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## Genetic Analysis of Effect of Heat Stress on Genomic DNA from Cowpea (*Vigna unguiculata* (L) Walp.)

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### Authors' contributions

This work was carried out in collaboration between all authors. Author OA designed the study, performed the statistical analysis and prepared the draft manuscript. Author AAO carried out the cluster analysis comparison of absorbance spectra optical density values. Authors MEA, ADP, FMO carried out the bench work, author TOO prepared the final manuscript. Authors BAA, AA and JAF carried out the comparison of absorbance spectra of DNA with different heat treatments. All authors read and approved the final manuscript.

### Article Information

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### ABSTRACT

**Aims:** Genetic analysis was used to study the effect of heat stress on young seedlings of cowpea (*Vigna unguiculata* (L) Walp.).

**Study Design:** Four different colors of cowpea seeds (white, dirty white, deep brown and light brown) were obtained from GeneBank of International Institute of Tropical Agriculture (IITA) Ibadan, Nigeria. Seeds from each of the cowpea four colors were first pre-germinated and young seedlings

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subjected to DNA extraction. Extracted DNA subjected to different temperature treatments at 75°C and 100°C for one hour and control not heated.

**Place and Duration of Study:** Department of Chemical Sciences Afe Babalola University Ado Ekiti, Nigeria between January 2015 and June 2015.

**Methodology:** UV wavelength absorption spectrum analysis ( $A_{200} - A_{960}$ ) was carried out on control DNA and DNA heated at 75°C and 100°C respectively. Cluster analysis of optical density (OD) data was carried out to establish the relationship between control DNA and heat treated DNA (75°C and 100°C).

**Results:** DNA concentrations of *Vigna unguiculata* (L) Walp. were between 0.40 to 1.15 mg/ml, 0.33 to 0.84 mg/ml, and 0.26 to 0.89 mg/ml for control and heat treatments of 75°C and 100°C respectively. DNA UV absorption spectra of control and heat treatments of 75°C and 100°C were generally different due to differential UV wavelength absorption. Cluster analysis revealed three different clusters (cluster 1, cluster 2 and cluster 3) among control DNA and heat treated DNA. Cluster 1 comprised of V1-control, V1-75°C and V1-100°C, with V1-75°C and V1-100°C having similar characters. Cluster 2 was made up of V4-control, V4-75°C and V4-100°C, with V4-75°C and V4-100°C having the same characters. Cluster 3 was largely characterized by dissimilar DNA extracts of V3-75°C, V2-control, V3-100°C, V2-100°C, V 3-control and V2-75°C.

**Conclusion:** Genetic diversity among individual *Vigna unguiculata* (L) Walp. accession DNA as obtained in this study could possibly be as a result of variations in heat tolerance among dissimilar cowpea genomic compositions.

*Keywords: Absorption; clusters; germplasm; spectra; diversity.*

## 1. INTRODUCTION

Cowpea (*Vigna unguiculata* (L) Walp.) plant has the ability to tolerate drought and fix atmospheric nitrogen in the soil enhanced by the *Rhizobium* symbiont [1]. Hence, it improves soil fertility, and consequently helps to increase the yields of cereal crops when grown in rotation and contributes to the sustainability of cropping systems [2]. The seeds are a major source of proteins and vitamins for man, feed for animals and also a source of cash income. The young leaves and immature pods are eaten as vegetables [3]. The economic uses of cowpea makes it a choice crop for serving food security needs of societies [4]. Some health benefits of cowpea include, toning the spleen, stomach and pancreas, inducing urination and relieving damp conditions like leucorrhoea [5].

All cultivated cowpeas are found within the universally accepted *V. unguiculata* subspecies classification, which is then commonly divided into four cultivar groups: *Unguiculata*, *Biflora*, *Sesquipedalis*, and *Textilis* [6,7]. Some well-known common names for cultivated cowpeas include black-eye pea, southern pea, yardlong bean, catjang and crowder pea.

Traditionally, diversity is estimated by measuring variation in phenotypic or qualitative traits such as flower colour, growth habit, or quantitative agronomic traits such as yield potential, stress

tolerance. Diversity has been used as a powerful tool in the classification of cultivars and also to study taxonomic status. Morphological variability in cowpeas abounds in the tropics suggesting adequate knowledge of the germplasm structure for the development of hybrids with specific ecology adaptation [8]. However, this approach is often limited and expression of quantitative traits is subject to strong environmental influence [9].

The specific objectives of the research study are to Carry out DNA extraction on young seedlings of *Vigna unguiculata* (L) Walp, Compare the genetic diversity of different *Vigna unguiculata* (L) Walp. accessions at various temperatures using UV absorption spectrum analysis, and Apply NTSYS software to reveal the effect of temperature on genetic diversity of different *Vigna unguiculata* (L) Walp. accessions.

## 2. MATERIALS AND METHODS

### 2.1 *Vigna unguiculata* (L) Walp. Seed

The *Vigna unguiculata* (L) Walp. seeds used in this study (Table 1) were obtained from GeneBank of International Institute of Tropical Agriculture, Ibadan October, 2014. The seeds were white, dirty white, light brown and deep brown. These were then planted under laboratory conditions to obtain the seedlings needed for DNA extraction.

## 2.2 Seed Treatment

Seeds of four cowpea accessions (Table 1) were pregerminated for 5 days at room temperature inside Petri dishes with filter paper soaked with sterile distilled water. Five day-old seedlings were collected and used for DNA extraction.

## 2.3 Genomic DNA Extraction

The genomic DNA extraction procedure used was according to [10] with some modifications. A total of 12 seedlings leaf samples were used for the DNA extraction. 2 g of leaf sample was grinded in 5 ml of 2xCTAB buffer (50 mM Tris, pH 8.0; 0.7 mM NaCl; 10 mM EDTA; 2% hexadecyltrimethylammonium bromide; 0.1% 2-mercaptoethanol) using mortar and pestle. The homogenate was transferred into sample bottles and incubated at 65°C for 20 min. After incubation, the homogenate was purified by two extractions with equal volume of chloroform. Using 2 ml Eppendorf tubes, the homogenate was centrifuged with a refrigerated centrifuge at 14,000 rpm for 10 min. The supernatant was then transferred into new 5 ml sample tube. DNA was precipitated with 2 times volume of -20°C absolute ethanol. This was transferred to new Eppendorf tubes and centrifuged at 12,000 rpm for 10 mins. The DNA was dried at room temperature and then re-suspended in 5 ml sterile distilled water.

## 2.4 Heat Treatment of DNA Extract

The genomic DNA extract was divided into three batches of which two of the three batches were subjected to different temperature treatments of 75°C and 100°C respectively for 1 hour inside water bath and the remaining batch served as control (Table 2).

**Table 1. List of *Vigna unguiculata* (L) Walp seeds used for the study**

S/N	Cowpea code	Cowpea seed name	Color
1	V1	IT98K-205-8	White
2	V2	T Vu – 3947	Dirty white
3	V3	IT99K-573-2-1	Deep brown
4	V4	T Vu – 8775	Light brown

## 2.5 Uv Absorption Spectrum Analysis of Genomic DNA Extract

The procedure used for UV absorption spectrum analysis of both genomic DNA extract and DNA standards was according to Mattley and Garcia-Rubio [11] and Grimsley and Pace [12].

**Table 2. List of *Vigna unguiculata* (L) Walp genomic DNA extracts and different temperature treatments**

S/N	Cowpea seed name	DNA sample	Heat treatment (Temperature °C)
1	IT98K-205-8	V1	Control
2	IT98K-205-8	V1-75C	75
3	IT98K-205-8	V1-100C	100
4	T Vu – 3947	V2	Control
5	T Vu – 3947	V2-75C	75
6	T Vu – 3947	V2-100C	100
7	IT99K-573-2-1	V3	Control
8	IT99K-573-2-1	V3-75C	75
9	IT99K-573-2-1	V3-100C	100
10	T Vu – 8775	V4	Control
11	T Vu – 8775	V4-75C	75
12	T Vu – 8775	V4-100C	100

## 2.6 Genomic DNA Extract

0.1 ml of DNA extract was added to 1.9 ml of sterile distilled water, mixed well, and the optical density (OD) absorbance value was taken from  $A_{200}$ - $A_{960}$  UV wavelengths using Spectronic 20 spectrophotometer. All the DNA extracts control and heat treated were analyzed.

## 2.7 DNA Standard

0.1 ml each of 0.007 mg/ml, 0.013 mg/ml, 0.020 mg/ml, 0.026 mg/ml, and 0.033 mg/ml of DNA standard (SIGMA) was added to sterile distilled water, mixed well and the optical density (OD) absorbance value was taken for each DNA standard from  $A_{200}$ - $A_{960}$  UV wavelengths using Spectronic 20 spectrophotometer.

## 2.8 Graphical Analysis of Optical Density (OD) Data

The procedure used for the graphical analysis of optical density (OD) data was according to Mattley and Garcia-Rubio [11] and Pavokovic et al. [13]. Using Microsoft Excel plot of optical density (OD) absorbance values against  $A_{200}$ - $A_{960}$  UV wavelengths was made to generate UV absorbance spectra profile for DNA standards (SIGMA). A Standard Curve was prepared by plotting the optical density (OD) absorbance values at  $A_{280}$  UV wavelengths against the concentrations of DNA standard (SIGMA). The

concentration of each genomic DNA extracts was obtained using the linear equation generated from the DNA Standard Curve. A plot of optical density (OD) absorbance values against  $A_{200}$ - $A_{960}$  UV wavelengths was also carried out to generate a combined UV absorbance spectra profile for DNA extracts and DNA standard (SIGMA).

### 2.9 Cluster Analysis of Optical Density (OD) Data

In order to establish the relationship between DNA extracts (control) and DNA extracts heat treated (75°C, and 100°C), cluster analysis of optical density (OD) data was carried out using numerical taxonomy and multivariate analysis system (NTSYS-PC), version 2.1 [14]. OD data were first converted to pairwise distance matrices using the Jaccard coefficient of similarity present in NTSYS-PC 2.1 and dendrogram cluster was created by Unweighted Pair Group Method with Arithmetic mean (UPGMA) cluster analysis [15].

## 3. RESULTS

### 3.1 Concentration of DNA Extracts

The final concentrations of DNA standard used were 0.007 mg/ml, 0.013 mg/ml, 0.020 mg/ml, 0.026 mg/ml and 0.033 mg/ml. A highly significant ( $P \leq 0.01$ ) standard linear curve was plotted with absorbance value at 260 nm against concentrations of the standard (Fig. 1). A standard linear curve equation was generated ( $y = 0.1511x - 1E-16$ ) and was used to deduce the concentration of the DNA extracts, where  $y$  and  $x$  represented the absorbance value at 260 nm and DNA concentration respectively (Fig. 1). The DNA content (control) of *Vigna unguiculata* (L) Walp was between 0.40 to 1.15 mg/ml. Other DNA contents (heat treated) in *Vigna unguiculata* (L) Walp were between 0.33 to 0.84 mg/ml, 0.26 to 0.89 mg/ml in DNA extracts heat treatments of 75°C and 100°C respectively (Table 3). DNA extract control (V3) of IT99K-573-2-1 seed has the highest DNA content of 1.15 mg/ml while DNA extract control V2 and V4 have the lowest DNA content with 0.40 mg/ml respectively (Table 2). The DNA purity index of each DNA extract was also obtained by dividing  $OD_{260}$  by  $OD_{280}$ . The DNA content (control) of *Vigna unguiculata* (L) Walp purity index was between 1.5 to 2.0, which was within the range of standard DNA purity index (Fig. 2). DNA purity index (heat treated) in *Vigna unguiculata* (L) Walp was

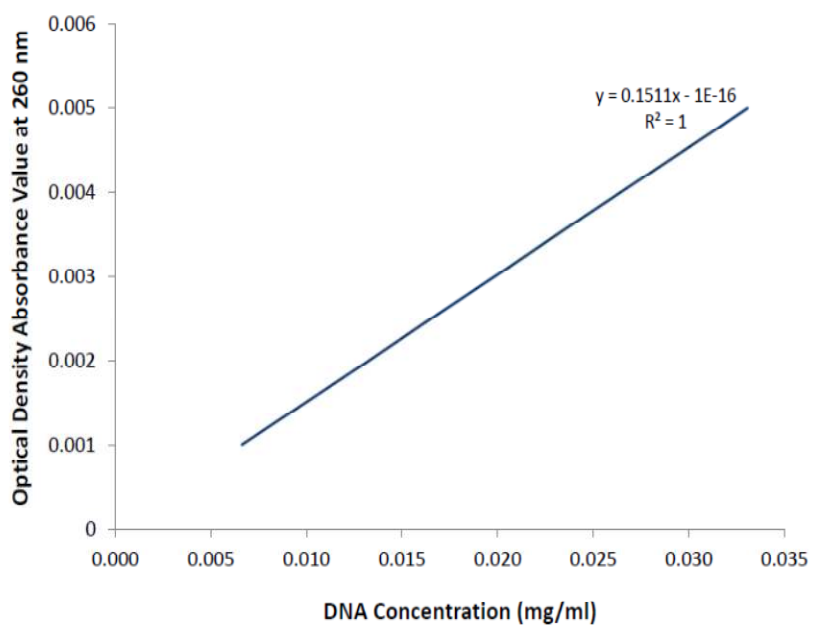
between 0.4 to 1.2 and 0.3 to 0.8 in DNA extracts heat treatments of 75°C and 100°C respectively (Fig. 2). The DNA purity indices at these temperatures are out of range of the standard DNA purity index (Table 3). This implies that at these temperatures, most of the DNA must have been degraded. The DNA content in IT98K-205-8 was 0.53 mg/ml (control), 0.33 mg/ml (75°C), 0.26 mg/ml (100°C), while 0.40 mg/ml (control), 0.57 mg/ml (75°C), 0.44 mg/ml (100°C), where obtained in TVu-3947 (Fig. 3). Also, DNA content in IT99K-573-2-1 was 1.15 mg/ml (control), 0.84 mg/ml (75°C), 0.89 mg/ml (100°C), while 0.40 mg/ml (control), 0.79 mg/ml (75°C), 0.53 mg/ml (100°C) was obtained in TVu-8775 (Fig. 3). All these concentration variations were obtained at 260 nm wavelength. Fig. 4 gave an absorbance profile of DNA samples of different heat treatments from 200 to 300 nm wavelengths.

### 3.2 Comparison of Absorbance Spectra of DNA Standards

There was comparative difference between the absorbance values of DNA standards. All the five standards, STD1, STD2, STD3, STD4, and STD5 had peaks at 330 nm respectively, with STD1 and STD5 having the highest peak (Fig. 5). At other wavelengths (340-960 nm), there were absorption of the DNA molecules at different peaks. STD4 was chosen as an absorbance spectrum reference DNA standard for the DNA extracts analyzed.

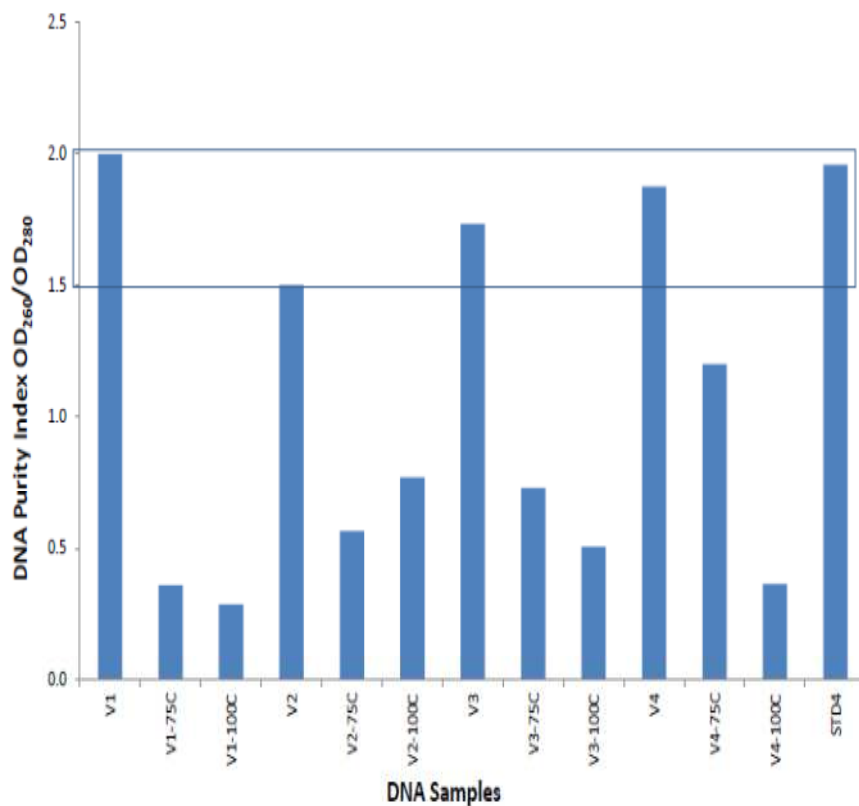
### 3.3 Comparison of Absorbance Spectra of Different Heat Treated DNA Samples from IT98K-205-8 with DNA Control

Comparative analysis revealed that there was difference between the absorbance of DNA heat treatment in IT98K-205-8 and DNA control. At the same wave length of between 280 nm and 300 nm, DNA concentrations in IT98K-205-8 DNA heat treated samples, V1-100°C and V1-75°C produced a peak, with V1-75°C heat treated sample having the highest peak, relative to control DNA (Fig. 6). V1-control produced a peak at wavelength between 300 nm and 320 nm, and other peaks at between 580 nm to 900 nm wavelengths. It was observed that V1-75°C and V1-100°C heat treated DNA samples had lower peaks at other wavelength between of 480 nm to 900 nm (Fig. 6).



**Fig. 1. DNA standard curve using sigma DNA ladder**

Standard linear curve equation:  $y = 0.1511x - 1E-16$ ; R: Regression value. \*\*=Highly significant ( $P \leq 0.01$ )



**Fig. 2. DNA purity index relative to different heat treatments of DNA samples from *Vigna unguiculata* (L) Walp.**

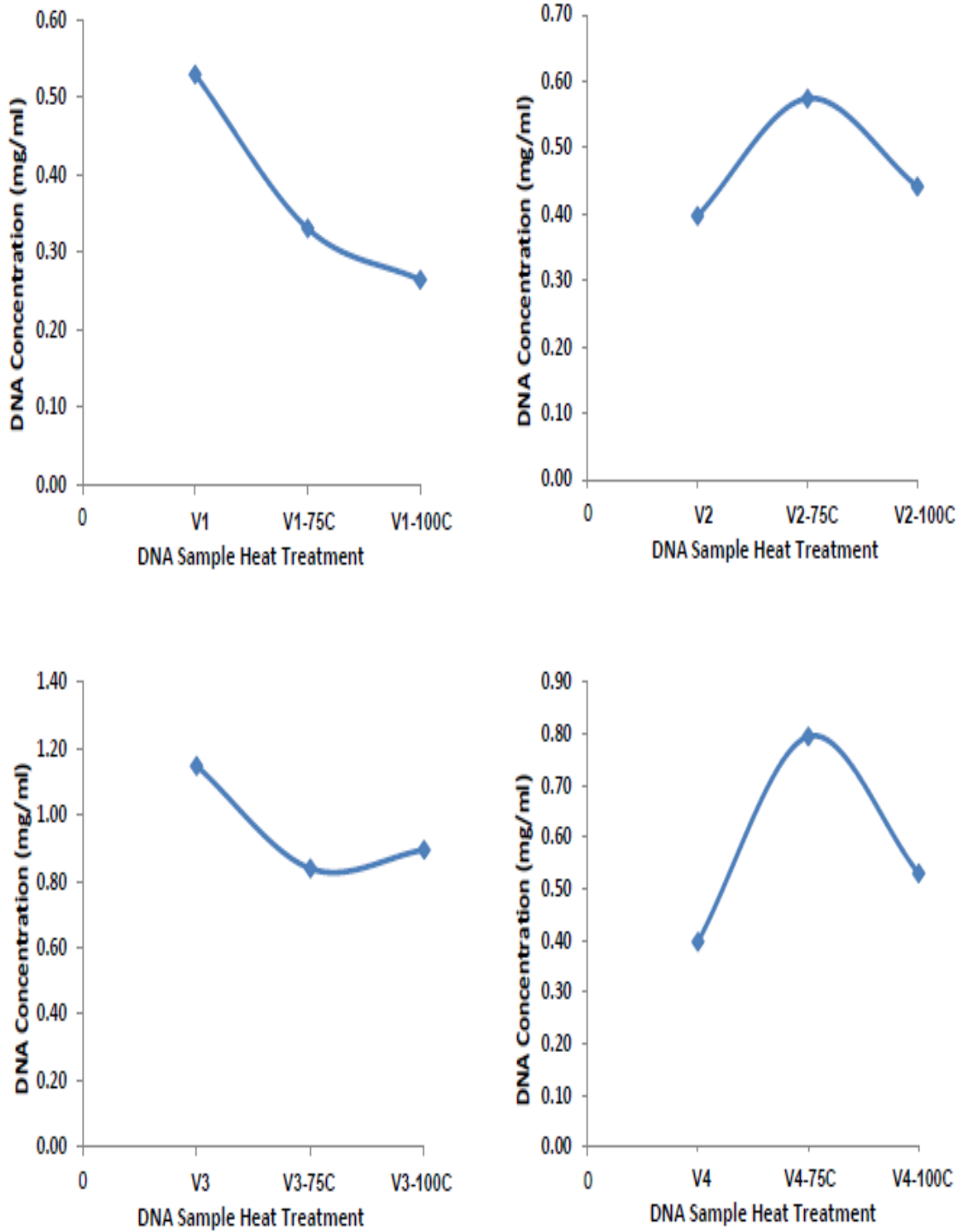
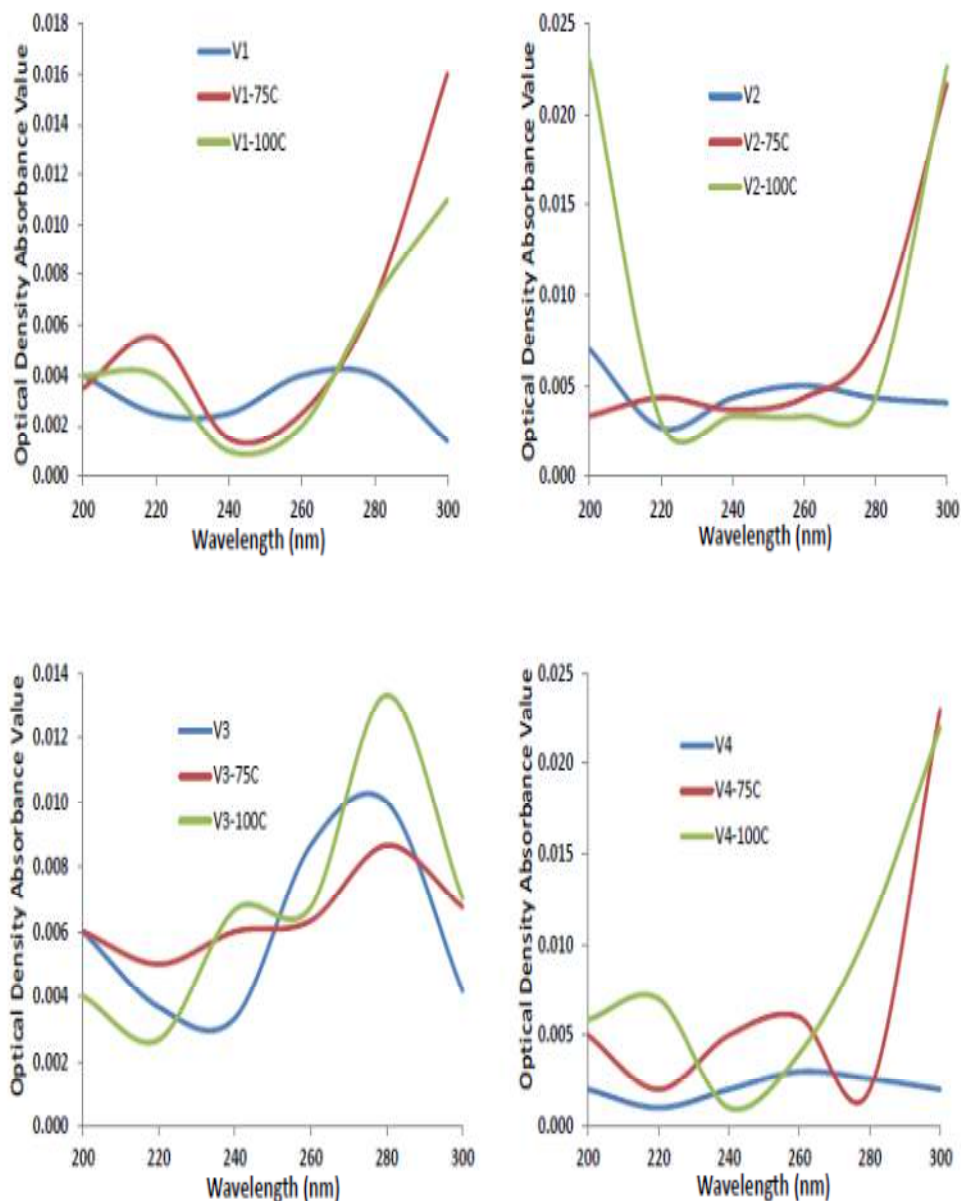


Fig. 3. DNA concentration variations at 260 nm wavelength due to different heat treatments of DNA samples from *Vigna unguiculata* (L) Walp.



**Fig. 4. Absorbance profiles of DNA samples of different heat treatments from 200 to 300 nm wavelengths**

**3.4 Comparison of Absorbance Spectra of Different Heat Treated DNA Samples from TVu-3947 with DNA Control**

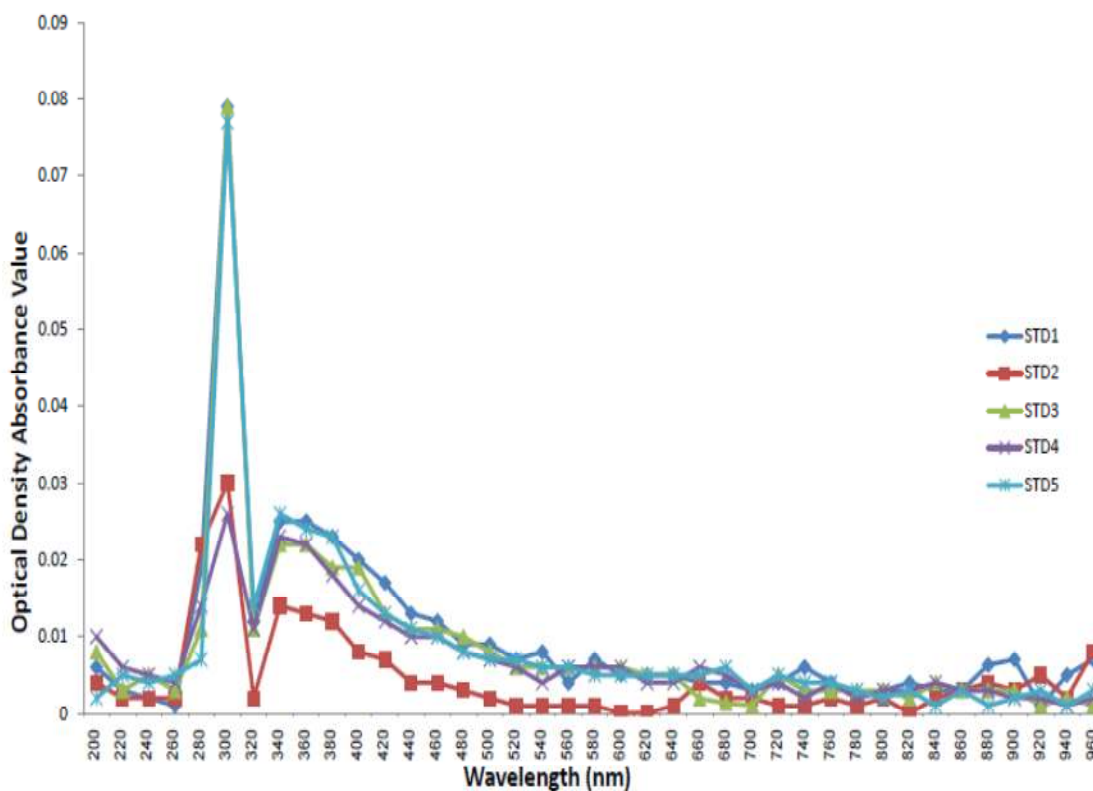
There was comparative difference between the absorbance values of heat treated DNA from TVu-3947 (Fig. 7). The comparison showed that V2-100°C heat treated DNA had the highest

peak at 340 nm wavelength and other lower peaks from between 400 nm to 900 nm wavelengths. V2-75°C and V2-100°C heat treated DNA had peaks at 300 nm respectively. V2-control DNA had a peak at 320 nm and along with V2-75°C heat treated DNA had peaks at other wavelengths of between 340 nm to 900 nm (Fig. 7).

**Table 3. Comparison of DNA content and purity with different heat treatments in *Vigna unguiculata* (L) Walp.**

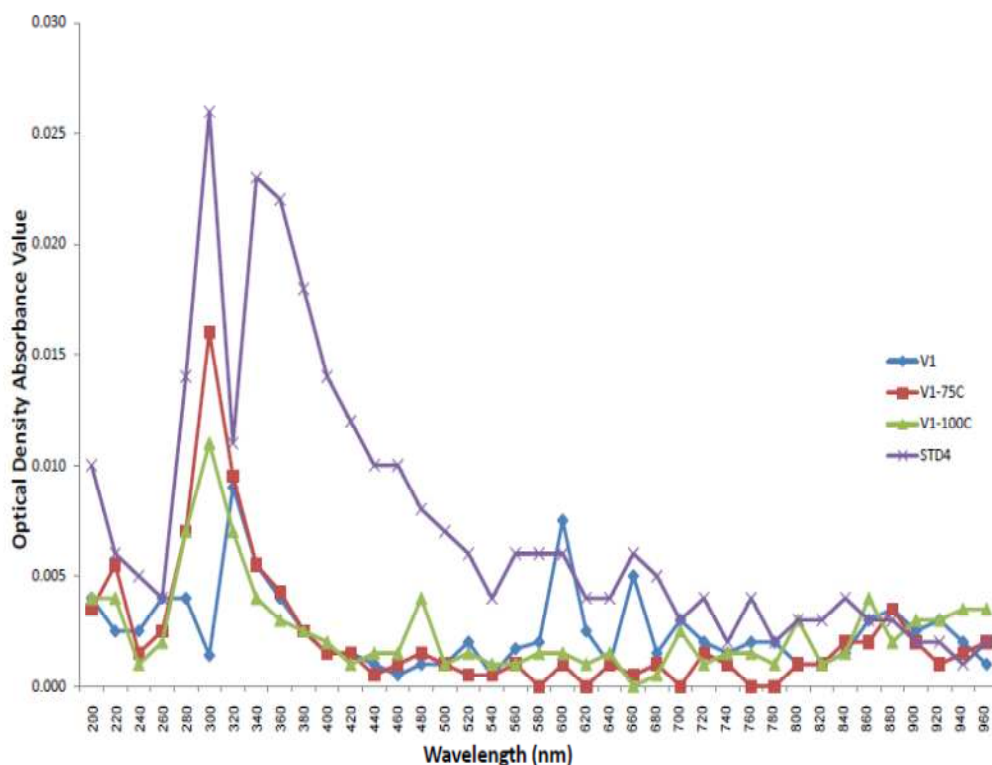
S/N	Cowpea seed name	DNA sample	Heat treatment (Temperature °C)	DNA concentration <sup>a</sup> (mg/ml)	DNA purity index <sup>a</sup> OD <sub>260</sub> /OD <sub>280</sub> (1.5-2)
1	IT98K-205-8	V1	Control	0.53**	2.0**
2	IT98K-205-8	V1-75C	75	0.33	0.4
3	IT98K-205-8	V1-100C	100	0.26	0.3
4	T Vu – 3947	V2	Control	0.40ns	1.5**
5	T Vu – 3947	V2-75C	75	0.57	0.6
6	T Vu – 3947	V2-100C	100	0.44	0.8
7	IT99K-573-2-1	V3	Control	1.15**	1.7**
8	IT99K-573-2-1	V3-75C	75	0.84	0.7
9	IT99K-573-2-1	V3-100C	100	0.89	0.5
10	T Vu – 8775	V4	Control	0.40*	1.9*
11	T Vu – 8775	V4-75C	75	0.79	1.2
12	T Vu – 8775	V4-100C	100	0.53	0.4

<sup>a</sup>T-test used to compare the mean value of DNA concentration of control DNA with that of heat treated DNA (75 °C and 100 °C) and the significant difference level (between control DNA and heat treated DNA) was indicated on each control DNA. ns=no significant difference. \*=significant difference at p≤0.10. \*\*=significant difference at p≤0.01



**Fig. 5. Absorbance spectra of different concentrations of DNA standard (DNA ladder)**  
 STD=Standard; STD1=0.007 mg/ml; STD2=0.013 mg/ml; STD3=0.020 mg/ml; STD4=0.026 mg/ml; STD5=0.033 mg/ml





**Fig. 6. Comparison of absorbance spectra of different heat treated DNA samples from IT98K-205-8 with DNA standard (DNA ladder)**

V1= IT98K-205-8 control DNA; V1-75C= IT98K-205-8 DNA heated at 75°C for 1 hr; V1-100C= IT98K-205-8 DNA heated at 100°C for 1hr; STD=DNA Standard; STD4=0.026 mg/ml

### 3.5 Comparison of Absorbance Spectra of Different Heat Treated Samples from IT99K-573-2-1 with DNA Control

The comparative study carried out on IT99K-573-2-1 revealed great difference in absorbance values of heat treated DNA. V3-control, V3-75°C and V3-100°C heat treated DNA showed very high peaks at wavelength of between 300 nm and 320 nm (Fig. 8). V3-control, V3-75°C and V3-100°C heat treated DNA showed peaks at wavelengths between 380 nm to 900 nm with V3-control DNA having peaks higher than V3-75°C heat treated DNA, which was in turn, higher than V3-100°C heat treated DNA (Fig. 8).

### 3.6 Comparison of Absorbance Spectra of Different Heat Treated DNA Samples from TVu-8775 with DNA Control

Analysis on the absorbance spectra of different heat treated DNA samples from TVu-8775 with

DNA control showed that V4-75°C and V4-100°C heat treated DNA had peaks at 280 nm wavelength (Fig. 9). V4-control, V4-75°C and V4-100°C heat treated DNA had peaks at wavelength between 320 nm to 900 nm, with V4-100°C heat treated DNA having higher peak at wavelength between 620 nm to 880 nm (Fig. 9).

### 3.7 Cluster Analysis Comparison of Absorbance Spectra Optical Density Values of DNA with Different Heat Treatments

Cluster analysis comparison of absorbance spectra optical density values of DNA revealed salient differences in the formation of clusters among control DNA extracts and heat treated DNA extracts at 75°C and 100°C respectively (Fig. 10). Three clusters (cluster 1, cluster 2 and cluster 3) have been identified among control DNA extract and heat treated DNA extracts. Cluster 1 comprised of V1-control, V1-75°C and V1-100°C, with V1-75°C and V1-100°C having

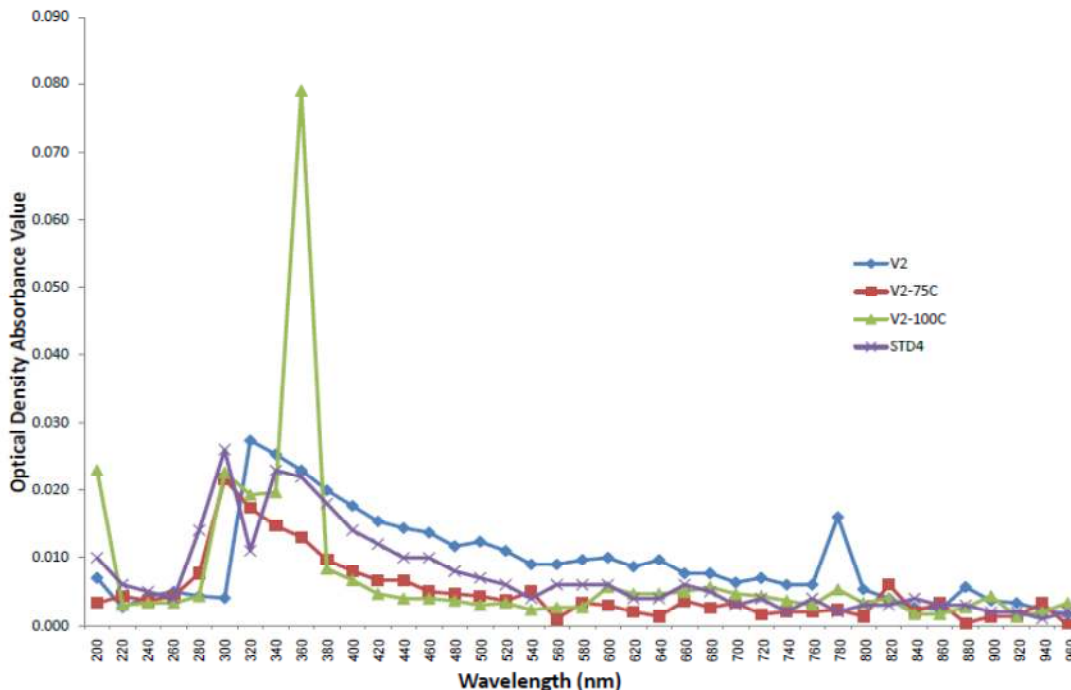
similar characters. Cluster 2 was made up of V4-control, V4-75°C and V4-100°C, with V4-75°C and V4-100°C having the same characters. Cluster 3 was largely characterized by dissimilar DNA extracts of V3-75°C, V2-control, V3-100°C, V2-100°C, V3-control and V2-75°C (Fig. 10).

#### 4. DISCUSSION

The objective of the study was to assess the extent of DNA diversity based on effect of heat stress on genomic DNA of cowpea (*Vigna unguiculata* (L) Walp.), using UV absorption spectrum analysis. The study showed that DNA content was between 0.04 to 1.15 mg/ml. Cowpea is known to have a better tolerance to drought and high temperature compared to other legumes [16]. However, this study showed that DNA content of cowpea exposed to heat (75°C and 100°C) was between 0.33 to 0.84 mg/ml and 0.26 to 0.89 mg/ml, which revealed some level of DNA variability in the different cowpea accessions used. It is believed that at these high temperatures, most of the DNA must have been degraded. Simões-Araújo (2002) identified transcripts that present similarities with those that

encode small molecular heat shock proteins in cowpea nodules subjected to heat stress. In heat stress conditions, analysis of transcripts expression showed 600 bands, among which 55 and 9 were up-regulated and repressed, respectively [17]. However, these transcripts showed homologies with low molecular weight heat shock proteins, wound-induced proteins, disease resistance protein, xylan endohydrolase isoenzyme and different housekeeping genes [18]. In addition, DNA content of different accessions of *Vigna unguiculata* (L) Walp. obtained from UV absorption spectrum analysis varied a great deal and could possibly as a result of different levels of heat tolerance among different chemical compositions of the seeds.

Diouf [18] and Garcia-Rubio et al. [19] opined that UV absorption spectrum assay is a powerful characterization tool with several applications because a lot of data could be generated from a single multi-wavelength measurement. Among the information derivable from a typical multi-wavelength UV spectrum are cell size, chemical composition, and shape [19]. This information is obtained from the spectroscopic analysis of a



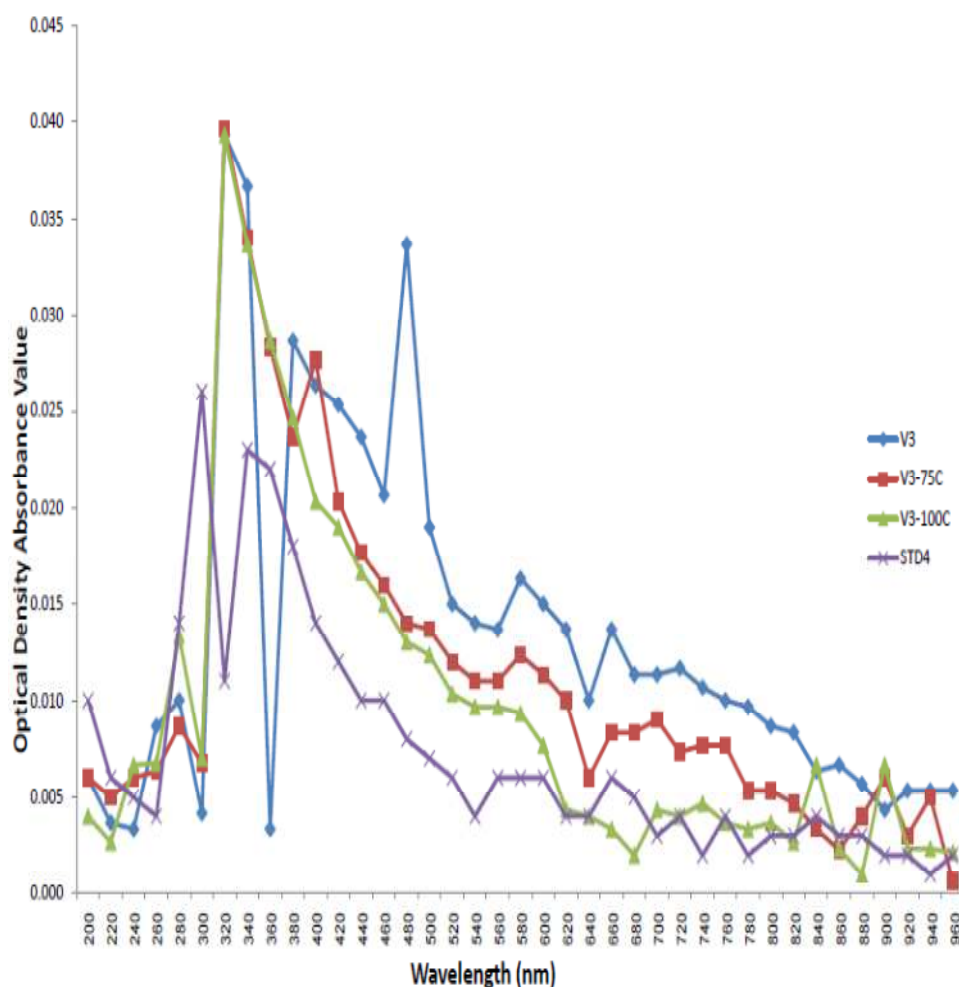
**Fig. 7. Comparison of absorbance spectra of different heat treated DNA samples from TVu-3947 with DNA standard (DNA Ladder)**

V2= TVu-3947 control DNA; V2-75C= TVu-3947 DNA heated at 75°C for 1 hr; V2-100C= TVu-3947 DNA heated at 100°C for 1hr; STD=DNA Standard; STD4=0.026 mg/ml

sample measured over a broad range of wavelengths (200 - 900 nm) [20]. In this study, absorbance spectra between 200-960 nm UV wavelengths have been used to compare DNA content from DNA extracts from *Vigna unguiculata* (L) Walp heated at 75°C and 100°C. Within the same *Vigna unguiculata* (L) Walp accession different DNA absorbance spectra were obtained from control DNA extract and DNA extract heated at 75°C and 100°C. For example, the comparative study carried out on IT99K-573-2-1 revealed great difference in absorbance values of heat treated DNA. V3-control, V3-75°C and V3-100°C heat treated DNA showed very high peaks at wavelength of between 300 nm and 320 nm. V3-control, V3-75°C and V3-100°C

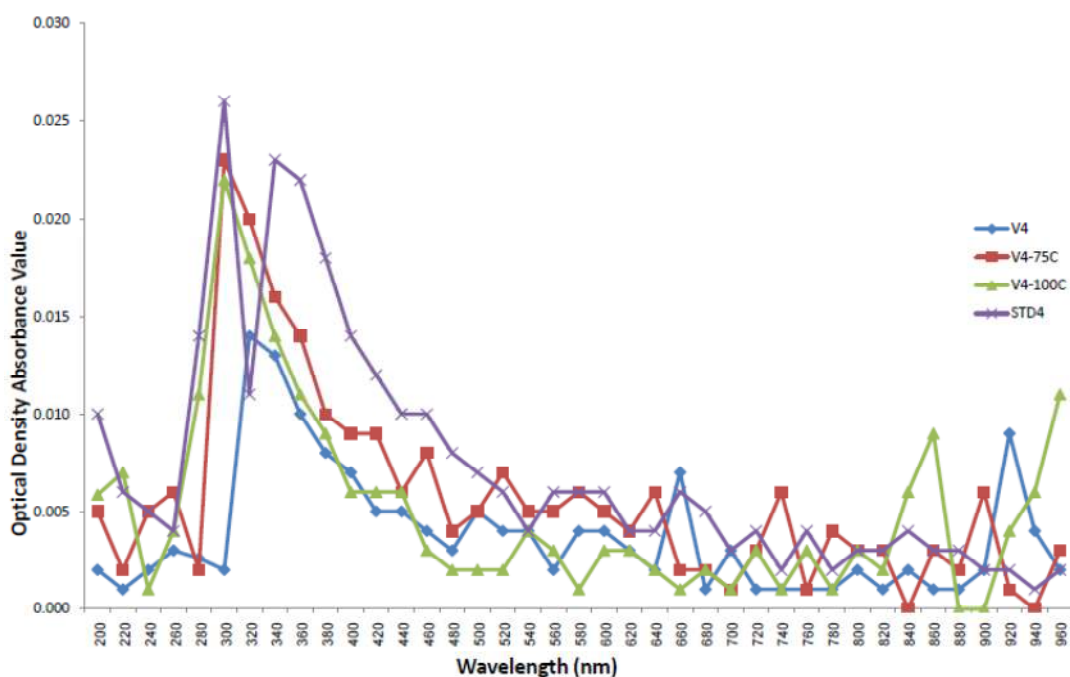
heat treated DNA showed peaks at wavelengths between 380 nm to 900 nm with V3-control DNA having peaks higher than V3-75°C heat treated DNA, which was in turn, higher than V3-100°C heat treated DNA.

Cluster analysis comparison of DNA multi-wavelength absorbance spectra for the characterization of *Vigna unguiculata* (L) Walp revealed the discriminating power of the spectroscopic technique [21]. Three clusters (cluster 1, cluster 2 and cluster 3) were identified among control DNA extract and heat treated DNA extracts. Heating DNA extracts at 75°C and 100°C altered genomic diversity in different accessions of cowpea.



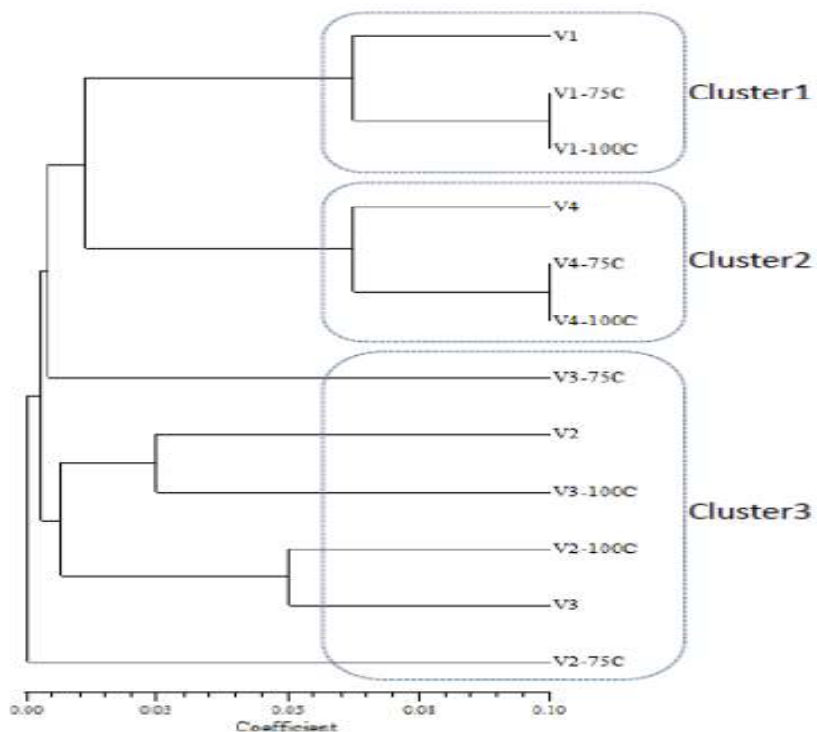
**Fig. 8. Comparison of absorbance spectra of different heat treated DNA samples from IT99K-573-2-1 with DNA standard (DNA Ladder)**

V3= IT99K-573-2-1 control DNA; V3-75C= IT99K-573-2-1 DNA heated at 75°C for 1 hr; V3-100C= TVu-3947 DNA heated at 100°C for 1hr; STD=DNA Standard; STD4=0.026 mg/ml



**Fig. 9. Comparison of absorbance spectra of different heat treated DNA samples from TVu-8775 with DNA standard (DNA Ladder)**

V4= TVu-8775 control DNA; V4-75C= TVu-8775 DNA heated at 75°C for 1 hr; V4-100C= TVu-8775 DNA heated at 100°C for 1hr; STD=DNA Standard; STD4=0.026 mg/ml



**Fig. 10. Cluster analysis comparison of absorbance spectra optical density values of DNA samples with different heat treatments**

## 5. CONCLUSION

The result of the present study showed that heat stress can greatly affect the genomic composition and cause genetic diversity in *Vigna unguiculata* (L) Walp. UV absorption spectrum genomic assay was used to study the effect of heat stress of DNA extract from cowpea seeds. UV absorption spectrum assay is a powerful characterization tool with several applications because a lot of data could be generated from a single multi-wavelength measurement. From our study using cluster analysis to compare multi-wavelength absorbance spectra of DNA for the characterization of *Vigna unguiculata* (L) Walp, three clusters were observed among the control and heat treated DNA extracts. This is an additional indication of the sensitivity and discriminating power of the spectroscopic approach. The ability to discriminate between crude DNA content with different heat-treatment levels from cowpea have been demonstrated with the light scattering and absorption interpretation model and optical properties developed in our laboratory. Heating protein extracts at 75°C, and 100°C have altered genomic diversity in different cowpea accessions. It is highly recommended that further studies be carried out on the interpretation of models for more cowpea varieties. Information gotten should be applied in the cultivation and subsequent methods for consumption of cowpea in ways that would retain its landraces. Also, the sensitivity of the multi-wavelength absorption technique in regards to genomic assay should be investigated.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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