International Journal of Innovative Horticulture. 7(1):50-53, 2018 **Original Article**

DNA extraction method from endosperm of *Areca concinna* Thwaites, a wild relative of arecanut

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ABSTRACT

In this study, a genotyping technique utilizing endosperm DNA sampled from *Areca concinna* Thwaites was optimized, which can be utilized as an alternative to leaf DNA-based genotyping for genetic studies and subsequent breeding applications. Extraction of DNA in pure form is important to undertake DNA based marker studies and optimization of extraction protocol is even more significant in members of *Areca* spp. with higher polyphenolic content. Embryo was scooped out from ripened fruit of *A. concinna* and endosperm was ground to a fine powder. SDS-based protocol did not yield DNA whereas genomic DNA could be extracted from endosperm with the addition of cellulase and mannitol to the extraction buffer. Amount of extracted DNA was more in protocol with cellulase in comparison to mannitol. The purity of the extracted DNA was validated by successful amplification of *A. catechu*-specific microsatellite markers. The protocol standardized could be applied to other large seeded plants. Also, DNA extraction from seeds facilitates long-distance transfer of plant materials (in the form of seeds) in comparison to leaf samples, which can perish faster unless kept on ice or are lyophilized.

Keywords: *Areca catechu*, *Areca concinna*, endosperm, genomic DNA, extraction, SSR.

INTRODUCTION

The genus *Areca* comprises of 76 species (Murthy and Bavappa, 1960) with *A. catechu*, an important cash crop in the Western Ghats, eastern coastal region and North Eastern regions of India, being the only cultivated species. *Areca concinna* is endemic to the low lands which is a wild relative of *A. catechu* and presumed to be a native of Sri Lanka (Prabhakaran Nair, 2010). It is a medium sized clustering palm, with attractive, bright green leaves and grows well in woodlands, scrub jungles, and along to water streams of wetlands and intermediate zones. Seeds are used as a betel nut substitute however domestic cultivation is rare. It is also used as masticatory similar to arecanut (Murthy and Pillai, 1982). Today, it is gaining popularity in many countries as an ornamental plant because of its beautiful fragrant flowers and coloured nuts. *A. concinna* finds a place in the IUCN (International Union for Conservation of Nature) red list of threatened species as its habitat is threatened by the expansion of human habitation. Most plants sold as *A. concinna* are actually *A. triandra*, which is another wild relative of arecanut. The difference lies in the number of stamens. *A. triandra,* as the name implies, being triandrous, while *A. concinna* is hexandrous.

Many factors determine the choice of a DNA extraction protocol: the nature of samples, the quality/quantity of DNA required, the presence of natural inhibitory substances that might interfere with the extraction process and subsequent analysis (restriction digestion or PCR). Several protocols have been developed for isolating DNA with improved quality and quantity from plants with higher levels of secondary metabolites which complicates the extraction procedures (Sahu *et al.*, 2012). Seed-based DNA isolation methods are rare (Sun *et al.*, 2010) and its optimization would help replace leaf DNA-based genotyping for both genetic studies and breeding applications (Gao *et al.*, 2008). Also, seed samples could be transported internationally, without deterioration, in comparison to leaf samples, which have to be kept on ice or lyophilized (von Post *et al.*, 2003).

Endosperm, a reserve tissue which ensures nutrition for the developing embryo, originates from the fusion between a polar male gamete and two female gametophyte nuclei and results in the generation of the triploid endosperm nucleus (Wang *et al.*, 2008).

DNA extraction has been standardized in *A. catechu*, from leaf tissues (Rajesh *et al.*, 2007) and many

molecular marker studies have been undertaken (Bharath *et al.*, 2015; Rajesh *et al.*, 2016 a, b). *A. concinna* is generally free from pests and diseases, while *A. catechu* is highly susceptible. Challenge inoculation on harvested immature nuts of *A. concinna* showed their resistance to *Phytophthora meadii* (Prathibha *et al.*, 2015), which cause fruit rot or mahali in A, catechu, leading to severe yield losses. Hence, it forms an invaluable material for breeding pest and disease resistance into *A. catechu* through interspecific hybridization. Unfortunately, reports indicate higher abortion rate of embryos in inter-specific crosses between *A. concinna* and *A. catechu*. Embryo rescue is a form of *in vitro* culture techniques by which non-viable embryos could be turned to viable (Iyer and Subramanyam, 1971; Sharma *et al.*, 1996; Sage *et al.*, 2010). In such scenario, DNA from endosperm could be used for the genetic characterization. However extraction procedure DNA from the endosperm from *Areca* spp. has not been optimized. With this background, the present study was conducted to optimize DNA extraction procedure from endosperms of *A. concinna* and also to evaluate the quality of extracted DNA for PCR using *A. catechu* specific SSR markers.

MATERIALS AND METHODS

Ripened nuts of *A. concinna* were harvested from bearing palms at ICAR-CPCRI, Kasaragod, Kerala State. Calyx and husk was removed and embryo was scooped out carefully. The endosperm was grounded to fine powder in a mortar pestle using liquid nitrogen. Five modified extraction protocols were followed (Table 1) to isolate DNA from the endosperm along with normal SDS based method (N). Tris-EDTA extraction buffer was used in all the protocols. Protocol 1, 2 and 5 made use of cellulase (SIGMA) whereas mannitol (0.2 and 0.4 M) was added in protocol 3 and 4. Cellulase (1.5%) was incorporated to the sample in extraction buffer and â-mercaptoethanol and SDS were added after an incubation period of 30 minutes (protocol 1), 60 minutes (protocol 2) and four hours (protocol 5).

Genomic DNA was run in 1.2% agarose gel stained with ethidium bromide following the protocol of Sambrook *et al.* (1989) to check the quality and quantified by reading the absorbance at 260 nm and 280 nm in a spectrophotometer as described by Sambrook *et al.* (1989). *Areca catechu* SSR primers AC07 and AC30 (Hu *et al.*, 2009) were used to confirm the quality of extracted DNA from *A. concinna* endosperm.

RESULTS AND DISCUSSION

Six protocols were tested to extract DNA from endosperm of ripened nuts of *A. concinna*. All the protocols were utilized same extraction buffer whereas cellulase and mannitol as additives in the modified protocols. The results obtained indicate that DNA could not be extracted following regular protocol where finely grounded powder of endosperm treated with lysis buffer. On the other hand, all modified protocols resulted in isolation of genomic DNA from endosperm samples with varied outputs. Cellulase (1.5%) was used in Protocols 1, 2 and 5 while mannitol (0.2 and 0.4M) in Protocols 3 and 4. Results indicate that amount of DNA was more in protocols with cellulase as compared to that of mannitol (Fig. 1). Genomic DNA from solid endosperm was found to be

Table 1. Protocols followed to extract DNA from endosperm of ripened *Areca concinna* nuts, a wild relative of *Areca catechu*

Protocol 1	Protocol 2	Protocol 3	Protocol 4	Protocol 5
Sample (200 mg)	Sample (200 mg)	Sample (200 mg)	Sample (200 mg)	Sample (200 mg)
EB (1 ml)	EB (1 ml)	EB (1 ml)	EB (1 ml)	EB (1 ml)
Cellulase (1.5%)	Cellulase (1.5%)	β-me (4 µl)	β-me (4 µl)	Cellulase (1.5%)
After 30 minutes	After 60 minutes	SDS (100 µl)	SDS (100 µl)	After 4 hrs
β-me (4 µl)	β-me (4 µl)	Mannitol (0.2 M)	Mannitol (0.4 M)	β-me (4 µl)
SDS (100 µl)	SDS (100 µl)	Incubate	Incubate	SDS (100 µl)
Incubate	Incubate			Incubate
overnight in RT	overnight in RT	overnight in RT	overnight in RT	overnight in RT

800 µl of chloroform: isoamylalcohol (24:1) and shaken for 10 min.
Centrifuged at 10000 rpm for 20 mins
Upper layer was transferred into fresh Eppendorf tube
Ice cold ethanol was added
Centrifuged at 10000 rpm for 20 mins
Pellet was washed with 70% alcohol
Pellet was dried and dissolved in TE buffer (200 µl)
EB: Extraction buffer; β-me : β-mercaptoethanol

poor quality because of high levels of lipid and galactomannan contaminants (Jorgegil *et al.,* 2005).

Incorporation of cellulase (1.5%) in to the extraction buffer and incubating for 30 minutes prior to the addition of SDS and â-mercaptoethanol significantly improved the yield and quality of synthesized DNA. However increasing the incubation period above 30 minutes in cellulase did not enhance the content of isolated DNA. A high-throughput extraction method was reported in rice to isolate DNA from endosperm utilizing 1.5% amylase (Liang *et al.*, 2016). The addition of mannitol in the extraction buffer has been reported to enhance the efficiency of soil DNA extraction (Fathima *et al.*, 2011).

The lower yield of DNA from endosperm tissues in general, when compared to leaf tissues commonly used for plant DNA extraction, could be elucidated by particular tissue variations between the two such as the chemical/ structural composition (seeds, being reserve tissues, are rich in carbohydrates and starches, lipids, proteins and polyphenols), tissues size and number of cells, rate of cell division, concentration of extracellular DNA etc. (Ramos *et al.*, 2006). Another major factor could be the DNA losses in the endo-spermatic tissue during the course of reserve accumulation phase in the seeds. During formation of starch, cell organelles get ruptured and modifications of cellular contents occur, resulting in cell death and complete filling of the starch reserves. These factors lead to diminishment in the amount of DNA (McDonald *et al.*, 1994).

Since *A. concinna* is an orphan crop with respect to molecular markers, we have tried cross-transferability studies using SSR primers from *A. catechu*. In this study both SSR primers *viz.*, AC07 and AC30 gave good amplification in *A. concinna* DNA. AC07 produced bands of around 300 bp and AC30 gave amplification of around 210 bp (Fig. 2). Thus, we have confirmed that the extracted DNA is of good quality and amenable for PCR amplification and other downstream applications.

The improved extraction protocol, comprising of enzymatic digestion, found to be the efficient DNA extraction method in *A. concinna* endosperm, capable to provide PCR quality DNA with high yield. This protocol can be utilized in extracting genomic DNA from inter-specific areca hybrids and can also be applied to other large seeded nuts.

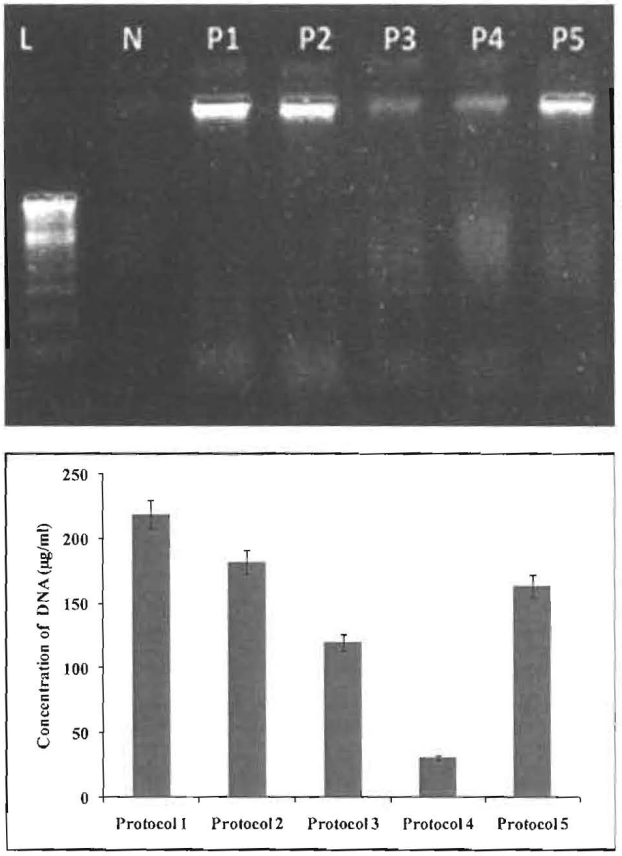

Fig. 1. Extraction of DNA from *A. concinna* endosperm. A: Gel image of the DNA extracted following normal protocol (N) and improved protocols (P1, P2, P3, P4 and P5). B: Concentration of DNA (µg/ml) collected following improved protocols (1, 2, 3, 4 and 5).

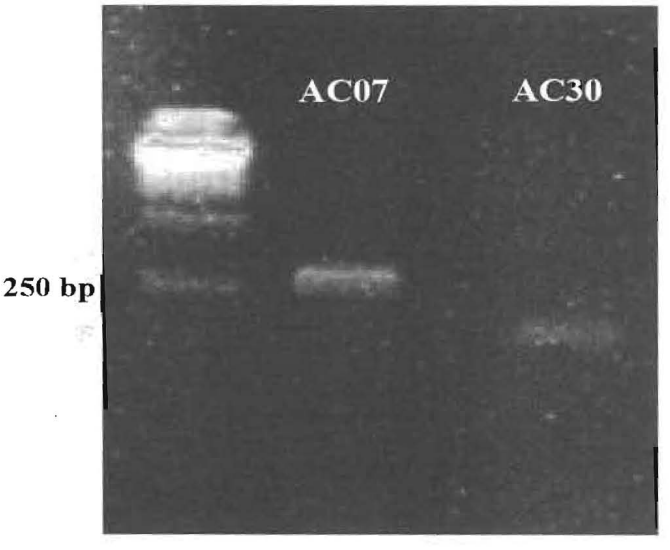

Fig. 2. PCR Amplification of *A. catechu* specific SSR primers in *A. concinna*. DNA extracted from ripened nuts. DNA extracted using Protocol 1 was employed in this study.

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