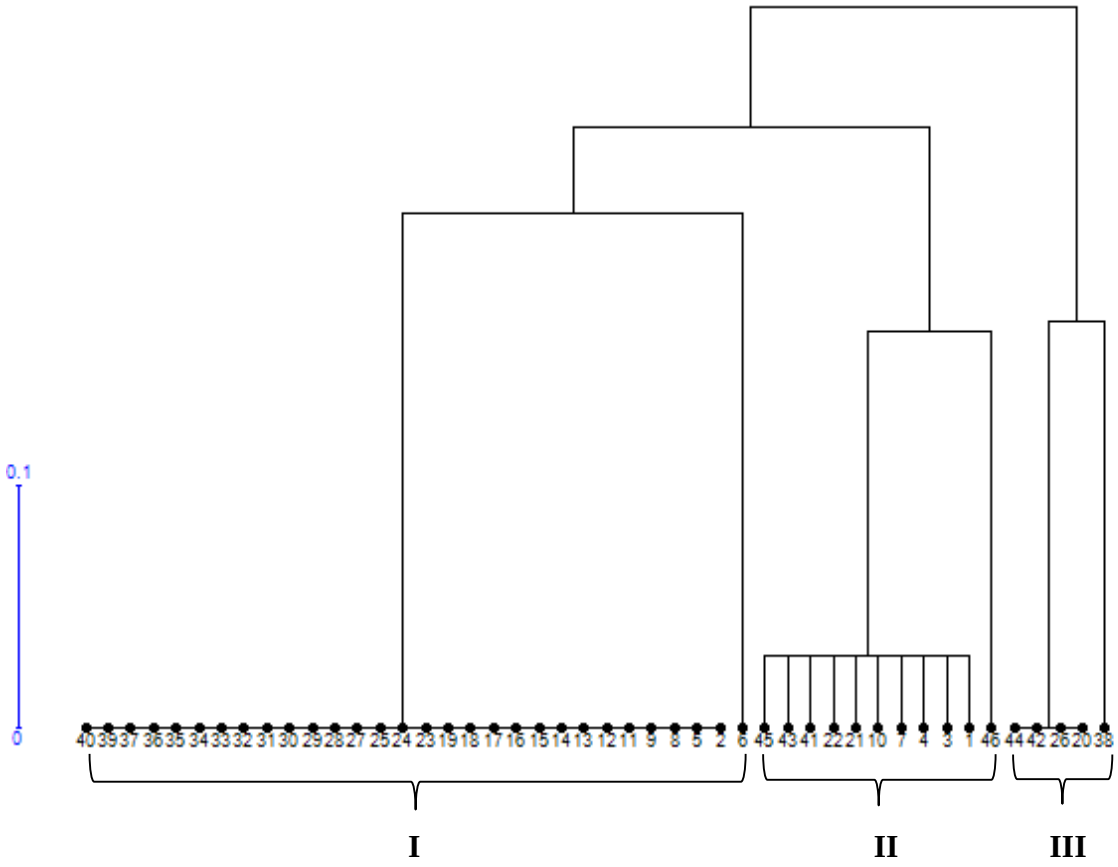

Figure 3. UPGMA dendrogram illustrating genetic similarities of 40 French bean accessions and six dry bean genotypes based on analysis of four gene pool specific markers using Jaccard distance.

germplasm into specific gene pools. The SHP1 markers were only useful in subdividing the Andean gene pool further which were already classified using the phaseolin protein marker. The phaseolin protein marker is therefore useful in classifying the French bean accessions into gene pools.

SHATTERPROOF (SHP) belongs to the family of MADS-box genes and is involved in fruit shattering in *Arabidopsis* (Liljegren et al., 2000). In common bean, pod dehiscence is characterized by the presence of pods suture strings and wall fibers (Singh and Singh, 2015). Koinange et al. (1996) mapped the *St* gene, which is responsible for the presence or absence of pod string, on linkage group Pv02. Hagerty et al. (2016) recorded a QTL for string: pod length (PL) ratio found on Pv02 in a dry bean × snap bean mapping population. However, Davis et al. (2006) located a QTL on Pv06 for pod strings using a mapping population involving two snap beans, suggesting that there may be additional loci for pod suture strings. Nanni et al. (2011) reported that the *PvSHP1* fragment did not map to the *St* gene (the *PvSHP1* was mapped on PV06) although the authors could not totally exclude that the *PvSHP1* is somewhat involved in genetic control of fruit shattering in *Phaseolus*. In this regard, further studies are recommended using a diverse and large French bean germplasm pool to understand the low polymorphism of the SHP1 markers in French beans. Contemporary French bean cultivars are stringless and lack pod fibers and this could be the reason for lack of variability when using the SHP1 markers.

The predominance of Andean gene pool among French bean genotypes has been reported previously (Metais et al., 2002; Wallace et al., 2018). In the present study, the predominance of the Andean gene pool was evident supporting the studies by Arunga et al. (2015) who characterized Kenyan French beans using microsatellite markers. The few entries that were classified as Mesoamerican were either landraces or breeding lines. The breeding lines were obtained from one breeding program, which utilized resistance genes from both gene pools (including dry beans). All commercial French bean cultivars are of Andean origin indicating the narrow genetic base of the commercial cultivars.

The consequence of having predominance to the Andean gene pool among French beans is the low diversity that exists among the Andean gene pool and their susceptibility to major pathogens in East Africa.

Studies on common bean diseases have reported association of plant pathogens with the common bean gene pool. Chilagane et al. (2016), while studying the interaction between the common bean host and *Pseudocercospora griseola*, found out that Andean genotypes were more susceptible compared with Mesoamerican genotypes. Similar studies on rust (*Uromyces appendiculatus*), angular leaf spot (ALS) and anthracnose (*Colletotrichum lindemuthianum*) in Kenya point towards the importance of using the Mesoamerican gene pool as sources of resistance (Arunga et al., 2012; Mogita et al., 2013; Kimno et al., 2016). The challenge of inter-genepool hybridization is the low success rate of transferring important quantitative traits from one gene pool to another (Johnson and Gepts, 1999). This has been observed especially in French beans where the French bean pod traits are usually affected. Breeders can therefore diversify the germplasm by collecting host plants from locations with high disease or with high pathogen diversity as recommended by Acevedo et al. (2008). Crosses within the same gene pool have been the most effective strategy to improve yield, adaptability and resistance to diseases in common bean (Kelly et al., 1998). In situations where this is not possible, French bean breeders can cross between the two gene pools using appropriate selection methods in combination with molecular markers to increase selection efficiency.

Conclusion

This study has revealed that most commercial French bean varieties grown in Kenya are of Andean origin and therefore breeders can harness the divergence between the two gene pools to broaden the genetic base of the crop. Furthermore, the phaseolin protein marker has shown to be informative for determination of the gene pool affiliations of Kenyan French bean accessions. Further studies are recommended on germplasm characterization using the SHP1 markers in a diverse and large French bean germplasm pool.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Exploring differentially expressed genes of microspore embryogenesis under heat stress in sweet pepper

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Stress is considered to be the inducer of microspore embyogenesis (ME), and heat stress is indispensible in the ME of sweet pepper. The aim of the study was to explore differentially expressed genes of microspore embryogenesis under heat stress in sweet pepper. The swollen rate of microspore was significantly affected by heat stress, while no green plant could be acquired without heat pretreatment. Anthers with or without heat stress were used for whole transcriptome analysis by RNA sequencing to provide new insights on how cells adapt to stress. A total of 5031 differentially expressed genes were identified, among which 2657 differentially expressed genes were up-regulated and 2374 differentially expressed genes were down-regulated in the early stage of heat stress. KEGG pathway analysis identified "plant hormone signal transduction" (67; 11.20%), followed by starch and sucrose metabolism (63; 10.54%). RNA-Seq data and quantitative real-time polymerase chain reaction showed that 224 genes related to glutathione metabolism, starch and sucrose metabolism, plant hormone signal transduction and phenylpropanoid biosynthesis were the most likely specific genes in ME under heat stress. This research provides new insights into molecular regulation during the early stage of ME in sweet pepper under heat stress.

Key words: Differentially expressed genes, heat stress, microspore embryogenesis, sweet pepper.

INTRODUCTION

Stress is considered as a kind of mutation on microspore embryos: unconstrained microspores form flower powder along the normal binding pathway (Touraev et al., 1997). Among various stress treatments, heat stress is widely used to initiate microspore embyogenesis (ME) in many crops (Asadi et al., 2018; Bhatia et al., 2018; Cimò et al., 2017; Dubas et al., 2014). The anthers of sweet pepper cultured under heat stress can be induced to develop into haploid embryos with complete functions instead of mature pollen (Bárány et al., 2005). Despite extensive advances have been made, compared to cruciferous and cereal species, sweet pepper is still considered recalcitrant to ME and doubled haploid production, which limit the use of this technology in breeding programs (Seguí-Simarro et al., 2011). In the past few years, most molecular biology researchers have focused on the culture system of sweet pepper (Popva et al., 2016; Heidari-Zefreh et al., 2019; Sánchez et al., 2020). We do not know the mechanism of cell fate transformation and the regulation of gene expression that initiates

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embryogenesis, and this knowledge gap is particularly obvious (Tsuwamoto et al., 2007). Many biochemical and morphological changes are closely related to the forced manipulation and alteration of gene expression patterns in embryos (Soriano et al., 2013).

Today, transcriptome analysis is an important means to study possible mechanisms and identify potential genes. Using this method, the roles of stress to induce the embryogenesis and alternation of gene expression have been examined in several crops (Elhiti et al., 2012; Liu et al., 2016; Zhang et al., 2019). However, it is still unclear for the differentially expressed genes in the early stage of ME under heat stress in sweet pepper.

In our previous work, an efficient ME system for pepper was established (Cheng et al., 2013). The present study aimed at exploring differentially expressed genes of microspore embryogenesis under heat stress in sweet pepper using high-throughput sequencing technology. This discovery will provide new insights into the molecular mechanism of sweet pepper micro-ecosystem.

MATERIALS AND METHODS

Plant material and heat treatment

Sweet pepper (*Capsicum annumm* L.) variety Jinjiao 203, a responsive genotype in ME, grew up in the greenhouse of Shanxi Academy of Agricultural Sciences, China. In February 2017, seeds were sown in the soil, and flower buds were collected from the donor plants. Anther pretreatment was performed as previously decribed by Cheng et al. (2013). Briefly, flower buds were sterilized immediately after collection. They were dissected and cultured on pretreatment medium (10 mM $CaCl_2$, 1 mM $MnSO_4 \cdot 7H_2O$, 1 mM KNO_3, 200 M KH_2PO_4, 1 M KI, 100 nM $CuSO_4 \cdot 5H_2O$, 0.37 M mannitol and 0.5% agar) for embryogenesis induction. Anthers cultured at 25°C were used as control group and anthers cultured at 34°C as heat treatment group. Each group was repeated four times. Anther load of each biological replica was isolated from at least five feed plants and divided into two groups on average. After 7 days of pre-culture, the microspores of 5 anthers were separated in 1 ml sterile water, and the microspore expansion rate (the total expanded microspores and the fraction of the total microspores in 5 microscope fields) of each treatment was analyzed. At the same time, a small amount of anthers (about 100 mg) were collected into test tubes by liquid nitrogen quick freezing method, and then stored at -80°C or RNA isolation. As described by Cheng et al. (2013), the remaining anthers were isolated and cultured for embryogenesis. After 2 months of culture, the number of green plants in every 100 anthers was counted.

RNA isolation and cDNA library construction

In order to separate RNA from pretreated anthers, frozen samples were ground into fine powder in a microcentrifuge tube. Total RNA was isolated by triazole reagent (Invitrogen, Carlsbad, California, USA), which produced about 10 micrograms of total RNA per sample. The nanodrop 2000 spectrophotometer (Thermo) was used to detect the concentration, and the RNA nano6000 detection kit of Agilent Bioanalyzer 2100 system (Agilent Technologies, California, USA) was used to evaluate the integrity. After adjusting their concentration to 10 nm, appropriate total RNA samples were collected in the same volume in 4 repeated pretreated anthers.

A total of 1 microgram of ribonucleic acid was used as input material in each sample to prepare a ribonucleic acid sample. Following the manufacturer's recommendations, the NEBNext UltraTM RNA Library Preparation Kit for Illumina (NEB USA) was used to create a sequencing library and the index code to the attribute sequence of each sample was added. Briefly, mRNA was purified from total RNA using magnetic beads and addition of oligonucleotide poly-T. At high temperature, divalent cations was used to adhere to the buffer of NEBNext synthesis reaction (5×). The first cDNA strand was synthesized with 6 polysilicon random seeds and mulv arz. Then deoxyribonucleic acid polymerase I and ribonuclease H were used to synthesize the second strand of deoxyribonucleic acid. The remaining overhangs were converted to blunt ends by exonuclease/polymerase activity. After adenylation at the 3' end of deoxyribonucleic acid fragment, NEBNext adapter with hairpin loop structure was connected to prepare for hybridization. To select a cDNA fragment with a length of 240 bp, the library fragment was purified by AMPure XP system (Beckman Coulter, Beverly, USA). Then, before polymerase chain reaction, 3 $mU \cdot L^{-1}$ of user enzyme (National Biological Laboratory, USA) was used to treat the cDNA ligated with the adapter selected in size at 37°C for 15 min, and then treated at 95°C for 5 min. Then polymerase chain reaction was carried out with polymerase, universal primer and index primer. Finally, the PCR products (Ample XP system) were purified on Agilent Bioanalyzer 2100 system, and the library quality was evaluated.

RNA sequencing and transcript analysis

According to the manufacturer's instructions, TruSeq PE Cluster Suite v4-cBot-HS (Illumia) was used to cluster the index coded samples on cBot cluster generation system. After the cluster is created, the preparation order of libraries on the Illumina Hiseq Xten platform is specified, and generates an end reading pair. The raw data in Fastq format (raw reading) was first processed by internal perl script. This step deletes the adapter-containing read, policy-containing read and low-quality read from the original data to obtain clean data (clean read). Q20, Q30, GC- content and sequence iteration level of clean data are also calculated. All downstream analysis is based on high quality and clean data. The adaptor sequences and low quality sequence reads would be removed from the dataset. The original sequence was converted to clean reading after data processing. These clean reads were then mapped to the reference genome sequence database Zunla-1. Only completely consistent or inconsistent readings are further analyzed and annotated according to the reference genome. Tophat2 tool software is used to map reference genomes. The Kyoto Encyclopedia of Genes and Genomes predicts metabolic and cellular pathways. The gene expression level was calculated by reading millions of Millennium bug (FPKM), and the fragments used were mapped to reference sequences. The two groups of DEGs were screened according to gene expression levels using the DESeq R software package (1.10.1). Binary negative sign distribution is used to identify differential expression in digital genetic expression data. More than twice the expression level changes and significantly different expressions ($P<0.05$) are considered as the differential expressions among different treatments.

Quantitative real-time PCR analysis

To verify the expression of SDR, 20 candidate genes identified by KEGG concentration analysis were randomly selected for real-time PCR quantitative analysis (QR-PCR). Selected gene names and primer information are listed in Table 1. Before assembling RNA sequences, the total RNA (1 µg) of each sample was used as a

Table 1. Primer information for qRT-PCR.

Gene id	Forward (5'→3')	Rewerse (5'→3')
*Actin*_GQ339766.1	GAAGCACCTCTCAACCCTAAG	GTACGACCACTAGCATACAAGG
Capana00g002630	CTAGGTTTGAGGGTGATAGGC	CTGAATGCAGGCTGGTAGTC
Capana01g000883	AGTCAAAGATGCGTGCTGAG	GACCCTGTATCACTGAGATTGC
Capana00g003106	GGATGTTTATGGGCTACTGTTG	TATCTCAGCTTTCCAGAATCG
Capana01g002457	TCAATGTTGCTCGGACTCTTC	CAGACCAAAACAATTAGAATAG
Capana00g001129	AACTTTCTCATGGTAACGATGC	AATCCTTAGTCGTGAATCGTGG
Capana01g001728	CTTGAAACAGCAAAGACCAGC	CATTGATGGTTGGAACAGCAC
Capana01g004182	AAAGGAATGTGGGCTGTTC	GGGTGAGAGAGTTTATGGGAG
Capana00g004135	TGTGTCTGCATTGTCTCATCC	TTTGAAGCTGGATCTGTTTCAG
Capana00g004867	TGGTTTGTTCAGGTAGGGAAG	ACCAGTTCGACAAGTTCCAG
Capana01g000278	ACCTTTTACACATTTTGGGCTG	GAGGACTATAGAGGCACAAAAC
Capana01g000279	GGACTACCCTTACAGCAACTTC	TCTCAGATCAGTCAAATGGCC
Capana01g000500	AGTCCATATTCAGAAGGCGG	TTGTACTACGTAGACTATCAC
Capana01g000731	AAGTACCAATGAAGAGGGCTG	AAAGCTCAGCGTACCATTAGG
Capana01g003125	GTGCTGATTGTGATTTCGGG	TGGGACTGGATTTGGATTTGG
Capana01g003124	CACCCTTCTCATCCTTCTCAC	CAGATCCACAGGCATTACAGG
Capana00g004543	ACCCACACCCTCTTGCTG	ACACCCAAATTCTCTGTCGAG
Capana01g004373	CGATGTCTGATATCTGTATTAG	CTTTGATAGGATCCGCTACCC
Capana00g005078	TCTGATACTGTGTTCTCTGGTG	GATATCCACCGCTACCTTGTG
Capana01g001352	AGCCCCAAGTTGTATGTGTC	TGGTGAAGTATCCGTTTCTTGG
Capana01g001414	CATCTGAGGCTACTTGGTGTC	GTTCCAGTTCATGCTTCCATTG

template for synthesizing the first strand cDNA. Gene names and information of selected seeds are shown in Table 1. Before compiling the nucleic acid sequence, the whole DNA [1 of each sample (1 microgram)] was used as a model for synthesizing the first cDNA strand. On the Bio-Rad CFX96 instrument, trans start top green qpcr super mix (AQ131) was used to replicate each biological sample for three times, and quantitative reverse transcription polymerase chain reaction analysis was carried out Using capsicum actin nanny gene as internal control. Quantitative comparison CT method ($^{\Delta\Delta}$CT method) is used to quantify the relative expression of specific genes (Livak and Schmittgen, 2001).

RESULTS

Effects of heat stress on the induction of ME in sweet pepper

In order to check the influence of thermal stress on electromagnetic induction, 7 days 34°C anther pretreatments were processed. In all four biological replicates, the anther volume increased after heat stress compared with the control group (Figure 1A). Moreover, more microspores in anthers were inflated, while fewer expanded microspores and no green plants were observed in the control (Figure 1B). The results indicated that both the swollen rate of microspore and green plants production were significantly affected by the heat stress (34°C, 7 days) in anther pretreatment, which means that heat stress could improve the embryogenesis initiation and possibility of microspore to develop into green plants.

Illumina sequence analysis and verification of selected genes by quantitative reverse transcription polymerase chain reaction

The cDNA library of embryogenic microspores of sweet pepper was sequenced on Illumina Hiseq Xten platform, and paired terminal readings were generated. A total of 143,541,722 and 151,420,406 original readings were obtained from two cDNA libraries composed of control group and thermal pressure group (Table 2). 92.91% of the readings were useful after data quality inspection and screening with quality greater than 30 (NQ30), among which 89.88% (128,990,381) of the control group readings and 82.90% (125,760,669) of the heat stress group readings were located in the pepper genome. About 86.39% of the readings were located on genes, and 13.61% were not located on genes, which indicated that most of the readings were located on reference genes.

In order to confirm the differentially expressed genes confirmed in sequencing and computational analysis, 20 DEGs concentrated in KEGG pathway were randomly selected for quantitative real-time polymerase chain reaction including 2 signal transduction mechanisms protein (*Capana00g002630*, *Capana01g000883*), 1 probable glutathione S-transferase (*Capana00g003106*), 1 agamous-like MADS-box protein (*Capana01g002457*), 1 peroxidase of carbohydrate transport and metabolism (*Capana00g001129*), 1 ent-copalyl diphosphate synthase

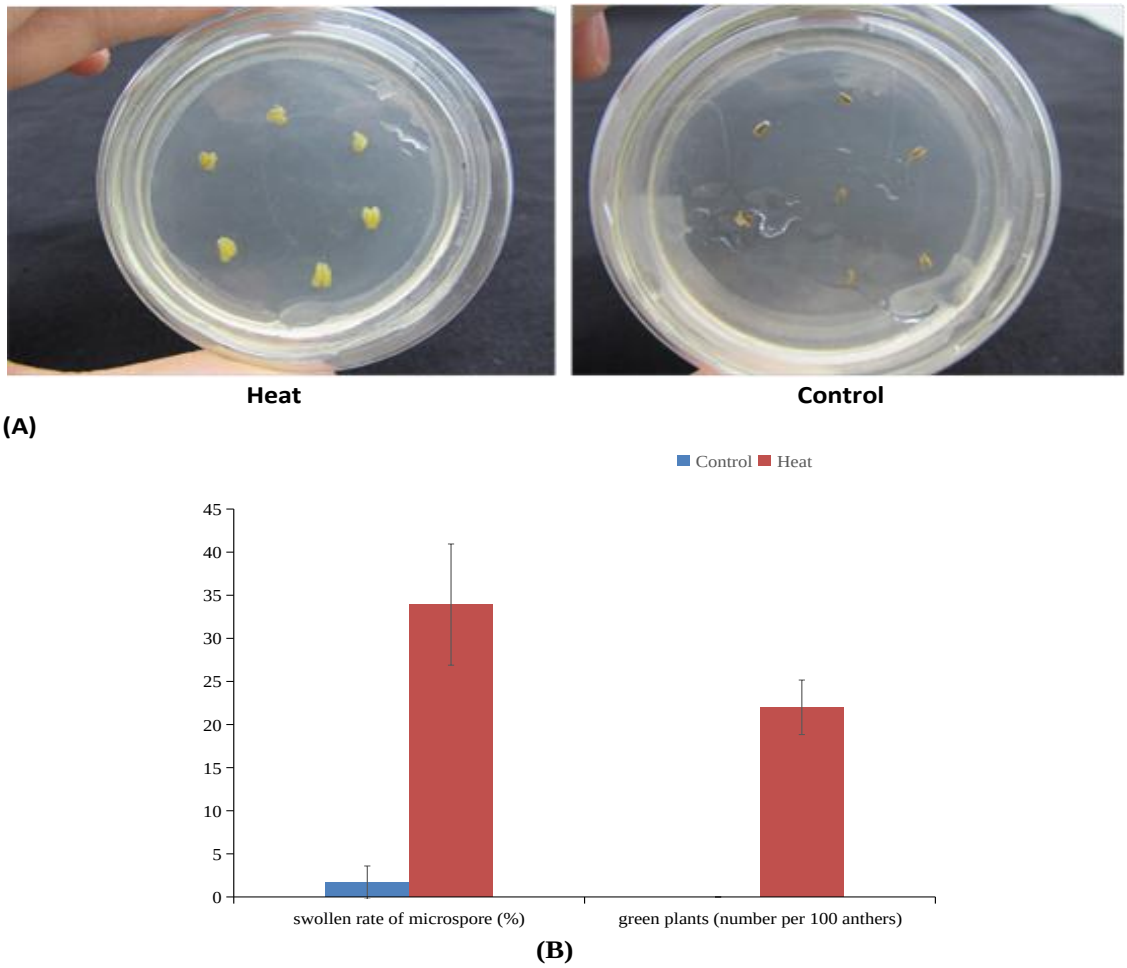

Figure 1. Comparison of ME of sweet pepper and resulted green plants produced between the control group (25℃, 7 days) and heat stress group (34℃, 7 days). (A) The pretreated anthers (7 days) between two groups. (B) The output of green plants is between two groups. Data represent the mean standard deviation (n=4). Compared with the control group, the single asterisk and double star numbers showed statistical differences (T test, P < 0.05 or 0.01, respectively)

Table 2. Summary of the illumina sequencing.

Sample	Reads	Raw reads	Clean reads	Q20 (%)	Q30 (%)	GC (%)
Control	Replicate1	47539426	23769713	100	93.04	42.71
	Replicate2	52194066	26097033	100	92.91	42.80
	Replicate3	43808230	21904115	100	93.26	42.65
	Total	143541722	71770861	100	93.07	42.72
Treatment	Replicate1	50034294	25017147	100	92.99	43.01
	Replicate2	52372404	26186202	100	93.07	42.56
	Replicate3	49013708	24506854	100	93.16	42.85
	Total	151420406	75710203	100	93.07	42.81

of coenzyme transport and metabolism (*Capana01g001728*), 1 cytokinin dehydrogenase of energy production and conversion (*Capana01g004182*), 1 GDSL-like Lipase/Acylhydrolase (*Capana00g004135*), 1 tetrahydrocannabinolic acid synthase (*Capana00g004867*), 1 thaumatin-like protein

Figure 2. Relative gene expression of 20 randomly selected genes detected by quantitative real-time polymerase chain reaction. Data represent the mean standard deviation (n=3). Compared with the control group, the single asterisk and double star numbers showed statistical differences (T test, $p<0.05$ or 0.01, respectively).

(*Capana01g000278*), 1 protein P21 of thaumatin family (*Capana01g000279*), 1 hydrophobic seed protein (*Capana01g000500*), 1 probable lipid transfer protein (*Capana01g000731*), 1 pollen-specific leucine-rich repeat elongation protein 1 (Capana01g003124), 1 C1-like domain (*Capana01g003125*), 1 serum kinase protein /su-aminotransfer LRR receptor (*Capana00g004543*), 1 shikimate O-hydroxycinnamoyltransferase (*Capana01g004373*), and 3 function unknown protein (*Capana00g005078*, *Capana01g001352, Capana01g001414).*

The results of quantitative reverse transcription polymerase chain reaction (Figure 2) on the expression of 20 selected genes showed that compared with the control group, the average expression level of 12 genes in the heat stress group was significantly increased. However, the average gene expression level of heat stress group was significantly down-regulated. The expressions of these genes were consistent in RNA RNA-Seq and qRT-PCR data.

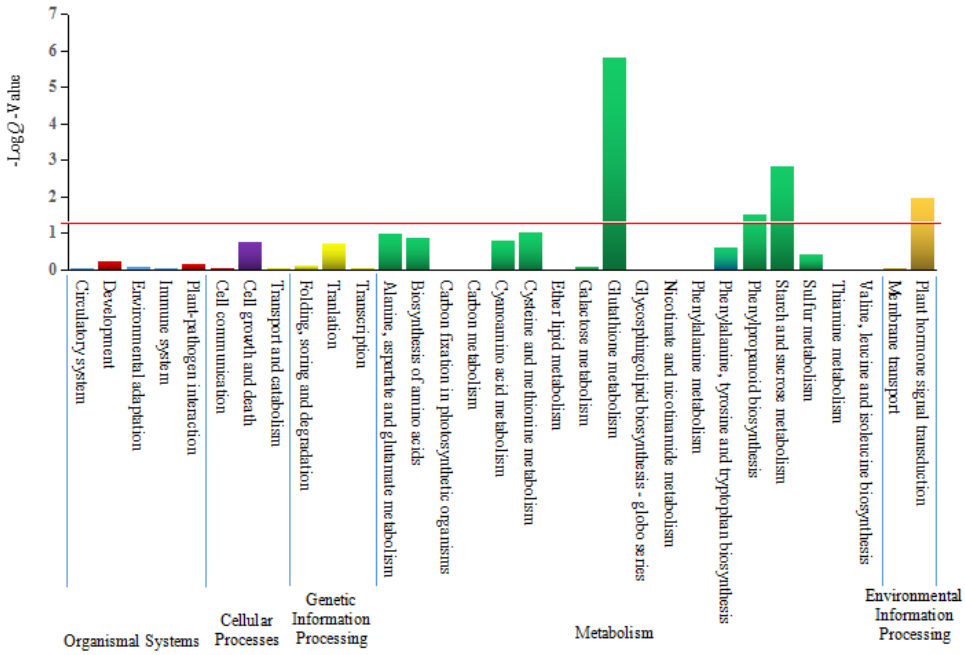

Figure 3. KEGG enrichment analysis identifying three possible main methods. The red line indicates the elevation of p=0.05.

KEGG enrichment analysis on DEGs

In addition to 32832 unchanged genes, 2657 up-regulated gene expression regions and 2374 down-regulated gene expression regions were found in the microenvironment between the control group and the hot group. In order to further identify the possible functional pathways, 598 DEGs were found in 20 KEGG functional pathways by KEGG pathway analysis. Among these pathways, the category of 'plant hormone signal transduction' (67; 11.20%) represented the largest group, followed by 'starch and sucrose metabolism' (63; 10.54%), 'biosynthesis of amino acids' (61; 10.20%), 'Carbon metabolism' (57; 9.53%), 'phenylpropanoid biosynthesis' (51; 8.53%) and 'Plant-pathogen interaction' (50; 8.36%). KEGG enrichment analysis showed that heat stress regulatory genes were enriched in four main pathways ($p<0.05$), including 'glutathione metabolism', starch and sucrose metabolism', 'plant hormone signal transduction' and 'phenylpropanoid biosynthesis' (Figure 3). Among the four main pathways, 224 estimated heat stress specific genes have been confirmed, of which 138 genes are up-regulated under heat pressure and 86 genes are down-regulated under heat pressure (Supplementary Table 1).

DISCUSSION

High stress tolerance could be presumed the first prerequisite for successful ME induction, for the reason that ME was induced or at least strongly stimulated by a stress pre-treatment (Zoriniants et al., 2005). One of the most important components of stress resistance is an effective antioxidant system composed of enzymes and low molecular antioxidants, which protects cells from the production of reactive oxygen species (Mittler, 2002). Glutathione was a major antioxidant in all forms of life and an indicator of cellular oxidative stress (Belmonte and Stasolla, 2009). In a reduced form, glutathione was metabolized in multiple ways leading to the biosynthesis of mercapturonate, glutamate, glycine, cysteine and other amino acids (Noctor et al., 2012). It also participates in the regulation of cell cycle, proliferation and programmed cell death as a signal molecule. Our results proved the importance of glutathione metabolism under heat stress in ME of sweet pepper. The key role of glutathione in embryo and meristem development was confirmed by analyzing the phenotype of glutathione-deficient Arabidopsis mutants (Noctor et al., 2012). The published results show that some glutathione response genes have encoded transcription factors and proteins, and are involved in cell division, redox potential (such as thioredoxin, glutamoren), auxin biosynthesis, transport and regulation of transcription reaction (Schnaubelt et al., 2013). It revealed that the initial environment of embryogenesis requires a reduced environment (high GSH/GSH + GSSG), which may promote cell proliferation by enhancing nucleotide synthesis and mitotic activity (Stasolla, 2010). More and more recently published data

indicate that ROS accumulation initiates signal transduction leading to microspore reprogramming and embryogenic development (Żur et al., 2019).

In this study, starch and sucrose metabolism were detected as the second major pathways in ME of sweet pepper under heat stress. The metabolism of starch and sucrose fuels all aspects of plant growth and development. The kinetics of starch synthesis is related to the differentiation process and the clear change of cell wall structure and organization, which is characterized by the de-esterification of pectin and the increase of RGII and XG components (Bárány et al., 2005; Satpute et al., 2005). The accumulation of plastid starch occurred in differentiated cells, which showed high levels of de-esterified pectin and rich RGII and XG, while the proliferation cells rich in esterified pectin did not show starch deposition (Bárány et al., 2010). Data show that one of the important changes of carbohydrate Daisy network is related to the transformation of embryogenesis and development program and cell fate (Corral-Martínez et al., 2019). The results reported in this paper show that many genes related to cell wall, major carbohydrate and starch metabolism are expressed differently during early embryonic development.

It was found that among the 20 KEGG functional pathways, plant hormone signal transduction accounted for the largest number of DEGs. Plant growth regulators are considered as the core signaling molecules for controlling plant growth and development, which respond to environmental stimuli and initiate signaling pathways (Kohli et al., 2013). Moreover, plant growth regulators interfere with the interaction between plant genotypes and environmental factors, and play a very important role in the micro-ecosystem, controlling the differentiation and development of embryos derived from microspheres and the regeneration of haploid/diploid plants (Divi et al., 2010). In the early stage of ME, numerous genes related to axins (Dubas et al., 2014), cytokinins, abscisic acid (Dubas et al., 2013), gibberellins, brassinosteroids, jasmonic acid, salicylic acid, or ethylene may produce translational regulation to adapt the stress. Hormone homeostasis seems to be one of the most important factors that determine the embryogenesis ability of cells, and a more comprehensive method is needed to understand the mechanism controlling this process, so as to break the barrier of self-resistance (Żur et al., 2015).

Phenylpropanoid biosynthesis was induced by several stresses (Dixon and Paiva, 1995), and became the main pathway in ME of sweet pepper under heat stress. In plants, the phenylpropanoid pathway was responsible for the synthesis of secondary metabolites, including lignin monomers, flavonoids, and coumarins, which play essential roles in determining plant structure, biomass recalcitrance, and stress tolerance (Vogt, 2010). It had been the significantly enriched pathways with specific common DEGs mainly including peroxidase, phenylalnine ammonialyase, and β-glucosidase (Zhang et al., 2019). The up-regulated DEGs related to phenylpropanoid biosynthesis in this study might have important function to keep microspore from death and promote embryonic microspore dedifferentiation.

Conclusions

Heat stress is indispensible in the ME of sweet pepper. In this study, transcriptome analysis of the anther of sweet pepper in the early stage of ME showed that the DEGs between heat treatment and control were mainly associated with glutathione metabolism, starch and sucrose metabolism, plant hormone signaling and phenylpropyl ester biosynthesis. Our research provides new insights into molecular regulation during the early stage of ME in sweet pepper under heat stress.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Supplementary Table 1. 224 putative heat stress-specific genes.

Pathway	Gene id	Expression	Annotation
Glutathione metabolism			
	Capana03g000768	up	PREDICTED: glutathione S-transferase U9 [Nicotiana sylvestris]
	Capana01g003296	up	PREDICTED: glutathione transferase GST 23-like [Solanum lycopersicum]
	Capana09g001742	up	PREDICTED: glutathione S-transferase U8-like [Solanum tuberosum]
	Capana01g002551	up	uncharacterized protein LOC543813 [Solanum lycopersicum]
	Capana09g001740	up	uncharacterized protein LOC543814 [Solanum lycopersicum]
	Capana09g001741	up	PREDICTED: probable glutathione S-transferase [Solanum lycopersicum]
	Capana02g000952	up	PREDICTED: probable glutathione S-transferase-like [Solanum tuberosum]
	Capana09g001858	up	PREDICTED: probable glutathione S-transferase [Solanum lycopersicum]
	Capana02g000950	up	PREDICTED: probable glutathione S-transferase-like [Solanum tuberosum]
	Capana00g002164	up	PREDICTED: LOW QUALITY PROTEIN: probable glutathione S-transferase parA-like [Solanum tuberosum]
	Capana00g003105	up	uncharacterized protein LOC543817 [Solanum lycopersicum]
	Capana00g004478	up	PREDICTED: probable glutathione S-transferase [Solanum lycopersicum]
	Capana03g004566	up	glutathione S-transferase 12 [Capsicum chinense]
	Capana07g002010	up	glutathione S-transferase/peroxidase [Capsicum chinense]
	Capana09g001764	up	probable glutathione-S-transferase [Capsicum annuum]
	Capana03g004562	up	glutathione S-transferase 12 [Capsicum chinense]
	Capana06g003058	up	glutathione S-transferase GST1 [Capsicum chinense]
	CapsicumannuumL_newGene_14186	up	PREDICTED: probable glutathione S-transferase [Solanum lycopersicum]
	Capana10g001792	up	PREDICTED: glutathione S-transferase L3-like [Solanum tuberosum]
	Capana08g001515	up	PREDICTED: glutathione S-transferase U8-like [Solanum tuberosum]
	Capana09g001763	up	PREDICTED: probable glutathione S-transferase [Solanum lycopersicum]
	Capana00g001895	up	PREDICTED: glutathione S-transferase U9-like [Nicotiana tomentosiformis]
	Capana09g001861	up	PREDICTED: probable glutathione S-transferase [Nicotiana sylvestris]
	Capana08g001520	up	unnamed protein product [Coffea canephora]
	Capana10g001806	up	PREDICTED: glutathione S-transferase L3-like [Solanum tuberosum]
	Capana09g001760	up	glutathione S-transferase [Capsicum annuum]
	Capana09g001860	up	PREDICTED: probable glutathione S-transferase [Solanum lycopersicum]
	Capana12g001177	up	PREDICTED: probable glutathione peroxidase 8-like [Solanum tuberosum]
	Capana12g001176	up	PREDICTED: probable glutathione peroxidase 8-like [Solanum tuberosum]
	Capana01g000225	up	PREDICTED: probable phospholipid hydroperoxide glutathione peroxidase-like [Solanum tuberosum]
	Capana09g001761	down	glutathione S-transferase [Capsicum annuum]
	Capana02g002285	down	glutathione S-transferase, partial [Capsicum annuum]
	Capana00g003106	down	PREDICTED: probable glutathione S-transferase parA [Solanum lycopersicum]
	Capana03g003600	down	PREDICTED: glutathione S-transferase PARB [Nicotiana sylvestris]
	Capana12g000354	down	PREDICTED: glutathione transferase GST 23-like [Solanum tuberosum]
	Capana09g002045	down	uncharacterized protein LOC543815 [Solanum lycopersicum]
	Capana02g000947	down	PREDICTED: probable glutathione S-transferase [Nicotiana sylvestris]
	Capana11g001532	down	PREDICTED: glutathione S-transferase U17-like [Nicotiana sylvestris]
	Capana11g001536	down	PREDICTED: glutathione S-transferase U17-like [Nicotiana sylvestris]
	CapsicumannuumL_newGene_14187	down	PREDICTED: probable glutathione S-transferase parC [Nicotiana tomentosiformis]
	Capana11g001525	down	PREDICTED: glutathione S-transferase U17-like [Solanum tuberosum]

Supplementary Table 1. Contd.

Capana07g002005	down	PREDICTED: probable glutathione S-transferase-like [Solanum tuberosum]
Capana02g002271	down	PREDICTED: probable glutathione S-transferase parC-like [Solanum tuberosum]
Starch and sucrose metabolism		
Capana08g002622	down	PREDICTED: endoglucanase 24-like [Solanum tuberosum]
Capana07g000730	down	PREDICTED: sucrose synthase-like [Solanum tuberosum]
Capana01g000086	down	PREDICTED: beta-amylase 1, chloroplastic-like [Nicotiana tomentosiformis]
Capana03g001539	down	PREDICTED: sucrose synthase 6-like [Solanum tuberosum]
Capana01g000524	down	PREDICTED: probable trehalose-phosphate phosphatase F-like isoform X1 [Solanum tuberosum]
Capana00g002284	down	PREDICTED: alpha,alpha-trehalose-phosphate synthase [UDP-forming] 1-like [Solanum tuberosum]
Capana03g001994	down	PREDICTED: soluble starch synthase 1, chloroplastic/amyloplastic isoform X1 [Solanum lycopersicum]
Capana03g004067	down	PREDICTED: probable sucrose-phosphate synthase 2-like [Solanum tuberosum]
Capana08g000100	down	PREDICTED: alpha,alpha-trehalose-phosphate synthase [UDP-forming] 6-like [Solanum tuberosum]
Capana00g000875	down	starch branching enzyme II, SBE-II [Solanum tuberosum]
Capana02g003365	down	granule-bound starch synthase 2, chloroplastic/amyloplastic precursor [Solanum tuberosum]
Capana10g000293	down	PREDICTED: probable alpha,alpha-trehalose-phosphate synthase [UDP-forming] 7 [Solanum lycopersicum]
Capana00g004617	down	PREDICTED: glucose-1-phosphate adenylyltransferase small subunit, chloroplastic/amyloplastic-like [Solanum tuberosum]
Capana01g002934	down	ADP-glucose pyrophosphorylase large subunit [Solanum lycopersicum]
Capana10g000025	down	PREDICTED: probable galacturonosyltransferase 12 [Solanum lycopersicum]
Capana03g003674	down	PREDICTED: cellulose synthase-like protein D5-like [Solanum tuberosum]
Capana00g004214	down	PREDICTED: probable galacturonosyltransferase 12 [Solanum lycopersicum]
Capana04g001640	up	PREDICTED: alpha-amylase [Solanum lycopersicum]
Capana01g001057	up	PREDICTED: alpha,alpha-trehalose-phosphate synthase [UDP-forming] 5-like isoform X1 [Solanum tuberosum]
Capana01g000074	up	PREDICTED: probable trehalase [Nicotiana tomentosiformis]
Capana03g003656	up	sucrose synthase [Solanum tuberosum]
Capana04g000118	up	PREDICTED: probable alpha-amylase 2 [Nicotiana sylvestris]
Capana01g003777	up	unnamed protein product [Vitis vinifera]
Capana09g000138	up	sucrose synthase [Solanum tuberosum]
Capana00g001331	up	PREDICTED: probable trehalose-phosphate phosphatase J-like [Solanum tuberosum]
Capana09g000101	up	PREDICTED: UDP-glucuronate 4-epimerase 1-like [Nicotiana sylvestris]
Capana01g000377	up	PREDICTED: UDP-glucuronate 4-epimerase 5-like [Solanum tuberosum]
Capana08g002001	up	PREDICTED: beta-xylosidase/alpha-L-arabinofuranosidase 2 [Solanum lycopersicum]
Capana00g003684	up	PREDICTED: UDP-glucuronate 4-epimerase 1-like [Solanum tuberosum]
Capana01g000079	up	PREDICTED: beta-amylase 1, chloroplastic-like [Nicotiana tomentosiformis]
Capana02g002280	up	PREDICTED: sucrose synthase 7-like [Nicotiana sylvestris]
Capana07g000366	up	sucrose-phosphate synthase [Lycium barbarum]
Capana03g002554	up	PREDICTED: trehalose-phosphate phosphatase A-like isoform X1 [Solanum tuberosum]
Capana02g001649	up	PREDICTED: probable alpha,alpha-trehalose-phosphate synthase [UDP-forming] 7-like [Solanum tuberosum]
Capana07g002060	up	PREDICTED: alpha,alpha-trehalose-phosphate synthase [UDP-forming] 1-like isoform

Supplementary Table 1. Contd.

		X1 [Solanum tuberosum]
Capana07g000086	up	trehalose-6-phosphate synthase [Solanum lycopersicum]
Capana03g004414	up	beta-amylase [Solanum lycopersicum]
Capana04g001731	up	RecName: Full=Alpha-1,4 glucan phosphorylase L-1 isozyme, chloroplastic/amyloplastic; AltName: Full=Starch phosphorylase L-1; Flags: Precursor [Solanum tuberosum]
Capana00g000646	up	PREDICTED: probable alpha,alpha-trehalose-phosphate synthase [UDP-forming] 11-like isoform X2 [Solanum tuberosum]
Capana12g001615	up	PREDICTED: 4-alpha-glucanotransferase, chloroplastic/amyloplastic isoform X1 [Nicotiana sylvestris]
Capana11g001362	up	PREDICTED: probable alpha,alpha-trehalose-phosphate synthase [UDP-forming] 9 [Nicotiana sylvestris]
Capana05g001390	up	PREDICTED: probable alpha,alpha-trehalose-phosphate synthase [UDP-forming] 9-like [Solanum tuberosum]
Capana10g000752	up	PREDICTED: probable trehalose-phosphate phosphatase F-like isoform X1 [Solanum tuberosum]
Capana01g000073	up	PREDICTED: probable trehalase [Nicotiana tomentosiformis]
Capana03g002047	up	PREDICTED: probable trehalose-phosphate phosphatase 2 [Solanum lycopersicum]
Capana02g000868	down	PREDICTED: uncharacterized protein LOC101249042 isoform X2 [Solanum lycopersicum]
Capana01g004209	down	PREDICTED: beta-amylase 8 isoform X4 [Solanum lycopersicum]
Capana06g001686	down	PREDICTED: probable trehalose-phosphate phosphatase F-like [Solanum tuberosum]
Capana07g001806	down	PREDICTED: probable alpha,alpha-trehalose-phosphate synthase [UDP-forming] 7-like [Solanum tuberosum]
Capana02g001891	down	PREDICTED: glycogen phosphorylase 1-like isoform X1 [Solanum tuberosum]
Capana08g000914	down	PREDICTED: beta-amylase 2, chloroplastic-like isoform X2 [Phoenix dactylifera]
CapsicumannuumL_newGene_14041	down	PREDICTED: probable alpha-amylase 2 [Nicotiana sylvestris]
Capana08g000917	down	PREDICTED: beta-amylase 7-like isoform X1 [Solanum tuberosum]
Capana09g001212	down	alpha-glucan phosphorylase, H isozyme [Solanum tuberosum]
Capana11g001904	down	alpha-1,4 glucan phosphorylase L-2 isozyme, chloroplastic/amyloplastic [Solanum tuberosum]
Capana00g001034	down	PREDICTED: alpha,alpha-trehalose-phosphate synthase [UDP-forming] 1-like [Solanum tuberosum]
Capana02g000320	down	PREDICTED: 4-alpha-glucanotransferase DPE2-like [Nicotiana sylvestris]
Capana04g000086	down	1,4-alpha-glucan branching enzyme [Solanum tuberosum]
Capana12g001588	down	PREDICTED: probable trehalose-phosphate phosphatase J [Solanum lycopersicum]
Capana02g003313	down	PREDICTED: probable galacturonosyltransferase 14-like [Solanum tuberosum]
Capana02g000897	down	PREDICTED: probable galacturonosyltransferase 13-like isoform X2 [Solanum tuberosum]
Capana08g002835	down	PREDICTED: probable galacturonosyltransferase 3-like [Solanum tuberosum]
Capana10g001175	down	PREDICTED: probable galacturonosyltransferase 7 isoform X1 [Solanum lycopersicum]
Plant hormone signal transduction		
Capana01g001914	down	hypothetical protein JCGZ_23114 [Jatropha curcas]
Capana10g002513	down	PREDICTED: ABSCISIC ACID-INSENSITIVE 5-like protein 2-like isoform X1 [Solanum tuberosum]
CapsicumannuumL_newGene_9344	down	PREDICTED: protein TIFY 4A-like [Solanum tuberosum]
Capana01g003720	down	PREDICTED: jasmonate ZIM-domain protein 3 isoform X1 [Solanum lycopersicum]

Supplementary Table 1. Contd.

Capana09g000285	down	IAA8 [Solanum lycopersicum]
Capana10g002328	down	PREDICTED: regulatory protein NPR5-like [Solanum tuberosum]
Capana06g003038	down	PREDICTED: protein TRANSPORT INHIBITOR RESPONSE 1-like isoform X1 [Solanum tuberosum]
Capana10g001576	down	PREDICTED: serine/threonine-protein kinase CTR1-like isoform X1 [Solanum tuberosum]
Capana12g002655	down	serine/threonine-protein kinase SAPK1-like [Solanum tuberosum]
Capana08g001056	down	PREDICTED: auxin response factor 9-like isoform X3 [Solanum tuberosum]
Capana08g001036	down	PREDICTED: probable indole-3-acetic acid-amido synthetase GH3.5 [Solanum lycopersicum]
Capana10g000405	down	PREDICTED: probable indole-3-acetic acid-amido synthetase GH3.5 [Solanum lycopersicum]
Capana10g001370	down	PREDICTED: auxin transporter-like protein 4 [Nicotiana tomentosiformis]
CapsicumannuumL_newGene_9164	down	PREDICTED: protein TIFY 4A-like [Solanum tuberosum]
Capana03g002801	down	PREDICTED: protein phosphatase 2C 37-like [Nicotiana sylvestris]
Capana11g002252	down	PREDICTED: transcription factor HBP-1b(c38)-like [Solanum lycopersicum]
Capana12g000990	down	PREDICTED: protein TIFY 10A-like [Solanum tuberosum]
Capana09g001555	down	PREDICTED: auxin transporter-like protein 4 [Nicotiana tomentosiformis]
Capana01g000511	down	PREDICTED: uncharacterized protein LOC101246270 [Solanum lycopersicum]
Capana06g000011	down	PREDICTED: protein TRANSPORT INHIBITOR RESPONSE 1-like [Solanum tuberosum]
CapsicumannuumL_newGene_13152	down	PREDICTED: indole-3-acetic acid-induced protein ARG7-like [Nicotiana sylvestris]
Capana00g005021	up	leucine zipper transcription factor [Solanum tuberosum]
Capana04g000600	up	CycD3;3 protein [Solanum lycopersicum]
CapsicumannuumL_newGene_11569	up	leucine zipper transcription factor TGA [Capsicum annuum]
Capana09g000028	up	F-box protein [Capsicum annuum]
Capana00g001638	up	PREDICTED: EIN3-binding F-box protein 1-like [Nicotiana sylvestris]
CapsicumannuumL_newGene_13200	up	PREDICTED: histidine-containing phosphotransfer protein 4-like [Nicotiana sylvestris]
Capana00g004470	up	PREDICTED: probable serine/threonine-protein kinase At5g41260 [Solanum lycopersicum]
Capana10g002376	up	PREDICTED: transcription factor HBP-1b(c1)-like [Solanum tuberosum]
Capana00g001152	up	PREDICTED: ABSCISIC ACID-INSENSITIVE 5-like protein 7-like isoform X1 [Solanum tuberosum]
Capana03g003488	up	PREDICTED: gibberellin receptor GID1B-like [Solanum tuberosum]
Capana06g000051	up	PREDICTED: histidine-containing phosphotransfer protein 1-like [Solanum tuberosum]
Capana06g001979	up	PREDICTED: indole-3-acetic acid-induced protein ARG7-like [Solanum tuberosum]
Capana06g000791	up	PREDICTED: uncharacterized protein LOC101265243 [Solanum lycopersicum]
Capana02g003021	up	PREDICTED: probable indole-3-acetic acid-amido synthetase GH3.1-like [Solanum tuberosum]
Capana08g001978	up	PREDICTED: G-box-binding factor 4-like isoform X2 [Solanum lycopersicum]
Capana03g004568	up	PREDICTED: auxin-responsive protein IAA3-like [Solanum tuberosum]
Capana06g002186	up	PREDICTED: ethylene receptor 2-like [Solanum tuberosum]
Capana09g001771	up	PREDICTED: auxin-induced protein 15A-like [Solanum lycopersicum]
Capana01g003077	up	PREDICTED: histidine-containing phosphotransfer protein 1-like isoform X1 [Solanum tuberosum]
Capana03g000310	up	IAA15 [Solanum lycopersicum]

Supplementary Table 1. Contd.

Capana05g001701	up	PREDICTED: ethylene-responsive transcription factor 1B-like [Solanum tuberosum]
Capana07g001662	up	PREDICTED: probable indole-3-acetic acid-amido synthetase GH3.5-like [Solanum tuberosum]
Capana06g003043	up	PREDICTED: gibberellin receptor GID1B-like [Solanum tuberosum]
Capana03g003343	up	PREDICTED: auxin-induced protein 22D-like [Nicotiana tomentosiformis]
Capana00g000222	up	PREDICTED: DELLA protein GAI-like [Solanum lycopersicum]
Capana02g000676	up	PREDICTED: probable indole-3-acetic acid-amido synthetase GH3.1-like [Solanum tuberosum]
Capana07g000391	up	PREDICTED: auxin-responsive protein IAA20-like [Solanum tuberosum]
Capana03g001922	up	Auxin-induced protein X15 [Glycine soja]
Capana12g001341	up	PREDICTED: transcription factor TGA1 [Solanum lycopersicum]
Capana03g000749	up	PREDICTED: protein TIFY 6B isoform X3 [Solanum lycopersicum]
CapsicumannuumL_newGene_701	up	PREDICTED: shaggy-related protein kinase eta-like [Solanum tuberosum]
Capana03g004532	up	PREDICTED: ethylene receptor 2-like isoform X1 [Solanum tuberosum]
Capana04g000808	up	Aux/IAA protein [Solanum tuberosum]
Capana08g002278	up	auxin and ethylene responsive GH3-like protein [Capsicum chinense]
Capana06g000110	up	PREDICTED: auxin-induced protein 22D-like [Solanum tuberosum]
Capana05g000206	up	PREDICTED: ethylene-responsive transcription factor 1B-like [Solanum tuberosum]
Capana07g000894	up	NPR1 [Capsicum annuum]
Capana03g001668	up	PREDICTED: uncharacterized protein LOC102598616 [Solanum tuberosum]
Capana04g000996	up	PREDICTED: serine/threonine-protein kinase SAPK1 isoform X1 [Nicotiana tomentosiformis]
Capana08g002366	up	abscisic acid-insensitive 5-like protein [Solanum nigrum]
Capana04g001165	up	PREDICTED: transcription factor TGA1-like [Nicotiana sylvestris]
Capana10g002278	up	PREDICTED: two-component response regulator ARR9-like [Solanum tuberosum]
Capana07g000264	up	PREDICTED: auxin response factor 1-like [Nicotiana sylvestris]
Capana05g000287	up	PREDICTED: serine/threonine-protein kinase SAPK2-like isoform X2 [Nicotiana tomentosiformis]
Capana03g001923	up	PREDICTED: auxin-induced protein 6B-like [Nicotiana sylvestris]
Capana08g002192	up	pathogenesis-related protein PR-1 precursor [Capsicum annuum]
Phenylpropanoid biosynthesis		
Capana03g001810	up	RecName: Full=Caffeic acid 3-O-methyltransferase; Short=CAOMT; Short=COMT; AltName: Full=S-adenosysl-L-methionine:caffeic acid 3-O-methyltransferase [Capsicum chinense]
Capana12g002757	up	PREDICTED: 8-hydroxygeraniol dehydrogenase-like [Solanum tuberosum]
Capana03g001805	up	RecName: Full=Caffeic acid 3-O-methyltransferase; Short=CAOMT; Short=COMT; AltName: Full=S-adenosysl-L-methionine:caffeic acid 3-O-methyltransferase [Capsicum chinense]
Capana12g002750	up	PREDICTED: probable mannitol dehydrogenase [Nicotiana tomentosiformis]
Capana09g000319	up	PREDICTED: aldehyde dehydrogenase family 2 member C4-like [Solanum tuberosum]
Capana03g000549	up	putative hydroxycinnamoyl transferase [Capsicum annuum]
Capana02g002632	down	PREDICTED: cytochrome P450 84A1-like [Solanum tuberosum]
Capana12g002758	down	sinapyl alcohol dehydrogenase 2 [Nicotiana tabacum]
Capana10g000692	down	PREDICTED: caffeic acid 3-O-methyltransferase-like [Solanum lycopersicum]
Capana02g000251	down	PREDICTED: probable cinnamyl alcohol dehydrogenase 6 [Solanum lycopersicum]
Capana12g002751	down	PREDICTED: 8-hydroxygeraniol dehydrogenase-like [Solanum tuberosum]

Supplementary Table 1. Contd.

Capana03g000476	down	putative cinnamoyl-CoA reductase [Capsicum annuum]
Capana08g002304	up	putative cinnamyl alcohol dehydrogenase [Capsicum annuum]
Capana06g001112	up	cinnamoyl-CoA reductase [Solanum lycopersicum]
Capana00g002455	up	PREDICTED: anthocyanidin 3-O-glucosyltransferase 5-like [Solanum tuberosum]
Capana03g000357	up	PREDICTED: caffeoylshikimate esterase [Solanum lycopersicum]
Capana09g000318	up	PREDICTED: aldehyde dehydrogenase family 2 member C4-like [Solanum tuberosum]
CapsicumannuumL_newGene_16529	up	PREDICTED: 8-hydroxygeraniol dehydrogenase [Solanum lycopersicum]
Capana03g001811	up	caffeic acid O-methyltransferase [Capsicum annuum]
Capana10g002482	up	PREDICTED: cytochrome P450 98A2-like [Solanum tuberosum]
Capana07g000164	up	PREDICTED: shikimate O-hydroxycinnamoyltransferase-like [Nicotiana sylvestris]
Capana08g001159	up	putative p-coumarate 3-hydroxylase [Capsicum annuum]
Capana03g001807	up	RecName: Full=Caffeic acid 3-O-methyltransferase; Short=CAOMT; Short=COMT; AltName: Full=S-adenosysl-L-methionine:caffeic acid 3-O-methyltransferase [Capsicum chinense]
Capana02g001298	up	PREDICTED: probable cinnamyl alcohol dehydrogenase 6 [Nicotiana tomentosiformis]
Capana03g002955	up	PREDICTED: aldehyde dehydrogenase family 2 member C4 [Nicotiana sylvestris]
Capana06g000179	up	PREDICTED: caffeic acid 3-O-methyltransferase-like [Solanum lycopersicum]
Capana12g002756	up	PREDICTED: 8-hydroxygeraniol dehydrogenase-like [Nicotiana tomentosiformis]
Capana03g001803	up	RecName: Full=Caffeic acid 3-O-methyltransferase; Short=CAOMT; Short=COMT; AltName: Full=S-adenosysl-L-methionine:caffeic acid 3-O-methyltransferase [Capsicum chinense]
Capana06g000180	up	PREDICTED: caffeic acid 3-O-methyltransferase-like [Solanum lycopersicum]
Capana02g002655	up	PREDICTED: anthocyanidin 3-O-glucosyltransferase 5-like [Solanum tuberosum]
Capana02g000041	up	PREDICTED: anthocyanidin 3-O-glucosyltransferase 5-like [Nicotiana tomentosiformis]
Capana09g000321	up	PREDICTED: aldehyde dehydrogenase family 2 member C4 [Solanum lycopersicum]
Capana01g002290	up	PREDICTED: cinnamoyl-CoA reductase 1 isoform X2 [Solanum tuberosum]
Capana06g001742	up	PREDICTED: caffeic acid 3-O-methyltransferase-like [Solanum tuberosum]
Capana09g000320	up	PREDICTED: aldehyde dehydrogenase family 2 member C4-like [Solanum tuberosum]
Capana04g002317	up	PREDICTED: cytochrome P450 84A1-like [Nicotiana tomentosiformis]
Capana02g000042	up	PREDICTED: anthocyanidin 3-O-glucosyltransferase 5-like [Nicotiana sylvestris]
Capana03g001804	up	PREDICTED: caffeic acid 3-O-methyltransferase [Nicotiana sylvestris]
Capana06g000178	up	PREDICTED: caffeic acid 3-O-methyltransferase-like [Solanum tuberosum]
Capana08g002072	up	PREDICTED: acetyl-CoA-benzylalcohol acetyltransferase-like [Nicotiana sylvestris]
Capana10g002485	down	PREDICTED: cytochrome P450 98A3-like [Solanum tuberosum]
Capana03g002214	down	PREDICTED: acetyl-CoA-benzylalcohol acetyltransferase-like [Nicotiana sylvestris]
Capana10g001996	down	PREDICTED: cytochrome P450 98A3-like [Nicotiana tomentosiformis]
Capana11g001541	down	acyltransferase 1 [Capsicum chinense]
Capana12g000381	down	PREDICTED: acetyl-CoA-benzylalcohol acetyltransferase-like [Nicotiana tomentosiformis]
Capana05g002521	down	PREDICTED: BAHD acyltransferase At5g47980-like [Solanum tuberosum]
Capana06g001583	down	PREDICTED: deacetylvindoline O-acetyltransferase-like [Nicotiana sylvestris]
Capana00g002716	down	PREDICTED: cytochrome P450 98A2-like [Solanum tuberosum]
Capana09g000119	down	PREDICTED: shikimate O-hydroxycinnamoyltransferase-like [Nicotiana sylvestris]
Capana02g002339	down	acyltransferase [Capsicum frutescens]
Capana00g002692	down	PREDICTED: deacetylvindoline O-acetyltransferase-like [Solanum lycopersicum]

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Germination studies on seeds of *Burkea africana* and *Erythrophleum africanum* from Kazuma Forest Reserve, Northern Botswana

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***Burkea africana* and *Erythrophleum africanum* are characterized by seed coat-imposed dormancy that prevents water entry and gaseous exchange, which are essential for the germination process. The objective of this study was to determine the best possible pre-sowing treatment method that maximizes seed germination of the two species. Seeds of both species were subjected to four experiments, containing 10 levels of presowing treatments: The control, mechanical scarification, soaking in concentrated sulphuric acid (for 15, 30, 45 and 60 min), immersion in boiling water (for 1, 3 and 5 min), and soaking in boiling water (and cooling down for 24 h). The germination data were subjected to ANOVA followed by Tukey's HSD Test to separate significantly different treatment means. The most promising results showed that seeds treated with mechanical, sulphuric acid and boiling water scarification had significantly higher mean percent germination than the controls for *B. africana*; whereas for *E. africanum*, mechanical scarification, exposure to sulphuric acid, boiling water (1 min) and immersion in boiling water (and cooling down for 24 h) had higher percent germination than the controls.**

Key words: *Burkea africana*, *Erythrophleum africanum,* germination percentage, pre-sowing treatment, seed dormancy.

INTRODUCTION

Over the past few years, Botswana has put considerable efforts into forest conservation and afforestation programmes, such as the annual national tree planting day. This day dates back to 1985 when the then President Sir Ketumile Masire launched the first national tree planting day and has since been commemorated on the last Saturday of November each year (BOPA, 2013). At inception of the tree planting day, exotic tree species were planted in community woodlots and distributed for planting by individuals. Exotic species were promoted

because they establish easily, grow fast and are highly productive, especially on harsh sites where native tree species do not perform well (Dodet and Collet, 2012). They are highly productive because pests from their native habitats are absent (Nair, 2001). These characteristics contribute to their ability to invade local ecosystems (Dodet and Collet, 2012) and are a threat to native biodiversity (Bellard et al., 2016). Afforestation using exotic species has long been beneficial to the environment, and the aim of using exotic species was to repair damaged ecosystems (Richardson, 1998).

The use of indigenous tree species in afforestation and reforestation programmes is increasing world-wide (McNamara et al., 2006; Shono et al., 2007a; Raman et al., 2009). Similarly, Botswana has also been promoting their use in recent years (Rasebeka et al., 2014) because they cope well with prevailing harsh environmental conditions. However, the use of indigenous species in planting programmes is limited by the availability of quality planting materials (Elliott et al., 2002; Meli et al., 2014). There is need to identify indigenous tree species with readily available seed and propagation techniques that are suited to local environments (Shono et al., 2007b; Doust et al., 2008; Lamb, 2011; Meli et al., 2014).

Burkea africana Hook. also known as *monato, mosheshe, Ohehe, nkalati* in Botswana (Setshogo, 2002), burkea red syringa, Rhodesia ash, sand syringa, wild seringa and wild syringa (English) (Setshogo, 2002; Maroyi, 2010) belongs to the family Fabaceae (Caesalpinioideae) (Palmer and Pitman, 1972; Palgrave, 2002; Neya et al., 2004; Maroyi, 2010). The species is distributed throughout tropical Africa (Neya et al., 2004; Mair et al., 2018), from Senegal to Sudan and as far as South Africa (Maroyi, 2010). It has a flat-top and grows up to 61 cm in diameter and 20 m high (Fanshawe, 1972). The species grows naturally in open, wooded grassland and open woodland (Maroyi, 2010; Tanko et al., 2011) on sandy soil and lower slopes on rocky hills in the high rainfall areas, occasionally in miombo woodland (Mulofwa et al., 1994). The wood of *B. africana* is hard, heavy and is used in constructional work such as bridges, sleepers, furniture, firewood, charcoal, fences and tool handles (Neya et al., 2004). The heartwood is very resistant to fungi (Neya et al., 2004). The bark, roots and leaves are used as medicine (Mulofwa et al., 1994; Mathisen et al., 2002). The bark has been used in medicine to treat colds, coughs, and constipation, gonorrhoea and syphilis (van Wyk and Gericke, 2007). *B. africana* is planted as a roadside tree and ornamental (Maroyi, 2010). It is host to caterpillars of Saturnid moths (*Cirina forda* and *Rohaniella pygmaea*), which are eaten by local people. The flowers produce nectar collected by honeybees (Mulofwa et al., 1994). The bark and leaves are eaten by elephants and the tree yields a semi-translucent gel or green gum of high quality (Roodt, 1998).

Erythrophleum africanum (Welw. ex Benth.) Harms is known as *mmako, mobaku, ununza, mopombo and mokong ochi* in Botswana (Setshogo, 2002) as well as African blackwood and ordeal tree in English and belongs to the family Fabaceae (Caesalpinioideae) (Burkill, 1995; Setshogo, 2002). It is a medium-sized to large tree growing up to 15 m high (Palmer and Pitman, 1972; Palgrave, 2002). It has a straight and cylindrical stem, up to 120 cm in diameter, and a dense and spreading crown (Kawanga, 2008). The bark is grey in colour and smooth in young trees and becoming red-brown, rough and fissured with age (Kawanga, 2008; Maroyi, 2019). The leaves are alternate, egg-shaped to oblong, finely velvety, particularly when young and on the under surface. The apex of the leaf is broadly tapering to rounded or notched and the base is broadly tapering with entire margins (Kawanga, 2008; Maroyi, 2019). Flowers are cream to yellow in colour, sweetly scented, occurring in dense spikes and often grouped together in large heads. The fruit is a pod, splitting along both sides simultaneously and each section curving backwards (Kawanga, 2008; Maroyi, 2019). It is indigenous to tropical Africa (Lock, 1989; Burkill, 1985; Germishuizen and Meyer, 2003; Smith and Allen, 2004; Hyde et al., 2020). The species grows naturally in hot and dry deciduous woodlands at 600 to 1400 m above sea level, and is absent from riparian woodlands and the dry savanna of the Sahel (Kawanga, 2008). It is indigenous to tropical Africa (Lock, 1989; Burkill, 1985; Germishuizen and Meyer, 2003; Smith and Allen, 2004; Hyde et al., 2020). The wood is used for furniture, heavy and light construction, posts, poles and tool handles. In addition, it is used for firewood and making high quality charcoal. The bark, roots and leaves are used in medicine. An infusion of the bark is drunk to treat stomach-ache or dysmenorrhoea. Steeped in water, the bark is applied externally and internally to cure cardiac diseases and epilepsy. The powdered root bark, mixed with urine, is applied to the skin to treat leprosy and a paste of root-bark is applied to the skin to cure scabies (Kawanga, 2008).

Germination of seed is important in propagating seedlings for mass planting of woody plant species. However, it can be a time-consuming process because seeds of some plants take a longer time to germinate, or may fail to do so under some culture regimes. According to Botumile et al. (2020), a high level of seed dormancy is a characteristic feature of many plants of dry regions, and

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it either completely prevents germination or allows very few seeds to germinate over a long period of time. Seed dormancy is an adaptive mechanism that blocks the germination of intact viable seeds under conditions when the chance of seedling survival and growth is low (Weibrecht et al., 2011; Smýkal et al., 2014; Long et al., 2015). Seeds of many leguminous plants have hard coats, which make it difficult for the seeds to imbibe water and prevent gaseous exchange (Bolingue et al*.,* 2010). In nature, hard seed coats are cracked or softened by fire (Mbalo and Witkowski, 1997; Walters et al., 2004), extreme temperatures, digestive acids in the stomachs of animals or the abrasion of blowing sand (Luna et al., 2009) that can promote germination.

Hard seed coat-imposed dormancy of leguminous species hinders their successful artificial regeneration (Teketay, 1996a, b; Mojeremane et al., 2017, 2018; Odirile et al., 2019; Setlhabetsi et al., 2019). Several pre-sowing treatments have been used to enhance germination of seeds characterised by hard coats. These include mechanical, acid, cold, hot and boiling water scarification (Teketay, 1996a, b, 1998, 2005; Alamgir and Hossain, 2005; Amri, 2010; Azad et al., 2011; Rasebeka et al., 2014; Fredrick et al., 2017; Kahaka et al., 2018; Opoku et al., 2018; Botumile et al., 2020), among others. These techniques can improve germination by overcoming seed dormancy within a relatively short period of time (Tadros et al., 2011; Mojeremane et al., 2017, 2018; Odirile et al., 2019; Setlhabetsi et al., 2019).

B. africana and *E. africanum* are among the excellent candidate species for introducing in planting programmes in dry regions, because of their multiple uses and adaptation to the local environment. The hard seed coat is seen as a hindrance to uniform and rapid germination of tree and shrubs species, hence, there is a need for pre-sowing seed treatments to enhance germination. Therefore, the objective of this study was to determine some of the best possible pre-sowing treatment methods that maximize the germination of *B. africana* and *E. africanum* seeds.

MATERIALS AND METHODS

Study site

The experiment was conducted in the laboratory at the Botswana University of Agriculture and Natural Resources (BUAN) from January to February, 2019. The university is located at Sebele (23°34' S and 25°57' E, altitude of 994 m), approximately 10 km from the Centre of Gaborone, the capital city of Botswana along the A1 North-South highway.

Seed source

Seeds were collected from Kazuma Forest Reserve (18. 4259° S and 25.4970 E, altitude 997 m) in the Chobe district during August 2018. Mature and healthy fruits/pods were collected from the tree crown by shaking with a long-hooked stick. The mature dry pods were placed in paper bags and transported to the Department of Range and Forest Resources Laboratory, Botswana University of Agriculture and Natural Resources. Seeds were extracted by crushing the pods by hand, followed by winnowing to separate the husk. Seeds were kept refrigerated at 5°C for four months awaiting commencement of experiments. Prior to sowing, seeds were tested for viability using the floating method, in which the floated seeds were considered unviable and discarded.

Experimental design and treatments

In this study, four experiments, containing 10 levels of treatments, including the control, were carried out. The four experiments were mechanical scarification, exposure to sulphuric acid, exposure to boiling water and exposure to hot water for 24 h. The experiments were laid down in completely randomized design having four replications.

Experiment 1: Mechanical scarification

In this experiment, 100 seeds of each studied species, with four replications of 25 seeds, were used. In all these seeds, a pair of scissors was used to cut way 1 to 2 mm of the seed coat on a convex edge opposite where the embryo is located and avoiding removal of endosperm as much as possible.

Experiment 2: Exposure to sulphuric acid

In this experiment, four periods of exposure of seeds of the studied species using sulphuric concentrated sulphuric acid (98%), that is, 15, 30, 45 and 60 min, were used by employing the method described by Teketay (1996a). For each period of exposure, the four replications of 25 seeds were put into four 100-ml, heat-resistant, non-corrosive glass beakers containing sulphuric acid by making sure that all the seeds were covered by the acid. Seeds were hand stirred every 5 min during the specific treatment time to ensure their uniform exposure to the acid. After the specified periods of exposure, the seeds were sieved out of the acid using an acid-resistant sieve, while the acid was drained off simultaneously into another beaker. Seeds were, then, thoroughly washed and rinsed to remove acid using running water tap first and subsequently using distilled water, successively.

Experiment 3: Exposure to boiling water

In this experiment, three periods of exposure of seeds of the studied species, that is, 1, 3 and 5 min, to boiling water were used. For each period of exposure, four replications of 25 seeds were put into four separate coffee filter papers and immersed into a cooking pot with boiling water for the specified period, after which they were removed and immersed in a small bucket containing room temperature distilled water to cool them down for a few minutes.

Experiment 4: Exposure to boiled water for 24 h

In this experiment, four replicates of 25 seeds were put into four separate coffee filters and placed into a 250 ml beaker. Boiling water was, then, poured into the beaker and left to cool with the seeds inside for 24 h.

Four replications of 25 untreated seeds were used as control for all the experiments. In all the experiments and the control, each replication, containing the 25 seeds, was placed in 8-mm closed Petri dishes lined with cotton wool. The cotton wool was

Table 1. Means and ranges of the cumulative germination of seeds of the study species subjected to different pre-sowing seed treatments (± standard error of the means).

Treatment	*Burkea africana*		*Erythrophleum africanum*	
	Germination (%)	Range	Germination (%)	Range
Control	5 ± 1^{e}	04 - 08	17 ± 3^{e}	12 - 24
Mechanical Scarification	80 ± 4^{abc}	72 - 92	90 ± 6^{abc}	72 - 100
Sulphuric Acid (15 min)	74 ± 8^{abc}	52 - 84	58 ± 4^{d}	52 - 68
Sulphuric Acid (30 min)	73 ± 9^{abc}	48 - 92	98 ± 1^{a}	96 - 100
Sulphuric Acid (45 min)	93 ± 4^{a}	84 - 100	96 ± 2^{ab}	92 - 100
Sulphuric Acid (60 min)	92 ± 2^{ab}	88 - 96	95 ± 3^{abc}	88 - 100
Boiling Water (1 min)	50 ± 6^{cd}	36 - 64	72 ± 4^{bcd}	64 - 80
Boiling Water (3 min)	62 ± 7^{bcd}	52 - 84	49 ± 6^{de}	32 - 60
Boiling Water (5 min)	66 ± 7^{bcd}	48 - 80	13 ± 3^{e}	08 - 20
Boiling Water (allowed to cool in 24 h)	37 ± 7^{de}	28 - 56	65 ± 12^{cd}	36 - 92

Means separated using Tukey's Honestly Significant Difference (HSD) Test at $P \leq 0.05$. Means within columns followed by the same letters for each species are not significantly different.

continuously kept moist by adding distilled water whenever necessary until the end of the experiments. Seeds were considered to have germinated when the radicle penetrated the seed coat and reached 1 to 2 mm. The germinated seeds were counted and recorded on daily basis. The germinated seeds were removed from Petri-dishes after counting and recording. The experiments were terminated after 30 days.

Data analyses

Data collected on germinated seeds were used to calculate germination percentage (GP), for each treatment using the equation:

Germination percentage=(Total number of seeds germinated/Total number of seeds sown)×100

The data collected were subjected to both descriptive statistics and one-way analysis of variance (ANOVA) using Statistix Software, Version 10 (Statistix 10, 1984-2003). Before the ANOVA, the germination percentage data were arcsine transformed to meet the requirement of normality (Zar, 1996). Significant differences of means were tested using Tukey's Honestly Significant Difference (HSD) at the significance level of $P < 0.05$.

RESULTS

Germination of seeds

The results indicated that seeds treated with mechanical scarification, sulphuric acid and boiling water had significantly higher mean germination percentages than the control in *B. africana* [(One Way ANOVA: ($F(9, 39) = 15.86$, $P = 0.00001$)]. For *E. africanum* mechanical scarification, sulphuric acid, boiling water (1 min) and hot water (boiling water allowed to cool for 24 h) had significantly higher mean germination percentages than the control [(One Way ANOVA: ($F(9, 39) = 22.19$, $P = 0.00001$)] (Table 1). The ANOVA also indicated that there were significant differences among the different treatment times and conditions further clarified by the HSD significant differences within and among the treatment means (Table 1) as explained more fully in the following paragraph.

The highest mean germination percentages (93 and 92%) for *B. africana* were found in sulphuric acid (45 and 60 min) treatments, followed by those exposed to mechanical scarification (80%), sulphuric acid for 15 (74%) and 30 (73%) minutes as well as boiling water for 5 (66%), 3 (62%) and 1 (50%) min, respectively. Results of seeds immersed in hot water for 24 hours showed no significant effect on the germination of seeds compared with the control (Table 1). For *E. africanum,* the sulphuric acid (30, 45 and 60 min) treatments had the highest mean germination (95-98%), followed by mechanical scarification (90%), those treated in boiling water (1 min) (72%), hot water (boiling water allowed to cool in 24 h) (65%) and sulphuric acid (15 min) (58%). Boiling water treatments (3 and 5 min) had no significant effect on the germination of seeds (Table 1).

Seed germination rate

The results showed that seeds of *B. africana* that were treated with sulphuric acid (for 45 and 60 min) exhibited the fastest and uniform germination, reaching > 90% cumulative germination within five days after sowing, followed by mechanical scarification, reaching > 78% within 8 days and those treated with sulphuric acid (for 30 and 15 min), reaching > 71% and > 65% within seven days, respectively (Figure 1). On the other hand, untreated seeds (control) and seeds treated with hot water exhibited, not only the lowest germination percentage, but also the slowest germination.

The results also showed that in the case of

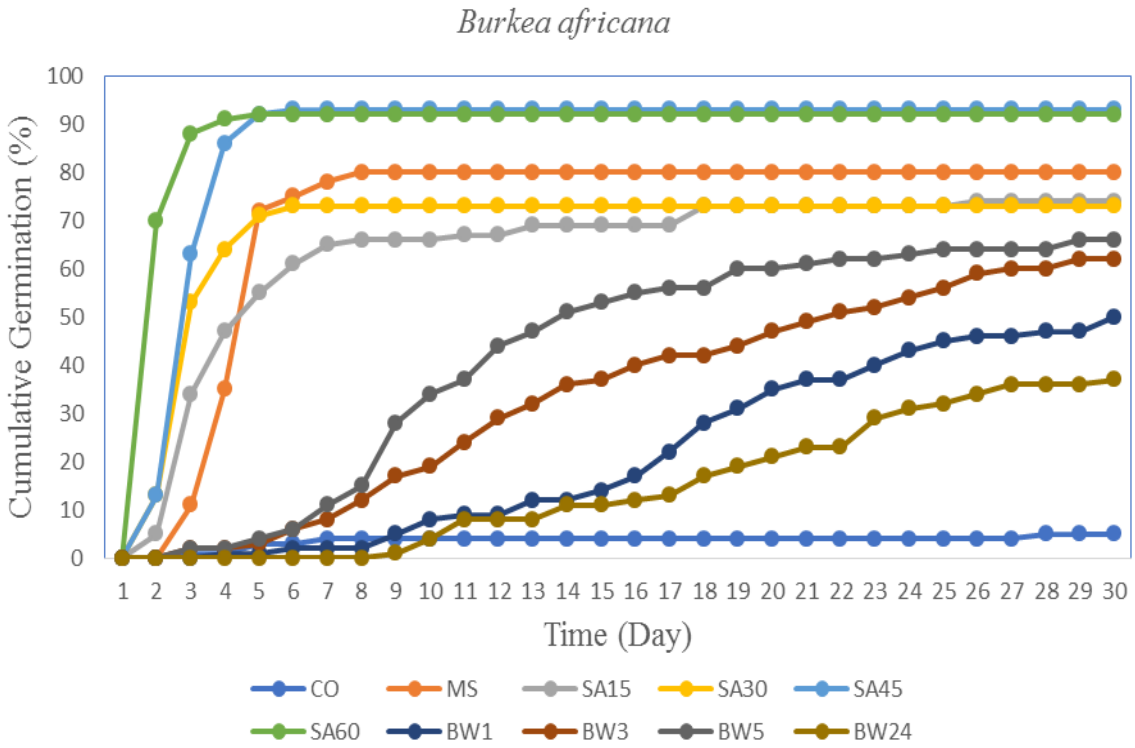

Figure 1. Cumulative germination percentage of *B. africana* recorded for 30 days (CO = Control, MS = Manual scarification, BW1 = Boiling water for 1 min; BW3 = Boiling water for 3 min, BW5 = Boiling water for 5 min, HW24 = Boiling water allowed to cool in 24 h, SA15 = Sulphuric acid for 15 min, SA30 = Sulphuric acid for 30 min, SA45 = Sulphuric acid for 45 min and SA60 = Sulphuric acid 60 min).

E. africanum, seeds treated with sulphuric acid (for 30, 45 and 60 min) and mechanical scarification exhibited the fastest and uniform seed germination, reaching > 95% and > 80% cumulative germination within five days, respectively (Figure 2). On the other hand, seeds treated with boiling water (5 min) and the control exhibited not only the lowest, but also the slowest germination.

DISCUSSION

Different techniques of breaking seed dormancy, in order to improve germination rate and speed up the germination process, have been suggested by other authors (Airi et al., 2009; Azad et al., 2010). Results of this study indicated that *B. africana* and *E. africanum* are characterized by physical seed dormancy imposed on the seeds by a water-impermeable seed coat. Mechanical scarification proved to be one of the most effective methods to break dormancy for both the two study species compared with the controls (Table 1). Removing 1-2 mm of the seed coat allows the seed to imbibe water, hence promoted radicle emergence. Once the seed imbibed water, the cumulative germination was improved significantly, and became more rapid and uniform. This result is consistent with work carried out on other leguminous plant species (Teketay, 1996a, 1998; Tigabu and Odén, 2001; Sy et al., 2001; Alamgir and Hossain, 2005; Rodrigues-Junior et al., 2014; Naim et al., 2015; Boateng, 2017; Fredrick et al., 2017; Mojeremane et al., 2017, 2018; Odirile et al., 2019; Botumile et al., 2020). Teketay (1996a) reported that mechanical scarification enhanced seed germination for most leguminous species. Tigabu and Odén (2001) recorded 100% germination in *Albizia gummifera* seeds and 80% in *Albizia grandibracteata* compared with <10% germination of the untreated seeds. Mackay et al. (1995) also recorded 100% germination for mechanically scarified *Lupinus havardii* seeds. Botumile et al. (2020) obtained 96% germination for *Vachellia robusta* (Burch.) Kyalangalilwa and Boatwright and 88% for *Senegalia galpinii* (Burtt Davy) Seigler and Ebinger seeds. Mechanical scarification is a safer and more practical technique for scarifying few seeds. The technique is simple and effective in promoting rapid and uniform germination (Odirile et al., 2019). However, it requires a lot of time, especially if scarifying many seeds (Mapongmetsem et al., 1999; Himanen et al., 2012; Baskin and Baskin, 2014; Müllera et al., 2017). According to Mmolutsi et al. (2020), it is also possible to damage the endosperm, cotyledons or embryo during nicking, which could result in low germination.

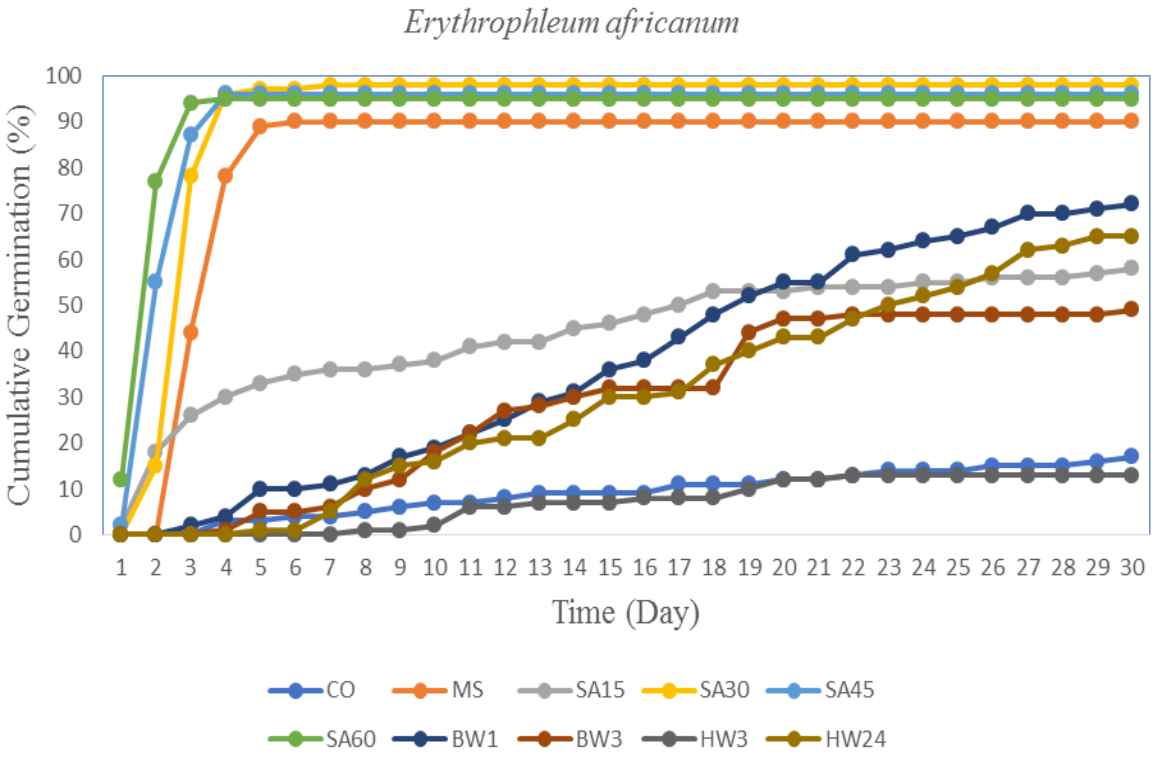

Figure 2. Cumulative germination percentage of *E. africanum* recorded for 30 days (CO = Control, MS = Manual scarification, BW1 = Boiling water for 1 min; BW3 = Boiling water for 3 min, HW5 = Boiling water for 5 min, HW24 = Boiling water allowed to cool in 24 h, SA15 = Sulphuric acid for 15 min, SA30 = Sulphuric acid for 30 min, SA45 = Sulphuric acid for 45 min and SA60 = Sulphuric acid 60 min).

Sulphuric acid enhanced germination in *B. africana* and *E. africanum* compared with the controls (Table 1). Sulphuric acid is one of the most effective pre-sowing treatments for seeds with very hard coats. The acid wears out the thick seed coat and allows water to enter the seeds and trigger germination, which is more rapid and uniform. The results of the sulphuric acid treatments on the two study species are supported by similar studies conducted on other leguminous species elsewhere (Teketay, 1996a, 1998; Sy et al., 2001; Rincón-Rosales et al., 2003; Cirak et al., 2004; Phartyal et al., 2005; Aref et al., 2011; Nasr et al., 2013; Fredrick et al., 2017; Mojeremane et al., 2017; Odirile et al., 2019). Although the sulphuric acid treatments are more effective methods for many tropical leguminous trees, the sulphuric acid used is expensive and a very dangerous and abrasive chemical to people and materials (Doran et al., 1983) as well as a potential pollutant of the environment unless properly disposed of. The acid needs to be handled with great care observing safety rules (Schmidt, 2007). Safety glasses, gloves and other protective clothing must be worn, and if possible, a fume cabinet used because inhaling the fumes is very harmful (Luna et al., 2009). There is also a possibility of damaging seeds by over soaking (Nasr et al., 2013). Disposing the waste acid safely can be serious challenge in some areas.

Hot water (boiling water allowed to cool for 24 h) increased the germination of *E. africanum* seeds compared with that of the control (Table 1). Soaking of seeds of *E. africanum* in hot water might have softened the seed coats and allowed for the imbibition of water. In contrast, *B. africana* seeds soaked in hot water (boiling water allowed to cool for 24 h) did not differ significant from the control. These contrasting results have been reported in other studies elsewhere (Albrecht, 1993; Teketay, 1996a, 1998; Sharma et al., 2008; Mwase and Mvula, 2011; Botsheleng et al., 2014; Fredrick et al., 2017; Mojeremane et al., 2017; Botumile et al., 2020). Studies have shown that the effectiveness of hot water in improving seed germination vary with species (Tigabu and Oden, 2001; Teketay, 2005). For seeds treated in hot water at 100°C, Sharma et al. (2008) reported germination of 94 to 100% in *Albizia lebbeck* (L.) Benth.), *Albizia procera* (Roxb.) Benth., *Peltophorum pterocarpum* (DC.) Backer ex Heyne, *Acacia auriculiformis* A. Cunn. ex Benth. and *Leucaena leucocephala* (Lam.) de Wit. Albrecht (1993) reported that treating seeds for 24 h in hot water at 100°C enhanced percent germination of *Adansonia digitata* L., *Calliandra calothyrsus* Meissner and *Sesbania sesban* (L.) Merr. Botumile et al. (2020) also reported that hot water improved percent germination in *Vachellia karroo* (Hayne) Banfi & Galasso

compared with the control. According to Mwase and Mvula (2011), hot water softens hard seed coats, leaches out chemical inhibitors and allows imbibition and gaseous exchange. Mojeremane et al. (2017) found that hot water was not effective in improving percent germination of *Vachellia rehmanniana* Schinz just like those of *B. africana* in the present study. According to Teketay (1996a) the degree of the seed coat hardness among different species is the cause of different responses to various treatments. The poor performance of *B. africana* in the hot water treatment could be due to the thickness of the seed coat, which failed to break before the water cooled down. The fact that boiling water treatments (experiment 3 in this study) improved germination is evidence that the species is characterised by hard coat-imposed dormancy.

Boiling water (for 1, 3 and 5 min) was effective in increasing percent germination in *B. africana* compared with controls (Table 1). Results indicated that percent germination in this species increased with exposure time, suggesting physical dormancy imposed by the hard seed coat. In the case of *E. africanum,* percent germination was increased by treating seeds in boiling water (1 min) compared with the control. There were no significant differences in percent germination among the boiling water (3 and 5 min) treatments and the control (Table 1). Results show that percent germination decreased with increase in exposure time to boiling water. This result is consistent with Botumile et al. (2020) who reported a decrease in percent germination with increasing exposure time up to 5 min with boiling water for *Senegalia galpinii* and *Vachellia robusta.* Similar results were also reported for *Vachellia karroo* (Mmolutsi et al., 2020). The decline in percent germination with increase in boiling time could be due to the sensitivity of seeds to the heat, which might have damaged the embryo.

Conclusion

Dormancy in the legume species is mainly caused by their hard seed coat covering which prevents water uptake and gaseous exchange. Therefore, the hard seed coat needs to be subjected to pre-sowing treatments before seeds can germinate. The study has shown that the hard seed coat in *B. africana* can be overcome by mechanical scarification, exposure to sulphuric acid and boiling water. Seed germination in *E*. *africanum* was significantly improved by mechanical scarification, exposure to sulphuric acid, boiling water (1 min) and hot water (boiling water allowed to cool for 24 h). The results also indicated that sulphuric acid and mechanical scarification treatments resulted in the highest, fastest and uniform germination percentages relative to the control and boiling water treatments. Therefore, extension agents and researchers that have plans to raise seedlings of *B. africana* should consider scarification treatments using mechanical scarification, sulphuric acid and boiling water before sowing. For *E. africanum,* they should subject seeds to mechanical scarification, sulphuric acid and boiling water (1 min) and hot water. Mechanical scarification and boiling water treatments are recommended for farmers and nurseries since they are safer and require less skill to administer; while sulphuric acid treatments can be used in research laboratories. When using mechanical scarification, care should be taken to ensure that the scarification treatment does not bruise the endosperm or the embryo since it could lead to fungal attack and death of the seed.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Earning trust and building credibility with a new paradigm for effective scientific risk-benefit communication of biotechnology innovations

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Biotechnology has tremendous potential to transform agriculture and contribute significantly to reduce hunger, malnutrition and food insecurity. Many benefits of biotechnology have been recorded over the years, yet the fears surrounding biotechnology adoption persist in the same measure. Unfortunately, the reasons for opposition to biotechnology have remained the same over the years. The developing world has become a battle ground up for grabs by the proponents and opponents of biotechnology adoption for years. Change in public perception and acceptance of biotechnology has been minimal in spite of the strides experienced by some countries in its adoption. The missing ingredient is inadequate targeted communication that relates to the risks and benefits of the technology to sway the decision making processes to accelerate adoption of biotechnology in the developing countries. To do this, a new paradigm that understands the need for effective strategy in and the role of scientists is needed. The new paradigm should also entail embedding of communication in curriculum and training courses. This paper offers the concepts of a new paradigm of risk benefit communication to enhance success of the technology adoption in Kenya and Africa by extension.

Key words: Risk communication, Biotechnology, Genetic Engineering, GMOs, Biosafety, Food Safety, Developing countries.

INTRODUCTION

Risk-benefit communication is an important component of the process of developing and commercialization of biotechnology products across several sectors; health, agriculture, environment, and industry. There has been mixed reactions to the adoption of the technology in different parts of the world and also based on the distinction of these industries. For decision, making both risk communication and balanced information sharing about the benefits of biotechnology is equally crucial. At the same time, for decision making and implementation of biotechnology especially in developing countries, the target audience whether policy makers, regulatory agency, technical players or the general public is crucial as this determines depth, nature, and method of delivering the information. The reality is that risk communication is and should be treated as a two way

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process that involves receiving fears and concerns of stakeholders and designing mechanisms and processes to deliver feedback and to address them (interested and affected parties) (Koch and Massey, 2011). Scientific and technical matters may be dominant themes in risk communication but wider concerns including socio-economic nature usually play an important role as well. There are 3 important entities in risk communication: government and regulatory agencies communicate risk as developers and implementers of ensuing regulations, whereas product developers do so with regard to introduction of new products, risk management measures, and possible liability. Activists on the other hand use risk communication to raise concerns and to support their positions on the activities and products they are opposed to. The media since they thrive on market ability of negative information (fears and controversy) become regular purveyors of risk communication. This is the more reason for credibility and trustworthiness of risk communicators. Scientists usually take an important slot in ensuring that there is evidence-based communication.

Society is going through a phase of so much mistrust. Biotechnological solutions have unfortunately fallen into this space of mistrust. This distrust has been infused into the biotechnology space and has wreaked havoc literally as it has led to diminishing of noble efforts geared toward poverty, hunger and malnutrition elimination especially in developing countries (Bailey et al., 2014; Caulfield et al., 2006). According to Koch and Massey (2011), risk communication around biotechnology has not been effective.

It remains true that scientists or organizations' ability to offer constructive communication is based on whether the audience perceives it as trustworthy and believable or not. According to Covello (1992), public assessment whether a source can be trusted and believable is asked on these key indicators: ability to express empathy, caring, competence, expertise, honesty, openness, dedication, and commitment. These factors act to build the foundation of trust. Trust and credibility are the most difficult to gain in this biotechnology discourse. Once lost, it is even more difficult to regain (Dunn, 2019). In deed the history of distrust of the biotechnology industry and its persistence is a very clear indication of this very fact that trust has never really been built (Kozubek, 2017).

In this paper, we critically look at what strategies to employ especially in delivering effective science and risk-benefit communication. Apart from taking communication lobbying, and consensus building as an integral part of scientists' research plan, we also advocate scientists to build architecture of communication in relation to, understanding the decision making process in the target organization or governments so as to achieve the necessary success (France and Gilbert, 2019). This strategic planning on communication cannot be un-ethical but a necessary mechanism to ensure that the technology finally achieves its intended goal of changing lives especially for small holder farmers in the developing countries (Besley et al., 2017). Finally, this paper presents a new paradigm for science communication aimed at dramatically improving the odds of success and adoption of modern biotechnology products in Kenya and Africa at large.

MATERIALS AND METHODS

Mixed multi-dimensional data collection methods and tools were used in this study. Primary data was collected through interactive semi-structured interviews and focused group discussions, and interviews with key informants. Secondary data collection was done through desktop reviews, and informally through the various activities (observing Lecturers, YouTube interviews with communicators) and events organized for Dr. Norman E. Borlaug Fellowship during a 3-month period at Michigan State University. Secondary data was collected through desktop collation and analysis of publications, documentaries and documents from the leading science communication experts and websites that were made available through the Michigan State University Library and other related organizations. Additional primary data was collected through a biotechnology industry survey using a semi-structured questionnaire. Key Informant Interviews (KII) was conducted with various players in including, farmers in Michigan State, Science communication and Biotechnology Professors from Michigan State University, biotechnology companies in USA, Biotechnology and related companies and NGOs in Kenya, Borlaug Dialogue delegates and other stakeholders at the 2019 World Food Prize (WFP) Symposium in Des Moines, Iowa. Direct observations were also employed to collect data on effective science communication from the numerous panelists and scientific papers' presenters at the 2019 WFP Symposium in Des Moines, Iowa, USA. The focus of the questions focused on understanding the difficulty of biotechnology communication with regard to: Facets of risk-benefit communication, the role of scientist, and premise of effective risk-benefit communication in biotechnology.

Organization of research questions in the framework for major issues in biotechnology

Food safety

Here, the study explored the question such as; are genetically modified (GM) foods and crops safe? Is the regulatory process effective and fool proof? Second is that would like to know what foods have been modified by this means? These are now available in several databases as Questions and Answers (QA). Unfortunately, many people are not aware or do not take the time to visit the websites to appreciate the scientific consensus about the evidence of safety of GM foods.

Environmental safety

While there are real and perceived risks of adoption of genetically modified organism (GMO) to the environment, the nature of risk managements put in place if well communicated could form an important basis for more acceptance of the GMO crops. As a matter of fact the general public should be invited into co-creating solutions and measures to ensure responsible adoption of the biotechnology. The study therefore explored the gaps and opportunities for

effective communication around this issue.

Third, socio-economic/ethical issues: many studies have shown that the most opposition to biotechnology has little to do with the scientific safety or view of the process. Much of the opposition is embedded in socio-economic and ethical matters disguised as science. This is why a communicator must delve beyond what is said to dig out these matters, bring them to the fore and address possible solutions together with the community. The study sought to understand the gaps in knowledge attitudes, and practice of scientists in communication and how to bridge any gaps.

The results of the study were organized as a new paradigm for successful scientific risk-benefit communication coalesced broadly into the facets of; risk communication in biotechnology, the role of scientists, and the underpinnings of risk technology debates.

RESULTS

Understanding the facets of risk-benefit communication in biotechnology

The results of this study revealed that there are basically two areas of risk-benefit communication that should be considered. The first is about the processes and regulatory framework in place to evaluate and manage the risks. For the developing countries and especially the Kenyan case, this involves communicating on the roles and regulations in place by government bodies to ensure that there is vigilance through the whole process of developing and commercialization of biotechnologies. The second area involves risk communication about the biotechnology itself (the science). This deals with specific risks-benefits about specific crops for specific applications in a specific part of the world.

Because of the foregoing, there is a tendency by the public and different members of the society even when focusing on the same aspects to draw differing conclusions and especially those that foster their position (Iraki-kipkorir, 2017). The mere fact that a communication has been made does not necessarily translate into an effective campaign. Failure to ensure success in communication is the reason many interventions have struggled. When scientists get alarmed that they communicated but nothing followed, the meaning may as well be that the audience was probably not touched and affected by the information given or that it was not the appropriate audience for that matter. It is therefore critical that an effective method for assessing effectiveness based on specific objectives be utilized by scientists involved in various biotechnology projects (ISAAA, 2020).

When disseminating information to the public, three things are important: it should be readily available, easily understandable, and interesting (with short and punchy content). Otherwise, the public will have limited interest in following through with the information. The choice of a medium is also critical: Whether one chooses television, radio, and print media such as newspapers, magazines or the internet and especially the escalating use of social media platforms (blogs, face book, twitter, and Instagram) is a key ingredient of success.

Understanding the role of scientists in earning credibility and building trust

The other result coming from the study noted that there has been a changing dynamic of public trust among different entities over the years. Scientists were once considered the most trusted and reliable source of information. However, this has changed over time; they are no longer primary source (Lemaux, 2003). Instead, the media asks politicians, clergy, and public activists on views even of the kind that require scientific knowledge that these organizations and people may be lacking. The other emerging and trusted source is the social media influencers. Which is only a recent phenomenon but whose impact is causing positive as well as negative ripples in the communication space. This is a key reason why science alone has not worn this battle of strategic information sharing. But on the other hand this offers a unique opportunity to enlist the concerns and fears of the public and thread these through the channels of communication. Gathering information and giving of feedback then become just as crucial if not more crucial than simply passing information. Scientists should accord equal amount of time if not more time to the aspect of collection of feedback from the target audience. Proactivity in communicating rather than reactivity is the key to exploiting the opportunity to take the public perceptions in and deliver influential information. Credibility and trust are earned and kept when scientist are proactive, communicate openly and respond adequately to every feedback required by the public.

Premise of the most risk communication debates

Finally, the results also revealed that the multifaceted issues characterize the debates around GMO. Public debates relating to GMO foods and crops are multifaceted. The most prominent components include discussions around: potential benefits, risk to humans health, ecosystems, farmers profits, food security, control and loss of control regarding decision making in the food system (Bailey et al., 2014). Whereas some of these challenges may be addressed through scientific data currently available, is important for scientists to be able to dig into the data and provide answers to the questions being raised on the food and environmental safety of new GMO crops and foods (Lemaux, 2003). Though we may determine scientific risks of GM crops and foods, this is only one side of the debate. Where possible, it is important to provide peer reviewed scientific facts and offer our opinion (clearly indicated as personal opinion) (Lemaux, 2003). Otherwise, real-life stories of people's experiences provide a better attention catching episodes and increases attention span of an average reader and listener (Box 1).

Box 1: Invaluable questions as a guide toward effective science communication an organization or a scientist should be prepared to ask the questions below:

- Why do we care at all about this: This is a great place for scientists to find very common grounds with their audience? In most cases, scientists have a noble relatable reason for venturing into the field with a hope to solving real societal problems. The audience will most likely be persuaded as this need becomes clear.
- Who am I (are we) and how did I/we get into the science? What is in stake for me in this: This is the place where authenticity integrity building is truly key.
- What do we know from what is available currently in the realm of the science: This allows the audience to explore with the scientists and hopefully allow them to come to independent but mutually beneficial conclusions?
- How does the public view and perceive me or our organization with regard to science being communicated: This true position of the public opinion and perception about scientist is the 1st step in drawing strategy to communicate effectively.
- What can we do together as a community: This encourages buy in, it's the place to get the fears and clarify any outstanding doubts in the minds and hearts of the audience?
- How shall we continue to partner far beyond the implementation of this project or product beyond the life cycle for sustainability: There are things we need to do and this is how we have responded. This is calling the audience to need for action and demonstrates scientists as a partner in offering a solution to a common enemy.

Box 1. Real-life stories of people's experiences.

DISCUSSION

Understanding the facets of risk-benefit communication in biotechnology

Why communicate and what is there to communicate anyway?

Millions of dollars go into the science research and technology innovation in the area of biotechnology only for the results and products to sit on the shelves for years because of lack of approvals by various governments and regulatory agencies worldwide. A good example is the Insect Resistant Maize for Africa (IRMA) Project in 1999 developed by the Kenya Agricultural Research Institute (KARI) and the International Maize and Wheat Improvement Center (CIMMYT) (Olembo et al., 2010). On the basis of pragmatism, it would make sense to balance out this expenditure by directing some of it to the bottleneck that has the potential to either delay or deny a technology from being utilized perpetually. Even when the scientist have budgeted well, it is prudent to know that success is not always about providing the people with more scientific facts but to use tried and time tested skills from the 'science of effective communication' to relay the information in a manner as to improve greatly, the odds for success (Iraki-kipkorir, 2017).

Strategies to help communicate with public audiences

The understanding of basic tenets of biotechnology provides a basis for effective communication. Finding ways to simplify the terms of biotechnology without losing meaning is critical. It is important to communicate in an easy to understand manner and terms. But we must have some basic means of stating role of genes and genetics in understanding the evolution of agriculture and foods today. The audience also needs to understand the evolution in the methods of yesterday and the ones used today verses those used centuries ago. Without these basic tenets, it will be very hard to discuss risk and benefits with the audiences (Iraki-kipkorir, 2017).

In organizing the content of training, scientists first need to put the biotechnology into the context. A good place to begin is to communicate a general theory of history of foods and agriculture. This will put biotechnology into perspective as one of the technologies that has been used by man to affect food supply such as domestication, mechanization, use of chemical inputs, processing, among others (Garvey, 2013). Evidently, each of these processes has raised questions of risk, and benefits in every era. The general understanding about how these changes have affected the food supply chain and types of foods available become critical. For example, people generally do not appreciate the reason why foods in restaurants and groceries taste the way it does. But this ignorance or lack of understanding become hindrance in raising the awareness about how the food of tomorrow may need to look and taste like. By not appreciating these changes, a majority of people then cannot appreciate biotechnology and genetically modified foods could lead to changes in our food system. The benefits of classical breeding as expressed on the basis of amount of land that would be required to produce the

Box 2: Enhancing effectiveness of biotechnology communication using analogies relatable to the audience.

Explaining genetics using simple analogies:

One important hurdle that exists between scientists and general public on risk communication is the difficulty of finding suitable and friendly terms to use for common scientific terminologies. For example, the idea of genes, DNA and genetic information presents a special challenge in many communities where communication comes in. According to pioneering work of (Lemaux, 2019), genetic information in a cell is recipe that determines what the cells can do and imparts the plants or animals' characteristics. That recipe is made up of chemical units. If each unit in a wheat plant were represented with a letter of the alphabet, it would take 1700 books of 1000b pages each to carry that information. Or if each gene in a plant is represented as a pop-it bead, the string would be about half a mile (800m). Such illustrations basing on daily relatable objects will help them to get the best understanding.

What happens when we do a genetic crossing?

The analogy can be extended to add that, in classical breeding, only half of the information is retained, and, randomly. Scientists can then enrich the information by back crossing but the breeder cannot read the information and hence cannot ascertain the kind of negative effects that may occur (Wieczorek and Wright, 2012).

The parallel idea on genetic engineering can also be communicated by intimating that 'in genetic engineering, it is possible to move only a small text like half a page (single pop-it bead) and that text can be read before it is moved'.

Of course, for classical breeding, the information must come from same species whereas in genetic engineering, the text may come from any living organism. This is only possible because all information in all organisms is written in the same language (Wieczorek and Wright, 2012). A frame work for effective sharing of the information could follow the steps below:

1. A look at how biotech is already helping and impacting agriculture (benefits and risks).
2. A look at the Biotechnology Pipeline: there are so many products of biotechnology in the market and many more are being churned out daily. There are unlimited opportunities for use especially in the pharma and medical field and there are significant experiences with this.
3. A look at regulatory structure (the testing of GMO food and environment) and the scientific organizations and international consensus regarding these crops' adoption.
4. Interjecting a bit of humour: humans receive information best if they can have an opportunity to be happy while also getting helpful feedback. Though most scientists consider science a serious and tough subject, the infusion of humour is not to water down the science but rather to build trust by relaying that scientists are human too and may even share common interests with the public including a sense of humour!

Box 2. Real matters with potential to help the scientists get a basis of relating benefits of biotechnology to the common public.

same amount of food in the USA based on the productivity of 1929, the land requirement is almost 10 times more (Fernandez-Cornejo et al., 2014). A projection of how much this can influence food and agricultural productivity in Kenya can offer some relatable insights. These are real matters that when discussed and shared with audience has potential to help the scientists get a basis of relating benefits of biotechnology to the common public (Box 2).

Starting with the end in mind: The need for communication strategy

Because of the multifaceted nature of debates around biotechnology, clarity and expected end of communication is important. It is evident that science and risk-benefit communication in particular require goal clarity about whom it is intended and for what results. Many times scientist attempt to give a summary of the

science but without taking the time to understand the audience to be targeted and the results this communication ought to achieve (Besley et al., 2017). Evidently, every player requires a unique messaging even from the same scientific data and results. For example, policy makers require different messaging, presentation, and delivery than farmer groups, lobbyists or even the media.

Having a goal for each communication will allow scientist's package targeted information to respond even to unspoken nuances by their target audience. It is also very important that scientists appreciate the fact that they are starting at a disadvantage mainly because the early part and introduction phase of biotechnology was mishandled and public was treated to tons of negative messaging that will take years to correct (Gassen, 2007). In Kenya, Health Ministry banned import of GMO foods because of an alarmist publication purporting GMOs cause cancer. Years later after the research informing the decision was retracted, and discredited, the decision still holds (MoSPH, 2012). It is very important to map out how decisions are made and who is making them whether it be advisors to the president, government ministers, or cabinet secretaries so that they can be appropriately targeted with relevant messaging to remove the fog and ease their decision-making prospects.

In the Kenyan case, communication targeting the political class with a message of how this technology would lead to solutions in the country's key primary area of food security and contribute to the manufacturing pillar of the Big 4 agenda seemed to be the kind of messaging required to allow government re-consider continued development of the biotechnology crops in the country (Vijida, 2019). The cabinet decision that allowed the country to proceed with commercialization of Bt. cotton was lauded as a positive step in the right direction. Well planned risk-benefit communication strategy will have multifaceted approach to address the specific needs of specific stakeholders and ensure that appropriate feedback is obtained.

Need for biotechnology and biosafety communication strategy more than in any other technological advancement

One wonders why in the technology space, we have accepted technological innovations some which have more proven direct harm to us with little protest as compared to the GMO crops and foods. Our study especially wanted to understand the reason why biotechnology in food applications has held such a polarizing position. Food is central, universal and almost sacred part of human beings. Anything modification affecting food touches on our beliefs, lives, culture, future, and all of these at the same time. Ignoring this nature of food and biotechnological innovations and purely focusing on the scientific benefits is the shortest route to raising resistance from the public.

For example whereas there are many technologies used in crop biotechnology including the tissue culture, marker assisted breeding, and mutation breeding, the gene insertion remains most controversial whereas the data available points that tissue culture introduces more genetic variability than the gene insertion. This experience also demonstrates that the technology is not the matter per se but rather perceptions shaped by anti GMO lobby groups and group think culture that has engulfed the world these days.

Nature of biotechnology debate and the role of scientists

Preparing scientists to drastically improve their effectiveness at risk-benefit communication

First, it is important to have in place the infrastructure for communication. Relying on traditional channels by governments like newspapers and other things may be suitable for a short while especially if they have good readability and a wider reach. In case these are missing, new channels will be in evitable to be created (Koch and Massey, 2011). This has to do with both the physical and technical structures that support ease of communication by identifying the appropriate platforms and facilitation for scientists to appear on these platforms with the right message and best possible delivery. It also involves identifying, capacity building, and training of key personnel who can be relied upon to communicate the science effectively. It involves mapping out, understanding and collecting data regarding, what kind of people, their values and fears. It encompasses the understanding of their values, needs, and interests, political as well as the socio-cultural ones. It is being able to discern what are the hidden worries that do not get to the media and yet remain extremely invaluable to the people or group (Joslyn, 2016). It may also mean mechanisms for collecting feedback on the perception of various stakeholders on the scientific community. It will also become very useful to review Biotechnology courses' offerings in degree and diploma courses with a view to infusing them with few chapters on effective communication.

From 2010 to 2012, Michigan State University (MSU) organized the *International Short Course in Science and Technology Communication*. This course at MSU stemmed from these participant concerns about the difficulty in making science communication understandable and accessible for non scientists and the general public. The course was organized as a one week intensive course which covered broadly four main areas. These courses are a good starting point as platforms for stimulating scientists to engage as

communicators of biotechnology and science in general. However, these short courses are not an end but rather a beginning and an accelerator toward a more rigorous and proactive scientific community. The aim should be to realign community to ensure that the scientists are a head of reliable, valuable and authentic information. It should be to ensure that the general public can turn to them in case of doubts or information overload.

Strategy on who is to communicate and the pillars of effective communication for scientists

There is a norm among scientific community that whoever produces the research communicates it. Most of the time, it could be the Principal Investigator (PI) or another top ranked scientist in the consortium. But who are the people to communicate? Just because one produced the research in a study, does not give one a mandate or the qualifications to be an effective communicator (France and Gilbert, 2019). Scientist must become strategic in this matter. The Institute of Food Technologists (IFT), U.S, for example have communication experts on their team that convey the IFT position on matters of food science and related industry. These communication experts must have certain characteristics of being able to connect with audience and deliver the message in an understandable and effective manner (IFT-Michele Perchonok, 2019).

Scientific knowledge alone however is powerful must not be considered as sufficient in ensuring proper risk communication (Koch and Massey, 2011). FAO (1998), had given the warning that scientific knowledge alone must not be considered flawless, value-free, and unbiased, nor should scientific knowledge be considered the only important criteria for making decisions on biotechnology adoption. For a time, scientists have been accused of arrogance and not caring about the people they aim to help with their science. The failure of biotechnology industry to introduce educational and awareness creation programmes to address the public perceptions early on, was a blunder that has ramifications to this day and may continue into the future (France and Gilbert, 2019). To succeed they must communicate respect for the concern of the beneficiaries their shared value about environment, our future, and even our children. In most countries, scientists and university professors were held in high esteem by the public even though this has been changing toward a declining trust over the years (Besley, 2017). This trust seems not to be utilized properly by scientists in communicating their research in the area of biotechnology. For effective science communication, the science community must endeavor to consistently demonstrate the following values: competence, integrity, authenticity, transparency, warmth, neutrality, and passion (Iraki-Kipkorir, 2017).

There must be careful planning to build these key aspects which together constitute the most crucial element of effective risk communication which is trust (Koch and Massey, 2011). Scientists must not just let the science speak for itself, they must be willing to share with the rest of the public who they are, their interests, and the reason they are involved in the science they do. Even so scientific results must be presented with the view that they are simply part of alternate framings and not necessarily the panacea for all our world problems. The concept of finding the correct framing has been found to be an important element and makes the difference in whether societies accept and adopt a technology or pass on it (MacArthur Foundation, 2019).

Scientists must truly understand the society's framework in order to provide a communication that is not just relatable but also actionable. For example, in some African societies, the way a technology is framed and presented will annul all the benefits. If a technology is packaged and presented as modern, western, it may simply be rejected on the framework that exists rather than by looking at its own merit. On the other hand, packaging a technology that communicates to people sense of belonging and ability to make their own independent choices without a nudging or a coercing may achieve better result even though the merits of the technology remain the same in both cases. In such a case, the framing makes all the difference (MacArthur Foundation, 2019). Where the scientists have failed to demonstrate the foregoing values, the chances of success are very dismal.

The other important aspect of communication is that the information must be accurate and evidence-based. At times the authors need to debunk the much miscommunication that has been circulated in the media and which have been taken as facts while they are inaccurate. For example, in many countries, the populace believes that the terminator gene is present in the GMOs, yet this is not true at all (Genetic Literacy Project, 2020). Sometimes starting from what your audience knows or has been convinced to believe becomes an important entry point for supplying accurate information on biotechnology.

Premise of most risk-benefit communication debates and navigation approach

Mitigating mistrust as a premise of risk communication debate

Mistrust has characterized most of the risk-benefit communication debates. Scientists can improve chances of success in communicating with their audience by drawing attention to successful examples in the neighboring countries and sharing testimonials demonstrating testimonials especially of farmers who have been successful. For example, crop biotechnology

has had over 15 years of successful implementation in some countries such as USA, Canada, India, China and in Africa, South Africa (Fernandez-Cornejo et al., 2014). This concept has been described as storytelling. Come to think of it, we all are captivated by stories (Sundin et al., 2018). There is nothing in endearing listeners and that works like telling stories that are not just as true but also captivating. Story telling is one of the best ways to take our audiences with communicators, to explore with them and to arrive at their own conclusion (Dahlstrom, 2014). According to the communication officers at the Feed the Future Innovation Lab at Michigan State University, storytelling was an important tool for reaching to audiences in biotechnology (Fierro, 2019) personal communication. Whereas story telling was part and parcel of some traditions, this art is one that is majorly either ignored or simply does not come naturally for most scientists. Yet, storytelling is one of the most effective ways to capture retain and inform and even educate audiences. In Africa, storytelling was part and parcel of the culture. The stories were so memorable as they were very informative. The stories do more than entertain and inform, they were powerful tools that shaped our morality, values, and contributed significantly to the betterment of the society. Story telling can therefore provide an effective tool to reach audiences with scientific information, through the narrative a context is provided and even complex scientific data can be synthesized (Dahlstrom, 2014).

At the same time, scientists must communicate the desire to help countries to build capacity especially where the technology to be adopted is a novel one. One also wonders whether in some cases, staggered introduction of technology: for example, firstly introducing Bt. cotton compared to maize in terms of adoption can give clear indication of where people are putting their fears and would cotton then perform a better job of introducing a GM technology in some cases. Starting off by introducing cotton rather than a staple food crop such as maize in East Africa, could be a better strategy in some cases. Finally, additional information sharing around letting audiences know about the foregone opportunities and including the loss of opportunity to observe the potential risks and fix them quickly, can form integral part of designed message (Wesseler et al., 2017).

In addition, the message too must be balanced in a manner that provides insights into suspected risks and addresses the important benefits that could accrue from the biotechnology. Careful wording is necessary to ensure a neutral voice in any risk communication. It may be helpful to distinguish risk assessment communication focused on the evaluation of risk and the decision documents that include risk assessment recommendations to help in the decision making process. This dichotomy can help scientists strive for a balanced risk communication. The information must also be sufficient and balanced. Balance is often very hard to strike. However, understanding the purpose, target audience, interest, and knowledge level, time constrains, and preferred mechanism of assessing information can be helpful factors to consider (Koch and Massey, 2011). It may be vital to follow-up any additional requested information through the provided contact list, or through virtual contact points present in websites, blogs or social media pages.

Communication in multi-institutional and multi-countries projects

Evidently, there are at any given point in time, somewhere in the world multimillion-dollar research on various biotechnologies. Sometimes these projects are funded by different organizations but within the same country. In other cases, the scientists may be working on the same thing but each one does independent work. Whereas it may not be possible at the beginning to have a centralized registry for all the different types of biotechnology projects going on, the messaging however should be same. It becomes very confusing especially in this era of interconnectedness when scientists give different messages and opinions on the same technology even when they are in different countries. Any mishap in one part of the world is instantly picked up and used as a blockade in the next country. Therefore, the least these institutions should do is to collaborate even if unofficially through their respective communication officers to realize a constant clear message to the public. Of course, there will be differences based on every country's specific requirement. But the differences are not in the science 'what to', but rather in the 'how to' and this is a marked difference.

It is also helpful to have access to a wider range of technical expertise that addresses some fears that may not be related to the technical aspects of risks of the technology. For example, some crops have passed all the biosafety tests and criteria yet concerns of the public may involve matters of possible inaccessibility of international markets through the adoption of such a technology. In such a case an expert in international trade may be the better resource to help technology adoption to overcome this hurdle (Koch and Massey, 2011).

The need for constant communication and data curating

Recently, the Kenyan Government has given approval for commercialization of Bt. cotton in Kenya. This has been received as very good news by the proponents of biotechnology and many organizations (Indeje, 2020). The fact that science has prevailed and farmers at last will be receiving good quality Bt. cotton seeds has not taken the opponents out of the way. They will be looking not just for any mishandled opportunity to raise their

voice but more so, they will be keen to explore any gaps in communication to dazzle the public and discredit all the effort being put in place to allow Bt cotton technology to work. The government must know that any gap in communication will be filled and any silence in the face of rising challenges will be interpreted in a manner that favors the opponents' course. This is the reason why the communication must be proactive, rather than reactive. This was clearly the case of the controversy leading to ban of GMOs in Kenya (Ministry of Public Health and Sanitation, 2012). The Science of GMOs came later and by then the damage had been done. The voice of farmers and the transformation they receive by growing Bt. cotton must be collected and presented in a manner that provides evidence. The result and the after stories of Bt cotton must be curated and preserved and presented in all relevant places. The government and the proponents need to receive challenges and constant feedback from the farmers and all players and address them as soon as they arise.

Conclusion

There is no technology that is 100% risk free. It is very unlikely that the various stakeholders especially (opponents) are looking for 100 risk proof technology, but rather that their concerns to be addressed in a manner that validates them. Scientists must have the understanding that the communication and the desired influence will be incremental rather than immediate. It is even probabilistic because of the many diverse, dynamic and divergent variables at play; but scientists increase their odds of winning when they are consistent and apply all the aforementioned values. Scientists can help the audience understand the need for the huge amount of biotechnology expenditure and the justification for venture capitalists and the scientific methods used to keep the cooperation and big business in check so as to stay with the scientific facts. Of late, there has been massive progress made toward public funding to allow for public-private partnerships to reduce the cost thus availing the technology more effectively at less cost. The public does not seem to be well informed concerning these new developments (Bailey et al., 2014). Yet this is an important fact that when stressed and made known and available, the government and the rest of the programmes will be well accepted. Whereas some of the topics around biotechnology innovations have experienced very heated debates, scientists must learn to neutralize the position of others without demonizing or viewing others as foolish (Gassen, 2007). Localizing data for risk-benefit communication is very important since every society offers an influence on how technologies will be perceived (Koch and Massey, 2011). In a nutshell, GMO adoption in any given country has been characterized by a champion which has been either a person or a team. It makes sense for scientists to identify this champion and team to leverage their engagement through effective communication to the public for successful adoption of a biotechnology innovations.

CONFLICT OF INTERESTS

The Authors declare that they have no conflict of interest whatsoever.

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