Indian J. Plant Genet. Resour. 31(3):315–327 (2018)
DOI 10.5958/0976-1926.2018.00037.2

REVIEW ARTICLE

Plant Genomic DNA Isolation – the Past and the Present, a Review

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(Received: 24 August 2015; Revised: 20 February 2017; Accepted: 29 May 2017)

Successful isolation of a good quality of plant genomic DNA in sufficient quantity is a necessary prerequisite for its downstream applications,that are aimed to examine and analyze the molecular intricacies of the plant kingdom. The quantity and quality of isolated genomic DNA often determines the accuracy and robustness of results derived from many DNA based applications.The choice of the method adopted for DNA isolation depends on a large number of factors like the amount, nature and age of the starting plant tissue, the infrastructure available in terms of laboratory facilities, ready availability of required laboratory chemicals, time at hand and the downstream applications of the obtained DNA. A large number of modifications have been suggested to optimize DNA isolation from a particular plant species or tissue. There cannot be a universal protocol for DNA isolation across all plant species or tissues owing to the highly heterogeneous nature of plant cells. Hence, efforts need to be made to carry out suitable variations in the extraction method, so as to develop a protocol which is best suited to a particular plant species or tissue or application. So far, a large number of DNA isolation protocols from plants have been published, but there is no comprehensive report on suitability of these methods over each other and the different modifications which have been attempted to optimize the yield and quality of DNA. Many review papers have described various DNA isolation protocols and their underlying principles, that have been adopted in order to optimize DNA isolation from different plant species and tissues (Varma *et al*., 2007; Tan and Yiap 2009; Kumari *et al*., 2012). However, there is no comprehensive study which gives details of modifications at different steps of the plant genomic DNA isolation protocol, that have been attempted by researchers to overcome the specie and tissue specific limitations. This review gives a detailed account of the steps involved in genomic DNA isolation from various plant species and tissues and how each of these steps have been modified to overcome and eliminate the problem of contaminants in the extracted DNA so as to obtain a good quality of DNA amenable to downstream molecular biology applications.

Key Words: CTAB, DNA, Extraction buffer, High throughput methods, Kits

Introduction

The isolation of genomic DNA from plants is a routine procedure widely carried out in a plant molecular biology laboratory. This procedure is aimed to isolate the total genomic DNA from the plant for subsequent molecular analysis. The downstream application of the isolated DNA is crucial in determining the choice of method for DNA isolation. For applications like carrying out PCR for SSR analysis, small amounts of crude DNA can be used. However, PCR for RAPD, AFLP and ISSR analysis requires very small amount of good quality DNA. A highly pure and large quantity of DNA is required for applications like RFLP, southern blotting, cloning, genomic library construction, high throughput genotyping and sequencing (Tefler *et al*., 2013). Most commonly the isolated DNA is found to be contaminated by the presence of residual polyphenols, polysaccharides and other secondary metabolites, which result in inhibition of restriction endonuclease activity, amplification by *Taq* DNA polymerase and ligation by ligase (Moyo *et al*., 2008). Plant tissues, unlike animal tissues, pose a far greater challenge for DNA isolation because of the extensive variation and heterogeneity in the internal structures and composition (polysaccharides, storage proteins and secondary metabolites) of plant cells. As a result it is near impossible to have a single DNA isolation protocol which is universally applicable across different plant species or even different tissues from the same plant (Weising *et al.,* 2005). The presence of components like polysaccharides, cellulose, phenols and tannins, necessitates the standardization of the protocol which is able to remove these contaminants from the genomic DNA preparation. There are following five fundamental steps involved in all the DNA isolation methods:

a) **Cell disruption:** This means degradation of cell envelope (cell wall and cell membranes). The cell wall (cellulose) is disrupted by mechanical force and the cell

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membrane is broken by the addition of a detergent in the extraction buffer .The detergent disrupts the membranes due to the amphipathic (both hydrophilic and hydrophobic regions) nature of both cellular membranes and detergent molecules. When detergent comes close to the cell, it captures the lipids and proteins. The end result of cell disruption or lysis is that the contents of the plant cells are distributed in solution (cell extract).

b) **Deproteinization or organic extraction:** This step involves the use of phenol/chloroform/isoamyl alcohol to remove the proteins from the DNA. Phenol denatures proteins and dissolves denatured proteins. Chloroform is also a protein denaturant and also removes excess phenol from the preparation.

c) **Precipitation:** The DNA is recovered from the solution by insolubilization or precipitation on addition of isopropanol or ethanol. Isopropanol induces a structural change in DNA molecules that causes them to aggregate and precipitate out of solution. Ethanol in the presence of salt prevents DNA from dissolving in water causing it to precipitate out. The precipitated DNA is thereafter pelleted by centrifugation.

d) **Wash:** The precipitated DNA is washed with 70% ethanol to remove salts and other water soluble impurities.

e) **Resuspension:** The DNA thus obtained is suspended in a buffer (1X TE10 mM Tris:1mM EDTA) or in sterile distilled water.

In this review, we have attempted to compile various published protocols of DNA isolation, the specie -specific and tissue-specific modifications that have been carried out at different stages in the DNA isolation protocol and how these have led to troubleshooting in DNA isolation.

Discussion

Variables involved in DNA isolation

The steps involved in the genomic DNA isolation protocol are subjected to a large number of modifications in order to optimize the quantity and quality of the DNA. Efforts have been undertaken to streamline these modifications to develop the most suitable protocol for a particular plant species. The variables in the DNA isolation methodology, are described hereunder-:

a) **Plant tissue – age and type**: The source of the plant tissue to be used for DNA isolation greatly affects the quality and quantity of DNA obtained. Preferentially young leaf tissues are used because these have less concentration of starch and secondary metabolites (Varma *et al.*, 2007). However, it has been shown that the leaves of *in vitro* grown Himalayan herb, *Aconitum balfourii*, contain the highest amount of polyphenols compared to other tissues like shoots and roots (Sharma and Gaur, 2014). In some latex containing plants, etiolated leaves are also used as a tissue of choice for DNA isolation (Michiels *et al*., 2003). It has been reasoned that the photosynthetically active tissues like leaves contain more phenolic compounds as compared to etiolated leaves. These phenolics get oxidized during extraction and form irreversible complexes with proteins. Therefore, the use of etiolated leaves for DNA isolation is preferred in some plants. Mature leaves have also been used for DNA isolation but have been shown to yield lesser quantity of DNA compared to fresh leaves (Ribeiro and Lovato, 2007). However in plants like citrus and sweet potato, hardened and mature leaves have been used for DNA isolation (Varadarajan and Prakash, 1991).

Other tissues like seeds, embryos, endosperm, cotyledons, roots, floral parts, fruit rind, callus, rhizomes, cambial cells, wood tissues etc., have also been used for DNA isolation with limited success (Asish *et al*., 2010). It was found that flowers yielded the maximum amount of DNA in *Daucus carota*, as compared to seeds, leaves, calli and tap roots (Boiteux *et al*., 1999). Newly harvested seeds yielded a greater quantity and quality of DNA in maize compared to DNA isolated from leaves. In commercial timber yielding trees, bark tissue, heatwood, sapwood, cambium etc., have been used for DNA isolation (Asif and Cannon, 2005; Tnah *et al*., 2012). Other than differences in the biochemical composition of the tissues, the tissue specific variation in DNA yield and quality can also be attributed to differences in the number and size of cells in the tissue, the ratio of mitotic to interphasic nuclei and the amount of extranuclear DNA. Floral meristems of medicinal plants and grasses have been used for DNA isolation because of an expected high concentration of genomic DNA due to the presence of rapidly dividing cells and extensive DNA synthetic activity in the cells (Ibrahim, 2011). It has also been observed that not all tissues are amenable to DNA isolation with the same protocol. DNA could be isolated from seedlings and leaves, but not from seeds, of jute plant (*Corchorus*) from a protocol which did not employ phenol in organic extraction whereas DNA could be isolated from all the

tissues, using a protocol that used phenol for organic extraction (Haque *et al*., 2004).

b) **Collection and storage of tissue:** The tissue once harvested can be used directly for DNA isolation or may be stored conveniently at -80°C till the time of use. Freezing at this temperature inhibits the activity of nuclease enzymes and hence allows successful preservation of plant tissues. Another way of preservation of tissues for DNA isolation is immersing the tissue in a NaCl/CTAB preservation solution (Storchova *et al*., 2000). This tissue is later rinsed with tap water before grinding it for DNA isolation. The tissues can also be sun dried or heat treated at 65°C for 30 min before grinding (Elias *et al*., 2004; Sharma *et al*., 2013). Silica gel has also been used to achieve desiccation of tissue. Leaves are cut into small pieces and placed in bags containing silica gel (Moeller *et al*., 2014). This method is based on the principle that the rate of tissue desiccation is rapid enough to prevent degradation of DNA. Leaf tissues of many medicinal plants can also be dipped in a fixing solution (absolute alcohol) at room temperature which arrests the activities of enzymes (Sharma *et al*., 2010; Sharma and Gaur, 2014).

c) Homogenization: Tissue disruption in a ceramic mortar with the use of physical forces is the most common method used for tissue homogenization, more precisely cell wall disruption. Liquid nitrogen is widely used to aid the grinding process. It acts by making the tissue brittle and dry, suitable for grinding and also arrests the biochemical reactions taking place in a cell. Alternatively, prechilled mortar and pestle (at -80°C) can also be used for grinding the tissue (Biswas and Biswas, 2011). This averts the use of liquid nitrogen. Sterile sand has been used for grinding leaves of date palm (Arif *et al*., 2010). Grinding of tissues is now frequently carried out directly in eppendorf tubes or 96 well plates using mechanical tissue grinders and other high throughput systems. This prevents the chances of cross contamination which can otherwise occur while grinding in a mortar and pestle. The use of mechanical tissue lyser for high throughput lysis in a 96 well plate has drastically reduced the time for cell lysis (Hill Ambroz *et al*., 2002). Cell wall removal has also been achieved by ways other than application of physical forces. Alternate cold and heat treatment of the ground tissue, is one of the proposed ways to achieve it (Biswas and Biswas, 2011; Sahu *et al*., 2012). Chemical disruption of cell walls has also been achieved by using hydrolytic enzymes, such as cellulases, pectinases or cell wall macerases (Rogsatd *et al*., 2001). The leaf disks from different plant species were immersed in an enzymatic cocktail derived from *Trichoderma longibrachiatum*, which digests the cell walls (Manen *et al*., 2005).

d) **Extraction buffer:** The extraction buffer is essentially composed of individual chemicals which are prepared separately as stock solutions and mixed later. These individual constituents facilitate cell membrane dissolution (detergents), inactivation of nucleases (metal chelators, reducing agents) and removal of contaminants (salts, PVP etc.). Other than the common ingredients of the extraction buffer such as a suitable detergent (CTAB, SDS etc.), a buffering system to maintain the pH (Tris HCl), a salt to prevent DNA denaturation (NaCl), a protein denaturant (β-mercaptoethanol), a metal chelator or nuclease inactivator (EDTA), the extraction buffer is supplemented with various other ingredients to remove the contaminating residues . The latter includes RNA, proteins, polyphenolics (flavonoids, terpenoids and tannins), polysaccharides and non-nuclear DNA which can bind to the DNA and get co-precipitated with it. LiCl is incorporated in the extraction buffer to remove RNA (Arif *et al*., 2010). Proteinase K, a proteolytic enzyme is sometimes incorporated in the extraction buffer to inactivate nuclease enzymes and remove protein contaminants when protein rich seed tissue is used for DNA extraction (Thangjam *et al*., 2003; Pervaiz *et al*., 2011). When large amounts of phenolic compounds are present in the plants, a reducing agent or a phenol compound absorbent is frequently incorporated into the extraction buffer. The most commonly used phenol absorbents are polyvinyl pyrrolidon (PVP) or polyvinyl polypyrrolidon (PVPP).These prevent oxidation of polyphenols into quinines which bind to nucleic acids and hinder isolation of high quality DNA. Without the addition of PVP or PVPP (the two only differ in water solubility), the precipitated DNA is brown due to the presence of oxidized phenols. Various antioxidants like β-mercaptoethanol, BSA, sodium sulfite, sodium azide etc. are used with PVP for an effective removal of polyphenolics. A high concentration of PVP and β-mercaptoethanol has been used to remove tannins and polyphenolics from different tissue extracts of medicinal plant, *Aconitum heterophyllum* (Srivastava, 2010). Similarly, Mathew *et al*., 2014, used 1-3 % PVP, 0.1-1% sodium metabisulphite, 0.04-2% β-mercaptoethanol while grinding cardamom leaves to

remove polyphenolics from the tissue extract. However, in some cases like forest tree *Corymbia citriodora*, the use of 1% and 4% PVP compromised the quality of DNA and did not result in isolation of DNA which is amenable to NGS platforms (Healey *et al*., 2014). They instead used β-mercaptoethanol and carried out other minor modifications in the protocol, to rid the DNA of polyphenolics. Diethylthiocarbamic acid (DIECA) is a phenoloxidase inhibitor which helps to reduce brown colouring due to oxidation of polyphenols to quinines (Anuradha *et al*., 2013). L-ascorbic acid also prevents oxidation of phenolic compounds. The presence of gelling polysaccharides makes the DNA viscous, causing it to stick to the wells during gel electrophoresis (Diadema *et al*., 2003; Varma *et al*., 2007). To counter the problem of polysaccharides, a high salt (NaCl) concentration in the extraction buffer is most frequently favored. The NaCl concentration was increased to as high as 5M for removal of polysaccharides from *Urginea indica* extracts (Harini *et al*., 2008). A high CTAB concentration has also been used to remove polysaccharides from tissue extracts of *Chenopodium* (Akhtar *et al*., 2013). A 10% (w/v) CTAB solution containing 3M NaCl was used to remove polysaccharides from DNA isolated from leaves of cardamom (*Amomum subulatum*) (Mathew *et al*., 2014). The principle behind this CTAB based removal of polysaccharides is the formation of CTAB-polysaccharide complexes at high ionic strengths. Hence, a mere tweaking of salt concentrations can result in different functions of CTAB i.e., cell membrane disruption and nucleic acid precipitation or polysaccharide removal. The homogenate can also be diluted two or three times with the extraction buffer to reduce polysaccharide contamination (Sharma *et al*., 2000). The composition of extraction buffer used also differs with the age and nature of the tissue . A higher concentration of CTAB and NaCl in the extraction buffer was used for isolating DNA from dry leaves of rice while a lower concentration of CTAB and NaCl was present in the extraction buffer when the source of DNA was fresh leaves (Ahmadikhah, 2009). Similarly, different concentrations of PVP and β-mercaptoethanol were used for tissue grinding when DNA was isolated from fresh and dry leaves of cardamom (*Amomum subulatum*) (Mathew *et al*., 2014). N-phenylacylthiazolium B (PTB) has been extensively used to extract DNA from hard and woody tissues of timber yielding trees and mycorrhizal root tips (Asif and Cannon, 2005; Tnah *et al*., 2012). DNA obtained from bark tissue of such timber yielding trees by the modified CTAB method and kits failed to amplify, due to poor quality of DNA due to the presence of Maillard products. These are the condensation products of reducing sugars and primary amines. PTB cleaves these glucose derived protein cross linkages and helps to free the DNA entrapped in these crosslinks (Asif and Cannon, 2005). Different protocols have been suggested, varying in extraction buffer compositions and various other steps, for different classes of plant species differing in the amounts of secondary metabolites and DNases (Lutz *et al*., 2011). Hence, it can be concluded that depending upon the nature of the starting plant material, the composition of the extraction buffer in terms of presence or absence of individual chemicals or their concentrations is varied, to optimize the DNA yield. Thus, it can be concluded that the composition of extraction buffer has been subjected to most of the variations in order to optimize the quantity and quality of the extracted DNA . The composition of extraction buffer has been modified mostly by changing the concentration of salt or NaCl in tissues with high polysaccharide content (eg. potato tubers, citrus leaves, chickpea seeds); or by addition of PVP to tackle the problem posed by the presence of polyphenolics in plant tissues (eg. leaf tissues from medicinal and aromatic plants).Other changes that have been widely attempted and standardized for troubleshooting include the addition of chemicals like LiCl, ascorbic acid, sarcosyl, diethyldithiocarbamic acid to the extraction buffer or separately after homogenization of the tissue.

A detergent is added to the extraction buffer to effect the disruption of cell membranes. Based on the detergent used for lysis, most DNA isolation protocols can be broadly categorized into two different classes:

- CTAB based methods
- SDS based methods

CTAB based methods: These methods employ a cationic detergent, hexadecyltrimethylammonium bromide (CTAB) in the extraction buffer for cell membrane disruption. When CTAB is used in extraction buffer, a low ionic strength of the buffer is maintained. Under these conditions, CTAB precipitates nucleic acids and the protein contaminants lie in the supernatant.

Chronology of CTAB based DNA Isolation protocols:

A large number of CTAB based methods are used by researchers, which have been suitably modified to suit a particular plant genus or tissue type. Most of the current

methods of DNA isolation are modifications of these classical methods.

- 1980–Murray and Thompson Protocol: first used CTAB and employed cesium chloride (CsCl) density gradient technique to eliminate the polysaccharides.
- 1982–Taylor and Powell miniprep method
- 1984–Rogers and Benedich protocol
- 1984–Maroof and Saghai protocol- used lyophilized tissue
- 1987–Doyle and Doyle protocol

SDS based methods: These methods use the anionic detergent, sodium dodecyl sulfate (SDS) for cell disruption.

Chronology of SDS based DNA Isolation protocols:

- 1983–Dellaporta *et al*., Method
- 1997–Aljanabi and Martinez Method

There are some protocols which have shown the use of both CTAB and SDS or each of these with a different combination of detergents, in the extraction buffer e.g., the extraction buffer used for isolation of DNA from rice leaves, was a combination of two different solutions; solution A (150 mM sorbitol, 125 mM Tris, 25 mM EDTA, 500 mM NaCl, 20 mM Na_2SO_3, 0.8% CTAB and 2% sarkosyl, pH 7.5) and solution B (100 mM Tris, 500 mM KCl, 18 mM $MgCl_2$ and 1% Triton X-100, pH 9.0) in a defined proportion with supplementation of β-mercaptoethanol (Chuan *et al*., 2010). In cotton, two types of buffers termed as homogenization and lysis buffers were used in combination or successively to facilitate DNA extraction (Haiwen *et al*., 2001). Another cationic detergent, dodecytrimethylammonium bromide (DTAB), has been used for DNA extraction from many microalgal species (Fawley and Fawley, 2004). This method commonly referred to as the CTAB-DTAB method increases the DNA recovery rate and quality of the DNA obtained. Interestingly, household detergents and shampoos have also been used by some researchers (Inuwa *et al*., 2011).

Tables 1 and 2 enlist the published modifications (last 15 years) in the extraction buffer composition, for isolation of DNA from various plant species and tissues.

The volume of extraction buffer used in proportion to the amount of plant tissue taken, also affects the quality and yield of DNA (Krizman *et al*., 2006; Chen *et al*., 2009). The incubation time of the homogenate at a temperature of ~60°C or less (at 37°C) has also been shown to affect the quality of DNA (Ginwal and Maurya 2010; Ansari and Khan, 2012).

Comparative Studies on various DNA Isolation Protocols

Table 3 gives a succinct comparison of the classical DNA isolation methodologies. Most CTAB based DNA isolation methods reported in literature are variations of the original Doyle and Doyle method and most SDS based methods are variations of the original Dellaporta method. About 70 % of the studies reported from the year 2000 onwards, have used CTAB methods for DNA isolation. These methods have been adopted for a wide range of plant species, both monocots and dicots. Biophysically, it has been established that the self aggregation behaviour (micelle formation) of CTAB is stronger than that of SDS as determined by ESR (Bahri *et al*., 2006).

e) **Deproteinization or organic extraction:** This is a two step procedure which aims to remove protein and lipid contaminants present in the cell lysate. The first step involves treatment of the cell lysate with organic solvents like phenol or mixture of phenol:chloroform:isoamyl (25:24:1) alcohol or mixture of chloroform:isoamyl alcohol (24:1) or mixture of chloroform: octanol (24:1). In cases where phenol is used, the DNA is reextracted with chloroform or chloroform:isoamyl alcohol to ensure the removal of any trace phenol from the nucleic acid preparation (Adhikari *et al*., 2011; Akhtar *et al*., 2013). This is followed by centrifugation to enable separation of DNA containing aqueous phase from the organic phase containing protein and lipid contaminants. Most researchers have used chloroform:isoamyl alcohol for organic extraction as compared to using phenol because of a better quality of DNA obtained while using the former. More than one extractions can be carried out with chloroform:isoamyl alcohol to remove the cloudiness caused by PVP. Sharma *et al*. (2013) isolated DNA from different tissues of plants (cowpea, soybean, maize, *Dioscorea* sp., cassava, mango, banana and okra) for PCR amplification without phenol-chloroform extraction. Rehman *et al*. (2007) proposed a high throughput method of DNA extraction from wheat and canola by protocols with and without the use of any of

Table 1. Details of composition of CTAB based extraction buffer used for DNA isolation from different plant species and tissues

Plant/Family	Tissue used	Extraction Buffer Composition					Additional components/ Features	References
		Tris HCl	EDTA	NaCl	CTAB	β-mercap		
Allium sps.	Leaf,stem, flowers.	100mM	25mM	1.5M	2.5%	0.2%	1% PVP	Khanuja *et al*., 1999
Gossypium sps.	Leaf	0.1mM	.005mM	-	-	-	Sodium bisulfite (0.4g), 0.35 M sorbitol.	Haiwen *et al*., 2001
		200mM	0.05mM	2M	.055mM	-		
Agavaceae	Foliar tissue	100mM	20mM	1.4M	2%	10mM	4% PVP, 0.1% ascorbic acid	Keb Llanes *et al*., 2002
		100mM	50mM	0.1M	-	10mM	-	
Asteraceae (*Cichorium intybus, Taraxacum officinale, Lactuca sativa*)	Etiolated leaves	100mM	20mM	1.4M	2%	0.2%	2% PVP	Michiels *et al*., 2002
Manihot esculenta	Tuber	100mM	30mM	1.2M	3%	3%	-	Elias *et al*., 2004
Litchi chinensis	Leaf	100mM	20mM	2M	2%	5%	2%PVP,10mM ammonium acetate	Puchooa 2004
Foeniculum vulgare, Origanum vulgare, Cannabis sativa, Humulus lupulus, Coffea arabica	Leaf, seed, dried cones, beans	100mM	20mM	2M	2%	-	1% PVP, 0.5% activated charcoal	Krizman *et al*., 2006
Terminalia arjuna	Leaf	100mM	20mM	1.4M	2%	N.M	Combination of CTAB and column based purification system	Sarwat *et al*., 2006
Medicinal plants (*Sclerocarya birrea, Barleria greenii, Huernia hystrix, Aloe polyphylla*)	Leaf	100mM	20mM	1.4M	2%	N.M	3% PVPP added separately	Moyo *et al*., 2008
Ipomea batatas	Leaf	100mM	20mM	1.4M	2%	0.4 -1%	-	Borges *et al*., 2009
Arid trees (*Acacia, Prosopis, Calligonum*)	Leaf	100mM	20mM	4M	3%	0.2%	6% PVP	Sablok *et al*., 2009
Garcinia sps.	Fruit rind and leaf	100mM	30mM	1.4M	2-4%	0.3%	1.5%PVP	Ashish *et al*., 2010
Phoenix dactylifera	Leaf	1.21g	0.4g	8.12g	2%	-	PVP (2g), LiCl (0.2g)	Arif *et al*., 2010
Dalbergia sissoo	Leaf	100mM	20mM	1.42M	2%	3 ul	4% PVP-40, 5mM ascorbic acid	Ginwal and Maurya 2010
Allium stracheyi	Seed	100mM	25mM	2.5M	2.5%	3%	3% PVP, 0.15% sodium sulfite	Ranjan *et al*., 2010
Aconitum heterophyllum	Leaves from field grown and tissue culture raised plants, seeds	100mM	20mM	1.4M	2%	3%	15mM ascorbic acid, 3% PVP.	Srivastava *et al*., 2010
Cereals (rice, wheat, maize)	Seed and leaf	100mM	20mM	1.4M	2%	1%	1% PVP, Proteinase K (50ug/ml)	Pervaiz *et al*., 2011
Legumes (*Cajanus*, *Cicer, Vigna*)	Leaf	100mM	20mM	4M	3%	2%	-	Agbagwa *et al*., 2012
Mangifera indica L	Leaf	100mM	20mM	3M	4 %	3%	0.025 g/ml PVP	Azmat *et al*., 2012
Chrysanthemum indicum	Seed and leaf	100mM	20mM	1.4M	2%	1%	-	Hasan *et al*., 2012
Gymnema sylvestre	Leaf	1000mM	500mM	4M	10%	N.M	-	Krishna *et al*., 2012
Abelmoschus esculentus	Leaf	100mM	20mM	1.4M	3%	0.006%	Tissue is ground in PVP before adding E.B.	Kumar *et al*., 2012
Mangroves sps.	Leaf	100mM	20mM	1.5M	2%	1%	-	Sahu *et al*., 2012
Boswellia serrata	Leaf	100mM	20mM	1.4M	2.5%	10 mM	1% PVP	Sharma & Purohit 2012
Reaumuria soongorica	Leaf	100mM	25mM	2mM	2%	5%	5% PVPP	Wang *et al*., 2012
Sorghum bicolor, Populus deltoids, Gossypium hirsutum, Triticum aestivum	Seed & leaf	100mM	20mM	1.2M	2%	0.1%	-	Xin & Chen 2012

Plant/Family	Tissue used	Extraction Buffer Composition					Additional components/ Features	References
		Tris HCl	EDTA	NaCl	CTAB	β-mercap		
Chenopodium album Lepidium latifolium	Plantlets	100mM	25 mM	1.5M	2.5%	0.4%	2%PVP	Akhtar *et al*., 2013
Morus sp.	Leaf	200mM	20mM	1.4M	2%	-	2%PVP, 5mM L-ascorbic acid, 4mM DIECA, 1% sodiummetabisulfite, 0.5% SDS.	Anuradha *et al*., 2013
Zingiberales sps.	Leaf	100mM	20mM	1.4M	2%	-	1% PVP used separately with E.B for grinding	Devi *et al*., 2013
Tropical plants (*Ficus carica, Hevea brasiliensis, Nicotiana tabacum, Carica papaya, Musa nana, Roystonea regia*,etc.)	Leaf	200mM	25mM	2 M	2%	-	2% PVPP, 1% sodium lauroyl sarcosine, 20 mM disodium tetraborate decahydrate	Huang *et al*., 2013
Cicer arietinum	Leaf	100mM	20mM	1.75 M	2%	0.5%	Tissue grinding using a genogrinder in a 96 well rack.	Kumar *et al*., 2013
Aconitum balfourii	Leaf	100mM	25mM	1.5 M	3%	0.2%	2.5% PVP	Sharma and Gaur 2014

Abbreviations: CTAB: Hexadecyltrimethylammonium bromide; DIECA: Diethylthiocarbamic acid; EDTA: Ethylenediaminetetraacetic acid; E.B: Extraction Buffer; HCl: Hydrochloric acid; LiCl: Lithium Chloride; NaCl: Sodium Chloride; PVP: Polyvinyl pyrollidone; PVPP: Polyvinyl polypyrollidone; SDS: Sodium dodecyl sulfate.

*The pH of Tris HCl and EDTA is maintained at 8, unless specified in the tables.
*Measurements given g/100ml of extraction buffer.
*N.M: not mentioned in the papers.

Table 2. Details of composition of SDS based extraction buffer used for DNA isolation from different plant species and tissues

	Tissue	Extraction Buffer Composition					Additional Components/ Features	References
		Tris HCl	EDTA	NaCl	SDS	B-mercap.		
Morus sp.	Leaf	12.11g	18.07g	29.20g	0.25g	-	-	Venkateswarlu *et al*., 2002
Triticum aestivum	Leaf	100mM	20mM	500mM	2%	0.1%	7 M urea	Nalini *et al*., 2003
Cocus nucifera	Solid endosperm	200M	70mM	2000mM	-	0.2M	PVVP, 20% SDS added separately with extraction buffer.	Angeles *et al*., 2005
Capsicum annuum	Fruit	100mM	50mM	500mM	20%	10mM	-	OgunKanmi *et al*., 2008
Brassica napus, Nicotiana tobaccum	Leaf	200mM (pH 7.5)	25mM	250mM	0.5%	-	-	Amani *et al*., 2011
Cicer arietinum, Trichosanthes dioica, Bacopa monnieri	Leaf	200mM	25mM	200mM	-	-	1% PVP, 10 % SDS added separately with extraction buffer.	Adhikari *et al*., 2012
Malus sp.	Seed	100mM	50mM	1.5M	1%	-	7.5 M Amm. acetate added to tissue homogenate.	Ansari and Khan 2012
Gossypium sps.	Root tips	30mM	10mM	100mM	-	-	200mM sucrose	Rao *et al*., 2012
		500mM (pH 9.2)	250mM	-	2.5%	-		
Cajanus cajan	Leaf	100mM (pH 7.4)	50mM	500mM	0.7%	-	52 mM sodium sulphite, 3.6 µg RNase A,36 µg Proteinase K	Singh *et al*., 2012
Eleusine coracana	Leaf	50mM	10mM	100mM	10%	N.M	PVP (N.M)	Gupta *et al*., 2013
Cowpea, soybean, maize, *Dioscorea* sp., cassava, mango, banana and okra	Seed, leaves, tubers, stems, tuberous roots	100mM	10mM	1 M	1%	1%	2% PVP 0.05 mg/ml proteinase K 4% (w/v) PEG	Sharma *et al*., 2013

Table 3. Comparison of the classical DNA isolation protocols

Step In Extraction Protocol	CTAB based methods				SDS based methods	
	Murray and Thompson	Rogers and Bendich	Saghai Maroof	Doyle and Doyle	Dellaporta	Aljanabi
Detergent Conc. in E.B	1%	2%	1%	2%	20%	20%
Organic Extraction -:						
Chemicals used	$CHCl_3$ / oct.	$CHCl_3$ / isoamyl alc.	$CHCl_3$ / oct.	$CHCl_3$ / isoamyl alc.	Phenol/$CHCl_3$	None
No. of times	>1	>1	Once	Once	Once	N.A
DNA precipitation -:						
Chemicals	CsCl gradients	CTAB ppt. buffer and ethanol	isopropanol	isopropanol	isopropanol	isopropanol
When	after org. ex.	before and after org. ex.	after org. ex.	after org. ex.	before org. ex.	N.A
Washing	removal of DNA with a syringe	80% EtOH	76 % EtOH and 10mM NH_4Ac	76 % EtOH and 10mM NH_4Ac	80% EtOH	70 % EtOH
Resuspension of DNA	N.A	0.1 X TE	(10mM NH_4Ac + 0.25 mM EDTA)	1 X TE	1 X TE	sterile d H_2O

these organic solvents. They examined the efficacies of protocols that involved use of NaOH, $NaOCl_4$, NaOCl and sorbitol and concluded that even though the DNA yield obtained by using phenol chloroform extraction was higher compared to others however the quality of DNA obtained was better using the other methods. Mostly the organic extraction is carried out using the supernatant derived after centrifugation of cell lysate. However, in some studies this step is carried out after the DNA pellet is suspended and dissolved in TE buffer (Hariprakash *et al*., 2010; Ansari and Khan, 2012; Azmat *et al*., 2012).

Precipitation: DNA is precipitated after organic extraction using ethanol or isopropanol in the presence of salts like sodium chloride, sodium acetate, potassium acetate or ammonium acetate, at -20°C for 1 hr to overnight. To reduce the binding and precipitation of residual contaminants with DNA, the time of incubation with isopropanol was decreased (Krizman *et al*., 2006; Srivastava *et al*., 2010). A temperature of 25°C was maintained for precipitation in the presence of isopropanol (Krizman *et al*., 2006). Isopropanol is found to be more effective for precipitation of DNA and has been more commonly used. However, the use of ethanol has been suggested to be better than isopropanol in precipitating DNA from seeds of apple (Ansari and Khan, 2012). Since polysaccharides have a similar solubility as DNA, they have a tendency to be co-precipitated with DNA in ethanol or isopropanol. The presence of salt (NaCl, NaAc., KAc.,NH_4Ac. etc.) increases their solubility in EtOH or isopropanol, thus enabling their removal once the DNA has been pelleted (Healey *et al*., 2014). Adhikari *et al*., 2011, used 2 M NaCl and ethanol for precipitation of DNA from the supernatant obtained after organic extraction, from leaves of chickpea and pointed gourd. 3 M Na acetate and chilled ethanol in defined proportionate volumes, have been used for precipitation of DNA from leaves of mango and cardamom (Azmat *et al*., 2012; Mathew *et al*., 2013). It has been observed that NH_4Ac. is capable of only partial precipitation of protein and polysaccharide components and hence was not found suitable for isolation of DNA from seeds of *Sorghum* (Chen *et al*., 2009). Some studies have reported the use of a CTAB-NaCl precipitation solution instead of or in addition to isopropanol. A CTAB precipitation solution (1% CTAB, 50mM Tris HCl (pH=8), 100mM EDTA) was used to precipitate DNA from tissues of medicinal plants (Moyo *et al*., 2008). Xin and Chen (2012), diluted the CTAB- DNA complex by the addition of a dilution buffer, resulting in a decrease of NaCl concentration from 1.2 M to 0.4 M which allows precipitation of CTAB-DNA complex . To reduce RNA contamination, Sharma and Purohit (2012), carried out DNA precipitation on diatomite suspensions.

Purification and resuspension: The precipitated DNA is washed with 70%-80% ethanol to remove salts and other water soluble impurities. The air or vacuum dried DNA pellet is then dissolved in 1 X TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8). An optional RNase treatment (at 37°C for 30 mins or more) can also be given to remove any contaminating traces of RNA.

Hariprakash *et al*. (2010), added a mixed solution of 2% polyethylene glycol and 1.5 mM NaCl, to the dissolved DNA pellet after RNase treatment. This was done to remove undissolved polysaccharide contaminants in the DNA. This was followed by organic extraction, precipitation and a final washing to remove PEG and NaCl.

Kit based DNA Isolation

There are a large number of commercial DNA isolation kits available in the market today. These methods are designed to remove contaminants and offer a simple, convenient procedure for rapid isolation of genomic DNA of high yield and purity from tissue samples. The use of these kits also averts the use of hazardous chemicals like phenol, chloroform for DNA isolation. Most modern kits are based on purification of DNA from crude cell lysate by selective binding to a support material. The support material is essentially chromatographic columns which are based on either anion exchange chromatography or adsorption based chromatography. The main steps in DNA isolation using kits are:

Cell lysis: Cells are broken down to release the DNA.

Binding: Genomic DNA gets selectively adsorbed on a synthetic column.

Washing: Contaminants are washed away.

Elution: DNA is eluted in a low salt buffer.

Anion Exchange Methods

These methods are based on electrostatic interactions between negatively charged phosphate groups of the DNA with positively charged immobilized surface molecules on the column. The DNA binds to the column under low salt concentrations and the other impurities are washed away using medium salt buffers. The bound DNA is then eluted using a high salt buffer. The eluted DNA is then precipitated in alcohol and can be used for further downstream applications.

Silica based Column Methods

These methods make use of spin columns made up of silica gel membrane, on which the DNA gets bound. The DNA is then eluted in the presence of high concentration of chaotropic salts. In these methods no alcohol precipitation is required and the eluted DNA is ready to use (www.qiagen.com). Sometimes there are problems of loss of DNA, due to subsequent column washes, particularly when only a small amount of tissue is available. These methods have been scaled upto a 96- well format to ensure simultaneous processing of many samples.

Paramagnetic Bead based Column Methods

These are based on reversible adsorption of DNA on paramagnetic beads (Czembor *et al*., 2014).The reagents provided with these kits are used with magnetic tools. The use of this kit averts the need for use of any organic solvents, repeated centrifugation or column separation (Tan and Yiap *et al*., 2009).

A large number of studies are reported where comparisons between the manual and kit based methods of DNA isolation are reported (Coyle *et al*., 2003; Michiels *et al*., 2003; Tan *et al*., 2013). Michiels *et al*. (2003), reported a better quality and quantity of DNA from etiolated leaves of latex plants, isolated by the optimized CTAB method than that isolated by kit based method. Yadav *et al*., 2012 reported a greater quantity of DNA isolated from cotton leaves by kits as compared to that isolated by CTAB method. Application based superiority of DNA isolated from kit has been documented by Tefler *et al*. (2013), wherein it was purported that DNA isolated from needles of *Pinus radiata* through kits is more amenable for high throughput genotyping platforms in terms of SNP call quality. Moeller *et al*., 2014 showed the superiority of DNA isolated from two different kits over modified CTAB method in terms of yield and protein contamination. Karaslan *et al*. (2014) have compared six different commercial DNA isolation kits for DNA isolation from seeds and leaves of wheat, in terms of extraction efficiencies, cost and time effectiveness. There appears to be a lack of consensus in determining the superiority of kit or manual method over each other for DNA isolation in terms of quantity and quality. However, it can be safely concluded that there are major differences in the cost of processing/ sample through kit based and manual methods, with the cost being higher for kit based method. The estimated price for most kits is calculated to be ~$250 to $ 300+ for 50 reactions. Many approaches have been worked out to bring down this price. Some studies have recommended the use of both the methods, i.e. isolation by manual method and purification by kit based method, in order to reduce the cost involved in exclusive use of kits for DNA isolation (Sarwat *et al*., 2006; Tefler *et al*., 2013). Another approach to make the use of kits more cost effective is to buy the adsorbent

resins in bulk and pack them in ordinary class columns or syringes for use.

High throughput DNA Isolation

The development of high throughput DNA isolation methodologies has made the procedure of DNA isolation less time consuming and less cumbersome. DNA isolation has been scaled up to multiples of 96 well/sample format. Most of these methods utilize kits for DNA isolation and do not involve organic extraction as it is difficult to achieve phase separation in plates (Tibbits *et al*., 2006). These technologies utilize mechanical tissue grinders (Genogrinders) to handle a large number of samples simultaneously (Kumar *et al*., 2013; Sharma *et al*., 2013). Lemke *et al*. (2011) used a tissue lyser with tungsten carbide beads for DNA isolation from grapevine (*Vitis* sps.) in 96 column plates. DNA was isolated from lyophilized leaf tissue and seeds of *Sorghum bicolor* after grinding in a tissue lyser by placing a tungsten ball in a tube containing the tissue. A 96 well plate could be processed in less than 2 hrs using this method (Xin and Chen, 2012). Xu *et al*. (2005), described a high throughput method in rice which is capable of processing 384 samples in 2 hrs. A rapid and convenient method of DNA isolation from shoot and leaf tissues of transgenic plants was proposed by HwangBo *et al*. (2010) which uses a chelating resin Chelex 100 while homogenization of the tissue in an eppendorf tube followed by boiling and centrifugation, which yielded a PCR ready DNA preparation. High throughput has been achieved by using fully automated robotic workstations. Zhang *et al*. (2012) have elaborated a quick and simple method for megabase sized DNA isolation from plants, animals and insects. They have enunciated a LMP agarose gel based system for DNA isolation from intact nuclei or protoplasts. The protoplasts or nuclei are embedded in LMP agarose plugs and further purification is carried out in these plugs itself. Czembor *et al*., 2014 have described a state of the art DNA isolation procedure from wheat and barley leaves. They used a robotic workstation where grinding is achieved by a grinder after placing a stainless steel ball bearing in each tube containing lyophilized leaf sample. The processing capability of this system was 8 x 96 samples . The workstation is also equipped with a monochromatic plate reader for the purpose of DNA quantification. Moeller *et al*. (2014) used a Magna Cel paramagnetic cellulose DNA isolation method in conjunction with a Maxwell 16 robot unit which can carry out purification and elution of the DNA.

Conclusion

A detailed analysis of the papers published in the last decade or so, clearly reveals that the CTAB based DNA isolation protocols are more frequently used for different plant materials as compared to SDS based and other protocols. Apart from these detergents, different chemicals such as polyvinylpyrolidone (PVP), pectinases, proteinase K, sodium perchlorate, sodium acetate, ammonium acetate etc., are used in varying concentrations and at different steps for improving efficiency of the DNA isolation protocols. With the availability of a large number of commercial kits and high throughput sophisticated DNA isolation technologies, DNA isolation has now become much easier and feasible than ever before. There are protocols for DNA isolation available not only from new and freshly harvested plant tissues but also from ancient preserved herbarium specimens.

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