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Full Length Research Paper

Leaf storage conditions and genomic DNA isolation efficiency in *Ocimum gratissimum* L. from Kenya

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Storage of plant tissues for DNA is important to avoid degradation of DNA. Preliminary studies were conducted on *Ocimum gratissimum* L. in order to establish the storage conditions for the collected samples before DNA extraction. Secondly, the aim was to determine the best protocol for the extraction of high quality DNA, which would later be used for molecular analysis. DNA was extracted from the samples one month after field sampling. During the DNA extraction, four protocols were used; the modified hexadecyltrimethyl ammonium bromide (CTAB) mini preparation method described by Doyle and Doyle (1990), with reductants either mercaptoethanol or dithiothreitol; the modified sodium dodecyl sulphate (SDS) mini preparation method of Edwards et al. (1991) with redundant either mercaptoethanol or dithiothreitol. The DNA was purified, treated with RNase, quantified and examined for intactness using gel electrophoresis method. Good quality and high yield DNA could only be extracted with the buffer containing the detergent SDS and the reducing agent dithiothreiotol.

Key words: *Ocimum gratissimum* L., Sodium dodecyl sulphate (SDS), hexadecyltrimethl ammonium bromide (CTAB), dithiothreitol, mercaptoethanol.

INTRODUCTION

Ocimum gratissimum L. under study in this case belongs to the Lamiaceae family, which has close to 252 genera and 6700 species (Mabberley, 1997) most of which are used as medicine (Wren, 1968). The leaves are often hairy and posses epidermal glands which secrete volatile oils giving characteristic scents to many of the species.

The essential oils found in leaves, seeds, flowers and roots of *Ocimum* species are used as medicine. Under *in vitro* conditions, the oils have shown to have antibacterial activity against gram positive bacteria: *Staphylococcus aureus* (ATCC 25923), *Bacillus* species and gram negative bacteria: *Escherichia coli* (ATCC 25922), *Salmonella typhi, Pseudomonas aeruginosae, Proteus mirabilis*, *Klebsiella pneumoniae* (ATCC 27853), *Salmonella enteriditis*, *Shigella flexineri,* and pathogenic fungus namely *Candida albicans* (Nakamura et al., 1999; Matasyoh et al., 2007).

Because of its potential as a traditional medicine, incorporation of *O. gratissimum* L. into agro forestry systems would not only make the species accessible to the majority of the rural population that uses it but also contribute to its genetic conservation. Like most countries in Sub-Saharan Africa, access to health services in Kenya is beyond the reach of most of the rural population. However, before widespread domestication of the species is implemented, it would be important to determine its genetic diversity in Kenya so that useful genotypes that could be used as cultivars by farmers can be selected thereby also facilitating the efficient conservation, management and utilization of the species genetic diversity. There are techniques available for assessing genetic diversity at molecular level. These techniques can be divided into three classes as described in litera-ture review: morphology, biochemical and more recent DNA technology.

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The application of DNA technology in agricultural research has progressed rapidly over the last twenty years, especially in the area of cultivar identification and characterization (Nybom, 1990) as well as determination of population diversity in many plant species (Lei et al., 2006; Chen and Yang, 2004; Nan et al., 2003; Ipek and Madison, 2001; Muluvi et al., 1999; Cardoso et al., 2000). The application of this powerful tool in some plant species has however been constrained by lack of efficient nucleic acids isolation techniques. The extraction of the nucleic acids is difficult in a variety of plants because of the presence of secondary metabolites that interfere with DNA isolation procedures and reactions such as DNA restriction, amplification and cloning (Sghaier et al., 2005). A large number of secondary metabolites such as tannins, alkaloids, phenolics and terpens responsible for the valuable pharmacokinetic properties of medicinal plants which interfere with the isolation process, tend to copurify with DNA and interact irreversibly with proteins and nucleic acids (Katterman and Shattuck, 1983). Problems encountered in the isolation and purification of high molecular weight DNA from certain medicinal and aromatic plant species include: degradation of DNA due to endonucleases, co-isolation of highly viscous polysaccharides, and inhibitor compounds like polyphenols and other secondary metabolites which directly or indirectly interfere with subsequent enzymatic reactions (Weishing et al., 1995). A good extraction procedure for the isolation of DNA should yield adequate and intact DNA of reasonable purity. Various protocols for DNA extraction have successfully been applied to many plant species (Doyle and Doyle, 1990; Rogers and Benedich, 1985; Edwards et al., 1991; Ziegenhagen and Scholz, 1993; Dellaporta et al., 1983).

The major differences in these protocols mainly concern the ingredients (and also the pH) of the extraction buffer. For example EDTA is generally included in DNA isolation buffers and storage solutions, since this compound chelates bivalent cations and thereby inhibits metal-dependent DNases. Reducing agents such as β-mercaptoethanol/dithiothreitol are also usually included in inhibiting oxidation process, which either directly or indirectly cause damage to DNA. Each plant species may require its relevant protocol depending on the demand of the level of DNA purity. For example, Doyle and Doyle (1990) have used CTAB to isolate DNA with the reducing agent β-mercaptoethanol in addition to proteinase K which removes protein. Others like Reichardt and Rogers (1993) have used high CTAB concentration which is an active detergent to deter DNase activity and also removes polysaccharides. Edwards et al. (1991) have used SDS and phenol instead of CTAB as a detergent for the same function of pure DNA isolation. These are sometimes further modified to provide DNA suitable for several kinds of analyses (Wang and Taylor, 1993; Ziegenhagen and Scholz, 1998). The biochemical composition of plant tissues and species varies considerably; therefore it is virtually impossible to supply a single isolation protocol which is optimally suited for each plant species. Thus, different plant taxa often may not permit optimal DNA yields from one isolation protocol (Weishing et al., 1995). Even closely related species may require quite different isolation procedures (Weishing et al., 1995). In addition to a reliable DNA extraction method, the storage of plant tissues for DNA extraction is also important. Most of the protocols recommend isolation of DNA from fresh tissues, but sometimes the samples collected from remote and rare locations (Khanuja et al., 1999) may consist of plant parts in dry or semi-dry conditions. This necessitates the development of the protocols for isolation DNA from different plant organs, including dry tissues. Therefore, under these conditions, a good extraction procedure and good plant tissue storage conditions are important in order to extract good quality and quantity of DNA. CTAB is a cationic detergent, which solubilises membranes and forms a complex with DNA (Sghaier et al., 2005).

SDS extraction protocol is just but a modified version of CTAB with various alterations to increase the efficiency of removing proteins from the extracted DNA. In the protocol, there is the use of dithiothreitol (DTT), which reduces proteins at millimolar levels requiring only a slight excess of iodoacetamide (or N-ethylmaleimide) to alkylate. SDS is a detergent. It is (or a close relative, of sodium lauryl sulphate) often found in everyday shampoos, where it solubilises grease and oils. In the DNA preparation, it breaks up the lipids in the membranes to free the DNA from the cell (Ask a Scientist Molecular Biology Archive, 2005).

DTT is the common name for a small molecule redox reagent known as Cleland's reagent. DTT is an unusually strong reducing agent, owing to its high conformational propensity to form a six-member ring with an internal disulfide bond. DTT is used as a reducing agent for thiolated DNA. The terminal surfur atoms of thiolated DNA have a tendency to form dimmers in solution, especially in the presence of oxygen.

DNA isolation protocols generally use CTAB to avoid co-purifying polysacharrides from plant tissues. Keeping this in mind and the fact that *O. gratissimum* L. samples carry high amounts of polyphenols and polysacharrides, the standard CTAB method (Doyle and Doyle, 1990) was tried in the experiments. Several modifications including use of dithiothreitol 6.5 mM instead of 2-mercaptoethanol were also evaluated. Other alterations tried included replacing CTAB with SDS (Edwards et al., 1991; Keb Llanes et al., 2002). Polyvinylpolypyrrolidone (PVPP) has also been used successfully to remove polyphenols along with a high molar concentration of NaCl to inhibit co-precipitation of polysaccharides and DNA. Therefore, SDS method was used also as proposed by Khanuja et al. (1999).

O. gratissimum L. contains an array of secondary metabolites dominated by two classes of compounds: flavo-

noids and terpenoids including monoterpenoids, sesquiterpenoids, diterpenoids and iridoid glycosides (The Bear's Byte, 2004). These compounds make DNA extraction difficult in addition to the problems of storing the plant tissue samples before DNA extraction.

In this study therefore, different *O. gratissimum* L. tissue storage conditions and four different DNA extraction protocols were compared for effectiveness and efficiency in yielding optimal DNA concentrations and quality prior to genetic variation studies using different genetic markers.

MATERIALS AND METHODOLOGY

Storage treatment for *O. gratissimum*

A natural population of *O. gratissimum* was identified at Mill House, Njoro, Nakuru district 0 °19.285'S 36 °E for sampling. Twenty four samples of this species were collected and exposed to four different storage conditions as follows: in each treatment, there were two experiments carried out; that is, the leaf tissues were separated into two batches: one batch was cleaned with sterile distilled water (SDW) while the other was not cleaned. From each batch, leaf tissues were sampled for the storage treatments i.e. storage in a freezer at –76°C; oven drying at 50°C for 48 h and then storage at room temperature in a dark room; air drying and then storage in a dark room at room temperature; rapid drying using silica gel followed by storage in a dark room at room temperature. All the samples were left under these conditions for a month and total genomic nucleic acids isolated.

DNA extraction

Genomic DNA was isolated from the *O. gratissimum* specimens using four protocols: the modified hexadecyltrimethylammonium bromide (CTAB) mini preparation method described by Doyle and Doyle (1990), with 1% 2-mercaptoethanol as a reductant; the modified Sodium dodecyl sulphate (SDS) mini preparation method of Edwards et al. (1991) with either 1% 2-mercaptoethanol or dithiothreitol as redundant. Genomic DNA was also isolated using the CTAB-extraction buffer which contained 6.5 mM dithiothreitol (DTT) as the reducing agent instead of the commonly used concentration of 1% 2-mercaptoethanol. The methods are as detailed below:

CTAB mini preparation method with 1% 2-mercaptoethanol

The leaves were weighed to about 500 mg per tube into an eppendorf tube and then dropped in liquid nitrogen for 2 min. The weighed leaves were ground in 600 ∝l extraction buffer (100 mM Tris-HCl pH 8.0, 20 mM EDTA pH 8.0, 1.4 M NaCl, 2% CTAB), and 1% 2-mercaptoethanol) preheated to 65°C and incubated for 45 min at 65°C and vortexed every 15 min. The mixture was homoge-nised with an eppendorf homogeniser and added about 10 - 20 mg of polyvinylpolypyrrolidone (PVPP). 500 ∝l of chloroform: isoamylalcohol (24:1) was added and mixed by constantly swirling for 10 min and centrifuging for 5 min at 14,000 rpm. The supernatant was removed to a clean tube. The previous step was repeated and the supernatant was transferred again to a clean micro tube. The nucleic acids were precipitated by addition of 600 ∝l ice cold isopropanol and centrifuging at 14,000 rpm for 5 min. The DNA pellet was washed with 1000 ∝l cold 70% ethanol. The pellet was then dried and later resuspended in 100 ∝l sterile distilled water and put in water bath overnight at 55°C.

CTAB mini preparation method with 6.5 mM dithiothreitol

The leaves were weighed to about 500 mg per tube into an eppendorf tube and then dropped in liquid nitrogen for 2 min. The weighed leaves were ground in 600 ∝l extraction buffer (100 mM Tris-HCl pH 8.0, 20 mM EDTA pH 8.0, 1.4 M NaCl, 2% CTAB), and 6.5 mM dithiothreitol) preheated to 65°C and incubated for 45 min at 65°C and vortexed every 15 min. The mixture was homogenised with an eppendorf homogeniser and added about 10 - 20 mg of polyvinylpolypyrrolidone (PVPP). 500 ∝l of chloroform: isoamylalcohol (24:1) was added and mixed by constantly swirling for 10 min and centrifuging for 5 min at 14,000 rpm. The supernatant was removed to a clean tube. The previous step was repeated and the supernatant was transferred again to a clean micro tube. The nucleic acids were precipitated by addition of 600 ∝l ice cold isopropanol and centrifuging at 14,000 rpm for 5 min. The DNA pellet was washed with 1000 ∝l cold 70% ethanol. The pellet was then dried and later re-suspended in 100 ∝l sterile distilled water and put in water bath overnight at 55°C.

SDS mini preparation method with 1% 2-mercaptoethanol

The leaves were weighed to about 500 mg per tube into an eppendorf tube and then dropped in liquid nitrogen for 2 min. The weighed leaves were ground in 400 ∝l extraction buffer (200 mM Tris Hcl pH 7.5, 25 mM EDTA pH 8.0, 250 mM NaCl, 10% SDS (sodium dodecyl sulphate), and 1% 2-mercaptoethanol). The mixture was homogenised with an eppendorf homogeniser and added about 10-20 mg of polyvinylpolypyrrolidone (PVPP). Another 400 ∝l extraction buffer was added and the homogenate vortexed followed by centrifuging for 2 min at 14,000 rpm in order to pellet the plant debris. The supernatant was removed to a clean tube. An equal volume of chilled phenol: chloroform: isoamylalcohol (25:24:1) was added. The samples were mixed well to emulsify and then centrifuged at 10 000 rpm for 10 min. The nucleic acids were precipitated by addition of 600 ∝l ice cold isopropanol and centrifuging at 14,000 rpm for 5 min. The DNA pellet was washed with 1000 ∝l cold 70% ethanol. The pellet was then dried and later resuspended in 100 ∝l sterile distilled water and put in water bath overnight at 55°C.

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Purification of DNA

The samples isolated using the above methods were purified as detailed. To each tube, 500 ∝l chloroform : iso-amylalcohol (CIA 24:1) was added and the contents mixed by shaking for 15 min, followed by centrifugation at 12000 rpm for 15 min. The aqueous phase was transferred to a new tube and then 200 ∝l 1M NaCl-TE added to the old tube and shaken for 15 min. The old tube was centrifuged for 15 min at 12000 rpm. The aqueous phase was transferred to the new tube and mixed, followed by centrifugation at 12000 rpm for 15 min in order to settle any remaining debris. The supernatant was then transferred to a new tube. Ice cold isopropanol (700 ∝l) was added to the sample and mixed gently, and centrifuged at 10,000 rpm for 5 min and the supernatant discarded. Cold 75% ethanol (1000 ∝l) was added to the pellet to wash it thrice, and contents centrifuged at 5000 rpm for 5 min. The ethanol was discarded and the pellet air dried. The pellet was re-suspended in 200 ∝l sterile distilled water (SDW) and incubated overnight at 55°C.

RNase treatment

The DNA was treated with DNase free Ribonuclease A (10 mg/ml). Large amounts of RNA in the sample can chelate Mg^{2+} and reduce the yield of the PCR (Padmalatha and Prasad, 2006). This step removes RNA from the isolated genomic DNA. RNase (10 µl of 10 mg/ml; Sambrook et al., 1989) was added to 100 µl of re-suspended DNA pellet and then incubated at 37ºC over night. Equal volume of ice-cold absolute ethanol was added to each sample and then centrifuged at 10,000 rpm for 10 min to re-precipitate the DNA. This was done twice. The supernatant was poured off and the DNA pellets air-dried and re-suspended in 100 µl double sterile distilled water (dSDW).

Evaluation of quality and quantity of DNA

The DNA was quantified and examined for intactness using the gel electrophoresis method (Qiagen, 1997). The yield of DNA per gram of leaf tissue extracted was also quantified using a Bio-photometer at 260 nm. The purity of DNA was determined by calculating the ratio of absorbance at 260 nm to that of 280 nm (Sambrook et al., 2001).

RESULTS

Four different DNA extraction protocols

The results from Figure 1a (i), showed that there was no DNA obtained in all the four storage treatments using CTAB with mercaptoethanol (Figure 1a. (i)). Rinsing of the leaves in water caused loss of DNA due to the soft velvet nature of the leaf which led to rotting of the leaf in the process of storage (Figure 1a (ii)). Samples that yielded some DNA only had degraded low molecular weight DNA.

Similarly, in Figure 1b (i) and (ii), where CTAB was used with dithiothreitol (DDT), efficiency of DNA isolation was inconsistent. However, with DDT more samples yielded some DNA. These results indicated some improvement in getting DNA when the reducing agent dithiothreitol (DDT) was used (Figure1b (i). Leaf cleaning with sterile distilled water prior to storage and isolation of the total genomic DNA also resulted in low molecular weight DNA (Figure 1b (ii).

The use of SDS with 2-mercaptoethanol to isolate genomic DNA from *O. gratissimum* gave very poor results with all the leaf treatments giving little or no DNA (Figure 1c (i) and (ii)). Even where some DNA was successfully isolated, this was only low molecular weight degraded DNA (Figure 1c (i)).

The use of SDS buffer with DTT gave good DNA yield in all the samples (Figure 1d (i)). However, the rinsed samples did not yield as much DNA (Figure 1d (ii)) as those samples which were not rinsed (Figure 1d (i)).

Tissue storage conditions

In Figure 1a (i), there was no storage condition that showed any good DNA extracted. However, Figure 1a (ii), samples which were air-dried and later kept in the dark cupboard showed some presence of low molecular weight DNA in lanes 11 and 12. In Figure 1b (i), there was some DNA in the samples which were silica gel dried, oven-dried and frozen samples. However, the entire DNA was low molecular weight DNA. Figure 1b (ii) showed good DNA in the some samples which were air-dried and frozen treated.

In Figure 1c (i), there is some low molecular weight DNA in the frozen samples. Figure 1c (ii) had no DNA extracted in all the four storage treatment.

Figures 1d (i) and (ii) showed very good DNA extracted in silica gel dried samples and frozen samples. Oven-dried samples also gave some DNA but it was mostly low molecular weight. The air-dried samples gave the poorest results, with no DNA extracted.

Good quality DNA would give a quantification of ratio of 1.8 – 2.0, thus, the spectrophotometric determination. However, none of the above methods gave such high values, the range obtained being 1.4 - 1.6.

DISCUSSION

Four different DNA extraction protocols

Extraction of DNA from *O. gratissimum* using CTAB and the reducing agent mercaptoethanol (Figure 1a (i) and (ii) yielded no DNA. Results on DNA extraction in Figure 1b (i) and (ii) yielded some DNA after extracting with CTAB and the reducing agent dithiothreitol instead of the previous mercaptoethanol. Although, the results were not consistent, this showed some improvement when the reducing agent dithiothreitol is used. In Figure 1c (i) and (ii) however, no high molecular weight DNA was extracted. SDS and the reducing agent mercaptoethanol gave very poor results. On the other hand, results in Figure 1d (i) and (ii) where genomic DNA was extracted using SDS and the reducing agent dithiothreitol gave good quality

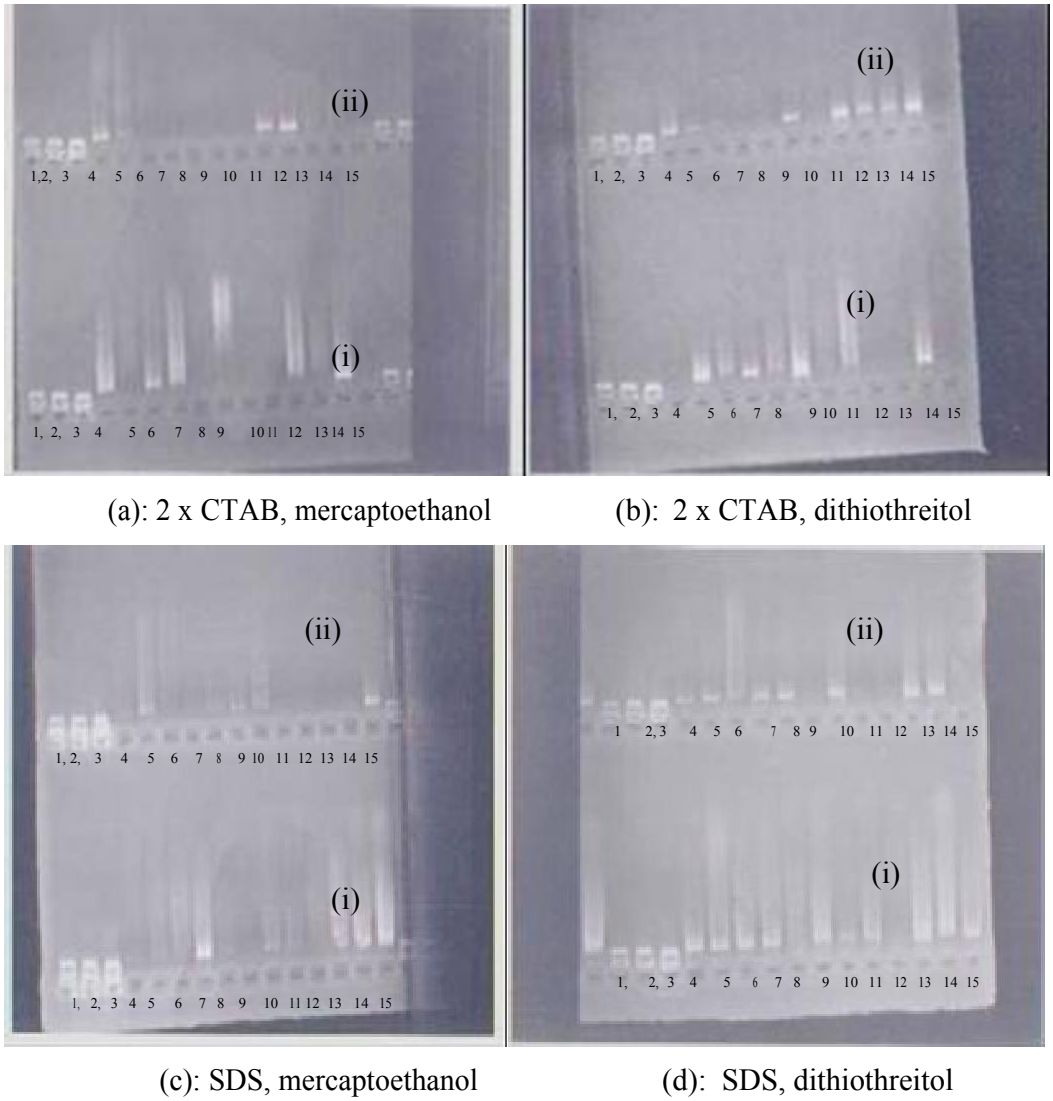

Figure 1. DNA isolation protocol a) CTAB with mercaptoethanol; b) CTAB with dithiothreitol; c) SDS with mercaptoethanol; (d) SDS with dithiothreitol. i) The non rinsed samples: lanes 1, 2, 3 were lambda (λ) DNA in concentration of 50, 100 and 200 ng/ respectively; lanes 4, 5, 6 were silica gel treated; lanes 7, 8, 9 were oven dried; 10, 11, 12 were air dried; and 13, 14, 15 were frozen samples. ii) The rinsed samples: lanes 1, 2, 3 were λ DNA in concentration of 50, 100 and 200 ng respectively; lanes 4, 5, 6 were silica gel treated; lanes 7, 8, 9 were oven dried; 10, 11, 12 were air dried; and 13, 14, 15 were frozen samples.

DNA. The genomic DNA extracted in Figures 1 a (i) and (ii) and 1 c (i) and (ii) had high levels of impurities while 1 b (i) and (ii) yielded low molecular weight DNA. The quality of DNA extracted in Figure 1d (i) and (ii) was better than that from the other methods due to the combination of SDS and the reducing agent dithiothreitol.

In higher plant tissues, particularly those with medicinal properties, secondary metabolic compounds generally get accumulated in the tissue, a problem that becomes severe as the material gets older. Polysaccharide contaminations are particularly problematic (Scott and Playford, 1996) as they can inhibit the activity of many commonly used molecular biology enzymes, such as polymerases (Fang et al., 1992), ligases and restriction endonucleases. This is because nucleic acids form tight complexes with polysaccharides creating a gelatinous pellet which makes it difficult for the embedded DNA to be inaccessible to the commonly used molecular biology enzymes (Sharma et al., 2002). Polyphenol contamination of DNA also makes it resistant to restriction enzymes as also shown in other taxa where polyphenols copurify with DNA (Katterman and Shattuck, 1983) and interact irreversibly with proteins and nucleic acids (Loomis, 1974). This phenomenon is mainly due to the oxidation of polyphenols to quinines and quinones by polyphenol oxidase followed by covalent coupling or by oxidation of

the proteins by the quinines. During homogenisation, polyphenols are released from vacuoles and they then react rapidly with cytoplasmic enzymes.

DNA extraction from leaves of *O. gratissimum* was complicated probably by the abundance of secondary metabolites. This is also true with many other medicinal plants for instance *Terminalia arjuna* (Sarwat et al., 2006) where isolation of purified DNA proved to be a major bottleneck. This has also been experienced with other species like *Theobroma cacao* (Haymes et al., 2004), *Vitis vinifera* (Hanania et al., 2004), *Tagetes minuta* (Hills and van Staden, 2002), *Eucalyptus* spp., *Pinus* spp., and *Araucaria cunninghamii* (Shepherd et al., 2002), *Davidia involuctata* (Li et al., 2002), *Anthurium andreanum* (Buldewo and Jaufeerally-Fakim, 2002), *Drosera rotundifolia, Artemisia dracunculus* (Pirttila et al., 2001).

In comparison of the four protocols analysed with four storage treatments namely, silica-gel dried leaves and frozen leaves, protocol Figure 1d, described by Edwards et al. (1991) with a few modifications gave good results (Figure 1d). According to Jobes et al. (1995), in the presence of PVPP, phenolics adhere to DNA in solution forming a coloured extract around the DNA that becomes cleaner after the addition of the detergent SDS. The addition of high molar concentration of NaCl increases the solubility of polysaccharides in ethanol, effectively decreasing co-precipitation of polysaccharides and DNA (Fang et al., 1992; Aljanabi et al., 1999). The addition of DTT also helps the removal of polyphenolics and other contaminants (Kumpatla et al., 2004).

Some protocols (results in Figure 1a (i) and (ii); Figure 1b (i) and (ii); and Figure 1c (i) and (ii)) did not exhibit satisfactory results for *O. gratissimum,* due to the low concentrations of and absence of some reagents. For instance, the use of CTAB which had low molar concentration of NaCl and the presence of the reducing agent mercaptoethanol which did not work well in all the extraction protocols where it was used (Figure 1a and 1c). The presence of different additives or reagents on the availability of high-quality genomic DNA is crucial during DNA extraction, because some plant tissues are rich in polysaccharides, secondary metabolites or polyphenols. It is also important to note that SDS is a negatively charged detergent and CTAB is a positively charged detergent and therefore, for most plant material the lysis efficiency is different due to this property.

In this study, it has been shown that SDS with DTT works better than any other combination of the reagents tested. Thus, the only extraction protocol that proved successful was when SDS based isolation protocol (Edwards et al., 1991) which had some modification was used with dithiothreitol as a reducing agent as also reported by Waldschmidt et al. (1997). This shows that some detergents work better in conjunction with specific reducing agents during DNA extraction for some plants.

This is also shown where mercaptoethanol was added instead of DTT and the DNA samples obtained were not as clear as those obtained from DTT protocols (Kumpatla et al., 2004) during the genomic DNA extraction of Sunflower. Only the DNA extracted from the use of SDS and DTT worked well for the subsequent PCR reactions using simple sequence repeats (Kumpatla et al., 2004).

Tissue storage conditions

The method of storage is also very important in order to obtain good quality DNA (Thomson, 2002; Bhattacharjee et al., 2004). The results in Figure 1a (i) and (ii) had no good DNA extracted irrespective of treatment. The DNA extraction procedure contributed a lot towards the results. It has been shown above that CTAB and mercapto-ethanol do not yield any DNA in this plant under all tested storage treatments. The results in Figure 1b (i) and (ii) showed the presence of DNA in some storage treatments. These results had partly to do with the DNA extraction procedure. In this case, the extraction buffer CTAB was used with the reducing agent dithiothreitol. The use of the reducing agent dithiothreitol might have contributed a lot to the results obtained.

In Figure 1c (i) and (ii) which resulted in low molecular weight DNA and no DNA, respectively, suggests the failure of the extraction buffer SDS with the reducing agent mercaptoethanol to release DNA from the plant. In Figure 1 d (i) and (ii) had good quality DNA extracted in those samples that were stored in silica gel and frozen. It is important to realise that these samples were extracted using SDS and dithiothreitol as the reducing agent. The results show that SDS buffer in combination with the reducing agent dithiothreitol gave the best method of extracting good quality DNA in this plant especially in those samples which have been stored under frozen condition or silica gel dried immediately after collection.

It is known that, success in the extraction of DNA depends also on the methods of sampling in the field and preservation of the samples in the laboratory (Drabkova et al., 2002; Feres et al., 2005). *O. gratissimum* species exhibited oxidation when left under humid conditions for some time after collection, which means that it is necessary to dry or freeze them as fast as possible to better preserve the DNA. Another important factor was the cleaning of the leaf samples prior to storage. Cleaning with distilled water proved very delicate as most of the leaf samples would rot in the process of storage. Therefore swabbing with some wet cotton wool was suggested as a better option in order to reduce loss of DNA due to rotting.

Although the spectrophotometric determination results were lower than the expected (1.8 – 2.0), the DNA obtained worked well for the subsequent studies in AFLP analysis.

Conclusion

Good quality DNA in *O. gratissimum* L. was obtained

when extracted form the non-rinsed and silica dried leaf samples or frozen leaf samples using the detergents SDS buffer with the reducing agent dithiothreitol. Cleaning of the samples before DNA extraction is recommended for good DNA yield (Thomson, 2002; Bhattacharjee et al., 2004), however, care must be taken when dealing with soft velvet feel-like leaves because the water may lead to rotting of the leaves as observed in this study. Probably, it would be recommended to swab the leaves with cotton wool wet with alcohol prior to preserving in order to dust off the leaf. Collecting conditions and preservation of samples are important for the quality of DNA (Ribeiro and Lovato, 2007) as also observed in this study. The storage treatments where the samples were either frozen or rapidly dried with silica gel gave higher DNA yields of better quality than oven dried or air-dried leaves. Therefore, in *O. gratissimum* plant, the methods of storage after leaf sample collection and the type of DNA extrac-tion reagents have a major influence on DNA quality and in the success of molecular studies.

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