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A comprehensive review of high-quality plant DNA isolation

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Abstract
The development of the Cetyltrimethylammonium Bromide (CTAB) protocol in plants paved the way for advances in DNA extraction. High quality plant DNA extraction is a prerequisite for molecular techniques and is crucial for crop improvement. Features of plant cell like presence of cell wall, polysaccharides and polyphenols, call for robust DNA isolation techniques. The approach utilized for extraction of DNA may vary with the age of plant sample and species. Numerous modifications have been reported for the extraction of high quality plant DNA which varies based on the sample tissue. To obtain uncontaminated high molecular weight DNA, the bulk of existing DNA extraction procedures rely on extended incubation and several precipitations or commercially available kits. Multiple methods for extracting high-quality DNA have been developed, however they are not widely used. The current review is an update on various advancements for isolating plant genomic DNA, and it encapsulates the various issues encountered and solutions devised to address them during DNA isolation from plant cells.

Keywords: DNA extraction, polysaccharides, cetyltrimethylammonium bromide (CTAB), polyphenols, genomic DNA

Introduction
Recent advancements in molecular biology and genome analysis has radically changed the ways in which complex genetic traits are approached for improving the agronomic traits in crop plants. Most require the genetic material of the organism in order to decipher and analyze that organism at the molecular level. The foremost step for doing so would be the rapid and low-cost isolation of good quality and quantity of genomic DNA.
Depending upon the purpose of isolation, numerous protocols for plant DNA extraction have been reported but there is no protocol that is universally accepted and befitting to all plant species. In most cases, alterations are required like merging two or more protocols to get the desired quality and quantity of DNA. High quality Plant DNA is an absolute necessity for further downstream analysis that have to be done on the extracted DNA. Single Nucleotide Polymorphism (SNP) analysis, Targeting Induced Local Lesions in Genomes (TILLING) and Next Generation Sequencing (NGS) are examples of modern genotyping techniques that can benefit from the use of high-quality DNA in a variety of high-throughput applications. High-quality DNA extracts are imperative for molecular studies. Extracted DNA can be used for gene amplification and genome editing. Emerging technologies such as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) have ushered in a new era of genome editing, largely owing to accurate genome sequencing.
Simple sequence repeats (SSR), in general, respond well to low quality and quantity of DNA, whereas marker systems such as Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Inter Simple Sequence Repeats (ISSR), and others require high quality DNA. Significant progress has been made in the development of various protocols that provide high-quality DNA for various plant types. This review not only addresses the numerous developments in plant genome extraction at each phase of DNA separation, but it also mitigates the difficulties that arise.

Collection and storage of source material
Fresh, young leaves are preferred for high-quality DNA extraction because they typically contain fewer secondary metabolites, polysaccharides, and other contaminants (Murray and Thompson 1980, Doyle and Doyle 1987, Bi *et al*. 1996, Peterson *et al*. 1997, Sika *et al*. 2015) [1, 3, 17, 32]. In contrast, DNA isolated from matured leaves may be of inferior quality due to presence of higher amounts of polysaccharides, secondary metabolites including tannins,

polyphenols and various other contaminants. The quantitative yield of DNA from matured leaves has also been reported to be low (Ahmad *et al*. 2004) [23].

Many studies have reported the cultivation of plant material in greenhouses (Bi *et al*. 1996, Michiels *et al*. 2003, Kotchoni *et al*. 2011, Liang *et al*. 2015) [30, 22, 33]. Seeds can also be grown in a seed germinator (Sharma *et al*. 2002) [21]. DNA extraction can be performed on leaves from germinated seedlings that are 14 to 20 days old. In addition to leaves, use of plant parts like seeds (Rogers and Bendich 1985, Sharma *et al*. 2002) [4, 2, 21], roots (Tel-zur *et al*. 1999) [19], embryo (Rogers and Bendich 1989) [4], tubers (Tapia-Tussell *et al*. 2005) [26], callus (Kang and Yang 2004) [24] and so forth have also been reported. Methods for extracting plant DNA from recalcitrant seeds and plant material that is several years old have been developed (Rogers and Bendich 1989) [4]. Ancient plant tissue samples are a valuable source of plant material for phylogenetic studies.

The plant tissue can be used instantly or it can be stored at -70 °C to -80 °C (Couch and Fritz 1990) [5]. Tissue can be dried at 50 °C for 48 hours if needed within a few days (Sharma *et al*. 2002) [21]. Tissues which have been lyophilized and ground to a fine powder are often used before moving on to the next step (Murray and Thompson 1980, Couch and Fritz 1990, Manen *et al*. 2005) [5, 1, 25]. It is critical that the lyophilized tissue is not thawed before use. The freezing of plant material prevents nuclease activity from rapidly degrading DNA.

In case field samples are being taken and immediate refrigeration is not possible, use of silica gel/silica gel beads has been reported to aid in the desiccation of plant material collected from the field. To ensure adequate desiccation, the plant material is left to desiccate in containers with silica gel/silica gel beads for at least 24 hours (Kim *et al*. 1997, Edge-Garza *et al*. 2014) [16, 31]. To collect a uniform tissue sample, the lid of an Eppendorf tube can be utilized to lacerate out a disc of plant material. It minimizes the contamination caused by manual handling of sample tissue (Edwards *et al*. 1991, Kim *et al*. 1997) [6, 16]. To prevent contamination, the sample tissue is surface sterilized with 70% ethanol. Following that, it is thoroughly washed with distilled water to effectively remove any dust, dirt, and other surface contaminants.

Disintegration of sample tissue

Grinding of the sample tissue is the most delicate step as inconsiderate grinding will cause abrasion of the DNA to be extracted, resulting in obtainment of smaller DNA fragments. As a result, the quantity and quality of isolated DNA would be strongly influenced by the method used to disrupt tissue. Due to the presence of a rigid cell wall in plant cells, the process of grinding and homogenization takes a significant amount of time for the entire procedure.

It has been reported that bruised leaves rapidly produce polyphenols, which eventually result in DNA degradation (Couch and Fritz 1990) [5]. Thus, the sample is preferably disrupted at low temperature which consequently minimizes polyphenolic activity while inhibiting nuclease activity.

The mechanical disruption of tissue is generally done using a mortar and pestle. Alternatively**,** metal balls in a mixer mill (Allen *et al*. 2006, Xia *et al*. 2019) [27, 34], sea sand (Bi *et al*. 1996), glass beads (Murray and Thompson 1980) [1] and hand operated homogenizer (Kang and Yang 2004, Kotchoni and Gachomo 2008, Ahmed *et al*. 2009) [24, 28], can also be used. Pre-chilling the mortar and pestle at -20 °C is recommended. The addition of liquid nitrogen causes the tissue to disintegrate into a fine powder (Bi *et al*. 1996, Sharma *et al*. 2002) [21]. Dry ice is used as a substitute for liquid nitrogen in some studies, but it is less effective in tissue disruption (Rogers and Bendich 1985, Couch and Fritz 1990) [4, 5]. The use of a rubber policeman has been suggested as a convenient way to transfer the homogenate from the mortar and pestle (Rogers and Bendich 1989) [4]. The main disadvantage of using mortar and pestle is that it is a time-consuming and labor-intensive procedure, and simultaneous extraction can only be performed on a limited number of samples. As it takes longer to grind the tissue with a mortar and pestle, there is a greater chance of enzymatic activity and polyphenol accumulation, which may degrade the quality of the extracted DNA. Mechanical disruption of tissue, while convenient and less expensive, poses a likelihood of DNA being sheared into small fragments. To overcome this shortcoming of mechanical disruption, many chemical disrupters have also been recommended. Hydrolysing enzymes make up the majority of chemical disrupters. Plant cells have a rigid cell wall that must be digested using a combination of carbohydrase enzymes. Enzymes derived from *Trichoderma longibrachiatum* have been shown to effectively digest the cell wall and liquefy the tissue without the need for mechanical disruption (Manen *et al*. 2005) [25]. Alternatively, Potassium ethyl xanthogenate, a chemical disruptor, has been studied to aid in the release of DNA without the need for tissue homogenization (Williams and Ronald, 1994) [10]. This method is especially useful for breaking down the cell walls of plants with fibrous leaves, such as rice.

Extraction buffer and incubation

The extraction buffer is made up of several constituents, the most important of which is detergent, which not only disrupts the cell membrane but also aids in the removal of polysaccharides. This is typically accomplished by mechanically grinding the tissue in the presence of the extraction buffer or by adding the extraction buffer preheated at 65 °C (Allen *et al*. 2006, Xia *et al*. 2019) [27, 34] after mechanical grinding. By removing lipid molecules from the cell membrane, the detergent denatures the cell membrane proteins and causes cell lysis. It also aids in the separation of polysaccharides from extracted DNA.

Cetyltrimethylammonium bromide (CTAB) is the most commonly used detergent for plant DNA extraction (Murray and Thompson 1980, Doyle and Doyle 1987, Allen *et al*. 2006) [1, 27]. CTAB is a type of cationic detergent (Allen *et al*. 2006) [27]. The majority of CTAB-based protocols being developed today eliminate the need for selective precipitation and the CsCl density gradient step in order to shorten the overall procedure. Sodium dodecyl sulphate (SDS) is an effective CTAB substitute (Edwards *et al*. 1991, Ahmed *et al*. 2009, Kotchoni *et al*. 2011) [6, 30]. SDS is an anionic detergent that is commonly used to extract DNA from animal cells; however, the isolation of DNA from plant cells has also been reported in several studies. The SDS-based method has been shown to be the most suitable in extracting pure DNA from genetically modified Soyabean seeds (Xia *et al*. 2019) [34].

Additionally, the extraction buffer also contains the following constituents –

1. **EDTA:** Ethylenediaminetetraacetic acid (EDTA) is a magnesium ion chelating agent. Magnesium ions are an important cofactor of the DNase enzyme. The enzyme DNase is inhibited by the addition of EDTA.

2. **Tris-HCl:** Tris is a buffering agent used to maintain a stable pH during the DNA extraction process. To keep DNA more stable, Tris maintains a slightly alkaline pH of 8.
3. **Salts like:** NaCl and Sodium acetate affect the solubility of DNA in the extraction buffer. Na^+ ions of the salt bind to the negatively charged phosphate ions, assisting DNA consolidation and preventing denaturation.
4. **Polyvinylpyrolidone (PVP) and polyvinylpolypyrrolidone (PVPP):** PVP and PVPP are added in an effort to reduce the number of polyphenolic compounds that contaminate the extracted DNA (Doyle and Doyle 1987, Couch and Fritz 1990, Sharma *et al*. 2000) [5, 20]. It has also proven to reduce the browning of the extracted DNA (Rogers and Bendich 1985) [2].
5. **Antioxidants:** Antioxidants such as β-mercaptoethanol and Bovine serum albumin (BSA) help in the prevention of polyphenol oxidation. The primary function of β-mercaptoethanol is to disrupt disulphide bonds and denature proteins. It acts as a reducing agent and aids in the elimination of tannins and polyphenols. BSA is frequently referred to as a polyphenolic absorbent. It improves DNA amplification, ligation, and restriction as well (Ahmed *et al*. 2009) [19].
6. **Charcoal:** According to some studies, the addition of activated charcoal aided in the purification of the DNA (Bi *et al*. 1996). Charcoal has been shown to bind and absorb resinous and coloured compounds found in some plants that are high in polyphenols, polysaccharides, and secondary metabolites.
7. **RNase:** The addition of RNase-A to the extraction buffer has been suggested to yield higher molecular weight DNA while also shortening the overall procedure time (Allen *et al*. 2006) [27].
8. **Commercial washing powder:** Most of the commonly available washing powders contain detergents and chelating agents as in case of standard buffers. Commercial washing powders are more economical, less toxic and easily available (Bahl, 1996) [12].

The amount of time required to complete the DNA extraction process varied greatly, from as little as 1 minute (Kotchoni *et al*. 2011) [30] to as much as 6 hours per extraction (Allen *et al*. 2006) [27]. In general, the CTAB method is more time consuming and hazardous as compared to the SDS method.

Phase formation and centrifugation

Conventionally, homogenate is treated with organic solvents to separate the aqueous phase containing DNA from the organic phase containing cell debris (lipids and denatured proteins). This treated homogenate is subjected to centrifugation process which separates the phases more efficiently and removes any potential contaminants. Organic solvents that are commonly used include phenol, chloroform, and isoamyl alcohol. Recent research suggests using a 25:24:1 ratio of phenol, chloroform, and isoamyl alcohol (Kang and Yang 2004, Allen *et al*. 2006) [24, 27] While some research have reported using a chloroform: isoamyl alcohol mixture to improve the quality of extracted DNA (Bi *et al*. 1996, Ahmad *et al*. 2004) [23]. Protein denaturation is aided by phenol and chloroform, but isoamyl alcohol inhibits foaming and facilitates phase formation during centrifugation. Chloroform and phenol have also been employed as alternatives (Tel-zur *et al*. 1999) [19]. Because DNA is more readily soluble in a 1:1 combination of chloroform and phenol, it is recommended over phenol alone. Since the latter solvent reduces the quality and quantity of DNA, chloroform: isoamyl alcohol (24:1) is preferred over chloroform: phenol (1:1). As octanol is more effective at isolating nuclei than isoamyl alcohol, a combination of chloroform and octanol is now recommended over chloroform and isoamyl alcohol (Lodhi *et al*. 1994, Richards *et al*. 1994) [8, 9].

Since organic solvents like chloroform are hazardous to the environment, expensive, and difficult to dispose of, a less toxic and less expensive substitute is required. Dichloromethane, according to recent findings, is less toxic, effective at removing proteins, and yields DNA of the same quality as chloroform (Chaves *et al*. 1995) [11]. When it comes to large protein molecules, phenol extraction alone appears to be ineffective in removing them. DNA shearing may occur as a result of multiple phenol extractions followed by centrifugation. As an alternative, Proteinase K can be used to digest proteins, breaking down large molecules into smaller ones that can then be removed using phenolic extraction (Aljanabi and Martinez 1997, Xia *et al*. 2019) [34, 15].

Precipitation of dna and washing of pellet

Following that, the DNA is precipitated with either ethanol (Allen *et al*. 2006) [27] or isopropanol (Xia *et al*. 2019) [34]. When the goal is to obtain high molecular weight DNA, isopropanol is more efficient in DNA precipitation, however, isopropanol was found to be more efficient in DNA precipitation when followed by a second precipitation using ethanol (Xia *et al*. 2019) [34]. Precipitation is usually performed in the presence of salts such as sodium acetate, sodium chloride, ammonium acetate, and so on. Spooling can be done with a glass rod if the amount of DNA obtained is large. For smaller amounts of DNA, centrifugation is performed later, and a pellet can be obtained. Following the formation of the pellet, the DNA pellet is washed with cold 70% ethanol to remove any residual salts.

Drying of pellet

To eliminate all traces of ethanol, the pellet is dried using several ways such as using a speedvac (Allen *et al*. 2006) [27], desiccator (Rogers and Bendich 1985, Ahmad *et al*. 2004) [2, 23], air drying at ambient temperature (Bi *et al*. 1996, Sharma *et al*. 2000, Allen *et al*. 2006) [20, 27], vacuum drying (Bi *et al*. 1996), or blotting away excess ethanol by placing the pellet on a clean paper towel (Ahmed *et al*. 2009, Sika *et al*. 2015) [32].

DNA preparation and resuspension of pellet

In either TE buffer or water, the DNA pellet is resuspended. If the DNA must be used immediately for downstream analysis, H2O can be used, however, TE buffer is recommended if the DNA must be preserved for an extended period of time (Allen *et al*. 2006) [27]. It is usually recommended to use sterile distilled or double distilled water (Sharma *et al*. 2002, Kotchoni *et al*. 2011, Sika *et al*. 2015) [21, 30, 32]

RNA is a significant contaminant that is isolated alongside DNA. While using UV absorption to measure yield, the presence of RNA causes the yield to be overestimated. It also produces DNA with a lower molecular weight (Allen *et al*. 2006) [27]. To eliminate RNA, a low concentration of RNase in TE buffer for 30 minutes to an hour at 37 °C is recommended (Bi *et al*. 1996, Kang and Yang 2004) [24]. Following that, DNA is extracted with organic solvents and re-precipitated

with ethanol or isopropanol in the presence of salts such as sodium acetate or ammonium acetate. To effectively remove RNA, modern studies use a combination of RNase A and RNase T1 (Murray and Thompson 1980, Rogers and Bendich 1985) [1, 4].

Currently, many studies use Lithium chloride to precipitate the RNA rather than RNase to digest it (Barnwell *et al*. 1998, Ahmad *et al*. 2004) [18, 23]. The RNA pellet formed is discarded and the DNA left in supernatant is precipitated again.

After that, the DNA is archived. Long term storage at -20 °C and short-term storage at 4 °C is recommended (Bi *et al*. 1996, Allen *et al*. 2006, Sika *et al*. 2015) [27, 32]. Long-term storage at -80 °C is recommended if the laboratory is well-equipped to maintain DNA quality (Sika *et al*. 2015) [32].

Problems in DNA extraction

Polysaccharides, polyphenols, RNA, extranuclear DNA, and proteins are among the contaminants found in the extracted DNA. Secondary plant products degrade the quality of isolated DNA, rendering it useless for further use. The diverse uses of extracted DNA, its varied types and origin of the tissue samples necessitate the use of different extraction protocols. Chaves *et al*. (1995) [11] reported that the beans that were grown in field conditions had more number of tannins than those grown in greenhouses. Cotton contains a high proportion of phenolic terpenoids and tannins, making it difficult to extract good quality DNA for downstream analysis (John, 1992) [7]. Members of the Euphorbiaceae family, such as cassava, have been found to contain latex and other compounds that interfere with quality DNA isolation (Chaves *et al*. 1995) [11]. Fibrous nature of rice and other monocots results in reduction in DNA yields. Some of these contaminants are discussed briefly below.

Polyphenols

Polyphenols pose a major problem in extraction of good quality DNA as they are frequently co-purified with DNA and cause DNA pellet to turn brown. Plants belonging to Malvaceae, Bombaceae, Moraceae (John 1992) [7], apple (Malus spp.), grape (Vitis spp.) (Lodhi *et al*. 1994, Kim *et al*. 1997) [8, 16], pear (Pyrus spp.), conifers (Kim *et al*. 1997) [16] tomato (Peterson *et al*. 1997) [17] etc. have been reported to be high in polyphenolics due to which DNA extraction becomes a tedious task.

Bruised or damaged leaves quickly begin to accumulate these polyphenolic compounds, causing oxidative changes. Leaves should never be allowed to thaw before coming into contact with the extraction buffer (Couch and Fritz 1990) [5]. The accumulation of polyphenols can begin with even minor freezing and thawing damage. Polyphenols are oxidised during cell disruption and irreversibly react with nucleic acids, resulting in browning of the DNA pellet (Couch and Fritz 1990, John 1992, Lodhi *et al*. 1994, Peterson *et al*. 1997) [17, 6, 8, 5]. Polyvinyl pyrollidone (PVP) and polyvinyl polypyrollidone (PVPP) are added as modification to the extraction buffer as they function as adsorbents and form hydrogen bonds with polyphenolic compounds such as terpenoids, tannins, flavonoids, and others (John, 1992, Lodhi *et al*. 1994, Sharma *et al*. 2000) [7, 8, 20] and latex (Michiels *et al*. 2003) [22]. In order to prevent oxidation of phenolic compounds, antioxidants such as Bovine serum albumin (BSA) (Couch and Fritz 1990, Ahmad *et al*. 2004) [5, 23], β-mercaptoethanol (Wang *et al*. 1996, Michiels *et al*. 2003, Ahmad *et al*. 2004) [23, 22], etc. are added to extraction buffer just prior to its use to prevent phenolic compound oxidation. Couch and Fritz (1990) [5] used a buffer of Sodium citrate and glucose in order to isolate the nuclei prior to lysis. The addition of glucose or sucrose (Ahmad *et al*. 2004) [23] is required because they act as osmoprotectants during nuclei isolation. Sucrose also contributes to the thermostability of BSA (Ahmad *et al*. 2004) [23].

Polysaccharides

Polysaccharides are a major contaminant because they make the extracted DNA very viscous and produce a gelatinous DNA pellet. Polysaccharides co-precipitate with the extracted DNA during the alcohol precipitation step, rendering the DNA unsuitable for processes such as southern hybridization, which requires good quality DNA free of polysaccharides and other inhibitors (Barnwell *et al*. 1998, Sharma *et al*. 2002) [18]. Polysaccharides have also been reported to inhibit DNA polymerase, resulting in irreproducible RAPD results (Bi *et al*. 1996). They also inhibit Taq polymerase activity during PCR (Tel-zur *et al*. 1999) [19] as well as other enzymatic activity.

The most effective and convenient method of removing polysaccharides is to dilute the DNA extracts, subsequently diluting the amount of polysaccharide inhibitors, but excessive dilution renders the DNA unsuitable for southern hybridization (Sharma *et al*. 2002). High concentrations of NaCl are effective in removing polysaccharides from DNA-containing solutions (Lodhi *et al*. 1994, Aljanabi and Martinez 1997, Sharma *et al*. 2002) [8, 15]. The high salt concentration reduces the solubility of polysaccharides in ethanol, thereby allowing DNA to be extracted alone. NaCl can be added after phenol extraction or in TE buffer. Sharma *et al*. (2000), reported that homogenising the sample tissue in a solution of 5M NaCl and Sarcosyl resulted in high-quality DNA yields. In other research, 6M NaCl was used to remove polysaccharides from polysaccharide rich plants like wheat, potato and barley (Aljanabi and Martinez 1997) [15].

High concentrations of CTAB have also been recommended for efficient polysaccharide removal (Doyle and Doyle 1987) [3]. The combination of a high concentration of CTAB and a high molar salt effectively removes polysaccharides from extracted DNA (Murray and Thompson 1980, Aljanabi and Martinez 1997, Tel-zur *et al*. 1999) [1, 15, 19]. Washing with extraction buffer several times further purifies the DNA and has been shown to be beneficial in the removal of polysaccharides (Tel-zur *et al*. 1999, Sharma *et al*. 2000) [19]. For polysaccharide reduction, Rogers and Bendich (1985, 1989) [2-4] described the use of buffers containing varying doses of CTAB. They utilised 2% CTAB to prepare the extraction buffer, 10% CTAB was added to the supernatant after centrifugation, and 1% CTAB was used to prepare the precipitation buffer. Following incubation and subsequent centrifugation, high salt TE buffer was used to precipitate DNA and separate it from polysaccharides and other impurities. A similar modification of this method was seen for the removal of polysaccharides from succulent plant *Sedum telephium* (Barnwell *et al*. 1998) [18]. Aside from that, Liang *et al*. (2015) [33] proposed using α-amylase to efficiently remove polysaccharides and improve PCR amplification rates.

Conclusion

Improved DNA extraction protocols that do not use hazardous reagents or organic solvents that are difficult to dispose of are

preferred because they are more feasible and less time consuming, especially when there are a large number of samples. Various new techniques are constantly being developed in order to obtain high-quality and quantity of the isolated DNA. Some procedures which make use of a micro needle (MN) patch are less time consuming. A micro needle patch consisting of polyvinyl alcohol can be used to isolate DNA from various plant and pathogenic species within a minute (Paul *et al*. 2020) [35]. DNA isolation kits are gaining popularity as they are less laborious and require minimal laboratory equipment. The wide heterogeneity observed in plant kingdom prompts us to analyse each and every species at the molecular level necessitating the development of plant genome extraction procedures suitable for varied, if not all, plant species. These methods need to be less threatening to nature, suitable for less equipped laboratories, and time and cost effective.

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