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Research Article

A RAPID, SENSITIVE AND ECONOMIC METHOD FOR ISOLATION OF HIGH QUALITY DNA FROM *BUTEA MONOSPERMA* AS COMPARED TO COMMERCIAL KITS

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ABSTRACT
Butea monosperma is a medicinal and aromatic plant which provides key sources for treatment of human ailments. Nucleic acid isolation is the prime step to understand plant genome in a better manner which helps in identification of plant species. In the present study DNA isolation protocol from *Butea monosperma* was optimized using different methods. Among the various methods tested a modified DNA isolation protocol was successfully developed which gave high yield and purity of DNA. In this method Triton-X 100 a non-ionic detergent was used instead of SDS for cell lysis and DTT an antioxidant was used in place of mercaptoethanol, which gave intact bands of genomic DNA in Gel electrophoresis. The commercial kit resulted in high optical density but no intact DNA bands on gel. This suggests that higher optical density may be due to more impurities in DNA samples. Therefore, this modified method is the better choice for studying features of plant up to genetic and molecular level.

INTRODUCTION
Butea monosperma (Lam.) is a species of genus *Butea koenig* which has high medicinal value. This plant belongs to the family Fabaceae (Patil *et al.*, 2006) which consists of thirty species. Among them three species are found in India viz, 1) *Butea monosperma* (Lam.) 2) *Butea capitata* and 3) *Butea superba* (CSIR Publication, 1988). It is geographically distributed in countries such as Bangladesh, Bhutan, Cambodia, China, India, Indonesia, Java, Laos, Myanmar, Nepal, Pakistan, Sri Lanka, Thailand, and Vietnam (CSIR Publication, 1988).

It is popular because of its medicinal and economic value. Secondary metabolites produced by this plant are very important for production of drugs which can fight against various human diseases. The ash of young branch is used in combination with other drugs in order to cure scorpion sting (Patil *et al.,* 2006). Young roots are used for making ropes. It is used to cure night blindness and other defects of sights. Spoonful of root powder mixed with water is drunk as antidote for snake bite (Bodakhe and Ahuja, 2004). Leaf extract is used as gargle to cure sore throat. Leaf extract about 3-4 spoons is drunk at night for 2-3 months in order to checks irregular bleeding during menstruation (Patil *et al.,* 2006). The ulcer index also decreased in dose dependent manner (Bhatwadekar *et al.,* 1999).

Since, *Butea monosperma* is used for production of drugs and pharmaceuticals; it is our prime duty to conserve particular variety of this plant in order to maintain natural wealth for future generation. The crude drugs obtained from sources of this plant are variety specific. Most of the raw material used for development of traditional medicine is collected from a particular variety. So, the time has come to meet the rising demand of the products and conserve the wild varieties developed in the natural environment. Understanding plant genomes of this plant will allow us to conserve wild varieties and also create plants with more medicinal and economic value. Thus, it becomes necessary to study features of this plant up to genetic and molecular level.

DNA isolation is the first step for studying plant genome. Hence, it should be optimized in better way to get the high yield and purity of DNA. Specific DNA isolation protocol which gives high yield and purity of DNA from *Butea monosperma* is not known. We have made an attempt to develop a DNA isolation

protocol using commercially available kits and other protocols used by different researchers, which fulfills the parameters required to precede the studies of plant genome of *Butea monosperma.*

MATERIALS AND METHODS

DNA was isolated from leaf tissue of *Butea monosperma* using DNA isolation kits named 1) CTAB Plant DNA Extraction Kit and 2) Ultrapure Plant Genomic DNA Prep Kit available from Banglore genei pvt. Ltd. DNA was also isolated using manual methods described for other plant species. DNA isolation protocol described by Lodhi *et al.,* (1994) in Vitis species was tested. Protocols described by HUGO *et al.,* (1998) and Zhang and Stewart (2000) in cotton species for DNA isolation were combined together and one protocol was developed from it. This protocol included lyophilized leaves tissue (250mg), crushed in chilled condition. CTAB-A (15µl) (0.1M Tris-Hcl (pH-8.0), 1M Nacl, 20mM EDTA, 4% PVP, 2% CTAB, 0.25% β – mercaptoethanol) was added to crushed material and incubated at 70˚C for 1hr. After incubation homogenate was centrifuged at 30,180 g for 20 min at 4˚C. Supernatant was collected and passed through PVPP column. Then, elute was purified by mixing equal volume of phenol: chloroform: isoamyl alcohol to it. Centrifugation was done at 20,000 g for 15 min. at 4˚C and aqueous phase was collected. Equal volume of CTAB-B (0.1M Tris-Hcl (pH-8.0), 1.4M Nacl, 20mM EDTA, 5% PVP, 6% CTAB, 0.25% β – mercaptoethanol) was added to aqueous phase and was incubated at 70˚C for 1hr. Further, sample was purified by mixing equal volume of chloroform: isoamyl alcohol (24:1) to the sample. Upper aqueous phase was separated by centrifugation and mixed with chilled isopropanol. Mixture was incubated overnight at 4˚C to precipitate out DNA. DNA was pelleted by centrifugation and washed twice with solution containing chilled 75% Ethanol and 3M Sodium acetate. Then, pellet was allowed to air dry and resuspended in 100 µl of TE buffer.

DNA isolation protocol described by Wilkins *et al.,* (1994) was modified. Lyophilized leaf tissue (2.0 g) was crushed with liquid nitrogen and 400.0 mg of polyvinylpyrrolidone (PVP) was added to it. Homogenized tissue was mixed with 8ml extraction buffer (20mM Na_2-EDTA, 100mM Tris base (pH-8), 1.4M NaCl, 0.2% β – merceptoehanol) pre-heated to 65^{o}C and incubated at 65^{o}C for 10 minutes. After incubation, 5M potassium acetate (2.68ml) was added and incubated in ice for 30 minutes. Centifugation was done at 20,000 Xg for 20 min at 4^{o}C. Supernatant was taken and 5.32ml isopropanol was added to it. Then, it was incubated at room temperature for 1.0 hour to allow precipitation of DNA. Precipitated DNA was separated by centrifugation at 20,000 Xg for 25 min at 4^{o}C. After centrifugation pellet was washed two times with 1ml of chilled 70% ethanol. Pellet was allowed to air dry and resuspended in 500 µL of TE buffer. Dissolved DNA pellet was further purified by addition of RNase (2.5 µl from 1.0 µg/µl stock) to it. Then, it was incubated for 1.5 h at room temperature and 30 min in ice. Precipitated RNA was separated by centrifugation at 20,000 Xg for 15 min at 4^{o}C. Supernatant was taken and equal volume of phenol: chloroform (1:1) was added to it. Centrifugation was done at 20,000 Xg for 10 min at 4^{o}C. Supernatant was taken and equal volume of chloroform: isoamyl alcohol (24:1) was added. Again, centrifugation at 20,000 Xg for 10 min at 4^{o}C was done. DNA was precipitated with 3.0 M sodium acetate (pH 5.2) and further washed with chilled 100 % ethanol and 70% ethanol. DNA pellet was air dried and resuspended in 200.0 µL of TE buffer. Concentration and purity of isolated DNA was determined by measuring its optical density at 260nm and ratio of A_{260}/A_{280} nm. DNA separation was carried out on 1 % agarose gel as per method suggested by Sambrook *et al.,* (1989). Gel was examined on UV transilluminator and photographed for documentation.

RESULTS

Five DNA isolation protocols were tested in order to produce high quality of DNA from *Butea monosperma*. Protocol 1 was a CTAB plant DNA extraction kit (Genei) which included CTAB an anionic detergent in extraction buffer to separate out polysaccharides from DNA. 1% (v/v) polyvinyl pyrolidon was added to the extraction buffer to remove phenolic compounds from the tissue. Phenol, chloroform and isoamyl alcohol was added in particular ratio in order to remove high and low molecular weight

proteins. This kit was especially for plant DNA extraction so, it was capable of giving high yield of DNA (50.14 □ g/ml). But, the percent purity (1.31 %) decreased and hence DNA sample cannot be used for further molecular marker methods. Protocol 2 was also, an Ultrapure Plant Genomic DNA Prep Kit (Genei). It included anion exchange columns on which clarified lysate is directly loaded and allowed to flow gravitationally. Genomic DNA binds to the resin under low salt and pH conditions. Then, a wash of medium salt buffer removes RNA, proteins and other impurities. Finally, pure genomic DNA was eluted in a high salt buffer which was desalted and concentrated with isopropanol and 70 % ethanol. Although, this kit also gave high yield of DNA (69.66 □ g/ml), it was unable to purify DNA up to satisfaction level (1.30 %). Protocol 3 was a modified method which included steps from DNA isolation methods described by HUGO *et al.*, (1998) and Zhang and Stewart (2000). It included □-mercaptoethanol as an additional reagent in lysate buffer which prevented DNA from degradation during isolation. The lysate was further passed through PVPP column to remove phenolic compounds present in the tissue. This protocol included sodium acetate along with 75 % ethanol in order to precipitate and aggregate the DNA molecules. This protocol gave better purity (1.55 %) of DNA as compared to protocol 1 and 2 but the yield of DNA decreased (18.48 □ g /ml). Protocol 4 was a DNA isolation method specific for *Vitis* species described by Lodhi *et al.*, (1994). It included the same chemical reagents which were used in protocol 3 except, SDS which is a cationic detergent used to remove lipids from the tissue. This protocol gave the similar purity (1.56 %) and concentration (7□ □ g/ml) of DNA as protocol 3. Protocol 5 was specific for cotton genomic DNA described Wilkins *et al.*, (1994). It was modified and included Triton-X-100 and DTT as an additional chemical reagents. Triton X-100 is nonionic detergent which induces cell lysis in the tissue and DTT is an antioxidant which prevents degradation of DNA during isolation. In this protocol □-mercaptoethanol was replaced by DTT. Average purity (1.42 %) and yield (17.44 □ g/ml) was obtainedby UV absorption method which was similar to protocol 3 and 4.

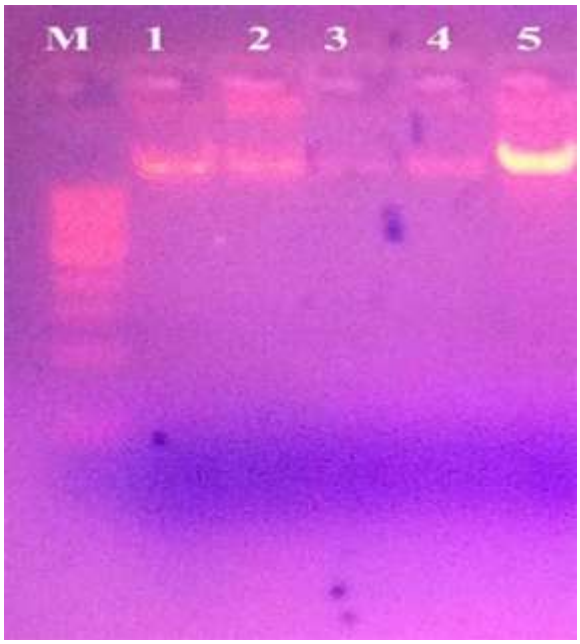

Figure 1: Comparison of DNA quality on agarose gel. Lane M - High range DNA Ruler from 33000 to 500bp; Lane 2 - CTAB plant DNA extraction kit (Genei); Lane 3 - Ultrapure plant Genomic DNA Prep kit (Genei); Lane 4 - Modified method which included steps from DNA isolation methods described by HUGO *et al.*, (1998) and Zhang and Stewart (2000); Lane 5 - DNA isolation method specific for Vitis species described by Lodhi *et al.*, (1994); Lane 6 - Modified DNA isolation method of Wilkins *et al.*, (1994)

However among the five methods tested, method of Ultrapure plant Genomic DNA Prep kit (Genei) yielded high quantity of DNA as compared to other methods tested by UV absorption method but purity of DNA molecule was negligible on agarose gel. However, modified method of Wilkins *et al.,* (1994) gave satisfied yield and better purity of DNA with intact bands on Agarose gel (Figure 1).

DISCUSSION

Molecular markers are essential tools in cultivar identification (DNA typing), assessment of genetic variability and relationships, management of genetic resources and biodiversity, studies of phylo-genetic relationships and in genome mapping. They show variability among individuals at the DNA level which is not influenced by the environment. Different genetic markers (e.g. RFLP, AFLP, RAPD, SSR, SCAR) have different properties, (dominant and codominant markers, different coverage of the genome) different advantages and disadvantages (e.g specificity, cost, ease of analytical interpre-tation of the resulting data). However, they are highly informative about genetic variability among individuals, populations and cultivars (Mizukami and Okabe, 1999). By looking directly at the genetic material itself, molecular markers represent a powerful and potentially rapid method for the characterization of diversity per se within the *in situ* and *ex situ* conservation (Ford-Lloyd, 2001). However, molecular studies dealing with medicinal and aromatic plants (MAPS) are rare in comparison with other cultivated plants. This is probably due to the presence of large amounts of secondary metabolites and essential oils in MAP tissues, which hinders DNA isolation. (Khanuja *et al.*, 1999)

High molecular weight DNA free from protein and RNA is essential for all molecular biology investigations. Thus, quality and not quantity play important role in DNA isolation. It should be the basic objective for DNA extraction protocol. The separation of DNA from cellular components can be divided into three stages: 1) Lysis of cells 2) separation of cell debris and 3) purification of DNA (Sambrook *et al.*, 1989). There are various methods for disintegration of cells on the basis of the type of cells used for extraction. Chemical, mechanical and enzymatic digestions are most commonly used methods for breaking cell wall of the cell in order to release cellular constituents. Among all this three methods chemical method is the cheap and effective method which ensures cell lysis. Hence, tissue should be pulverized using dry ice or liquid nitrogen and further, treated with better quality of extraction buffer capable of extracting DNA molecules in a stable manner without degradation. Extraction buffer should include EDTA to protect DNA from nucleases activity. It should also contain β-mercaptoethanol or DTT to prevent DNA from degradation during isolation (Sambrook *et al.,* 1989). The solution containing disintegrated cell can be purified by separating the cellular debris from it. This cellular debris can be removed from the solution by two ways: 1) Filtration and 2) Centrifugation (Pirttila *et al.*, 2001). Centirfugation is considered as an effective method for separation of cellular debris because it pellets out all high and low molecular weight particles with the help of relative centrifugal force at once. However, filtration requires various pore sized filters for removal of different size particles. The crude lysate obtained after removal of cellular debris can be purified for DNA by using various chemicals capable of removing unnecessary biomolecules, hindering the purity and yield of DNA. Treatment of SDS and CTAB (detergents) is given in order to remove lipids from the lysate (Liber *et al.*, 2006). A specific ratio of phenol and chloroform is used to treat lysate, as it is capable of removing high and low molecular weight protein from it. Lysate is also treated with chloroform alone to remove excess phenol from the lysate treated with phenol: chloroform (Pirttila *et al.*, 2001). DNA can be separated from lysate by precipitating it with isopropanol and further molecule can be aggregated and concentrated by washing it with ethanol or sodium acetate (Pirttila *et al.*, 2001).

As molecular marker technique rely on highly purified DNA than the high yield of DNA, this protocol is desirable for isolation of DNA from *Butea monosperma*. Thus, molecular marker studies from *Butea monosperma* can be successfully done by using this modified DNA isolation method which support purity of DNA.

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