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Optimization and Assessment of Different DNA Extraction Protocols in Cowpea (*Vigna unguiculata* L. Walp.)

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ABSTRACT: The first and the most essential step in any genetic analysis is the extraction of good quality DNA. In this study, comparison of five different protocols of isolating total genomic DNA from Cowpea plant (*Vigna unguiculata*) was carried out. A total of 10 varieties of cowpea (*V. unguiculata*) were collected from IITA and planted in pots in a screenhouse in the Department of Botany, University of Ibadan, Nigeria. After two weeks, DNA was extracted from their leaves using five different DNA extraction methods namely; CTAB protocol, Modified Dellaporta protocol, DNAzol kit, ZR MiniPrep Kit and Epicenter kit (for Blood) and were compared and analysed using nanodrop spectrophotometer and agarose gel electrophoresis. The results from the spectrophotometer showed that CTAB protocol yielded the highest concentration of DNA with a mean value of 2136.51ng/µl, followed by Dellaporta 1707.77ng/µl and least was ZR MiniPrep kit with concentration of 103.64ng/µl. Assessing the purity of DNA, Dellaporta protocol demonstrated DNA purity with mean value of 2.031 indicating slight RNA contamination followed by CTAB protocol 1.966 and the least was ZR MiniPrep kit at 1.263. The results showed that CTAB protocol was the best for further analysis as it provided good DNA quality and quantity.

Keywords: CTAB, Dellaporta, Extraction Kits, Cowpea, Gel Electrophoresis.

Introduction

The primary and the most essential step in any genetic analysis such as gene mapping, population studies, Phylogentic analysis, finger printing and polymerized chain reaction (PCR) is the extraction of good quality DNA (Huaqiang *et al*., 2013). Different organisms require different treatment as to how to extract DNA from their tissue. A particular extraction protocol that gives rise to an optimal result in a particular organism may not yield the same result in another organism due to diversity in the chemical composition of these organisms (Porebski *et al.*, 1997). Secondary metabolites are the major challenge encounter during extraction of DNA from plant tissues. Sometimes they act as an inhibitor against the reagents used in the extraction of DNA or they contaminate the extracted DNA and prevent it from amplification and other downstream analysis (Porebski *et al.,* 1997). Different plant species often may not allow optimal DNA production from one extraction protocol (Behrooz *et al.*, 2012). Thus, for each plant species, an efficient protocol for extraction of DNA as well as the optimization of the PCR conditions is required.
Cowpea as an essential staple crop with a wide range of distribution and uses which ranges from human consumption to livestock folder and to improvement and reclamation of land (Wikipedia). Cowpea is a highly economic plant, it is the most cultivated of its family and it has high nutritional value. Due to its enormous benefits there is therefore need for us to know how best to extract its DNA for genetic study, better understanding and improvement of the plant to further meet human's daily need (Agbicodo *et al.*, 2009).

Materials and methods

Collection of cowpea genotypes: Ten genotypes of cowpea were collected from International Institutes of Tropical Agriculture (IITA), Ibadan for this study. These genotypes are TVu 1185, TVu 1193, TVu 2013, TVu 2027, TVu 14818, TVu 10843, TVu 15206, TVu 8650, TVu 1177, Ife Brown.

Study location and planting procedure: The study location used for this study are the Screen-house, Department of Botany, University of Ibadan and Biotechnology laboratory of National Centre for Genetic Resource and Biotechnology. Black polyethyl bags containing five kilogram of loamy soil was used as nursery pot and four to five seeds were planted per pot. Germination started three days after planting. The seedlings were watered once every day for two weeks before harvesting was done for DNA extraction.

Harvesting of cowpea leaves for DNA extraction: Young leaves were harvested from the seedling two weeks after germination, this was done around 7 am. About 4-6 leaflets were plucked, placed in a polyethyl bags and kept on ice bath before transporting it to the laboratory for DNA extraction.

CTAB extraction protocol of DNA: The CTAB extraction protocol used was a modification of Doyle *et al.* (1990). 0.2g of young cowpea leaves were weighed and ground in a Mortar and pestles which has been preheated in a water bath at 65°C for 20minutes. 1000µl of CTAB buffer was added into the mortar and mixed properly together with the ground tissue before transferring it into an eppendorf tube. The eppendorf tube containing the tissue mixture was then incubated at 65°C for 10minutes. 1000µl of SEVAG reagent was then added into the tube containing the tissue mixture and then vortex vigorously to ensure uniform mixture. The caps were opened to release gas and close and shook again before the tubes were put on an orbital shaker at 450rpm for 1hour. The tubes containing the mixture were then centrifuged at 12000rpm for 5minutes. After centrifugation, the supernatant containing DNA was transferred into another labelled eppendorf tubes and 1000 µl of absolute ethanol was added into the tubes which immediately turns milky. The tubes were gently inverted for 6 times and put in a deep freezer at -20^{o}C for few hours so as to pellet DNA. After few hours, DNA pellet begins to appear at the bottom of tubes. The tubes were then centrifuged at 12000 rpm for 10mintes to precipitate DNA pellet at the bottom of the tube. The supernatant was gently discarded and the DNA is allowed to dry by arranging tubes on a tissue paper and opening the eppendorf tubes exposing the DNA to air. After few minutes, 100µl of ddH_2O (double distilled water) was added to the DNA to dissolve it. The tubes were then arranged in a rack and stored in the refrigerator at -20^{o}C.

Modified Dellaporta protocol: The protocol reported by Ogunkanmi *et al.* (2008) was used with the following modifications. 0.2g of young cowpea leaves were weighed and transferred into an already labelled mortar and pestle which has been preheated in a water bath at 65°C for 20minutes. 1000µl of Dellaporta buffer was added into the mortar containing the leaves and ground well to paste. The ground tissue was then poured into an already labelled eppendorf tube. 200µl of SDS (sodium dodecyl sulphate) was added into the ground tissue in the eppendorf tube. The tubes were then homogenized by shaking properly until a foamy solution is seen. The tubes were then incubated at 65°C in a water bath for 20mins. At every 5mins interval, the tubes were mixed gently. 300µl of ice-cold 5M of potassium acetate was added into the eppendorf tube and the tubes were then incubated on ice for 30mins and centrifuged at 12,000rpm for 10mins. The supernatants were carefully transferred into two separate eppendorf tube using a micropipette. 1ml of ice-cold Iso-propanol was added into the tubes containing the supernatant and mixed until strands of DNA appeared. The tubes were then centrifuge at 12,000rpm for 10mins. This precipitates the DNA at the bottom of the tube. Supernatants were discarded carefully so as not to dislodge pellet of DNA. Tubes were placed upside down on a paper towel for 30mins to drain last drop of iso-propanol. The tubes were then allowed to dry completely by arranging them flat on paper towel. 100µl of double distilled water (ddH_2O) was added into each tube to dissolve the DNA pellet. The tubes were then arranged in a rack and stored in the refrigerator.

DNAZOL extraction kit for DNA: This protocol was as instructed by the manufacturer of the extraction kit and it goes thus; 0.2mg of young leaves of the same samples used previously was weighed and each leaves sample were placed in 10 different labelled pre-heated mortars, and 1.5ml of DNAzol buffer was pipette into the mortar per sample and grind well into pestle. Grinding was done until a fine homogenate is seen, so as to break down cell walls and allow access to genomic DNA. The homogenate was then transferred into well labelled eppendorf tubes. The solution was thoroughly mixed by gentle inversion for about 10 times and then centrifuged twice at 10,000g for 10mins at 10^{o}C, so as to ensure proper sedimentation of the solutions. Following centrifugation, the supernatant was then transferred into another labelled tube and the DNA was precipitated by adding 1ml of ice-cold absolute ethanol into the labelled tube. Samples were mixed by gentle inversion of about 10times, ensuring that the DNAzol and ethanol mix well to form homogenous solution and were stored at room temperature for 30mins. It was then centrifuged again at 5000rpm for 5mins at 25°C to precipitate the DNA. At this stage, DNA becomes visible as a cloudy or milky precipitate. The supernatant is then removed by pouring it out of the tube leaving only the cloudy precipitate. The DNA precipitate was then washed with 70% ethanol. The tubes were then left for 2mins to allow DNA precipitate at the bottom of the tube and the ethanol was removed by decanting. The DNA pellet was then air dried by opening tubes and exposing to air for 10mins after decanting the ethanol. The DNA was then dissolved in 50µl of double distilled water so as to dissolve pellet and store in the refrigerator.

ZR plant/seed DNA miniprep by Zymo research: The protocol used was as instructed by the manufacturer with slight modification based on availability of resources. The kit contains customized, colour coded tubes. Below are the steps employed in the extraction process,

Pre-extraction Processes: Beta-mercaptoethanol was added to the binding buffer in the final dilution of 0.5% (v/v) i.e. 250 µl per 50 ml and Zymo-Spin IV-HRC Spin Filters in a customized microcentrifuge tube code with green caps was centrifuge at 8000rpm for 3 min and then placed in a rack.
DNA extraction: 0.2 g of finely cut leaves of previously used cowpea sample was placed in a customized microcentrifuge tube containing bashing beads. 750µl of lysis solution was added into the tube and the mixture was vortex vigorously on a vortex machine for five minutes. The mixture was centrifuge at 10,000rpm for 1min. 400µl supernatant was then transferred into another customized microcentrifuge tube coded with orange cap and has a filter and a collection tube. The tube was centrifuge at 7000rpm for 1min and the filtrate was collected in the attached collection tube. 1200 µl binding buffer was added to the filtrate in the collection tube and mix. 800 µl of the mixture was transfer into another customized tube (column) with a filter and then centrifuge at 10,000rpm for 1min. The filtrate was discarded and the remaining mixture was transferred into the filter and centrifuge at 10,000rpm for 1minute. 200µl of DNA Pre-wash buffer was added to the column and centrifuge at 10,000rpm for 1min and the filtrate was discarded. Then 500µl of DNA wash buffer was added into the column and then centrifuged at 10,000rpm for 1min, and the filtrate was discarded. 75µl of elution buffer was added into the column matrix and centrifuge at 10,000rpm for 1min. The filtrate was collected in a new, clean and sterile microcentrifuge. The Eluted DNA was transfer into the customized microcentrifugetube with a filter matrix which was centrifuge in the beginning of the extraction with a green colour code, it is designed for ultra-purification of the DNA. It was then centrifuge at 8,000rpm for 1min. The filtered DNA was collected in another microcentrifuge tube and stored in 10^{o}C refrigerator.
Epicentre kit: This Kit was initially designed for Animal tissues (Blood to be specific). The protocol for this Kit was modified in other to optimize its usage for plant tissue and described below.
DNA extraction: 0.2 g of young cowpea leaves (the same plant used in the protocols above) were weighed and transferred into an already labelled mortar and pestle which has been preheated in a water bath at 65°C for 20 mins. 600µl of Lysis buffer I was added into the mortar containing the leaves and ground well to paste. The paste was transferred into an eppendorf tube and incubated at 65^{o}C for 10min and the tubes were shook at 5min interval. 300µl of Lysis Buffer II was added to the tube and vortex briefly and then incubated on ice for 10mins. 175µl of precipitation buffer was added and vortex. The mixture was then centrifuged at 12,000 rpm for 10mins. The supernatant was divide into two tubes and 500 µl of ice-cold isopropanol was added to each tube. The tube was then centrifuged at 12,000rpm for 10min to pellet the DNA and the supernatant was discarded from the pelleted DNA. 70% ethanol was used to wash the pelleted DNA, mixed gently and air dried on a paper towel. The DNA was then dissolved in 50 µl of TE buffer and the stored in the refrigerator.
DNA quality confirmation: The quality of the extracted DNA was confirmed using Agarose gel Electrophoresis. This is done by running the extracted DNA through a Matrix of 1% Agarose gel in a buffer with the aid of an electric current and also by spectrophotometer check.

Results

Agarose gel profile of DNA extracted from the five DNA extraction protocol: The agarose gel profile of the extracted DNA from the five DNA extraction protocols is shown in Plate 1. Both CTAB and Dellaporta protocols showed clear and distinct gel products. The presence of smears was observed in DNAzol, Zymo and Epicentre kits in sampled DNA extracted in the study (Plate 1).

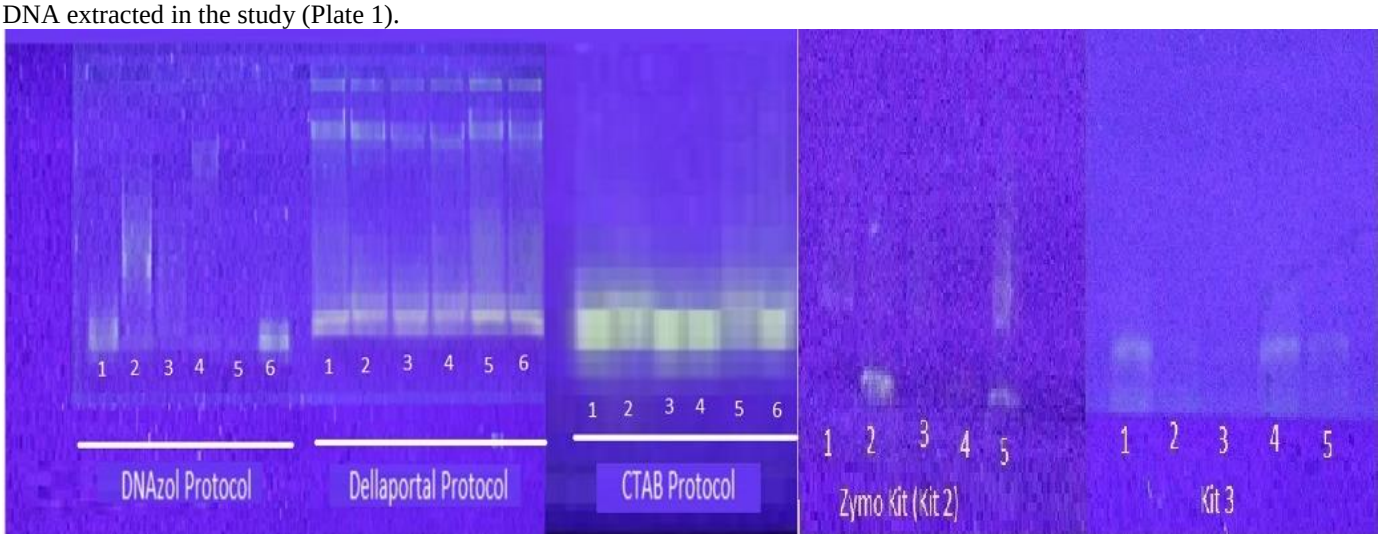

Plate 1: Agarose Gel Profile of DNA extracted from five DNA Extraction Protocols**.**

Comparison of DNA concentration among the five different extraction protocols: Comparison of the five DNA extraction protocol were made and plotted on graph below. Fig. 1 shows in comparison the concentration of extracted DNA from each sample (samples 1-10). CTAB protocol has the highest concentration in sample 6 containing 4368.5ng/µl while sample 9 has the least concentration of 744.2ng/µl. In Dellaporta protocol, the highest concentration was obtained in sample 10 containing 3905.1 ng/µl and the least was in sample 7 containing 803.7ng/µl. DNAzol kit has the highest concentration of the extracted DNA in sample 9, containing 378.5ng/µl and the concentration of the extracted DNA was lowest in sample 5 which contain 66.1ng/µl. in Zymo and Epicenter kit the highest concentration was observed in samples 3 and 5 with the values 346.7 and 289.3 respectively. While the least values were observed in samples 1 and 6 with the values 21.2ng/µl and 25.3ng/µl respectively.
Comparing the concentration of the extracted DNA from the five protocols, CTAB protocol has the overall highest concentration of extracted DNA with the mean value of 2136.51ng/µl and the least was observed in Epicenter kit with the mean value of 99.14ng/µl. Both CTAB and Dellapota protocols gave the highest DNA concentration output among the evaluated extraction protocols when used on the ten samples in the study. These two protocols were found to perform better in level of DNA production than commercialized DNA extraction kits used in the study that gave DNA products below 500 ng/µl in samples evaluated (Fig 1).

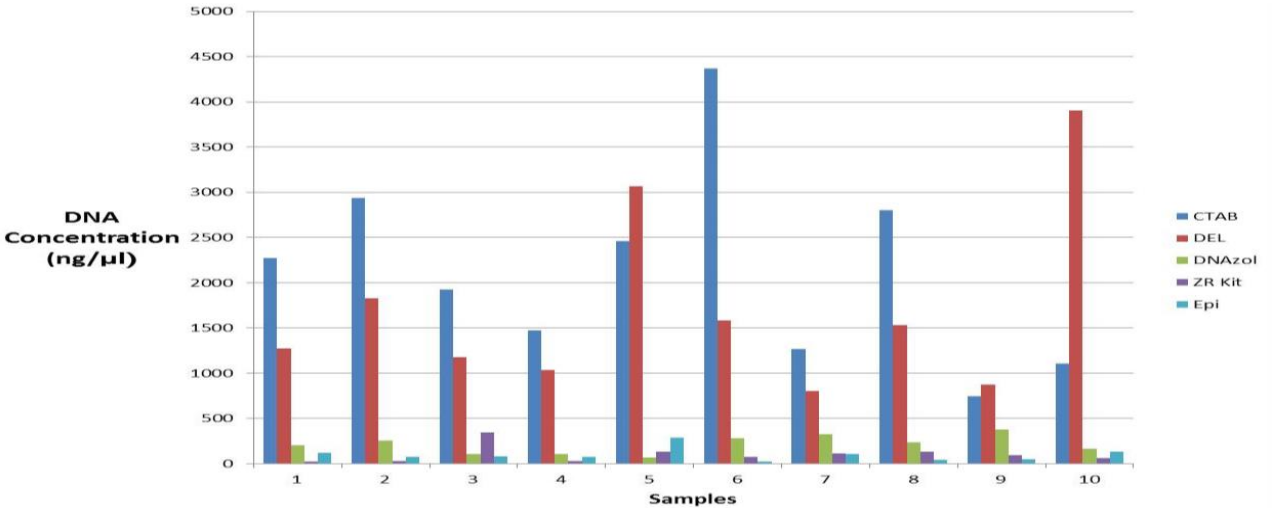

Fig 1: Comparison of ten genotypes of cowpea from five different extraction protocols against Concentration.

Comparison of DNA purity among the five-different extraction protocol: The purity of the extracted DNA from the 10 plant samples and the five protocols were made and plotted on graph below. Fig. 2 shows in comparison the purity of extracted DNA from each sample (samples 1-10). Dellaportal protocol has the highest purity in sample 3 with the purity value of 2.17 while sample 1 has the least purity value of 1.56. In CTAB protocol, the highest purity value was obtained in sample 4 with the value 2.47 and the least was in sample 9 having 1.60. DNAzol kit has the highest purity of the extracted DNA in sample 5, having 2.37 and the purity of the extracted DNA was lowest in sample 10 which has the purit value of 1.45. In Zymo and Epicenter kit the highest purity value was observed in samples 6 and 4 with the values 1.52 and 1.72 respectively. While the least values were observed in samples 10 and 5 with the values 0.83 and 1.08 respectively.
Comparing the purity of the five protocols, Dellaporta protocol has the overall highest purity of extracted DNA with the mean value of 2.01 and the least was observed in Zymo kit with the mean value of 1.18. The level of purity among extraction methods was observed to be least in ZR kit in samples evaluated in the study. However, CTAB, Dellaporta and DNAzol methods consistently gave purity levels above 1.5 (Fig 2).

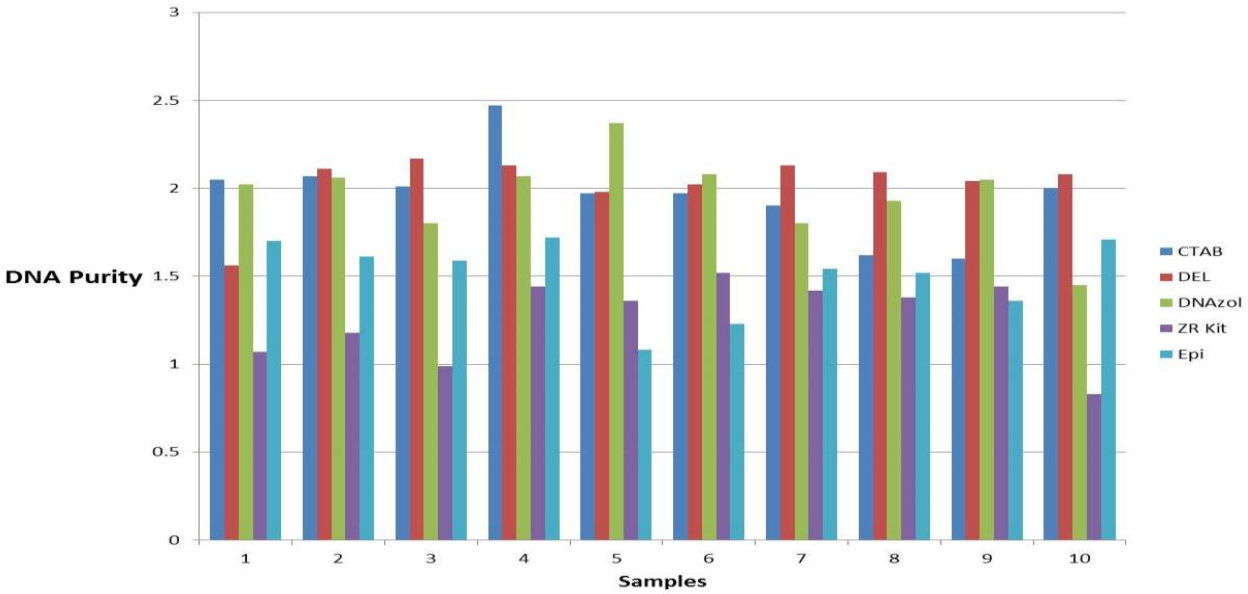

Fig 2: Comparison of ten genotypes of cowpea from five different extraction protocol against Purity

The Concentration and Purity of the extracted DNA: The mean of the DNA extracted by CTAB, Dellaporta, DNAzol, ZR DNA MiniPrep and Epicentre kit is shown in table 1. The concentration of the extracted DNA from CTAB protocol had the highest yield with a mean value of 2136.51ng/µl follow by Dellarpota protocol (1707.77ng/µl), DNAzol (212.43ng/µl), ZR MiniPrep Kit (103.64ng/µl) while Epicenter Kit (99.14ng/µl) was the least. Table 1 also shows that in terms of purity, Dellaporta protocol gave the highest value of 2.01 and Zymo kit gave the least value of 1.18.

Table 1: Mean Concentration and Purity of DNA extracted from each protocol.

Protocol	**Concentration (ng/µl)**	**A_{260}**	**A_{280}**	**$A_{260/280}$**
CTAB	2136.51	42.73	21.93	1.94
Dellapota	1707.77	34.15	16.94	2.01
DNAzol	212.43	4.25	2.25	1.88
Zymo	103.64	2.07	1.75	1.18
EPI	99.14	1.98	1.42	1.39

Summary of the five protocols in ten genotypes of cowpea: The overall performance of each protocol is summarized in table 2. Each protocol was score base on position (1^{st}, 2^{nd}, 3^{rd}…) with the first position scoring 10 points, second 8 points, third 6 points, fourth 4 point and fifth 2 points out of 10 points. In terms of yield and purity, CTAB protocol has the highest points while Epicenter and ZRminiPrep kit has the lowest points respectively. In terms of availability, Dellaportal is more readily available than the rest while ZR MiniPrep is the most time saving. In the overall performance, CTAB protocol is the most preferred with 75% while ZR Miniprep and Epicenter Kit are the least preferred. The time estimate used here exclude the time for grinding and drying of the extracted DNA (Table 2).

Table 2: Overall performance of each protocol

Protocols	**Yield**	**Purity**	**Availability and Cost**	**Time**	**Total**
CTAB	10/10	10/10	8/10	2/10	¾ or 75%
Dellaporta	8/10	6/10	10/10	4/10	2.8/4 or 70%
ZNAzol Kit	6/10	8/10	6/10	6/10	2.6 or 65%
ZR MiniPrep Kit	4/10	2/10	2/10	10/10	1.8 or 45%
Epicentre Kit	2/10	4/10	4/10	8/10	1.8 or 45%

Discussion

The concentration of DNA in a sample, and its condition, are often estimated by running the sample on an agarose gel, however, for a more accurate determination of the concentration of DNA in a sample or solutions, a UV spectrophotometer is commonly used (Clack *et al*. 2000). The quality, integrity or concentration of the extracted genomic DNA in this research was confirmed by both Agarose Gel Electrophoresis and Spectrophotometer check. 1% Agarose Gel was used in quantifying the Quality of the extracted genomic DNA, the Dellaporta protocol shows clear and high concentration of the DNA and RNA as well as CTAB protocol. This is in agreement with the findings of Ogunkanmi *et al.* (2008). Other protocols also show the presence of DNA but very faintly i.e. in lower concentration. The Spectrophotometer check also confirms the results observed in the Agarose gel. The evaluation of the purity of DNA can be easily quantified by absorbance of UV light. This procedure was first described by Warburg and Christian (Warburg *et al*., 1942) in measuring the purity of protein in the presence of nucleic acid contaminant. Absorbance at 260 wavelength (A_{260}) indicate the presence of Nucleic acids i.e. DNA and RNA, and Absorbance at 280 wave-length (A_{280}) indicate the presence of protein and phenolic compounds.

According to Nanodrop Incorporation (2007), purity of DNA samples can be measured by ratio of Absorbance at 260 wavelength (A_{260}) to Absorbance at 280 wavelength (A_{280}). Ratio between 1.7- 2.0 is generally accepted as pure DNA while ratio greater than 2.0 (>2.0) is said to contains RNA as impurity and ratio less than 1.7 (<1.7) is said to contain Proteins and Phenolic compounds as impurities which tend to affect the quality and behavior DNA in DNA downstream analysis.

However, there are two school of thought to the presence of RNA in the extracted DNA samples; The first believed that the presence of RNA contaminant often inhibit restriction endonucleases digestion and/or PCR amplification (Couch *et al*., 1990; Guillemaut*et al*., 1992; Richards *et al*., 1994) while some scientists believed that the presence of RNA in DNA extraction is not a major problem as this does not usually interfere with PCR or restriction digestion (Murry*et al.*, 1980; Vinod, 2004) because RNA is by nature transient and unstable unlike DNA.

In CTAB protocol sample six gave the highest yield of 4368.5ng/µl and an excellent purity of 1.97 while sample nine gave the least concentration and purity of 1.60 which shows the presence of contaminants such as proteins and/or phenol. Only two of the samples (sample 8 & 9) are contaminated with protein and/or phenol while four of them show presence of RNA.Dellaporta protocol gave the second highest DNA yield of the five protocols. It has a mean concentration of 17077ng/µl and mean purity of 2.031. Sample ten has the highest yield of 3905.1ng/µl and purity of 2.08 showing slight contamination of RNA while Sample seven gave the least concentration of 803.4ng/µl and Sample one gave the least purity of 1.56. The contamination in Sample one may be as a result of poor handling as it is the only one that is contaminated with protein of all the samples in this protocol (Ogunkanmi*et al.*, 2008).DNAzol Kit is the next in terms of yield and quality. It has a mean concentration of 212.43ng/µl and mean purity if 1.963. Sample nine has the highest yield of 378.5ng/µl and slight RNA contamination (purity of 2.05) while sample six has the least concentration (66.1ng/µl) and Sample ten has the least purity (1.45), which may also be due to poor handling of the sample as it is the only one with protein and or phenol contamination. ZR MiniPrep Kit gave a mean concentration of 103.64ng/µl and mean purity of 1.263. Sample three gave the highest yield of 346.7ng/µl but with very high protein/phenol contamination with a purity value of 0.99. Sample one gave the least concentration of 21.2ng/µl and Sample ten gave the least purity value 0.83 and also a very low yield. All extracted DNA has purity value that is less than 1.7 because they are all heavily contaminated with protein and or phenol. This poor performance observed in this kit may be due to the presence of some secondary metabolites that inhibit the activities of some of the buffers or loss of strength of the buffer if the date of manufacture is too far from date of usage (Huaqiang*et al.*,2013). Optimization of Epicentre Kit gave mean concentration of 99.14ng/µl and mean purity of 1.506. The result shows that with further improvement on this kit it can be used to extract good quality DNA as it has three samples with purity value above 1.7 (Ogunkanmi *et al.*, 2008).

Conclusion and Recommendation

In conclusion, this study shows that three out of the five protocols are suitable for extraction of DNA from Cowpea leaves. The best been CTAB with 75% preference followed by Dellaportal with 70% and then DNAzol with 65%, the other two are not advisable to be used as they both have only 45% preference. However Epicentre Kit shows possibility of been suitable for use if it is well modified. With respect to yield (DNA concentration), purity and availability CTAB protocol is preferred to other protocols used but when all the materials are readily available and time is the only factor to be considered, DNAzol is the most preferred.

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