Simple and efficient DNA isolation protocol for small cardamom [*Elettaria cardamomum* (L.) Maton]

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Abstract

Small cardamom [Elettaria cardamomum (L.) Maton] is one of the most economically valuable spice crops since ancient period. The spice has been used in culinary, traditional as well as modern pharmaceutical applications. A remarkable variation is existing within the species which increases the chance for misidentification and inadvertent duplication of varieties in many cardamom collections. Selection of parents without knowing its pedigree often leads to negative results. Therefore, information about germplasm diversity and genetic relationships among accessions or cultivars is of fundamental importance for breeding and the management of germplasm. Morphological characterization is influenced by various environmental factors. Hence, molecular characterization of cardamom accesions is essential identification of genotypes at molecular level. Currently, there is no efficient and cost effective DNA isolation protocol available due to presence of an diverse array of seondary metabolites, polyphenols and polysaccharides present in cardamom leaves. Therefore, this study aims to develop a DNA isolation protocol from the leaves of vazhukka cardamom accesions.

Emerging leaves of ten vazhukka type cardamom accesions were selected and used for genomic DNA isolation. A highly efficient DNA extraction protocol from freshly collected emerging leaves of cardamom was developed with modified cetyl trimethyl ammonium bromide (CTAB) method which is a collective stragegy of 2-mercaptoethanol, PVP (poly vinyl pyrrolidone), 1% sodium metabisulfide and 5M NaCl were used for removing polyphenols and polysaccharides. This protocol was pertinent to isolate genomic DNA from emerging leaf tissues of cardamom. Genomic DNA isolated by this method is highly suitable for characterization of cardamom accessions using molecular markers.

Keywords: CTAB, *Elettaria cardamomum*; DNA isolation; Polyphenol.

Introduction

Elettaria cardamomum (L.) Maton belongs to family Zingiberaceae, commonly known as small cardamom or green cardamom or true cardamom. It is mostly cultivated in India, Guatemala, Sri Lanka, Nepal, Indonesia, Costa Rica, Mexico and Tanzania[4]. In India, cardamom is cultivated in higher altitudes ranging from 900-1400 m above sea level of southern states of Kerala, Karnataka and Tamil Nadu. For centuries, cardamom capsules have been used for both culinary as well as traditional medicine applications mainly controlling asthma, teeth and gum infections, digestive and kidney disorders[6,16] and cataracts, nausea, diarrhea and cardiac disorders[5,7].

Small cardamom consists of wide array of genetic accessions and has a wider variability within the germplasm which is pre-requisite for efficient and sustainable germplasm management. Information on molecular characterization of small cardamom is very limitted. Isolation of good qualtiy DNA is an improtant step in molecular studies of all plant speices and especially, crops with polyphenols and polysaccarides. An array of DNA isolation protocols was available and is being used for the extraction of nucleic acids for various crops[18]. DNA isolation from small cardamom is a difficult task because of naturally occurring polyphenolic compounds and polysaccharides[9,12]. These secondary metabolites hinder the isolation of good quality DNA from cardamom leaf tissues while employing routine DNA extraction protocols. Procurement of good quality and yield of genomic DNA is found to be very challenging in any molecular analysis.

Available DNA extraction methods are applicable for model species and these protocols are often not suited for non-model species[19]. In addition to this, different DNA isolation protocols are required for closely related species. Hence, each species required separate standardized protocol for nucleic acid isolation. Keeping in view of above facts, in this study we have attempted standard CTAB protocols with several modified versions.[2,3,8,13]

Conversely, DNA appeared brownish yellow and highly viscous rendering it unsuitable for subsequent manipulations. In this situation, development of easy and reliable DNA isolation protocol suitable for small cardamom for especially removing polyphenols and polysaccharides is essential.

Presntly, a few protocols have been described for isolation of DNA from polysaccharide and polyphenol rich plant materials[10,15]. Therefore, present study aim was to develop DNA isolation protocol for emerging leaves of *vazhukka* type cardamom accessions.

Material and Methods

Plant samples: Emerging leaves of ten promising accessions of *vazhukka* type small cardamom [*Elettaria cardamomum* (L.) Maton] were collected from the germplasm of Cardamom Research Station, Kerala Agricultural University, Pampadumpara and stored at -20ºC. The accessions details were listed in the table 1.

Table 1
List of *vazhukka* type cardamom acessions used for this study

S.N.	Name of the entry	Accession number
1.	BABU-3	CRSP 129
2.	ASK-1	CRSP 19
3.	ASK-4	CRSP 22
4.	ASK-5	CRSP 23
5.	SAM-3	CRSP 181
6.	SAM-8	CRSP 34
7.	NS 34	CRSP 154
8..	CHETTI-1	CRSP 117
9.	PPK-2	CRSP 5
10.	PV1 x GG Type 1	CRSP 77

DNA isolation protocol: 2g leaf tissue was weighed and ground finely using pre-chilled pestle and mortar using 10ml of pre-heated (60ºC) CTAB extraction buffer (100mM Tris HCL pH 8, 20mM EDTA pH 8, 1.4M NaCl and 2% w/v CTAB) and homogenized after the addition of 50 mg PVP, 5µl of 2-mercaptoethanol and 0.1% sodium metabisulphite. The homogenate was transferred into 30 ml oak ridge centrifuge tubes and incubated at 60°C for 45min and shaked intermittently at every 10 minutes and cooled to room temperature (RT). 10 ml of Chloroform: Isoamyl alcohol (24:1) mixture was added to it and mixed carefully by inverting the tubes ~25-30 times to form an emulsion.

Centrifugation was performed at 5000 rpm for 15 minutes at 4ºC and after centrifugation, transfer the top clear aqueous phase to fresh 30 ml oak ridge centrifuge tubes using cut tips [If cloudy, one more addition of 6 ml of Chloroform: Iso-amyl alcohol mixture is required before repeating this step], 2.5 ml of 5M NaCl was added to the supernatant and mixed with it. 10 ml cold isopropanol was added to it and mixed gently until the precipitation of DNA followed by 1hr or overnight incubation at –20°C. DNA was pelleted at 3000 rpm for 3 min, then increase the speed to 5000 rpm for 3 min at RT. Poured off the supernatant and remaining pellet was washed with 2 ml 80% ethanol, centrifugation as above for 3 min followed by washing twice with 100% ethanol.

Decant the ethanol without complete drying DNA by leaving tubes uncovered at 37°C for ~30 min or dry at RT. DNA pellet was re-suspended with 750 - 1000µl of TE buffer and pooled using cut tips. In order to remove RNA, 5µl of RNAase (10µg/ml) was added and incubated at 37°C for 45 min for immediate analysis or kept at –80°C for long term storage.

Purification of DNA: The total DNA was purified by adding 1 ml of 7.5 M ammonium acetate followed by 10 ml of ice cold ethanol. After careful mixing, it was kept for 1hr at -20ºC for precipitation of DNA. Centrifugation at 8000 rpm for 20 min at 4ºC was done to decant the supernatant. The DNA pellet was air dried at 37ºC for 15 min and dissolved in 1ml of TE buffer.

Quantification of DNA: The quality of the isolated DNA sample was analyzed by agarose gel electrophoresis technique. 0.8% agarose was prepared in 1x TAE buffer and ethidium bromide (EtBr) was added to the gel prior to solidification and casting[17]. 3.5 µl of each sample was mixed with gel loading dye and loaded into the wells immersed in the tank of 1x TAE buffer and electrophoresed at 60V for 1 hr. The gel was visualized using gel documentation system (Bio-Rad, USA). The purity of genomic DNA was assessed by measuring the absorbance measured at 260nm and 280nm using UV Biophotometer (Eppendorf, Germany). The concentration of DNA (µg/ml) was calculated by following formulae of OD at 260nm x 50 x Dilution factor/1000. Total yield is obtained by multiplying the DNA concentration by the final total purified sample volume.

Results and Discussion

The genomic DNA isolation from plants generating polyphenols and polysaccharides has been a challenging task in various crop species[18]. When we did DNA isolation with CTAB extraction buffer based protocol along with 2-mercaptoethanol, the product obtained was brownish yellow in colour due to presence of high levels of polyphenols in the leaves. Recent studies reported accumulation of substantial quantity of polysaccharides and polyphenols in leaves of small cardamom[12] while the presence of major monoterpenes like, 1, 8-cineole, α-terpineol and α- terpinyl acetate was reported to be in the range of 10-34% [1].

PVP has been often used in CTAB extraction protocols for DNA to prevent the polyphenol oxidation[2]. Besides, polyphenolic compounds, the DNA was extracted highly viscous, which indicated the presence of polysaccharides; those are apparently water soluble and tend to co-isolate with DNA[18]. The key role of polysaccharides is interfering the enzymes like restriction endonucleases, polymerases and ligases and thus resulted in unsuccessful amplification[11]. DNA damage/ shearing is protected either directly or indirectly through addition of 2-mercaptoethanol which acts as a reducing agent that inhibits the oxidation process[18].

In the present study, PVP (50mg), 0.1% sodium metabisulphite (10µl) and β-mercaptoethanol (5µl), were administered during DNA extraction procedures and they encountered detrimental effect of polysaccharides and polyphenols. In our experiment, 2% CTAB as well as 5M NaCl also removed the polysaccharides. RNA was removed by addition of 3µl of 10µg/ml RNAase enzyme and further DNA was purified by adding 1 ml of 7.5M Ammonium acetate. This procedure yields high level of good quality

DNA. The scheme depiction or flow chart for detailed DNA extraction procedure is presented in figure 1.

The DNA obtained in the present study was documented with Bio-spectrophotometer optical density (OD) readings. The OD ratio of A260/280 was 1.7-1.9 indicating the absence of any contaminants[14]. The yield of DNA obtained from fresh emerging leaves of ten cardamom accessions ranged between 11-15.5µg/g tissues (Table 2). In 0.8% agarose gel, DNA formed a thick intact single band of high molecular weight which confirmed it of the good quality (Figure 2). In future, the isolated genomic DNA could be used for further characterization of cardamom accessions by PCR amplification and genetic diversity analysis by molecular markers.

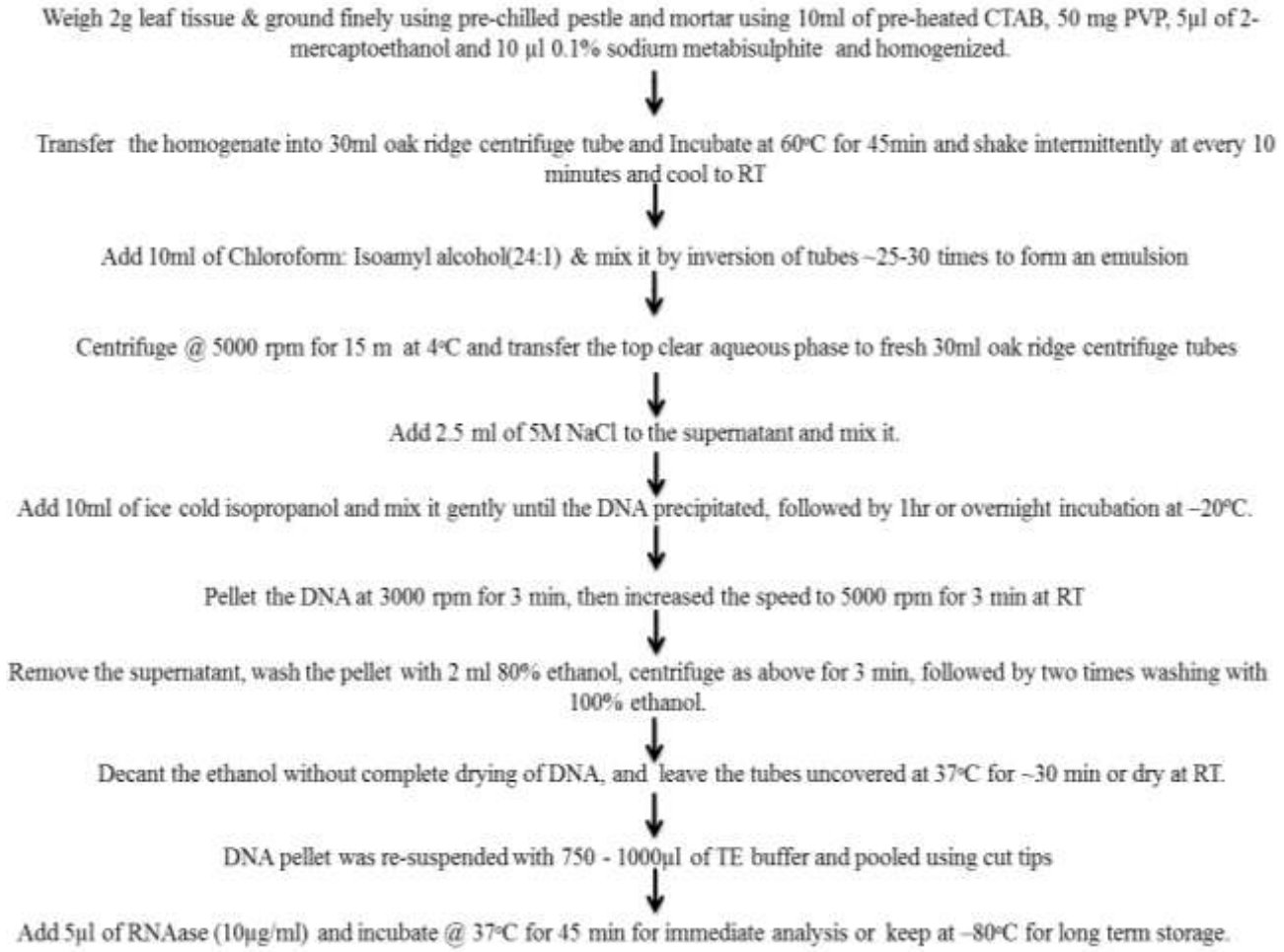

Figure 1: Scheme of genomic DNA extraction procedure for emerging leaves of *Elettaria cardamomum*

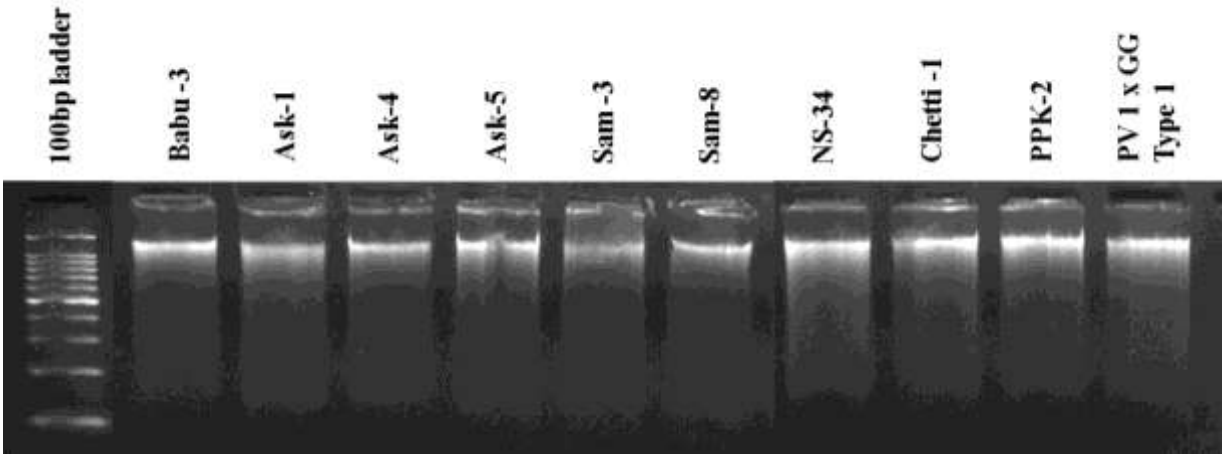

Figure 2: Isolated DNA from 10 *vazhukka* type small cardamom accessions

Table 2
Analysis of DNA purity and yield for 10 *vazhukka* type cardamom accessions

S. N.	Name of the entry	DNA Purity (A260/280 ratio)	DNA yield (µg/g tissue)
1.	BABU-3	1.7	12.1
2.	ASK-1	1.8	12.0
3.	ASK-4	1.7	14.5
4.	ASK-5	1.7	14.0
5.	SAM-3	1.8	11.0
6.	SAM-8	1.8	11.7
7.	NS 34	1.6	14.2
8.	CHETTI-1	1.8	13.1
9.	PPK-2	1.9	14.5
10.	PV1 x GG Type 1	1.7	15.5

Conclusion

The proposed DNA extraction method with the addition of PVP, 0.1% sodium metabisulphite, 2-mercaptoethanol, 5M NaCl and 7.5M ammonium acetate is highly suitable for extracting genomic DNA from emerging leaves of plants that contained substantial quantity of polyphenols and polysaccharides in cardamom.

In our study, the DNA purity and DNA yield from all cardamom accession evaluated were high and ranged from 1.7 - 1.9 and 11 - 15.5 µg/g tissue respectively. Genomic DNA isolated by this method could be useful for PCR amplification, molecular characterization and genetic diversity analysis. Also, this proposed protocol is less time consuming and cost effective to extract DNA from emerging leaves of cardamom.

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