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EFFECT OF GRINDING AGENTS AND DETERGENTS ON THE QUALITY OF EXTRACTED DNA FROM DIVERSE PLANT SPECIES

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ABSTRACT

The present work was aimed to study the effect of various grinding agents and detergents on the amount and purity of the extracted DNA from diverse plant samples. Agents supporting grinding process were liquid nitrogen, acid washed sand and glass beads, along with a number of concentrations of two detergents i.e. SDS (Sodium Dodecyl Sulphate) and CTAB (Cetyltrimethylammonium bromide). Six plants species Croton (*Codiaeum variegatum*), Tomato *(Lycopersicon esculentum*), Orchid (*Orchis militaris)*, Date palm *(Phoenix dactylifera* L.), Pineapple (*Ananas cosmosus)* and *Aloe (Aloe vera);* were selected for DNA isolation and these plant species differ in their leaf texture as well as polysaccharides and phenolics content. The results obtained showed the presence of linear relationship between detergent and yield of the DNA with a little influence on the purity. The type of grinding agent did not significantly affect the purity of the extracted DNA but the amount of extracted DNA is greatly influenced. The purest DNA samples of all six plants species were selected and subjected to PCR amplification.

Key words: DNA extraction, detergents, Sand, Glass Beads, Liquid Nitrogen, Croton, Tomato, Orchid, Date palm, Pine apple and *Aloe vera*.

INTRODUCTION

Plant molecular biology is a rapidly developing field. The good quality nucleic acid is a prerequisite for most of the molecular biology experiments. Extraction of nucleic acid is the major bottleneck in molecular studies of the plants (Karakousis and Langridge, 2003). The degree of purity and quantity varies between treatments during extraction procedure. A good extraction procedure for the isolation of DNA should yield adequate amount of intact DNA with a reasonable purity. The procedure should also be quick, simple and cheap with less hazardous chemicals.

Plant tissues contain high level of polysaccharides and polyphenolic compounds, which serve as a major source of contamination for plant DNA extraction. When cells are disrupted, the cytoplasmic compounds can come into contact with nuclei and other organelles and causes severe problems (Loomis, 1974). In their oxidized forms, polyphenols covalently bind to DNA giving it a brown color and making it useless for most research applications (Guillemaut and Maréchal-Drouard, 1992). One method commonly used to avoid problems of polyphenols is to freeze the tissues during or prior to homogenization (Leutwiler *et al*., 1984). The presence of these compounds renders studies difficult due to long and tedious extraction procedures and often does not result in good standards in terms of yield and quality. Certain polysaccharides are known to inhibit Random Amplified Polymorphic DNA (RAPD) reactions (Pandey *et al.*, 1996). Restriction Fragment Length Polymorphism (RFLP) analysis, cloning, creation of gene banks and various other techniques are also sensitive to DNA quality. The quality and quantity of the extracted DNA is usually determined by the UV spectrophotometer. The absorbance at 260 nm indicates the amount of DNA present in the sample where as the absorbance ratio at 260/280 nm shows the purity of the sample. If the value at 260 nm is 1 then the sample contains 50 µg/ml of DNA while the ratio of 1.8 shows the DNA of high purity. The ratio above 1.8 shows the presence of RNA and the value below 1.8 indicates the sample is contaminated with phenolics or polysaccharides (Sambrook *et al.*, 1989).

The extraction process involves breaking of cell walls in order to release the cellular constituents, followed by disruption of the cell membranes to release the DNA into the extraction buffer. This is normally achieved by using detergents such as SDS or CTAB. The released DNA should be protected from endogenous nucleases. EDTA is often included in the extraction buffer to chelate magnesium ions, a necessary co-factor for nucleases, for this purpose. The initial DNA extracts often contain a large amount of RNA, proteins, polysaccharides, tannins and

pigments which may interfere with the extracted DNA and difficult to separate. Most proteins are removed by denaturation and precipitation from the extract using chloroform and/or phenol. RNAs on the other hand are normally removed by treating the extracts with RNase. Polysaccharide-like contaminants are, however, more difficult to remove. They can inhibit the activity of certain DNA-modifying enzymes and may also interfere in the quantification of nucleic acids by spectrophotometric methods (Wilkie *et al.,* 1993). NaCl at concentrations of more than 0.5 M, together with CTAB is known to remove polysaccharides (Murray and Thompson, 1980; Paterson et al., 1993). The concentration ranges mentioned in literature varies between 0.7 M (Clark, 1997) and 6 M (Aljanabi *et al.,* 1999) and is dependent on the plant species under investigation. In some protocols, NaCl is replaced by KCl (Thompson and Henry, 1995).

This study was targeted towards the optimization of a DNA extraction protocol that is effective against a variety of plants; differ in their leaf texture along with polysaccharides and polyphenolic contents. To study the effect of type of detergent used and the concentration of grinding agent on the purity and yield of DNA was also the aim of this experiment.

MATERIALS AND METHODS

Plant Material

Six diverse plant species have been selected for this study named as Croton (*Codiaeum variegatum*), tomato *(Lycopersicon esculentum*), orchid (*Orchis militaris)*, date palm *(Phoenix dactylifera* L.), pineapple (*Ananas cosmosus)* and *Aloe vera*. All the plant material used in this study is obtained from the green houses of the H.E.J. Research Institute of Chemistry, University of Karachi. Different concentrations of two detergents i.e., CTAB and SDS were tested along with the three different types of grinding agents which includes liquid nitrogen, glass beads and sand. Twenty eight different treatments (with respect to grinding agents and extraction buffers) were applied to all six plant species and results were recorded and analyzed.

Solutions and Buffers

- ***Extraction Buffer-A***: 100 mM Tris-HCl, 1.4M NaCl, 20 mM EDTA, **2% CTAB**, 0.2% β-mercaptoethanol, 1% PVP, pH 8.0.
- ***Extraction Buffer-B***: 100 mM Tris-HCl, 1.4M NaCl, 20 mM EDTA, **4% CTAB**, 0.2% β-mercaptoethanol, 1% PVP, pH 8.0.
- ***Extraction Buffer-C***: 100 mM Tris-HCl, 1.4M NaCl, 20 mM EDTA, **6% CTAB**, 0.2% β-mercaptoethanol, 1% PVP, pH 8.0.
- ***Extraction Buffer-D***: 100 mM Tris-HCl, 1.4M NaCl, 20 mM EDTA, **2% SDS**, 0.2% β-mercaptoethanol, 1% PVP, pH 8.0.
- ***Extraction Buffer-E***: 100 mM Tris-HCl, 1.4M NaCl, 20 mM EDTA, **4% SDS**, 0.2% β-mercaptoethanol, 1% PVP, pH 8.0.
- ***Extraction Buffer-F***: 100 mM Tris-HCl, 1.4M NaCl, 20 mM EDTA, **6% SDS**, 0.2% β-mercaptoethanol, 1% PVP, pH 8.0.
- ***Extraction Buffer-G***: 100 mM Tris-HCl, 1.4M NaCl, 20 mM EDTA, 0.2% Marcaptoethanol, 1% PVP, pH 8.0.
- ***T.E. Buffer***: 10 mM Tris-HCl, 1mM EDTA, pH 8.0.
- ***Washing Buffer***: 10 mM Ammonium acetate, 76% Ethanol.
- Chloroform:Isoamyl alcohol (24:1).
- Sand was washed with 1 N HCl and autoclaved prior to use.
- Glass beads were autoclaved before use in extraction process.

DNA Extraction

Leaf samples of Croton (*Codiaeum variegatum*), Tomato *(Lycopersicon esculentum*), Orchid (*Orchis militaris)*, Date palm *(Phoenix dactylifera* L.), Pineapple (*Ananas cosmosus*) and *Aloe (Aloe vera)* were washed with tap water and dried completely before weighing. All the extraction buffers were preheated at 60^0C and then all the treatments (Table-1) were applied to each plant sample. Homogenization was the next step in which powdered leaves and the extraction buffer was incubated at 60^0C for 40 minutes, with continuous gentle shaking followed by the addition of 2 ml freshly prepared Chloroform:Isoamyl alcohol solution that was invert mixed 50 times, centrifuged at 18000 g for 10 minutes at 4^0C. After that, 3 ml of ice cold Isopropanol was added to the supernatant and the mixture incubated at 4^0C for 30 minutes. The DNA pellet obtained spooled out or in case of trace amount was centrifuged at 18000 g for 10 minutes at 4^0C and the obtained DNA pellet washed with 5 ml of washing buffer. In the end, centrifugation at 18000g was done to separate the pellet and dissolved in 1000 µl of TE Buffer.

Amount and Purity of DNA

0.8% agarose gel was run to check the presence of DNA in the samples (Figure-1). The dilution of the samples was made by dissolving 5 µl of sample in 995 µl of TE buffer to determine the OD first at 260 nm then at 260/280 nm to check the amount and purity of the extracted DNA, respectively.

Table 1. The O.D. Ratios (260/280 nm) of all the samples of six different plants.

		260/280 Ratios					
Code	Treatment	Date Palm	Orchid	Pineapple	Croton	Tomato	*Aloe vera*
T1	No detergent + liquid nitrogen	1.08	1.02	1.08	1.73	1.2	1.08
T2	2% CTAB + liquid nitrogen	1.23	1.02	1.21	1.7	1.06	1.26
T3	4% CTAB + liquid nitrogen	0.84	1.02	1.21	1.7	1.76	1.26
T4	6% CTAB + liquid nitrogen	0.82	1	1.2	1.68	1.08	1.25
T5	2% SDS + liquid nitrogen	1.02	1.3	1.2	0.76	1.1	1.25
T6	4% SDS + liquid nitrogen	0.91	1	1.2	2.59	1.13	1.83
T7	6% SDS + liquid nitrogen	0.95	0.99	1.15	2.5	1.2	1.85
T8	No detergent + sand	1.25	0.95	1.03	1.69	1.09	1.41
T9	2% CTAB + sand	1.48	0.85	1.08	1.77	1.03	1.55
T10	4% CTAB + sand	1.08	1.1	1.1	1.81	1.06	1.47
T11	6% CTAB + sand	1.05	1.01	1.25	1.85	1.04	1.23
T12	2% SDS + sand	1.03	0.97	1.15	1.71	1.09	1.5
T13	4% SDS + sand	1.05	1.15	1.01	1.74	1.09	1.52
T14	6% SDS + sand	1.04	1.2	1.05	1.8	1.1	1.55
T15	No detergent + glass beads	1.13	0.9	1.17	1.84	1.25	1.89
T16	2% CTAB + glass beads	0.96	1.01	1.2	1.74	1.24	1.42
T17	4% CTAB + glass beads	1.5	1.2	1.24	1.55	1.33	0.48
T18	6% CTAB + glass beads	1.55	1.24	1.3	1.58	1.55	0.45
T19	2% SDS + glass beads	0.92	1.06	1.17	1.77	1.22	1.47
T20	4% SDS + glass beads	0.89	1.1	1.02	1.89	1.17	2.56
T21	6% SDS + glass beads	0.9	1.16	1.1	1.82	1.54	2.54
T22	No detergent & grinding agent	1.18	1.1	1.18	1.67	1.14	0.39
T23	2% CTAB + no grinding agent	0.92	1.06	1.19	1.1	1.08	1.22
T24	4% CTAB + no grinding agent	1.05	0.97	1.19	1.72	1.13	0.68
T25	6% CTAB + no grinding agent	1.02	0.95	1.12	1.8	1.25	1.82
T26	2% SDS + no grinding agent	1.22	1.26	1.2	1.41	1.17	1.29
T27	4% SDS + no grinding agent	1.03	1.21	1.13	1.56	1	1.35
T28	6% SDS + no grinding agent	1.06	1.29	1.2	1.42	1.5	1.42

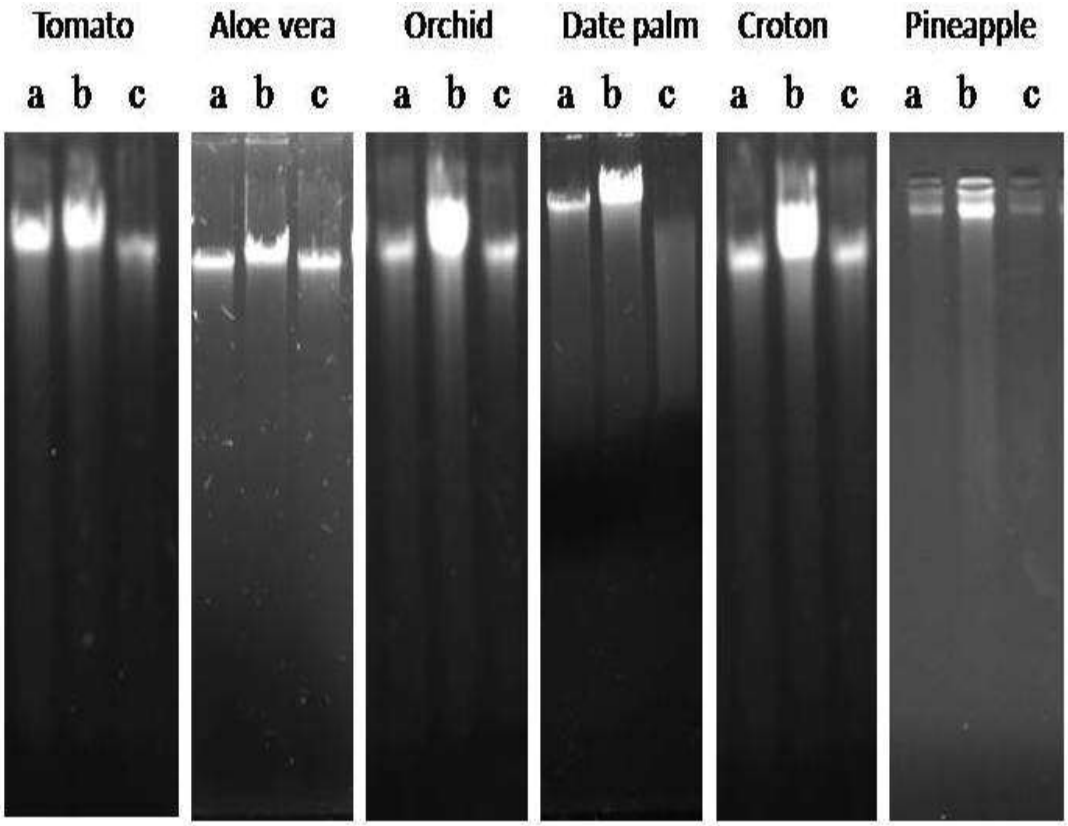

Fig. 1. 0.8% Agarose gel for the detection of DNA in the plant samples extracted by using various grinding agents. a- Acid washed sand, b- Liquid Nitrogen and c- Glass beads.

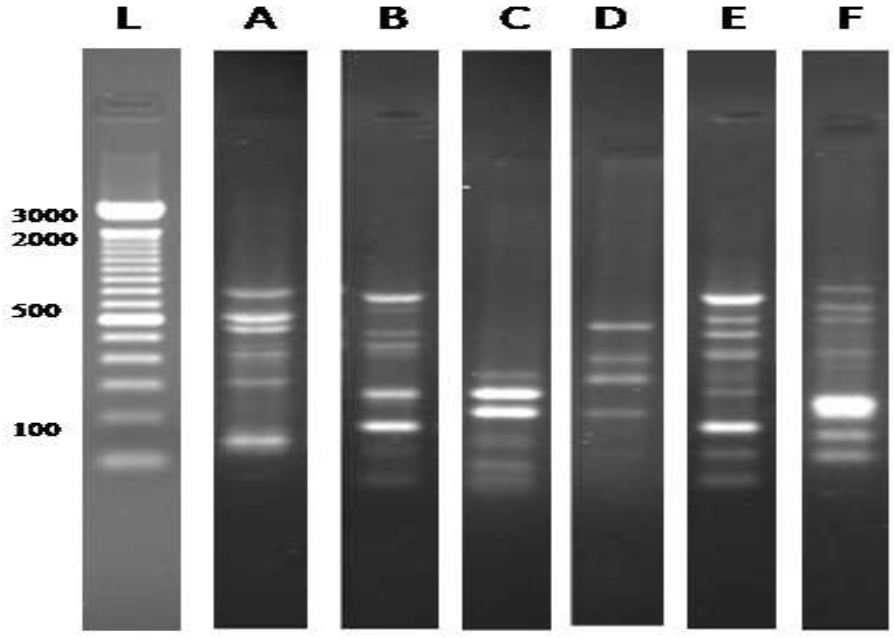

Fig. 2. RAPD-PCR products of extracted DNA samples. L- Ladder, A-Croton, B- Tomato, C-Orchid, D-Date palm, E- *Aloe vera*, F-Pineapples.

PCR Analysis

PCR analysis of the extracted DNA samples was done by using 25 µl PCR reaction mixture having 12.8 µl distilled water (PCR Grade), 1x PCR Buffer (10 mM Tris-HCl, 50 mM KCl, 0.1% Triton X-100), 2 mM $MgCl_2$, 0.1 µM of each dNTP, 0.5 µM of primer and 0.25 U of *Taq* polymerase. Each reaction mixture contains 20ng of DNA. The thermocycler was programmed for initial denaturation at 95^0C for 2 minutes, with 40 cycles of 30 seconds of denaturation at 95^0C, 30 sec for annealing at 60^0C and 40 sec for extension at 72^0C, followed by a final extension for 7 min at 72^0C. The lid of the thermocycler was preheated and maintained at 105^0C during the reaction. Electrophoresis was performed to separate the amplified PCR products on 1.2 % agarose gel. A series of OPF primers for RAPD were used and only the best-amplified products are shown in Figure-2.

Abbreviations: SDS: Sodium Dodecyl Sulphate, CTAB (Cetyltrimethylammonium bromide), OD (optical density)

RESULTS AND DISCUSSION

Effect of Grinding Agent

The grinding agents are used simply to aid the process of grinding of the plant tissues to rupture the cell wall. Several different types are reported including Liquid Nitrogen, Sand and Glass beads, depending upon the type of plant, availability and cost. The availability of liquid nitrogen to all parts of the world along with the hazards associated with its use makes liquid nitrogen unsuitable for many labs, especially in the developing world (Sharma *et al*., 2003). On the other hand, the DNA extraction protocols involving sand or glass beads cause the problems of purity and the overall yield of DNA.

In this experiment, all three methods were used and results were analyzed to select the most effective and economical DNA extraction method among them. In this experiment, liquid nitrogen is proved as the most effective grinding agent, as shown in Table-1, three (Orchids, Tomato, *Aloe vera*) out of six plants showed DNA yield of high purity and OD ratios on 260/280 nm was near to 1.8. The second most effective grinding agent was glass beads, date palm and pineapples showed DNA of maximum purity. Sand is proved least effective as only one sample of DNA (crotons) extracted and showed high purity. These result showed that the type of plant greatly influence the type of grinding agent to be used and all of the three grinding agents are capable of producing DNA of high purity.

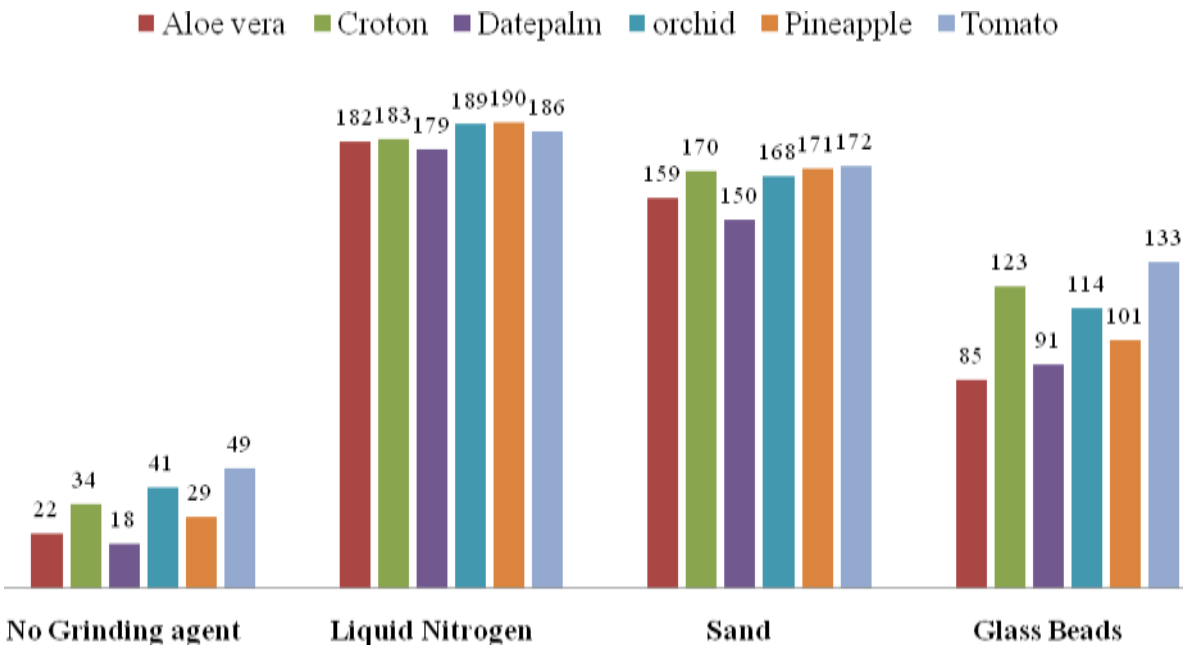

Fig. 3. Effect of grinding agent on average yield of DNA (µg/2gm of leaf).

Apart from purity, the amount of DNA is also the main concern while extracting the plant DNA. Figure 3 represents the average µl of DNA yields from 2 g leaves from plant samples and it can be easily suggested that the DNA yield is highly influenced by the use of grinding agent when compared to the control. The control sample with

no grinding agent is failed to produce the high yield of pure DNA. Thus, the use of a proper grinding agent is proved to be crucial. All the six plant samples gave maximum amount of DNA when extracted using Liquid Nitrogen. The Figure 3 also suggests that acid wash sand is good substitute of liquid nitrogen as far as yield of DNA is concerned. The texture of leaf also affect the yield as soft leaves (tomato and croton) gave higher yield as compare to other hard and succulent leaf samples. Liquid Nitrogen yields maximum amount of DNA irrespective of leaf texture (Figure 3).

All the samples of six plants that showed maximum purity are subjected to PCR analysis and all the six samples were being amplified (Figure 2) showing that grinding agents are ineffective in making the extracted DNA unsuitable for the further molecular analysis.

Effect of Detergent

Plant samples are considered to be the most difficult contenders for DNA extraction simply because of the presence of rigid plant cell wall (Sperisen *et al.*, 2000). The presence of polysaccharides and phenols causes main contamination problem and their presence simply makes the extracted DNA unable to be amplified by PCR amplification. The breaking or digesting the cell walls in order to release the cellular constituents is basically the main function of the detergent, along with the disruption of the cell membranes to release the DNA into the extraction buffer. Two detergents are normally used in this regard, which are SDS or CTAB.

In this experiment, different concentrations of SDS and CTAB (2%, 4% and 6%) were used to observe the effect of type and concentration of a detergent on the yield and purity of DNA. A control with no detergent is also used to identify the significance of the use of detergent.

The purity of the DNA is very much affected by the type and concentration of detergent. As Table-1 indicates, CTAB is proved to be more effective in the concentration of 4% as four (Date palm, Pineapple, Croton and Tomato) out of six purest samples have been extracted by the use of 4% CTAB. SDS is effective only in the case of remaining two plants (Orchid, *Aloe vera*) in the concentrations of 2% and 4% respectively. The control sample shows brown colored DNA, indicating the presence of phenolic and polysaccharides.

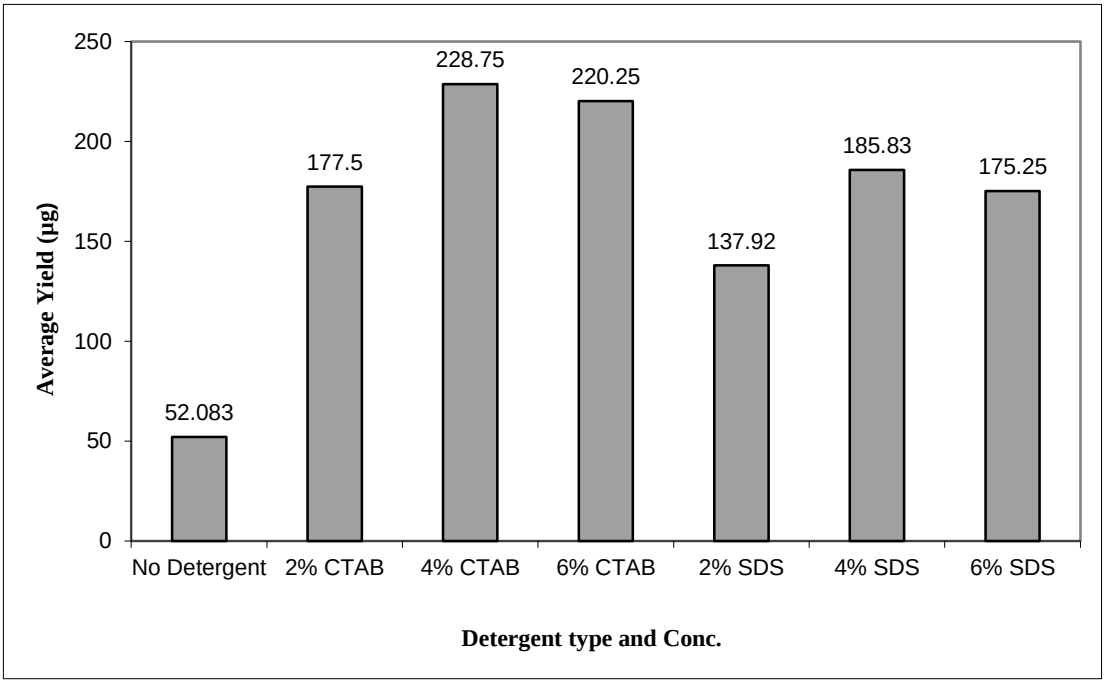

Fig. 4. Effect of type and concentration of detergent on the average yield of DNA (µg/2gm of leaf).

The average yield of DNA was found to be highly affected in comparison to control (Figure 4). Table 3 showed that there was a linear relationship between the average yield and concentration of detergent. CTAB (4%) provided the maximum average yield followed by the SDS (4%). Very interestingly both the SDS and CTAB, in the concentration of 6%, failed to produce higher amount of DNA in comparison to 4% concentration mainly because the amount of detergent present in 4% solution is sufficient enough to extract the DNA from 2 g of leaves. The very

low average yield of DNA in the control reveals the importance of the use of detergent while extracting DNA from plants.

CONCLUSION

In the light of the above results, it is concluded that all the grinding agents are equally effective in producing high quality DNA as average purity of all the samples extracted by different grinding agents is not very much different. DNA extracted by the use of liquid nitrogen, sand and glass beads, can be amplified by the PCR and suitable for the further molecular analysis. The average yield of DNA was although affected by the use of grinding agent. The concentration and type of the detergent not greatly influenced the purity of DNA but a linear relationship has been found between the concentration of detergent and the amount of DNA.

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