Araştırma Makalesi/ Research Article

An Efficient DNA Isolation Method from *Nigella sativa* L. (Ranunculaceae) Seeds for RAPD and ISSR Analysis

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Abstract- ***Nigella sativa* L. (Ranunculaceae) seeds (black cumin) that contain high oil, terpene, protein, alkaloids are an economically important for medicine and food industry. Lack of an efficient DNA isolation procedure is a limiting factor for any molecular studies of these plant. We have used a genomic DNA isolation protocol for *Nigella sativa* seeds based on a hexadecyltrimethylammonium bromide (CTAB) method described for other plant species. The method is a modified CTAB extraction using high salt concentrations and polyvinyl pyrrolidone (PVP), and successive isoamyl alcohol chloroform extractions and involves mortar grinding of black seeds. Black seeds include little amount of DNA despite high amounts nutrients as polysaccharides and oils. Therefore, the yield was approx., 20 ng DNA per 150 mg of dry seed material. The genomic DNA obtained by this method was suitable to be used in inter-simple sequence repeat (ISSR) and random amplified-polymorphic DNA (RAPD) analyses. This extraction method should facilitate the molecular analysis as genotypic selection via use of dry seeds instead of germination of *N. sativa* seeds.**

Keywords: **CTAB, DNA Extraction, ISSR, *Nigella sativa* L., RAPD, Seeds.**

I. INTRODUCTION

Nigella sativa L. (Ranunculaceae), known commonly as black cumin, is a herbaceous plant and their seeds have been used traditionally in the Middle East, Northern Africa and India for the treatment of asthma, cough, bronchitis, headache, rheumatism, fever, influenza, eczema, as a diuretic, lactagogue and vermifuge. [1, 2, 3]. The black seeds of *N. sativa* contain 0.4–2.5% essential oil, 30-38% fixed oil, protein, alkaloids, saponin and some new flavonol triglycosides [1, 2, 4]. The essential oil of black seeds contains 54% thymoquinone and many monoterpenes such as p-cymene and a-pinene, dithymoquinone and thymohydroquinone [3]. The many studies as medicinal potential [3], antimicrobial potential [5], antifungal potential [6], protective effect [4, 7], antioxidant activity [1, 8], antiepileptogenic effect [8], acute and chronic toxicity [2], and effect against liver damage [9] of *N. sativa* seeds and especially their oil have been accomplished. Furthermore, there are some studies as neuropharmacological activity [10], cytotoxic and immunopotentiating [11] effects of ethanolic extracts of *N. sativa* seeds. The crude oil prepared from the seeds produce a variety of pharmacological actions such as antihistaminic, diuretic and antihypertensive, hypoglycemic, antioxytocic, antinociceptive, respiratory stimulation, hematological, hepatoprotective and immunopotentiating effects [3].

The presence of high amounts of polysaccharides and secondary metabolites such as alkaloids, flavonoids, phenols and terpenes in some plants and their seeds are caused problems in extraction of genomic DNA and the growing number of DNA isolation protocols for specific plant species suggests that extraction of DNA is not always simple [12, 13]. Many earlier studies discuss problems faced during genomic DNA isolation from medicinal plants [14]. Currently, some studies on N. sativa have been reported at molecular level, employing germinated seeds for DNA isolation [15]. However, DNA isolation directly from seeds would save time. In order to begin an extensive genotypic study using seeds, an improved DNA isolation methods and optimized polymerase chain reaction (PCR) conditions, for example, for randomly amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) analysis are necessary. In order to isolate quality DNA from N. sativa black seeds, we have used a standard CTAB technique described by Doyle and Doyle (1987) [16] with some modifications. We obtained DNA that was consistently amplifiable by PCR using the ISSR [17] and RAPD techniques [18].

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II. MATERIALS AND METHODS

A. Plant Material

In this study, *N. sativa* black seeds were used as plant material. Dry seeds were obtained from commercial seed vendor and directly used for DNA isolation.

B. DNA Isolation

Approximately 150 mg of dry seeds were ground in liquid nitrogen to a fine powder. Ground seeds were transferred to a 2 ml eppendorf tube and added about 900 µl freshly prepared and preheated (65^{o}C) extraction buffer [100 mM Tris-HCl (Tris [hydroxymethyl] Aminomethane-Hydrochloride), pH 8.0, 25 mM EDTA (Ethylenediaminetetraaceticacid), 1.4 mM NaCl (Sodium Chloride), 2% CTAB (w/v), 1% PVP (Polyvinylpyrrolidone) (w/v) and 0.4% β-mercaptoethanol added to buffer prior to use]. The tube was inverted several times and incubated at 62 ^{o}C for 1 h with regular inversion every 10 min. After incubation 900 µl chloroform/isoamyl alcohol (24:1, v/v) was added and mixed gently by inversions for 15 min. The tube was centrifuged at 5300 g for 10 min. The upper dusky aqueous layer was transferred to a new tube and 600 µl isopropanol (-20 ^{o}C) was added. The mixture was incubated at -20 ^{o}C for 2 h. The tube was centrifuged at 9300 g for 10 min. and the supernatant was discarded. 300 µl sterile dH_2O was added gently to the dusky pellet and incubated at room temperature for 30 min. After incubation 150 µl upper compartment was taken gently with micropipette and discarded. 150 µl remainder aqueous layer was taken gently with micropipette without to touch dusky pellet and transferred to new tube. 100 µl isopropanol (-20 ^{o}C) was added and incubated at -20 ^{o}C for 1 h. The tube was centrifuged at 9300 g for 10 min. The supernatant was discarded and the pellet was washed with 300 µl 70% ethanol (-20 ^{o}C). The pellet was air-dried and dissolved in 40 µl DNase RNase-free water. The quantity and purity of DNA sample were determined by using a Nanodrop® ND-1000 spectrophotometer (Wilmington, Delaware USA).

C. DNA Amplification

Amplification of RAPD fragments from genomic DNA was carried out in a total reaction volume of 25 µl containing 10 ng of genomic DNA, 1X *Taq* polymerase reaction buffer, 2 mM $MgCl_2$, 0.1 mM each of dNTPs (dATP, dCTP, dGTP, and dTTP), 0.4 mM primer (ACCGGCTTGT) and 1 U of *Taq* DNA polymerase (Fermentas, Maryland, USA). Amplification of ISSR fragments from genomic DNA was carried out in a total reaction volume of 25 µl containing 5 ng of genomic DNA, 1X reaction buffer, 1.5 mM $MgCl_2$, 0.1 mM each of dNTPs, 0.2 mM primer (AGCAGCAGCAGCAGCAGCC) and 1 U of *Taq* DNA polymerase. Amplifications were performed in a Progene thermocycler (Techne Inc., Burlington, USA). Thermal cycler programmed for RAPD as follows: 15 sec first denaturation at 85 °C, 5 sec second denaturation at 94 °C and 45 cycles of 1 min each denaturation at 94 °C, 1 min annealing at 32 °C and a 2 min extension at 72 °C, followed by a final extension at 72 °C for 7 min; for ISSR as follows: 4 min pre-denaturation at 94 °C and 45 cycles of 45 sec each denaturation at 94 °C, 45 sec annealing at 50 °C and a 1.5 min extension at 72 °C, followed by a final extension at 72 °C for 7 min. Amplification products were separated on 1.4% agarose gel containing ethidium bromide (0.5 µg/ml). Gels were visualized under UV light and digitally photographed with an UVIpro gel documentation system (UVItec, Cambridge, UK).

III. RESULTS

In this study, we obtained a sufficient yield of good quality DNA from *N. sativa* seeds, using the modified CTAB method. The standard CTAB technique described by Doyle and Doyle produced a dusky pellet, while the opaque DNA pellet was obtained by our modified method (Figure 1).

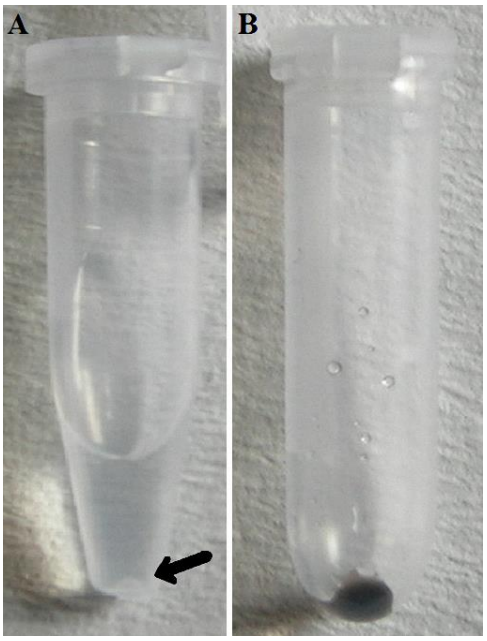

Figure 1. DNA pellets in eppendorf tubes. A: the opaque DNA pellet (black arrow) obtained by modified method, B: dusky pellet obtained by standard CTAB technique.

This modified method is very quick and inexpensive without germination process and provides quality genomic DNA from seeds. The method yields adequate amounts of DNA (14-20 ng/µl from 150-180 mg ground seeds) enough to conduct numerous PCR amplifications as RAPD and ISSR. The modified method was repeated three times. The DNA purity was confirmed with an A260/280 ratio of 1.70 – 1.72 for all samples tested (data not shown) and indicated that DNA samples were practically free from polysaccharides and other contaminants. DNA samples were run on 0.9% agarose gel to check their quality. A slim and single band was observed without any signs of DNA degradation (Figure 2).

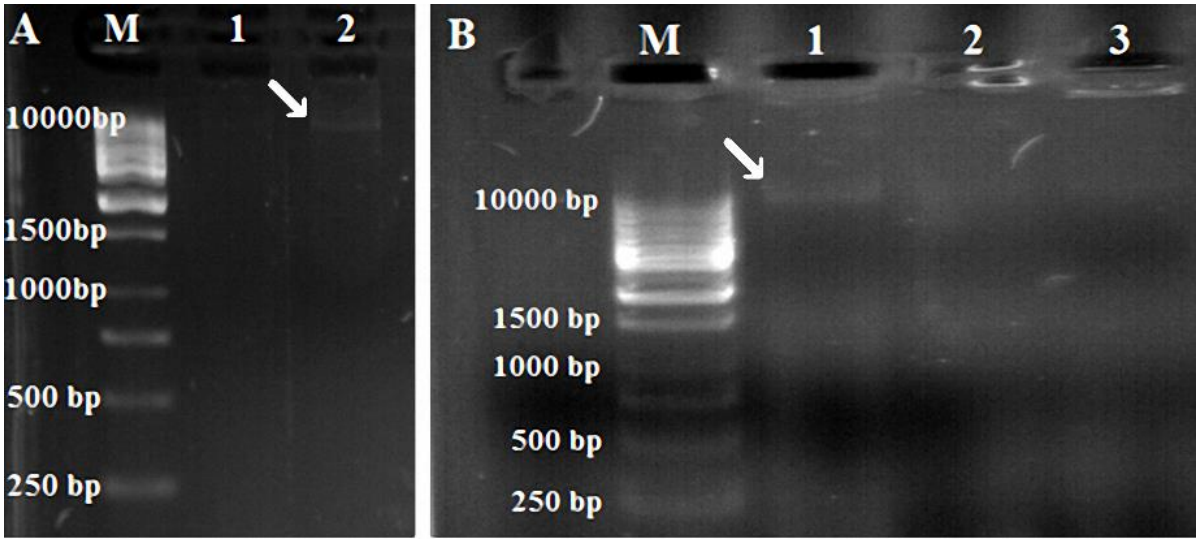

Figure 2. Electrophoretic analyses of total DNA samples isolated from *N. sativa* seeds by the CTAB method. A- Lane M: 1 kb DNA ladder (Fermentas), lane 1: 5 µl DNA sample, lane 2: 10 µl DNA sample; B- Lane M: 1 kb DNA ladder, lane 1: 10 µl DNA sample, lane 2: 5 µl DNA sample, lane 3: 2.5 µl DNA sample (white arrows show DNA bands).

In addition, DNA quality was checked with PCR using RAPD and ISSR primers. The results showed the distinct amplification of genomic DNA at the molecular weight range of 250 bp to 2.5 kb (Figure 3).

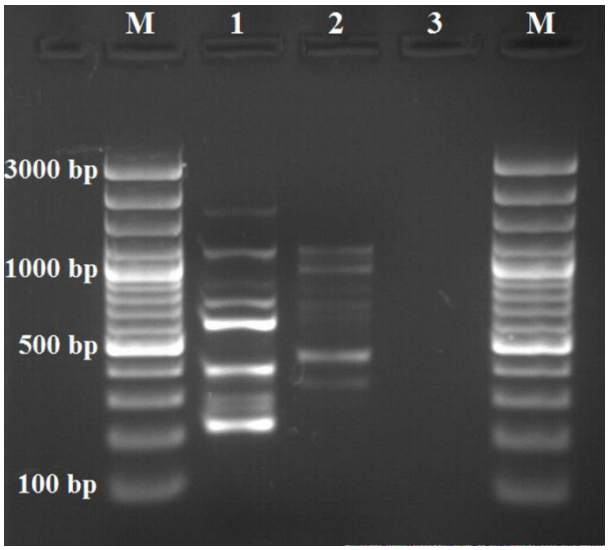

Figure 3. Representative gel fractionation of ISSR and RAPD-PCR amplification products of *N. sativa* genomic DNA. Amplification products were fractionated in 1.4% agarose gel. Lane M: molecular marker (100 bp plus DNA ladder, Fermentas), lane 1: ISSR products amplified using ISSR primer, lane 2: RAPD-PCR products amplified using RAPD primer, lane 3: negative control.

IV. DISCUSSION

Molecular analyses used PCR applications as RAPD and ISSR require isolation of quality genomic DNA. Isolation of DNA from fresh leaves may not be possible for every plant species, especially for those difficult to access in the field and possessing germination problems. DNA isolation from seeds without germination provides a time-saving for plant molecular studies. Therefore, optimization of DNA extraction procedure from seeds brings an important advantage for many researchers especially those working with important agricultural plants.

The presence of certain metabolites has been observed to interfere with DNA isolation procedures and downstream reactions such as DNA restriction, amplification and cloning [14]. The *N. sativa* seeds are very rich and diverse in chemical composition. They contain amino acids, proteins, carbohydrates, fixed and volatile oils [3, 19]. Therefore, DNA isolation from *N. sativa* seeds is complicated. Polysaccharides are also important contaminants in DNA samples and interfere with the PCR by inhibiting *Taq* polymerase activity [20]. The DNA isolated from various plant species by using the original CTAB method of Doyle and Doyle (1987) [16] is usually found to be contaminated with high levels of proteins and polysaccharides [21, 22, 23]. Therefore, after isopropanol precipitation stage we used an extra step to overcome this problem. A DNA extraction method was developed by Yu et al. (2010) [24] for peanut seed and the concentration of the DNAs was estimated at 0.3-0.5 ng/µl from only 3-5 mg cotyledonary tissue and reported that the amount of DNA was enough for more than 50 PCR reactions. Similarly, our method yields adequate amounts of DNA for RAPD and ISSR-PCR amplifications. The RAPD and ISSR-PCR are very important and popular techniques used in DNA polymorphism, genotype identification, genetic diversity and phylogenetic analyses for various organisms [25-28].

In conclusion, although the CTAB DNA isolation procedure [16] is simple and efficient, there is need for some modifications to isolate DNA free from materials including high levels of proteins and polysaccharides, especially when using seeds plant material. An advisable DNA isolation method for *N. sativa* yields adequate amount of DNA suitable for PCR without any ultra centrifugation or column purification steps. This modified method, especially for PCR amplifications, can be practiced with seeds of diverse medicinal and aromatic plants, which include proteins, polysaccharides, essential oils and secondary metabolites.

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