Short Communication

A simplified protocol for the recovery of high quality DNA from nutmeg

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Abstract

High levels of secondary metabolites even in the immature tissues leave the process of quality DNA isolation very difficult in the genus *Mysristica*. This difficulty is often characterized in the form of lower levels of DNA recovery and poor absorbance ratios due to RNA and protein contaminations, while analyzed in a spectrophotometer. The quantity and quality of DNA obtained through different methods were comparatively evaluated and the conditions for suitable recovery were optimized. A CTAB based simple and reliable method, standardized with necessary modifications on the protocol given by Doyle and Doyle was found to yield the best in terms of DNA quantity and quality. This method when compared with other protocols such as Rogers and Bendich and the Murray and Thompson, which are reported by the previous workers, was proven superior. The DNA recovery from the tender leaf tissues was high in the mean range of 83.55µg/g with a purity of 1.79. The standardized protocol was further experimented in other phenolic-rich plants such as cashew and cocoa and was found to yield high quality DNA.

Keywords: DNA isolation; Extraction; Genomics; Molecular markers; *Myristica fragrans*.

Nutmeg (family: Myristicaceae) is an important tree spice indigenous to Indonesia. The early detection of true female trees (Sheeja et al., 2006; Shibu et al., 2009), genetic diversity analysis, determination of population structure (Sheeja et al., 2013) and isolation and characterization of genes for specific characters are the major molecular biological challenges in this genus.. The foremost requirement in all these studies will be the availability of sufficient amount of quality DNA (Lele and Deshpande, 2011).

Since the biochemical composition of plant tissues vary with the species, DNA isolation protocols need to be optimized for each (Weising et al., 1995). Though the basic idea behind the DNA extraction is not very complicated, growing number of DNA isolation protocols for specific plant species suggest that the extraction procedures are not always simple and published protocols are not necessarily reproducible for all species (Porebski et al., 1997). This necessitates attempting various reported methods and to bring necessary modifications so that a suitable protocol may be arrived at. High percentage of polyphenols and other secondary metabolites in nutmeg make the DNA isolation difficult. The male plants are reported to have higher level of polyphenols compared to the females (Nybe et al., 2007). These metabolites negatively interfere with the downstream reactions such as DNA restriction, amplification and cloning (Bryant, 1997).

For the conduct of this experiment, third leaf from the shoot tip as suggested by Sudhamayee (2010) and emerging pale green leaves from six nutmeg

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trees were collected from the experimental field of the Department of Plantation Crops and Spices of College of Horticulture, Kerala Agriculture University. Samples separated from the mother trees were quickly wrapped in aluminium foils, transported to the laboratory in ice flasks and stored at 86 °C till being used for the extraction of DNA. DNA extraction using the procedures reported by Murray and Thompson (1980), Rogers and Bendich (1988), Doyle and Doyle (1987) and two commercially available kits GenElute™ Plant genomic DNA Miniprep kit (Sigma-Aldrich®) and HiPurA™ Plant DNA isolation Kit (CTAB method, HIMEDIA®) were performed and the results were compared. For performing the Rogers and Bendich method, 0.2 g of clean leaf tissue was ground with the extraction buffer concentration of 2X [2 % CTAB, 1.4 M NaCl, 20 mM EDTA and 100 mM Tris-HCl, pH 8.0] and 10 % CTAB and the protocol was followed. The Murray and Thompson method (10 % and 5 % CTAB levels) and the Doyle and Doyle methods were also followed as per the original protocols and using same amount of plant material at similar maturity level. The commercial kits were taken from the industries and directly used for the extraction by following the protocols supplied along with them.

Nutmeg is very rich in polyphenols that might influence the DNA extraction negatively. The general understanding is that with the maturity of leaves, the status of the phenols will also increase. To understand the most suitable maturity level of leaves to be used, the recovery from the third leaf from shoot tip was compared with that from pale green emerging leaves. Further, the DNA recovery is understood to be substantially influenced by the strategy adopted for its precipitation. The time requirement for the process varies with the species. While standardizing the protocol for *Myristica*, the recovery from precipitation through overnight incubation was compared with that with two hours incubation. Another factor influencing the DNA recovery is the volume with which the extraction procedure is executed. DNA recovery was compared among the 1.5 ml and 50 ml extraction tubes.

The quantity and quality of the extracted DNA were assessed using a spectrophotometer (Nanodrop ND-1000) at the absorption wavelength of 260 and 280 nm and the ratio of the corresponding absorbance values (A_{260}/A_{280}) was used to determine the protein and RNA contamination. In order to verify DNA integrity, 4 µl each of DNA was subjected to gel electrophoresis on 0.8% agarose gel, stained with ethidium bromide and visualized under a UV trans-illuminator. For confirmation of the results, the entire set of experiments was repeated four more times and the quantifiable data were pooled for further analysis and conclusions. The quantitative data were analyzed for descriptive statistics and the comparison among treatments were made using the ANOVA followed by Tukey's multiple comparison test at 0.05% significance level. Subsequent to the standardization of the protocol, the suitability of this protocol had to be experimented in the other phenolics-rich tress and for this, the cocoa and cashew were selected. The red and emerging tender leaves from these plants were collected and the protocol was attempted to prove the suitability of this protocol to be recommended as a general protocol for DNA extraction from phenolics- rich plant species.

DNA recovery in terms of quantity and quality, from the immature leaves of *Myristica fragrans* was found to be very much dependent on the methodology adopted in the extraction. The mean concentrations of DNA obtained using different methods are presented in Table 1.

The Rogers and Bendich method for DNA extraction is reported successful in many of the plant species (Choudhary et al., 2008; Matasyoh et al., 2008). This methodology when employed in *Myristica fragrans* using 2X CTAB extraction buffer resulted in 17.26 µg g^{-1} of DNA. Apart from the low yield, this procedure has not given a consistent result with the yields varying from 4.3 to 28.3 µg g^{-1} and the pellet obtained had a viscous

Table 1. Recovery and purity of DNA from the immature leaves of *Myristica fragrans* using different methods

Isolation method	DNA recovery (Mean±SD µg/ g of plant material)	Mean A_{260}/A_{280}	Remarks
Rogers and Bendich (2X with 10 % CTAB)	17.26±14.73[cd]	1.38	No consistent DNA recovery. DNA pellet viscous in nature and difficult to dissolve in distilled water. High protein contamination
Murray & Thompson (3X with 10 % CTAB)	18.52±5.33[cd]	0.92	Pellet was brown in color and was difficult to dissolve in distilled water. High protein contamination
Murray & Thompson (3X with 5 % CTAB)	11.96±3.18[d]	0.98	High protein contamination
Doyle and Doyle (Overnight Incubation in 1.5 ml tube)	24.85±4.67[c]	1.62	DNA yield was lower when compared to normal method
Doyle and Doyle (2hrs incubation in 1.5 ml tube)	83.55±19.60[a]	1.79	Higher concentration and intact DNA bands were obtained
Doyle and Doyle (Overnight incubation in 50 ml Okridge Tube)	60.82±12.19[b]	1.45	Higher settling of phenolic compounds makes phase separation difficult. High protein contamination
GenElute™ Sigma-Aldrich Plant genomic DNA Miniprep kit	0.00	0.00	No evidence of DNA
HiPurA™ HiMedia Plant DNA isolation Kit	0.00	0.00	No evidence of DNA

[a-d] Tukey's mean separation. Mean values represented by the similar letters do not differ significantly at 0.05%

nature. It was difficult to dissolve the pellet of DNA in sterile distilled water and had a high level of protein contamination. However, there was no visible RNA contamination. None of the previous researchers have experimented the suitability of this methodology in *Myristica* spp.

The Murray and Thompson method was reported to yield good quality DNA in *Myristica fragrans* (Sheeja et al., 2008; Sudhamayee, 2010). DNA recovery from the nutmeg leaves has been reported to be highly varying and it ranges from 25-175 μgg^{-1} (Sheeja et al., 2008) and 8-20 μgg^{-1} (Sudhamayee, 2010) and that from nutmeg seeds was 4-6 μgg^{-1} (Lele and Deshpande, 2011). The reported procedure when repeated in our laboratory has yielded brown coloured pellet which was very difficult to dissolve in water and had high protein contamination. The method yielded a concentration of only 18.52 μgg^{-1} and the higher levels of phenolics and proteins deprived its quality for molecular marker assays.

Many modified Doyle and Doyle DNA isolation protocols are reported in different plant species such as black pepper (Dhanya et al., 2007), *Castanea dentate* and *Vaccinium macrocarpon* (Stewart and Laura,1993). With many trials, we have modified this methodology for the species *Myristica fragrans* and the most satisfactory procedure is as follows.

Grind 0.2 g of fresh leaf tissue along with 1 ml extraction buffer, 50µl β-mercaptoethanol and a pinch of PVP in a mortar and pestle preheated to 60°C. The modifications recommended at this phase is to use PVP and β-mercaptoethanol additionally to increase the quality of pellet by degrading the phenolics. Subsequently incubate the ground material in a water bath at 60 °C for 30 minutes, with intermittent mixing. Add equal volume of Chloroform: Isoamyl alcohol (24:1) and centrifuge at 7200 rpm for 10 minutes at 25 °C. After centrifugation, transfer the aqueous phase on the top to a sterile 1.5 ml tube and add 2/3rd volume of ice cold isopropanol. Invert the tube gently to precipitate the DNA. Centrifuge the samples at 7200 rpm for 3 minutes at 25°C. Discard the supernatant and add 1ml of wash buffer (76% ethanol, 10 mM ammonium acetate) and keep for incubation at room temperature for 20 minutes. Again centrifuge at 7200 rpm for 10 minutes at 25 °C. Discard the supernatant carefully and dissolve the pellet in 30 µl of sterile distilled water (Fig. 1).

While working on this protocol, we have observed that the treatment of the pellet with wash buffer had removed the phenolic compounds completely and yielded a cream coloured pellet, which was dark before the wash. Due to the observed higher recovery in 1.5 ml tubes over the conventional 50 ml tubes, we recommend a scale down to 1.5 ml tubes. The same procedure gave a lower concentration (60.82 µg g^{-1}) and purity of DNA in 50ml tubes when compared with smaller tubes. When the samples were kept for overnight incubation after the addition of wash buffer, the DNA yield was found to be low (24.85 µg g^{-1}). This may be due to settlement of polyphenolic content during the incubation and hence we recommend a reduced incubation period during isolation.

The Doyle and Doyle method for DNA extraction was followed for mucilage rich *Abelmoschus esculentus* (Singh and Kumar, 2012), *Allium sativum*, *Catharanthus roseus*, *Mentha arvensis* etc. (Suman et al., 1999). The modifications involving the addition of PVP and β-mercaptoethanol are crucial to reduce DNA degradation by oxidized polyphenols formed during cell lysis. PVP acts as an adsorbent for poly phenol (John, 1992) and β–mercaptoethanol inhibit the oxidation of the same (Sa et al., 2011). This methodology has consistently yielded a high quantity of DNA up to 137.26 µg g^{-1} with an average of 83.55 µg g^{-1} . The capability to yield a high quality DNA was the most important advantage of this methodology. The mean value of absorbance ratio (A_{260}/A_{280}) was 1.79 against the recommended range of 1.80-2.00. On agarose gel electrophoresis, DNA was found to be intact with negligible RNA and no protein contamination.

Sheeja et al. (2008) have reported that the third leaf from the shoot tip is the best plant material for DNA extraction in *Myristica*. Our experiments have shown that DNA from the third leaf will be very much contaminated with polyphenols and proteins and emerging pale green leaves are best for the isolation, yielding a high quality of DNA with negligible protein and RNA contamination. The DNA isolated from the Rogers and Bendich and Murray and Thompson methods were only having a concentration in the range of 17.26 µg g^{-1} and 18.52 µg g^{-1} respectively. The isolations using the commercial kits were found to be ineffective to yield DNA.

This protocol was found to yield as much as 82.3 µg g^{-1} and 76.0 µg g^{-1} of good quality DNA with negligible RNA and protein contamination from the tender leaves of cashew and cocoa respectively. This clearly had shown that this protocol could be a general protocol for the phenolics-rich plant species. In the phenolics and other secondary metabolites rich tree spice nutmeg, the leaves were found to accumulate these secondary metabolites at a very early stage of development itself. This necessitates that the leaves selected for the DNA extraction should be the pale green and emerging ones, unlike the previous reports. The Rogers and Bendich as well as Murray and Thompson methods were

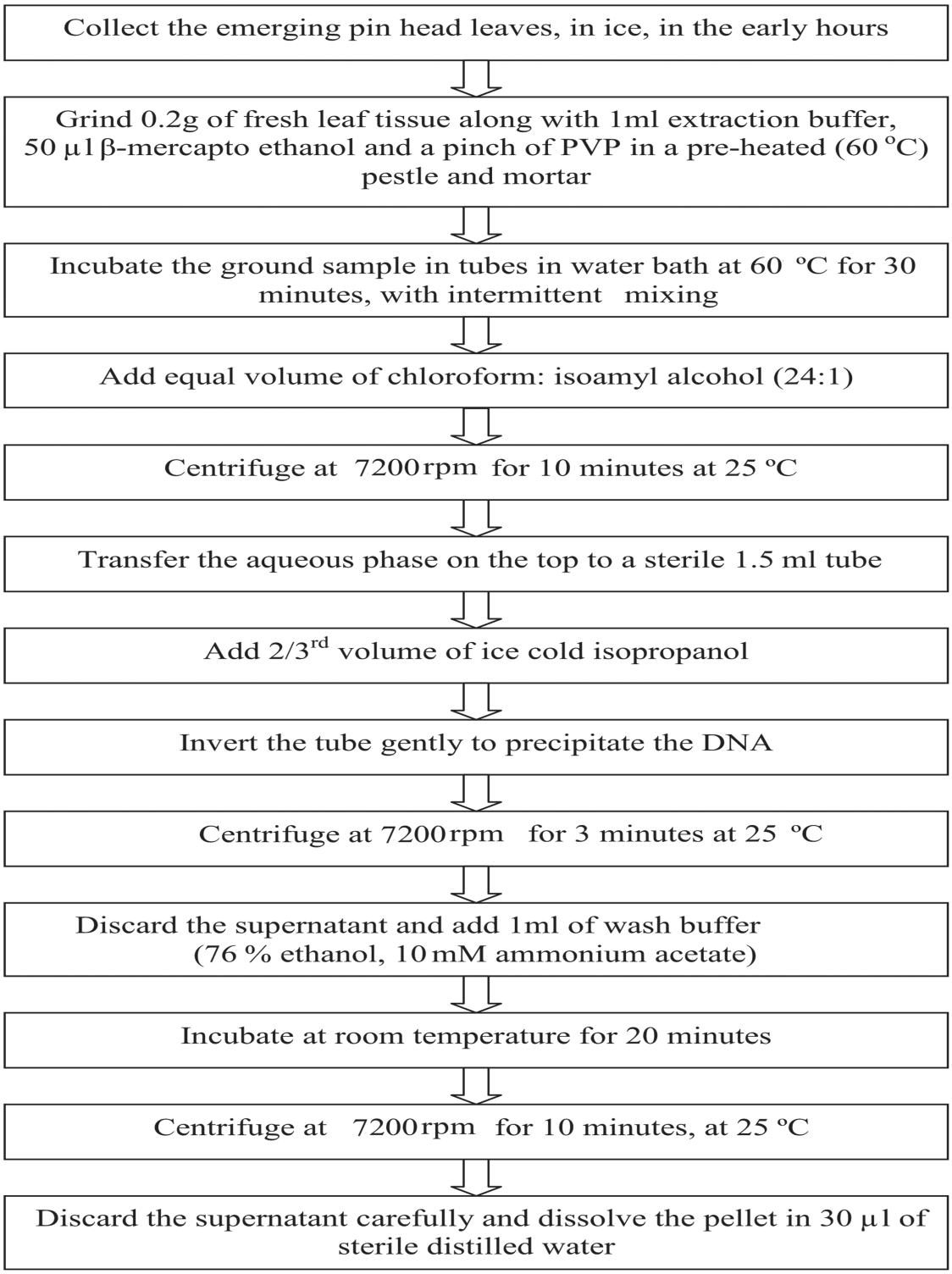

Fig. 1. The modified Doyle and Doyle protocol for the extraction of high quality DNA from nutmeg

inferior to the modified Doyle and Doyle CTAB method that we described in this paper. Due to the peculiar character of this species, the commercially available general purpose DNA extraction kits will not be generally useful. For maximum DNA recovery, the modified Doyle and Doyle protocol must be practiced in 1.5 ml tubes, using 2X extraction buffer, β–mercaptoethanol and PVP, with 2 hours incubation gave better quality and concentration of DNA in the range of 61.88 to 137.26 $\mu g g^{-1}$, which is far better when compared with the other protocols. Apart from these, this methodology has the added advantage of lesser time requirement of just one and half hours against the 2-3 hours demanded by others. Further, the non-requirement of liquid nitrogen makes the Doyle and Doyle protocol the best strategy in this genus. Experiments in cashew and cocoa had proven that this could be recommended as a general protocol for isolation of quality DNA from phenolics-rich plants.

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