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Extracting gDNA Suitable for Molecular Applications from Sugarcane, and its Phytopathogens

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ABSTRACT

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DNA fingerprinting, estimation of molecular genetic diversity, marker assisted breeding and molecular diagnostic tools are the need of the hour for sugarcane crop improvement. Efficient extraction of genomic DNA (gDNA) free from polysaccharides, polyphenols, RNA and other major contaminants is a key to all these molecular activities. We present herewith, a modified DNA extraction protocol that has been optimized for isolating gDNA from endergonic and hardy sugarcane plant and its phytopathogens and endophytic fungi. The protocol has worked equally well in a number of monocot and dicot plants including rice, maize, linseed, *Sesbania*, opium poppy, *Hyoscyamus*, phytoplasma infected sugarcane leaves and smut infected sugarcane whip. The success of this modified protocol was assessed by polymerase chain reaction (PCR)-based amplification and sequencing of amplicons, universality and reliability.

Introduction

Sugarcane sustains the second largest organized agro-industry in India. About 65% of sugarcane is cultivated in subtropics while 35% is in tropics. Sugarcane is most endergonic and hardy crop, grown in almost all the states of the country having diverse agro-climate situations both in tropical and subtropical regions and as such, the problems of sugarcane crop are of distinct and diverse nature. Sugarcane is also known as “Multi-utility crop” due to its use as raw material in many industries. Several major diseases caused by various phytopathogens such as

phytoplasma (prokaryotes) and fungal pathogens (eukaryotes) adversely affect sugarcane crop growth. These pathogens are responsible for cane yield loss and total crop failure, which may in turn lead to dwindled crop productivity. The various disease epidemics seriously require the attention of pathologists to find out the details about the pathogen and the epidemiology of the disease in order to develop suitable disease management practices. The economically important sugarcane diseases are mostly seed borne like red rot, smut, wilt, leaf scald, ratoon stunting, grassy shoot disease, mosaic etc. Sugarcane grassy shoot (SCGS)

phytoplasma is associated with ultra-structural changes in chloroplast, chlorophyll, changed orientation of vascular bundles, xylem, sieve tubes and companion cells, hypertrophy, hyperplasia and lignifications of sclerenchymatous cells in leaves.

Breeding for disease resistant varieties is a common practice to manage disease outbreak in sugarcane. The process takes several years and new isolates of pathogens emerge in due course. Marker assisted breeding is essential to cut short the long time span of releasing a resistant variety. Molecular characterization and incipient detection of pathogens on the other hand are essential for disease management and healthy seed cane production. DNA fingerprinting, estimation of molecular genetic diversity, trait linked markers for marker assisted breeding and molecular diagnostic tools are thus the need of the hour for sugarcane crop improvement. Candidate gene identification and sequencing of desired amplicons are the ways to achieve the target, and require high quality genomic DNA (gDNA) that should be free of polysaccharides, polyphenols, RNA and other major contaminants. DNA extraction plays an important role in the field of sugarcane molecular biology as for all such activities, the genomic DNA is the base which should be of high quality and free of major contaminants.

Extracting DNA from leaves of certain plant species (Bramwell *et al.*, 1995; Baker *et al.*, 1990), from conifer wood (De Filipis and Magel 1998; White *et al.*, 2000; Deguilloux *et al.*, 2002), including wild relatives of crop species, and sugarcane has been found to be particularly difficult. Marechal Drouard and Guillemaut (1992) devised protocol to extract DNA from spruce needles and also from mature tree leaves including fir (*Abies alba*), maple (*Acer saccharum*), ginkgo (*Ginkgo biloba*), beech (*Fagus sylvatica*), date

(*Phoenix dactylifera*), apple (*Malus sylvestris*) and peach (*Prunus persica*), leaves, hypocotyls or dried embryos of bean (*Phaseolus vulgaris*), leaves or tubers of potato (*Solanum tuberosum*), leaves of grapevine (*Vitis vinifera*) and hop (*Humulus lupulus*), cell suspension cultures or calli, seeds of millets (*Setaria italica*), barley (*Hordeum sativum*) and wheat (*Triticum aestivum*), Lichens and fern (*Pteridium aquilinum*).

To eliminate contamination problem, several methods have been employed such as sedimentation in cesium chloride gradients or extraction with CTAB and other procedures (Rether *et al.*, 1993; Lodhi *et al.*, 1994; Maguire *et al.*, 1994). However, it is not necessary that these methods are successful in all the crop plants as Porebski *et al.*, (1997) reported unsuccessful attempt to amplify *Fragaria* DNA by PCR using reported methods such as Dellaporta *et al.*, (1983), Saghai-Marooof *et al.*, (1984), Doyle and Doyle (1992), La Roche (1992), Oard and Dronavalli (1992), Wang *et al.*, (1993), Richards *et al.*, (1994) and Davis *et al.*, (1995), and hence, these methods necessitate modifications as per requirement depending upon the plant species.

Commercial sugarcane cultivars belong to the highly complex heterozygous polyploid genus *Saccharum*, which is characterized by the high chromosome numbers. Sugarcane tissues are rich in polysaccharides and polyphenolic compounds, which is a major hindrance in the purification of sugarcane genomic DNA. Several DNA extraction protocols have been described for such plants *e.g.* extraction of nuclei by Hamilton *et al.*, 1972, cesium chloride method by Murray and Thompson 1980, reproducible genomic DNA isolation method by Chiong *et al.*, 2017, rapid DNA extraction from sugarcane by Honeycutt *et al.*, (1992) and Aljanabi *et al.*, (1999),

microprep protocol for sugarcane by Srivastava and Gupta (2001) and genomic DNA from dry leaf samples of sugarcane by Vaze *et al.*, (2010). Seven rapid protocols of DNA isolation in sugarcane, differing with respect to the composition of extraction buffers and DNA precipitation methods were compared by Srivastava and Gupta 2006 to assess the quantity and quality of genomic DNA extracted from leaf material of different sugarcane genotypes and they suggested some modifications for improvement. Based on the above background, here we present a single modified protocol from Doyle and Doyle 1990, protocol that has been optimized for isolating DNA from the host plant sugarcane (*Saccharum* spp. hybrid), phytopathogens and endophytic fungi, with properties such as efficient extraction in high quantity of high molecular weight gDNA, its PCR amplifiability and appropriateness for sequencing based applications.

Materials and Methods

Plant material

Young, healthy and fast growing leaves of diverse plant species viz. healthy and SCGS phytoplasma infected plants of sugarcane, linseed, rice, maize, *Sesbania*, *Hyoscyamus*, *Papaver*, and mycelial cultures of different fungal isolates on PDA (potato dextrose agar medium) viz. *Colletotrichum falcatum*, *Sporisorium scitamineum*, *Fusarium moniliforme* and *Trichoderma viride* were taken as experimental material for genomic DNA isolation.

Solutions required

Extraction Buffer: 200 mM Tris, pH 7.4, 70 mM EDTA, 0.2% 2-mercaptoethanol, 2 M NaCl, 2% CTAB (hexadecyl trimethylammonium bromide), 1% PVP (polyvinylpyrrolidone)

TE buffer: 10mM Tris pH 8.0, 1 mM EDTA, pH 8.0

SDS Solution: 10% w/v

NaCl Solution: 5 M

DNA Extraction Protocol (Step by step)

Grind 1.0 gm of leaves in liquid nitrogen to fine powder. In case of fungus, harvest the mycelium of fungi grown on PDA medium, with the help of cover slip and grind with liquid nitrogen for DNA extraction.

Transfer the ground material in a small beaker containing 5 ml pre-warmed DNA extraction buffer. Mix it properly.

Add 500 µl of 10% SDS in case of leaves, 750 µl of 10% SDS in phytoplasma infected tissues and 250 µl of 10% SDS in fungal cultures. Mix it properly.

Transfer the mixture in a 20 ml centrifuge tube & incubate it at 65°C in water-bath (Memmert Model 200) for 30-60 min with gentle swirling.

Add equal volume of phenol: chloroform: Isoamyl alcohol (25:24:1). Mix gently to emulsify.

Centrifuge at 8000 rpm in high speed cold centrifuge (Biofuge Stratos) for 15 min at 10°C.

Transfer the aqueous phase containing DNA with a wide-bore pipette to an autoclaved new centrifuge tube.

Add equal volume of chloroform: Isoamyl alcohol (24:1). Mix gently to emulsify.

Centrifuge at 8000 rpm in high speed cold centrifuge (Biofuge Stratos) for 15 min at 10°C.

Transfer the aqueous phase containing DNA with a wide-bore pipette to an autoclaved new centrifuge tube.

Add 0.6 volume of cold isopropanol and mix gently to precipitate the DNA.

Spool out DNA with a glass hook if large strands of DNA appear. Otherwise centrifuge at 8000 rpm for 5 min.

Gently pour off the supernatant and wash the DNA pellet in chilled 70% ethanol for 20 min.

Centrifuge again at 8000 rpm for 10 min & discard the solution.

Dry the pellets and dissolve in 500 µl TE buffer. Here is your crude DNA sample. RNA, protein and polysaccharides are major constituents in crude DNA extraction. Degraded protein, polysaccharides and cell debris are removed during extraction of chloroform: isoamyl alcohol. The RNA is removed by treating the dissolved DNA with RNase in following steps.

Add 5 µl of boiled RNase (10 mg/ml) to crude DNA sample and incubate at 37°C for one hour.

Add equal volume of phenol: chloroform: Isoamyl alcohol (25:24:1) and mix gently.

Centrifuge at 8000 rpm for 15 min at 10°C. Pipette out aqueous phase in a fresh autoclaved centrifuge tube.

Extract again with chloroform: Isoamyl alcohol (24:1). Centrifuge at 8000 rpm and pipette out aqueous phase in fresh tube.

Add 5M NaCl to a final concentration of 0.2 M & equal volume of 100% chilled ethanol to precipitate pure DNA.

Centrifuge at 10,000 rpm for 5 min at 4°C. Rinse the DNA pellets with 70% v/v ethanol and allow air-drying up to 30 min.

Resuspend the pellets in TE buffer.

Checking quality and quantity of DNA

The purified DNA was visualized in 1 % agarose gel stained with ethidium bromide to check its purity and to estimate its quantity. The molecular weight of isolated DNA was ~20 Kb and uniform concentration of 5 ng/µl was standardized for the polymerase reaction. Aliquots of DNA in a uniform concentration of 5 ng/µl were prepared and DNA was kept at 4°C until further use and then stored at -20°C. DNA was kept at 4°C until use and then stored at -20°C.

PCR Amplification of genomic DNA

The genomic DNA (gDNA) templates of sugarcane, fungal pathogens viz. *Sporisorium*, *Colletotrichum*, *Fusarium* and endophytic fungus *Trichoderma* were amplified by PCR using universal oligonucleotides ITS 4 (5'-TCC TCC GCT TAT TGA TAT GC - 3') and ITS 5 (5'- GGA AGT AAA AGT CGT AAC AAG G - 3') (White *et al.*, 1990) to amplify nuclear rRNA gene fragment containing ITS1, ITS2 and the 5.8S rRNA gene. Oligonucleotide primers P4 (5'- GAA GTC TGC AAC TCG ACT TC- 3') and P7 (5'-CGT CCT TCA TCG GCT CTT- 3') were used to prime the amplification of 16S and 23S rDNA sequences from phytoplasma infected leaves. PCR amplification reactions were performed in a total volume of 50 µl. Each reaction mixture contained 10 to 100 ng of template DNA, 2 mM MgCl₂, 20 pmol of each primer, 200 mM of each dNTP, and 2 U of DNA polymerase (Thermo) in a reaction buffer containing 75 mM Tris HCl (pH 9.0), 50 mM KCl, 20 mM (NH₄)₂ SO₄, and 0.001% BSA. The polymerase chain reaction was

carried out in a MJ PTC 200 Thermal Cycler (BioRad, U.S.A.) programmed to perform a initial denaturation step of 95°C for 5 min, followed by 35 cycles consisting of 30 s at 95°C for denaturation, 30 s at 55°C for annealing, and 1 min at 72°C for extension with 10 min elongation at 72°C with final cooling at 4°C for 15 min. PCR products were loaded in a 2.0 % agarose gel containing 1 mg/ml ethidium bromide in TAE buffer.

Electrophoretic separation was performed in BioRad SubCell GT electrophoresis unit at 80 V for 30 min. The resulting DNA fragments were visualized by UV trans-illumination and analyzed using AlphaImager™ Gel Documentation System (Protein Simple, USA).

Clean-Up and sequencing of PCR Products

Desired PCR products were purified with Ultrafree DA Gel Extraction Kit, MILLIPORE, USA and got sequenced using an automated DNA sequencer ABI 3100. Annotated sequences were submitted to Genbank. A sequence homology search for each product was conducted through Internet Database programme BLASTn algorithm (www.embl.ac.uk).

Results and Discussion

Purified DNA has been obtained from sugarcane and its phytopathogens following this efficient and universal procedure. This method was further applied to some other crops to check its efficiency and universality, and was found suitable for linseed, rice, maize, *Sesbania*, *Hyoscyamus* and *Papaver* too. The ITS4 and ITS5 primer pairs were used to amplify the ribosomal region containing noncoding and 5.8S rRNA gene, in *Saccharum* hybrid cultivar CoLk 8102 and fungal isolates of diverse strains belonging to *C. falcatum* (Cf07) causing red rot disease, *S.*

scitamineum (cv. CoSe 92423) causing smut, *F. moniliforme* (Fm01) causing wilt, and *T. viride* (Tv01) - an endophytic fungi. The PCR products showed a single amplicon of ~600 bp in *Saccharum* spp. hybrid cv. CoLk 8102 (Figure 1a) & other fungi (Figure 1b). An amplicon of ~500 bp was obtained in grassy shoot disease (SCGS) affected leaves using primer pairs P4 & P7 which amplified 23S rRNA & spacer region of phytoplasma (Figure 1c). Desired PCR products were purified and sequenced using an automated DNA sequencer ABI 3100. Bioinformatics analysis conducted through BLASTn program to hit upon homology search representing reasonable E value and Score is given in Table 1.

Annotated sequences of *Saccharum* hybrid CoLk 8102, *Colletotrichum falcatum* pathotype Cf 07, *Fusarium moniliforme* isolate Fm 01, *Trichoderma viride* isolate Tv 01, *Sporisorium scitamineum* isolate CoSe 92423 and SCGS phytoplasma from var. CoLk 8102 were submitted directly to GenBank through WEBIN and SAKURA (a World Wide Web sequence submission servers available at EMBL and DDBJ). The sequences are available on line and can be located by accession numbers AJ880278, AB242410, AF333333, AT444444, AB246362 and AM085764.

Various plants contain high levels of polysaccharides and many types of secondary metabolites that affect DNA purification. Certain polysaccharides and secondary metabolites such as polyphenols, terpenoids and resins are known to inhibit PCR reactions (Pandey *et al.*, 1996). To overcome these problems, several methods have been employed. The current study is also a part on this line. The sugarcane plant tissues are in general a difficult material for DNA isolation due to the presence of various secondary plant products as polyphenols and polysaccharides.

Fig.1 Genomic DNA amplification of rDNA. (a) ITS region of *Saccharum* hybrid cultivar CoLk 8102, (b) ITS region of pathogenic and endophytic fungi, and (c) 16S-23S rRNA region of phytoplasma DNA in SCGS disease affected *Saccharum* hybrid cultivar CoLk 8102

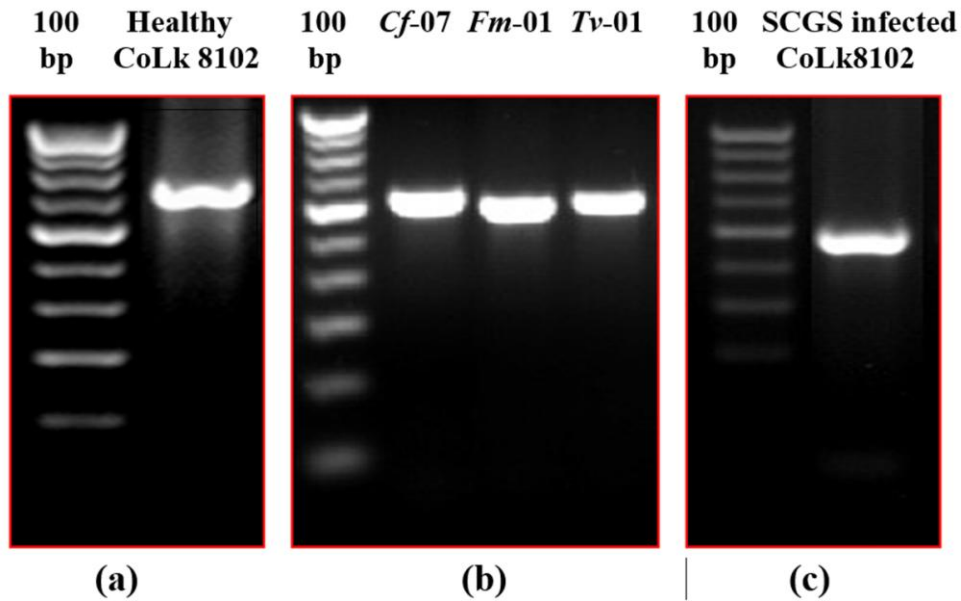


Table.1 Sequence homology search and comparison of rDNA sequences from sugarcane and its phytopathogens with GenBank database

		GenBank Accession no.	GenBank Accession no.	GenBank Accession no.	GenBank Accession no.
<i>Saccharum</i> spp. hybrid CoLk 8102 (AJ880278)		(AF345226)	(AF345223)	(AF345224)	(AF345239)
	Score	1112	1112	1112	1096
	E value	0.0	0.0	0.0	0.0
Red rot pathogen Cf 07 (AJ876759)		(AY944753)	(AY944748)	(AY944747)	(AY944743)
	Score	1031	1031	1031	1017
	E value	0.0	0.0	0.0	0.0
Wilt pathogen Fm 01 (AF333333)		(AF455450)	(AY533376)	(AF158314)	(AF158313)
	Score	1017	1017	1013	1013
	E value	0.0	0.0	0.0	0.0
Endophytic fungi Tv 01 (AT444444)		(AF510496)	(AB194281)	(DQ200258)	(AF176660)
	Score	1013	1009	1005	995
	E value	0.0	0.0	0.0	0.0
Smut pathogen <i>S. scitamineum</i> -92423 (AB246362)		(AY345007)	(DQ004831)	(DQ004829)	(AF135433)
	Score	301	272	258	256
	E value	5e-79	5e-70	7e-66	3e-65
SCGS Phytoplasma CoLk 8102 (AM085764)		(DQ380345)	(DQ380344)	(DQ380341)	(DQ380340)
	Score	361	361	361	361
	E value	1e-96	1e-96	1e-96	1e-96

The protocols available in literature were used for DNA extraction using reported methods, including those of Dellaporta *et al.*, 1983, Maroof *et al.*, 1984, Oard and Dronovalli 1992, Wang *et al.*, 1993. However, as the results of a single protocol were not suitable for host tissue as well as pathogens, we found it necessary to devise a single and simple cost effective protocol for DNA extraction from leaves of sugarcane and their phytopathogens. As the phytoplasma is present in hard midrib of sugarcane leaves, the protocol described here was relatively quick, inexpensive and effective for phytoplasma as well. The quality of DNA isolated by this method was evaluated by performing universal PCR-based applications. The yield of pure DNA obtained through current protocol ranged from 20-160 µg/gm of material with A²⁶⁰/A²⁸⁰ ratios in the range of 1.8 to 1.95 and the DNA is easily able to amplify as tested by rRNA region amplification and their sequencing. There is no visible contamination of RNA in electrophoresed gel. This method was evaluated for universality, speed, and reliability using PCR based applications. It was found suitable for both small and large scale extraction. RFLP analysis, cloning, creation of gene banks and various other techniques are also sensitive to DNA quality.

We have presented here a modified protocol of Doyle and Doyle 1990 as described above that has worked equally well in a number of plants including sugarcane, rice, maize, linseed, *Sesbania*, opium poppy, *Hyoscyamus* as well as phytoplasma infected sugarcane leaves, smut infected sugarcane whip and mycelial cultures of different strains belonging to red rot pathogen *C. falcatum*, wilt pathogen *F. moniliforme*, smut pathogen *S. scitamineum* and endophytic fungi *T. viride*. In short, this modified procedure is able to efficiently produce purified gDNA from sugarcane, its phytopathogens as well as diverse monocot and dicot plant species that

is suitable for PCR based applications as well as for sequencing purposes.

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