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Morphological and Molecular Assessments of *Sphenostylis stenocarpa* (Hochst. Ex A. Rich.) Harms. Accessions Induced Using Sodium Azide

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Abstract

Sphenostylis stenocarpa is a valuable, highly nutritious and adaptable crop. The main drawback towards its cultivation and utilization is narrowness of the germplasm available to plant breeders for its genetic improvement due to low seed yield, hard seed coat, and presence of antinutritional factors. There is limited information on its genetic improvement through mutation induction, hence the study was conducted to evaluate molecular characteristics as well as vegetative and yield performances of two accessions of *S. stenocarpa* in response to treatments. Seeds of two accessions (TSs 10 and TSs 86) of *S. stenocarpa* were sterilized, and induced with (0, 0.01, 0.03, 0.05, 0.07, 0.1, and 0.2%) concentrations of sodium azide. Treated seeds were washed, planted in Petri dishes and subsequently transferred to appropriately labeled pots. Increases in plant heights, number of leaves and number of branches were observed in both accessions while reductions were observed in plant heights, days to peduncle initiation as well as days to first and 50% flowering. Reduction observed in days to first and 50% flowering and days to peduncle initiation can lead to early maturing varieties. Twelve randomly selected primers were tested and 7 polymorphic ones were used. The number of amplicons varied from 7 to 19. The highest PIC was observed in primer OPT-07 (92%). Hence, these markers can be explored in *S. stenocarpa* breeding programs. Sodium azide at 0.07% induced increase in vegetative characters such as plant height, number of leaves as well as number of branches/plant in TSs 10 while in TSs 86 dosage of 0.01% induced reductions in reproductive characters such as peduncle length as well as days to first and 50% flowering. These concentrations can be used in future for inducing variability in these accessions. The test crop can further be genetically improved for utilization and conservation in future.

Key words: African yam bean; Morpho-agronomic traits; RAPD marker; Cluster analysis; Sodium azide.

Introduction

The genetic diversity inherent in crop species is due to natural selection on the wild parents and human intervention. An adequate knowledge of the genetic diversity inherent within crop species as well as the nature of their breeding systems is essential for crop improvement [1]. Plant improvement cannot be carried out without genetic variability, yet naturally occurring beneficial mutations are very rare, and mostly unnoticed. Therefore, it is possible to artificially induce mutations at frequencies much higher than the natural rate through the application of radiation or by chemically inducing the seeds [2]. The genetic diversity resulting from the induced mutations can be evaluated through the use of morphological traits and molecular marker techniques such as RFLPs, RAPDs or AFLPs [3]. *Sphenostylis stenocarpa* (Hochst. ex A. Rich.) Harms, known as African yam bean, is an underutilized leguminous crop in Africa capable of growing in adverse or extreme environment and produces pods and seeds that are very rich in proteins [4]. Hence, it is capable of meeting the ever-increasing protein demands of the people in sub-Saharan Africa when it is cultivated on a commercial scale [5]. It also possesses dual crop advantage in that it produces both roots and tubers [6]. The main drawback towards the cultivation and utilization of *S. stenocarpa* is the narrowness of the germplasm available to plant breeders for its genetic improvement [7], the low seed yield of the crop [8],

the hard seed coat responsible for its long cooking time [9], and the presence of antinutritional factors such as tannins, trypsin inhibitors, oxalate, phytic acid, saponins, and hydrogen cyanide [10-11].

Mutation breeding has been used to improve a number of ornamental plants, fruit crops and field crops such as tobacco, barley, rice, soybean, wheat, cotton, sunflower, groundnut, banana, grapefruit, pear etc. [12-13]. Several traits improved through this method include yield, lodging resistance, disease resistance, maturity, culm length, etc. [12]. The utilization of chemical mutagens had been described as a simple method of inducing mutation in plants for the improvement of potential agronomic traits [14]. Sodium azide is a chemical that had been reported to be highly mutagenic in plants as well as animals [15]. This mutagenicity has been reported to be mediated through the production of an organic metabolite of azide compound [16]. Presently, there is limited information on genetic improvement of *S. stenocarpa* through induced mutation with the aim of developing new germplasm.

Therefore, the main objective of this study is to assess the genetic diversity in two (2) sodium azide-induced accessions of *S. stenocarpa* using morpho-agronomic traits and RAPD analysis.

Materials and Methods

Plant material

The seeds of two accessions of *S. stenocarpa* (TSs 10 and TSs 86) were obtained from the germplasm at the Genetic Resources Centre of the International Institute for Tropical Agriculture (IITA), Ibadan, Nigeria.

Seed treatment

The uniform and healthy seeds of each accession of *S. stenocarpa* were randomly selected and treated with sodium azide using the method described by Asad *et al.* [17]. The seeds were divided into seven groups, comprising 150 seeds each, sterilized and pre-soaked in distilled water for six hours before treating with sodium azide (SA) at the concentrations of 0, 0.01, 0.03, 0.05, 0.07, 0.1, and 0.2% for six hours in phosphate buffer (pH 7.0). After the SA treatment, the seeds were washed in running water for 30 minutes in order to eliminate the residual effect of the chemical.

Germination tests

Germination tests were carried out on the air-dried treated seeds in the laboratory at room temperature. This was done by arranging 6 seeds in sterilized Petri dishes each of which was lined with double layer of Whatman No 1 filter paper. The Petri dishes were arranged in four replications, moistened with distilled water and labelled according to the concentration of sodium azide and variety of *S. stenocarpa*, in order to observe germination.

The seeds that were observed to have protrusions of the radicle were considered to have germinated as described by Ikhajiagbe *et al.* [18]. The seeds were observed daily until maximum germination was attained on the 9th day after sowing (DAS). On the tenth day, the sprouted seeds were counted, recorded and the germination percentage was determined using the method of Agbogidi [19]:

$$\text{Germination \%} = \frac{Total\ number\ of\ Sprouted\ Seedlings}{Total\ number\ of\ Planted\ Seedlings} X \frac{100}{1}$$

The seedlings were then transferred into pots containing sandy loam soils in an open field.

Field experiments

Field experiments were carried out using 18L plastic buckets (filled to about 80% capacity). These pots were arranged in a randomized complete block design (RCBD) with a total of three pots per treatment. Three weeks after planting (WAP), the plants were staked with sticks of about 2m in length in order to provide support. Watering was done on *ad-libitum* basis until the termination of experiment. In order to reduce insect attack, the plants were sprayed with an insecticide (Cypermethrin (10% EC)) when the flower buds were being initiated.

The qualitative characters of the accessions were determined by visual evaluation i.e. by scoring the variations in leaf colour, flower colour, and pattern of pigmentation on stems, branches, petioles, and peduncles (Table 1), while the quantitative characters were obtained by measurement in centimetres and counting on characters identified from morphological characterization list by Adewale and Durmet [20] (Table 2).

Table 1. Qualitative morphological characters studied on the AYB accessions.

Character description	Abbreviations	Procedure
Pigmentation intensity on main stem	**MSP**	Scored as: 1= NP, 2 = SP, 3 = S
Pigmentation intensity on branches	**BP**	Scored as: 1= NP, 2 = SP, 3 = S
Pigmentation intensity on petiole	**PP**	Scored as: 1= NP, 2 = SP, 3 = S
Pigmentation intensity on peduncle	**PdP**	Scored as: 1= NP, 2 = SP, 3 = S
Leaf colour	**LC**	Scored as: 1= Pale Green, 2= Green, 3 = Dark Green.

NP- Non-pigmented; SP-Slightly pigmented; S- Solidly pigmented;
G-Green; P.green- Pale Green; D.green-Dark Green.

DNA extraction and RAPD assay

Young healthy fresh leaves were obtained from two weeks old plants for DNA extraction. These leaves were collected from the different treatment groups of each accession and placed on ice in hermetically-sealed plastic bag before transportation to the laboratory. The samples were subsequently stored in freezer at -20°C and total genomic DNA was extracted from the frozen leaves using the modified mini preparation protocol described by Dellaporta *et al.* [21].

The concentration and purity of the extracted total genomic DNA was estimated according to Sambrook and Russed [22] using NanoDrop Spectrophotometer (ND-8000). The concentration of the DNA was additionally determined by electrophoretic analysis in a 1.5% agarose gel in TBE-buffer using standard markers of 1kb as DNA Ladder.

PCR amplification and subsequent electrophoretic analysis were performed using method described by Virk *et al.* [23]. The amplification was performed in a 10 µL reaction mixture containing a single incubation buffer, $MgCl_2$ (50mM), DNTPs (2.5 mM of each), 0.6 units Taq DNA Polymerase (BioBasics) and 30 ng genomic DNA. Thermocycler (Bio Oven III) was programmed for initial denaturation at 94^{o}C for 3 mins followed by 44 cycles (annealing (36^{o}C) for 1 min, extension (72^{o}C) for 2 mins, and denaturation (94^{o}C) for 3 mins) followed by a final elongation at 72^{o}C for 7 mins.

Table 2. Quantitative characters studied on the AYB accessions.

Character description	Code	Procedure and time of recording
Germination percentage	**GP**	Days from sowing till the protrusion of radicle.
Hypocotyl length	**HL**	Mean length of 10 hypocotyl seedlings measured from base to the tip when first primary leaves have fully expanded
Radicle length	**RL**	Mean length of 10 radicles measured from the base to the root tip.
Lateral root number	**LN**	Number of roots that extend horizontally from the primary root (radicle).
Terminal leaflet length	**TLL**	The average metric distance from the pulvinus to the apical tip of 10 fully developed terminal leaflets taken from 5 different plants a the peak of flowering
Terminal leaflet width	**TLW**	The average metric distance measured along the widest part of 10 fully developed terminal leaflets taken from 5 different plants at the peak of flowering
Petiole length	**PL**	Mean length of 10 petioles from 5 sample plants, measured from the base to the point where the three leaflets join
Peduncle length	**PdL**	Mean length of 10 peduncles measured from fully grown, flower/pod-bearing peduncles from 5 sample plants
Number of leaves per plant	**NL**	Number of leaves present on 5 sample plants, measured 8 weeks after planting.
Number of branches per plant	**NB**	Number of branches per plant, 10 plants as sampling unit.
Plant height	**PLH**	The vine length measured in cm at 8WAP, 5 plants as sampling unit.
Days to 50% peduncle initiation	**DPI**	Days from seedling emergence until 50% of the plant stands begin to initiate peduncle; 5 plants as sampling unit
Days to first flowering	**DFF**	Estimated as the period between germination and the appearance of first flower.
Days to 50% flowering	**D5F**	Days from seedling emergence until 50% of the plant stands begin to anthesize.

Twelve RAPD markers were utilized for optimization, among which only seven markers produced clear and bright fragments, while the others had poor amplification and produces smeared/complex bands or no PCR product at all. The amplified DNA was analyzed using the seven decamer oligonucleotide RAPD primers that showed distinct and scorable DNA bands (Table 3). These

primers were obtained from Operon Technology (Alameda, CA, USA). The products obtained were analyzed in 1.5% agarose gel in TBE- buffer. The gels were stained with ethidium bromide (5 mg/ml) and photographed under UV light. Electrophoretic profiles were examined visually by assessing the number of bands for each sample. The presence of a DNA band was scored as present (1) or absent (0), and each band was regarded as a locus.

Table 3. Nucleotide sequence and G+C content of the seven primers used for RAPD-PCR amplification.

Primer Identification	Nucleotide sequence (5'-3')	G+C content (%)
OPB-01	GTTTCGCTCC	60
OPH-05	AGTCGTCCCC	70
OPH-09	TGTAGCTGGG	60
OPT-01	GGGCCACTCA	70
OPT-06	CAAGGGCAGA	60
OPT-07	GGCAGGCTGT	70
OPT-20	GACCAATGCC	60

Statistical analysis

Quantitative morphological data were subjected to analysis of variance using SPSS software package in order to test the significance of variation among the treatments. Significant means obtained were separated using Duncan's Multiple Range Test. The collected data was also analyzed for mean, coefficient of variation (CV %). The quantitative data were also used for cluster analysis to study the relationship among the treatments. Correlation analysis was carried out to establish associations/relationships between the parameters.

The binary data obtained from scoring the RAPD bands were used to generate genetic (pairwise) similarity matrix using Jaccard's similarity coefficient [24], in order to evaluate the effect of treatment on the genome of the two accessions. Phylogenetic relatedness of the treatment groups in each accession was determined by constructing a dendrogram, to show a phenetic representation of the genetic relationship revealed by the similarity coefficient, using unweighted pair-group method with arithmetic averages (UPGMA) with the NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System) program version 2.02 (Exeter Software, New York, USA) [25]. This was done through the SHAN (sequential, hierarchical, agglomerative and nested clustering) clustering and tree sub-routine of NTSYS. Polymorphic information content (PIC) value was calculated for each RAPD primer according to Botstein *et al.* [26] using the formula: $PIC = 1-\Sigma(P_{ij})^2$.

Where Pij = the frequency of the i^{th} pattern revealed by the j^{th} primer summed across all patterns revealed by the primers.

Results and Discussion

Sodium azide treatment induces a non-linear stimulatory effect on the germination of the seeds of both accessions of *S. stenocarpa*. The highest germination percentage (68.75%) was observed in groups treated with 0.01 and 0.07% NaN_3 in TSs 10 unlike the 56.25% germination percentage observed in the 0.015 NaN_3 treated group of TSs 86. The lowest germination percentage (25.0%) came from the control groups in both accessions. The non-linear increase in the germination percentage in both accessions of *S. stenocarpa* may be due to enhancement of physiological and biological processes necessary for seed germination by sodium azide. The increase in germination may also be due to the stimulation of RNA or protein synthesis by sodium azide [27]. This observed effect was contrary to the decrease in germination percentage observed by Ali *et al.* [28] in two varieties of *Lens culinaris* exposed to increasing concentrations of Sodium azide. It also contradicts the reduction in germination observed in two dwarf varieties of *Phaseolus vulgaris* L. treated with Sodium azide by Ali *et al.*, [29].

The two accessions showed a non-linear stimulatory and inter-varietal differential response to hypocotyl length, radicle length, and lateral root number, compared to the control. A non-linear stimulatory effect on hypocotyl length, radicle length and lateral root number, with increasing concentration of sodium azide, was observed in TSs 10 while a concentration-independent reduction in hypocotyl length and lateral root number was observed in TSs 86 (Table 4). The observed increase in terminal leaf length and width (especially in 0.01% NaN_3-treated group of TSs 86 and 0.05% and 0.07% NaN_3-treated group of TSs 10) may be due to the stimulation of various biochemical factors involved in the expansion of leaves and cells [30].

Sodium azide-treatment induced polymorphism in the seedlings of *S. stenocarpa* judging by an intra-varietal differential response to increasing concentration of sodium azide observed on the terminal leaflet length, terminal leaflet width, and petiole length in both accessions of *S. stenocarpa* as well as in the number of leaves and plant height of TSs 86.

A non-linear stimulatory effect on number of leaves, number of branches, and plant height, with increasing concentration of sodium azide, was observed in TSs 10 while it was only observed in number of branches in TSs 86 (Table 5).

Table 4. Effect of sodium azide on seedling and morphological characters of both accessions of *S. stenocarpa* grown under laboratory conditions.

Variet	NaN_3Concn (%)	GP (%)	HL	Reduction (%)	Increment (%)	RL	Reduction (%)	Increment (%)	LN	Reduction (%)	Increment (%)
V1	**0.00**	25.00±13.23	2.83±0.61	-	-	1.56±1.77	-	-	1.70±2.89	-	-
	0.01	68.75±7.66	5.05±4.91	-	78.45	5.15±3.04	-	230.13	5.05±5.10	-	197.06
	0.03	56.25±2.88	4.85±3.53	-	71.38	4.78±2.05	-	206.41	4.70±4.56	-	176.47
	0.05	43.75±4.21	6.75±3.77	-	138.52	2.75±1.60	-	76.28	4.00±2.36	-	135.29
	0.07	68.75±7.95	4.53±4.00	-	60.07	4.75±3.76	-	204.49	2.25±2.91	-	32.35
	0.1	62.50±10.90	3.15±4.82	-	11.31	3.28±2.72	-	110.26	2.55±3.97	-	50.00
	0.2	56.25±6.96	5.73±2.98	-	102.47	6.80±2.12	-	335.90	5.00±3.43	-	194.12
V2	**0.00**	25.0±5.00	6.18±2.87	-	-	4.25±3.14	-	-	3.75±2.63	-	-
	0.01	56.25±5.58	5.15±2.48	16.67	-	5.90±1.67	-	38.82	3.58±3.00	4.53	-
	0.03	37.50±6.61	3.13±3.03	49.35	-	4.48±2.94	-	5.41	3.50±4.18	6.67	-
	0.05	37.50±2.50	5.00±2.30	19.09	-	6.03±1.35	-	41.88	2.13±2.40	43.20	-
	0.07	50.0±6.25	4.35±2.09	29.61	-	5.18±1.90	-	21.88	3.75±3.20	0.00	-
	0.1	43.75±5.45	2.23±4.21	63.92	-	3.65±3.26	14.12	-	1.95±3.92	48.00	-
	0.2	37.50±5.27	3.55±2.39	42.56	-	3.00±2.50	29.41	-	3.45±3.45	8.00	-

Table 5, Effects of Sodium azide treatment on the quantitative vegetative characters of both accessions.

Variety	NaN_3 Concn (%)	TLL (cm) (x±SD)	RED (%)	INC (%)	TLW (cm) (x±SD)	RED (%)	INC (%)	PL (cm) (x±SD)	RED (%)	INC (%)	NL (cm) (x±SD)	RED (%)	INC (%)	NB (x±SD)	INC (%)	PLH (cm) (x±SD)	RED (%)	INC (%)
V1	0.00	8.58±0.96	-	-	3.35±0.40	-	-	6.19±1.64	-	-	25.17±8.61	-	-	2.83±2.36	-	54.00±14.42	-	-
	0.01	7.95±2.69	7.34	-	3.31±0.89	1.19	-	5.60±1.22	9.53	-	48.90±7.39	-	94.28	5.33±1.15	88.34	78.10±5.60	-	44.63
	0.03	8.45±0.90	1.52	-	3.28±0.37	2.09	-	6.93±1.66	-	11.95	39.50±15.17	-	56.93	4.57±2.14	61.48	64.80±11.60	-	20.00
	0.05	9.13±1.76	-	6.41	3.72±0.76	-	11.04	6.10±1.15	1.45	-	49.83±9.31	-	97.97	6.17±2.02	118.02	72.30±8.90	-	33.89
	0.07	9.59±2.36	-	11.77	3.77±0.75	-	12.54	5.78±0.83	6.62	-	58.50±5.50	-	132.4	6.33±0.35	123.67	80.43±10.77	-	48.94
	0.1	8.36±1.68	2.56	-	3.47±0.92	-	3.58	6.44±0.98	-	4.04	43.40±10.84	-	72.43	4.33±3.16	53.00	65.33±16.33	-	20.98
	0.2	8.73±0.94	-	1.75	3.53±0.32	-	5.37	4.77±1.07	22.94	-	41.50±13.10	-	64.88	4.97±2.17	75.62	77.47±2.36	-	43.46
V2	0.00	10.28±1.01	-	-	4.20±0.61	-	-	5.98±1.03	-	-	48.50±15.02	-	-	5.00±2.65	-	88.83±20.00	-	-
	0.01	10.91±1.56	-	6.13	4.36±0.70	-	3.81	6.38±0.77	-	6.69	68.50±0.87	-	41.24	7.00±0.87	40.00	83.43±12.67	4.53	-
	0.03	9.55±0.77	7.10	-	3.75±0.35	10.71	-	5.82±1.17	2.68	-	48.00±14.50	1.03	-	5.00±2.00	0.00	80.17±14.02	6.67	-
	0.05	9.34±1.70	9.14	-	3.97±0.83	5.48	-	5.96±1.30	0.33	-	55.33±4.16	-	14.08	5.50±0.87	10.00	95.33±23.59	-	7.32
	0.07	9.63±1.30	6.32	-	3.72±0.52	11.43	-	5.67±1.12	5.18	-	50.17±14.44	-	3.44	5.43±1.25	8.60	82.23±13.19	0.00	-
	0.1	9.74±1.41	5.25	-	4.00±0.75	4.76	-	6.01±0.68	-	0.50	52.17±3.62	-	7.57	6.33±0.76	26.6	63.17±12.47	28.89	-
	0.2	10.75±1.02	-	4.57	4.34±1.21	-	3.33	6.73±0.92	-	12.54	48.17±18.33	0.68	-	5.50±3.04	10.00	77.33±15.50	8.00	-

RED: Reduction; INC: Increment. x±SD = Mean ± Standard deviation

The application of chemical mutagens to crops results in a lot of physiological variation hence the observed reductions in morphological characteristics such as lateral root number, terminal leaflet lengths and widths, petiole lengths, plant height, and peduncle lengths within the different treatment groups may be as a result of physiological/ metabolic disturbance caused by sodium azide. It may also be due to growth inhibition as a result of chromosomal damage, or injury to meristematic cells.

Reduction in plant height observed in TSs 86 may be partly due to the cells possessing more chromosomal damage hence, are at a disadvantage due to diplontic selection preventing them from competing well with other normal cells and making further contributions. The reductions observed corroborates the reduction in morphological characteristics observed in *Pisum sativum* L. exposed to Sodium azide by Saad-Allah *et al.* [31], as well as that observed by Ali *et al.* [28] in two varieties of *Lens culinaris* Medik.

A non-linear stimulatory effect on number of leaves, number of branches, and plant height, with increasing concentration of sodium azide, was observed in TSs 10 while it was only observed in number of branches in TSs 86 (Table 5). Similarly, an intra-varietal differential response to increasing concentration of sodium azide was observed on days to peduncle initiation, peduncle lengths, days to first flowering and days to 50% flowering in TSs 86 (Table 6). The reduction in days to first flowering ranges from 2.83 to 15.09% while the reduction in days to 50% flowering ranges from 1.85 to 13.80%. The observed increase in morphological characteristics (e.g. plant height, number of leaves, number of branches, hypocotyl length, radicle length, and lateral root number) as well reduction in days to peduncle initiation, and days to first and 50% flowering as a result of sodium azide treatment may be due to stimulatory effects and/or physiological changes caused by the mutagen. It may also be as a result of enhancement of auxin production in the affected plants. This effect was similar to the increase in number of branches per plant, number of pods per plant, number of seeds per pod and number of days to maturity in M_1 generation of *Arachis hypogaea* L. treated with sodium azide by Mensah and Obadoni [32]. It also corroborates the increase in plant height, and number of branches and reduction in time of flowering of *Vigna unguiculata* treated with Colchicine by Essel *et al.* [33].

The reduction observed in days to first-flowering, days to 50% flowering and days to peduncle initiation, in the treated groups compared to the control, can lead to early-maturing varieties. It also showed that sodium azide treated plants can change their reproductive characters in a positive way (i.e. towards earliness). This is very valuable because it offers an opportunity to obtain varieties that possess the ability to escape from pest, drought and other stress injuries that occur in the late growing period. It has been proposed that this observation may be related to seed metabolism and onset of DNA synthesis [34].

The means and coefficient of variation of the quantitative morphological characters measured in TSs 10 and TSs 86 are presented in table 7 and Table 8 respectively. In TSs 10, significant differences were found among the treatment applied for germination percentage, petiole length and number of leaves. The coefficient of variation (CV) ranged from 15.36% (for germination percentage), to 59.02% (for lateral root number). High to moderate CV values were observed for lateral root number (59.02%), hypocotyl length (53.82%), and number of branches (41.93%). On the other hand in TSs 86, significant differences were found among the treatment applied for germination percentage, terminal leaflet length, peduncle length, as well as days to first and 50% flowering. The Coefficient of variation ranged from 5.22% (for days to 50% flowering), to 83.52% (for lateral root number). Very high to moderate CV values were observed for lateral root number (83.52%), hypocotyl length (55.36%), and radicle length (49.95%). Similarly, low values of CV (ranging from 4.2 to 6.3) for days to 50% flowering were obtained by Adewale *et al.* [35] in 79 accessions of *S. stenocarpa*

The results of the relationship between the quantitative morphological characters observed in TSs 10 and TSs 86 were determined through correlation analysis in table 9 and table 10 respectively. In TSs 10, germination percentage was significantly and positively correlated with number of leaves (0.77), plant height (0.76), radicle length (0.69), and number of branches (0.67). Number of leaves was significantly correlated with number of branches (0.96), plant height (0.87), germination percentage (0.77), and hypocotyl length (0.51).

Table 6. Effects of Sodium azide treatment on some reproductive characters of TSs 86.

NaN_3 Concn (%)	DPI (x±SD)	Reduction (%)	Increment (%)	PdL (x±SD)	Reduction (%)	Increment (%)	DFF	Reduction (%)	Increment (%)	D5F	Reduction (%)	Increment (%)
0.00	82±1.50	-	-	12.69±3.46	-	-	106	-	-	108	-	-
0.01	82±2.20	0.00	-	14.17±3.87	-	11.66	90	15.09	-	93	13.8	-
0.03	82±1.50	0.00	-	20.90±6.25	-	64.70	103	2.83	-	103	4.63	-
0.05	81±4.00	1.22	-	11.67±6.04	8.04	-	102	3.77	-	102	5.56	-
0.07	85±2.50	-	3.66	9.41±1.91	25.85	-	94	11.32	-	96	11.11	-
0.1	81±1.00	1.22	-	19.77±3.40	-	55.79	109	-	2.83	110	-	1.85
0.2	83±2.50	-	1.22	11.78±2.47	7.17	-	105	6.25	-	106	1.85	-

RED: Reduction; INC: Increment. x±SD = Mean ± Standard deviation

Table 7. Quantitative vegetative characters of TSs 10 exposed to different levels of sodium azide.

NaN_3 Concn (%)	GP	HL	RL	LN	TLL	TLW	PL	NL	NB	PLH
0.00	25.00^{c}	2.83	1.56	1.70	8.58	3.35	6.19^{ab}	25.17^{c}	2.83	54.00
0.01	68.75^{a}	5.05	5.15	5.05	7.95	3.31	5.60^{bc}	48.90^{ab}	5.33	78.10
0.03	56.25^{ab}	4.85	4.78	4.70	8.45	3.28	6.93^{a}	39.50^{bc}	4.57	64.80
0.05	43.75^{b}	6.75	2.75	4.00	9.13	3.72	6.10^{ab}	49.83^{ab}	6.17	72.30
0.07	68.75^{a}	4.53	4.75	2.25	9.59	3.77	5.78^{bc}	58.50^{a}	6.33	80.43
0.1	62.50^{a}	3.15	3.28	2.55	8.36	3.47	6.44^{ab}	43.40^{abc}	5.23	65.33
0.2	56.25^{ab}	5.73	6.8	5.00	8.73	3.53	4.77^{c}	41.50^{abc}	4.33	77.47
Mean	54.46	4.70	4.15	3.61	8.68	3.49	5.97	43.82	4.97	70.35
CV (%)	15.36	53.82	30.49	59.02	20.16	19.32	20.95	23.85	41.93	15.60
SEM±	4.83	5.77	1.06	5.36	0.55	0.21	0.40	6.04	1.20	6.33
F test	**	**Ns**	**Ns**	**Ns**	**Ns**	**Ns**	*	*	**Ns**	**Ns**
LSD 0.05	14.65	3.71	1.63	3.13	1.56	0.60	1.12	18.31	3.65	19.19
LSD 0.01	20.34	5.06	2.26	4.26	2.08	0.80	1.49	25.41	5.07	26.63

*, **, Significant at 5%, and 1% respectively, ns = non-significant

Similarly, plant height was significantly positively correlated with number of leaves (0.87), germination percentage (0.76), radicle length (0.74), and number of branches (0.73). On the other hand, petiole length recorded significant but negative correlation with plant height (-0.63), and radicle length (-0.59). The positive correlation observed between plant height and number of branches is similar to that observed by Vijayalakshmi *et al.* [36] in Pigeon pea (*Cajanus cajan* L.)

In TSs 86, germination percentage was significantly and positively correlated with number of branches (0.77), number of leaves (0.69), while it was significantly negatively correlated with days to first flowering (-0.76) and days to 50% flowering (-0.75). Number of leaves was significantly and positively correlated with number of branches (0.85), germination percentage (0.69), and radicle length (0.65), while it exhibited significant but negative correlation with days to first flowering (-0.69), and days to first flowering (-0.66). The negative correlation observed between plant height and days to 50% flowering contrasts with that obtained by Gul *et al.* [37] in Mungbean (*Vigna radiata*).

Dendrogram result showed that the fourteen treatment groups in both accessions can be delineated into four clusters (Fig. 1). Cluster 1 consists of one member (control group of TSs 10). Cluster II had eight members (the seven treatment groups in TSs 86 and 0.07% group in TSs 10). Cluster III consist of 0.01% and 0.2%NaN_3-treated group of TSs 10 while cluster IV consists of 0.03%, 0.05% and 0.1% NaN_3-treated group. This result is similar to the 5 clusters observed by Adewale *et al.* [35] in 79 accessions of *S. Stenocarpa*.

Qualitative analysis revealed that none of the treated groups showed the same pattern and frequency of pigmentation as the control group. The control group of TSs 10 only had two types of

pigmentation patterns while majority of the treated groups exhibited three types. In TSs 86 on the other hand, the control group exhibited two types of pigmentation pattern, yet some of the treated group in this accession (0.03% and 0.2% NaN_3-treated group) exhibited slight pigmentation pattern. Three different types of leaf colour (dark green, green and pale green) were exhibited by the control groups in TSs 10 while the treated groups exhibited two types of pigmentation patterns. In TSs 86, the control group exhibited two types of pigmentation pattern (green and pale green). The pattern and frequency of peduncle pigmentation observed in the treated groups is the same as that in the control group except at 0.05 NaN_3-treated group. The variations observed in pigmentation patterns is similar to the result obtained by Adewale *et al.* [35].

The result of molecular analysis, using RAPD molecular marker, showed that genetic variation was induced in the genome of the *S. stenocarpa* accessions based on number of amplified fragments (82). Eighty of the bands obtained were polymorphic (11.43 bands/primer). The number of polymorphic bands varies from 7 for the OPT-06 primer up to 19 for the OPT-07 primer (Table 11).

This showed that OPT-07 primer was the most discriminatory among the primers used. The PIC value ranges from 0.84 for OPH-05 to 0.93 for OPT-07 while the gene diversity ranges from 86% to 93%. This high PIC value shows that variability has been induced in the genome of this accession. This effect was similar to the 62% polymorphism in popcorn populations based on 26 RAPD primers observed by Munhoz *et al.* [38]. Dendrogram result showed the delineation of the fourteen treatment groups in both accessions into five clusters (Fig. 2). The cluster group 1 consist of one member, 0.2% NaN_3-treated group of TSs 86 at a genetic distance of 0.489. The second cluster consists of the control group and 0.1% NaN_3-treated group of TSs 86. These two groups linked together at about 0.375 genetic distance level. The third cluster contained 0.03% and 0.05% NaN_3-treated group of TSs 10 linked together at 0.355 genetic distance level. The fourth cluster consisted of seven members' namely 0.01%, 0.03%, 0.05%, and 0.07% NaN_3-treated group of TSs 86 as well as 0.07%, 0.1% and 0.2% NaN_3-treated group of TSs 10. The fifth cluster consists of control group and 0.01% NaN_3-treated group of TSs 10 linked together at a genetic distance of 0.348.

The RAPD genetic distances based on Jaccard coefficient showed that the treatment groups are genetically dissimilar (Table 12). The 0.2% group of TSs 86, with genetic distance ranging from 0.923 and 1.00 (equivalent to 92.3% and 100% variation), is the most distant from other groups. About 92.3% variation was observed between this group and control group of TSs 10 while 100% variation was observed between the group and 0.03% and 0.05% NaN_3-treated group of TSs 10 respectively, as well as between the group and both control group and 0.07% NaN_3-treated group of TSs 86. Therefore, these markers can be utilized in *S. stenocarpa* breeding programs to detect genetic variability within the populations.

Table 8. Quantitative vegetative characters of TSs 86 exposed to different levels of sodium azide.

NaN_3 Concn (%)	GP	HL	RL	LN	TLL	TLW	PL	NL	NB	PLH	DPI	PdL	DFF	D5F
0.00	25.00^{d}	6.18	4.25	3.75	10.28^{abc}	4.20	5.98	48.50	5.00	88.83	82.00	12.69^{bc}	106.00^{a}	108.00^{a}
0.01	56.25^{a}	5.15	5.90	3.58	10.91^{a}	4.36	6.38	68.50	7.00	83.43	82.00	14.17^{b}	90.00^{c}	93.00^{c}
0.03	37.50^{c}	3.13	4.48	3.50	9.55^{c}	3.75	5.82	48.00	5.00	80.17	82.00	20.90^{a}	103.00^{ab}	103.00^{ab}
0.05	37.50^{c}	5.00	6.03	2.13	9.34^{c}	3.97	5.96	55.33	5.50	95.33	81.00	11.67^{bc}	102.00^{ab}	102.00^{abc}
0.07	50.00^{ab}	4.35	5.18	3.75	9.63^{bc}	3.72	5.67	50.17	5.43	82.23	85.00	9.41^{c}	94.00^{bc}	96.00^{bc}
0.1	43.75^{bc}	2.23	3.65	1.95	9.74^{bc}	4.00	6.01	52.17	6.33	63.17	81.00	19.77^{a}	109.00^{a}	110.00^{a}
0.2	37.50^{c}	3.55	3.00	3.45	10.75^{ab}	4.34	6.73	48.17	5.50	77.33	83.00	11.78^{bc}	105.00^{a}	106.00^{a}
Mean	41.07	4.23	4.64	3.16	10.03	4.05	6.08	52.98	5.68	81.50	82.3	14.34	101.3	102.57
CV (%)	13.10	55.36	49.95	83.52	12.83	15.39	16.76	22.69	32.52	20.13	6.62	29.32	5.26	5.22
SEM±	3.11	1.17	1.16	1.32	0.41	0.20	0.32	6.94	1.07	9.47	3.15	1.33	3.08	3.09
F test	**	ns	ns	ns	*	ns	ns	ns	Ns	Ns	ns	**	*	*
LSD 0.05	9.42	3.44	3.41	3.88	1.15	0.56	0.91	21.05	3.24	28.73	9.54	3.76	9.93	9.38
LSD 0.01	13.08	4.68	4.64	5.28	1.53	0.74	1.21	29.22	4.49	39.87	13.20	4.99	13.8	13.02

*, **, Significant at 5%, and 1% respectively, ns = non-significant

Table 9. Correlation Coefficient among Morphological characters in TSs 10.

	GP	HL	RL	LN	TLL	TLW	PL	NL	NB	PLH
GP										
HL	0.20									
RL	0.69**	0.45								
LN	0.37	0.72**	0.70**							
TLL	-0.04	0.27	-0.05	-0.39						
TLW	0.14	0.41	0.01	-0.26	0.89**					
PL	-0.20	-0.36	-0.59*****	-0.30	-0.13	-0.31				
NL	0.77**	0.51*****	0.42	0.20	0.45	0.66**	-0.22			
NB	0.67**	0.53*****	0.23	0.16	0.44	0.67**	-0.04	0.96**		
PLH	0.76**	0.64**	0.74**	0.48	0.30	0.51*	-0.63**	0.87**	0.73**	

*, ** = significant at 0.05 and 0.01 probability levels.

Table 10. Correlation Coefficient among Morphological characters in TSs 86.

	GP	HL	RL	LN	TLL	TLW	PL	NL	NB	PLH	DPI	PDL	DFF	D5F
GP														
HL	-0.22													
RL	0.42	0.53*												
LN	0.01	0.42	-0.03											
TLL	0.18	0.27	-0.23	0.48										
TLW	-0.03	0.33	-0.19	0.12	0.89**									
PL	0.06	-0.02	-0.38	0.08	0.82**	0.87**								
NL	0.69**	0.29	0.65**	-0.07	0.41	0.44	0.26							
NB	0.77**	-0.12	0.26	-0.26	0.45	0.46	0.38	0.85**						
PLH	-0.26	0.83**	0.65**	0.25	-0.09	0.03	-0.15	0.16	-0.35					
DPI	0.29	0.08	-0.05	0.67**	0.10	-0.26	-0.13	-0.24	-0.23	0.03				
PDL	-0.04	0.64**	-0.28	-0.31	-0.21	-0.21	-0.14	-0.06	0.11	-0.58*	-0.56*			
DFF	0.76**	-0.39	0.71**	-0.46	-0.26	-0.02	0.03	0.69**	-0.44	-0.35	-0.47	0.40		
D5F	0.75**	-0.34	0.75**	-0.41	-0.17	0.06	0.07	0.66**	-0.39	-0.38	-0.45	0.38	0.99**	

*, ** = significant at 0.05 and 0.01 probability levels.

Table 11. Primers used, number of amplified and polymorphic bands for the two accessions of *S. stenocarpa*.

Primer ID	Primer Sequence	Major Allele Frequency	Sample Size	No of Polymorphic DNA Bands	Allele Number	Gene Diversity	Polymorphic Information Content (PIC)
OPB01	GTTTCGCTCC	0.21	14.00	9.00	11.00	0.89*	0.88*
OPH05	AGTCGTCCCC	0.29	14.00	8.00	10.00	0.86*	0.84*
OPT06	CAAGGGCAGA	0.14	14.00	7.00	10.00	0.89*	0.88*
OPH09	TGTAGCTGGG	0.29	14.00	11.00	11.00	0.87*	0.86*
OPT20	GACCAATGCC	0.29	14.00	15.00	11.00	0.87*	0.86*
OPT01	GGGCCACTCA	0.29	14.00	13.00	11.00	0.87*	0.86*
OPT07	GGCAGGCTGT	0.07	14.00	19.00	14.00	0.93*	0.92*
Mean		**0.22**	**14.00**	**11.71**	**11.14**	**0.88**	**0.87**

Table 12. Matrix of genetic distance between the treatment groups in both *S. Stenocarpa* accessions estimated by RAPD markers based on Jaccard coefficient.

	V1T0	V1T1	V1T2	V1T3	V1T4	V1T5	V1T6	V2T0	V2T1	V2T2	V2T3	V2T4	V2T5	V2T6
V1T0	0													
VIT1	0.696	0												
V1T2	0.708	0.806	0											
V1T3	0.778	0.781	0.710	0										
V1T4	0.545	0.593	0.742	0.633	0									
V1T5	0.619	0.654	0.667	0.538	0.391	0								
VIT6	0.733	0.667	0.750	0.618	0.467	0.464	0							
V2T0	0.850	0.885	0.800	0.815	0.808	0.833	0.879	0						
V2T1	0.765	0.703	0.805	0.814	0.611	0.657	0.526	0.794	0					
V2T2	0.826	0.776	0.780	0.740	0.760	0.771	0.692	0.767	0.642	0				
V2T3	0.857	0.714	0.830	0.882	0.833	0.822	0.784	0.854	0.596	0.528	0			
V2T4	0.900	0.755	0.830	0.745	0.740	0.723	0.673	0.851	0.569	0.534	0.537	0		
V2T5	0.971	0.833	0.838	0.714	0.811	0.794	0.810	0.750	0.800	0.680	0.800	0.736	0	
V2T6	0.923	0.947	1.000	1.000	0.952	0.944	0.963	1.000	0.968	0.952	0.973	1.000	0.960	0

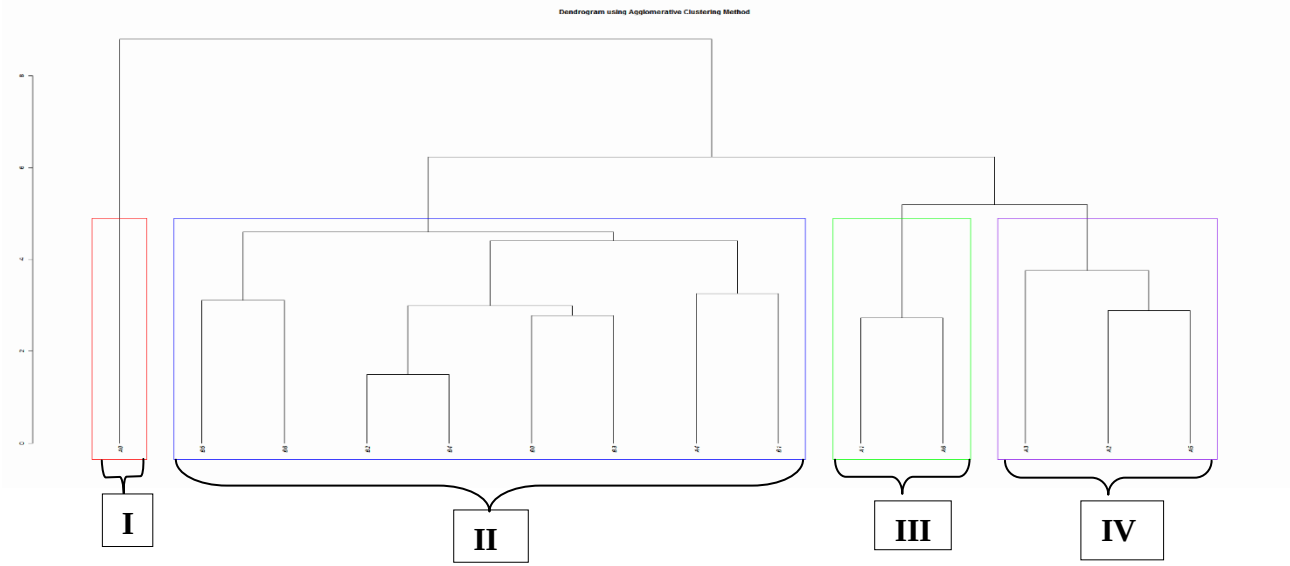

Figure 1. Dendrogram obtained by analysis of quantitative morphological data of the 7 treatment groups in both accessions of *S. stenocarpa*.

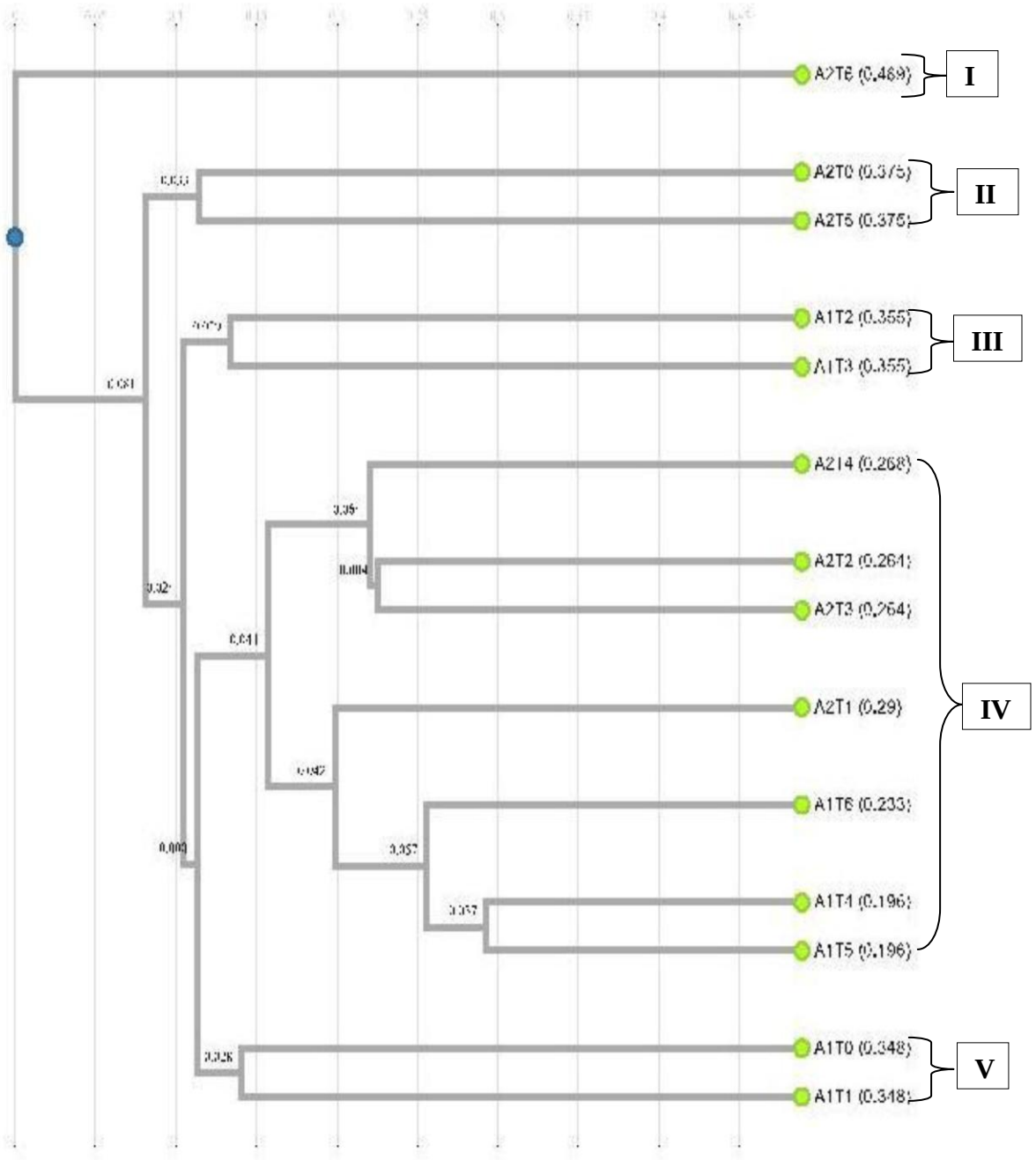

Figure 2. Dendrogram constructed from RAPD Data, by UPGMA clustering of the treatment groups in both accessions of *S. stenocarpa* based on Jaccard genetic similarities.

Conclusion

The result of this study showed that significant morphological and molecular variability was induced in the two accessions at all concentrations. Among the two accessions, TSs 10 is more effective at exhibiting mutant improvements in morpho-agronomic traits such as plant height, number of leaves as well as number of branches/plant at a dose of 0.07% of Sodium azide and the effect observed was more predictable than that observed in TSs 86. In TSs 86, sodium azide is more effective at

inducing mutant improvements in reproductive characters such as peduncle length as well as days to first and 50% flowering at a much lower dose of 0.01%. Since, the primary aim of most breeding programs is to produce cultivars with better agronomic/yield characters, it is hereby recommended that mutation study involving *S. stenocarpa* should be carried out at lower dosage of 0.01%. Furthermore, the two accessions can further be genetically improved with respect to tolerance to the chemical mutagen for utilization and conservation in future breeding.

The differential response to mutagens observed in this study implies that further studies needs to be carried out on induced mutagenesis especially in underutilized crops due to the narrowness of their germplasm.

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