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Research Note

Use of RAPD marker to confirm mutation in morphological variants on Neem tree

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Abstract:
Neem (*Azadirachta indica* A. Juss.) is commercially valuable medicinal plant studied for molecular analysis. In the present study, a morphological variant of neem was observed near our department. The leaf pattern was abnormal (crimpled or curly) morphologically observed and was against normal leaves. This curly leaves containing neem tree considered as mutant against normal. It was considered as either environmental/ chemical influence or certain variation that might lead to mutation in plant genome. To confirm mutation, in this experimentation, this morphological analysis was confirmed by molecular analysis. For that, the genomic DNA was extracted from both the plants and subjected to RAPD analysis. The morphological variants were shown distinct variation in DNA pattern by selected primers. Thus, RAPD profile proves that there was mutation in plant genome. This result supports the initiative to utilize morphological variants in plant breeding applications, and DNA fingerprinting.

Key words: Neem, mutation analysis, RAPD marker

Neem (*Azadirachta indica* A. Juss.), a member of Maliaceae, is one of the most beneficial plant species for the mankind as a source of medicines and biopesticides. The Medicinal properties of Neem have been known to Indians since long time immemorial (Biswas and Gupta, 2007). It has been used against a wide variety of disease which includes heat-rush, Boils, Wounds, Jaundice, Skin disorders, Chiken pox, Small pox, Malaria, etc (Chiranjib et al, 2010). Dried Neem leaves are burned as Mosquito repellents. Neem tree is not only medicinally useful but it is also commercially useful. The different parts of Neem tree are being used extensively in manufacturing of soaps, skin creams/ lotions, shampoo, toothpastes and beauty products (Biswas *et al*., 2002).

Genetic diversity has been created at inter- and intraspecific levels in a plant germplasm (Stebbins, 1957), that due to environmental influence, seasonal variation or genotoxical agents. Physical and chemical mutagens could effectively cause mutation (Dhakshanamoorthy and Selvaraj, 2010). The types of DNA damages such as protein cross-links, DNA strand breaks, deletion or insertion of base pairs can lead to DNA mutation (Hollosy, 2002). Information on genetic diversity is valued for the management of germplasm and plant breeding. Therefore, there is need to adopt marker assisted selection for early selection and recognition of desired types in tree species. DNA markers can be applicable for the detection of DNA alterations (Kumar *et al., 2009*). Different types of marker systems have been used for genetic analysis and characterization studies. These include morphological, cytological, biochemical and DNA marker systems (Mandaliya *et al.*, 2010c).

DNA based various markers, including restriction fragment length polymorphism (RFLP), Randomly amplified polymorphic DNA (RAPD), Amplified fragment length polymorphism (AFLP), and Simple Sequence repeats (ISSR) have been utilized to determine taxonomic identity (Nakum *et al*., 2011), to estimate genetic diversity and draw evolutionary histories (Ramanuj et al, 2010). RAPD markers have been used to characterize genetic diversity in a number of plants due to its simplicity and efficiency (Williams *et al.*, 1990; Vieira *et al.*, 2003). RAPD markers have been used for mutation analysis to detect DNA polymorphism in plant species (Dhakshanamoorthy and Selvaraj, 2010).

Neem is believed to be highly cross-pollinated. Inter-provenance variations have been reported in

neem in case of morphological and physiological characters. In order to have an idea about the extent and/or nature of genetic (DNA) variation in neem, the powerful molecular technique need to be employed. Molecular techniques like AFLP, RAPD, ISSR and RFLP banding patterns are recently being used to assess neem ecotypes for genetic diversity 8–10 (Kota et al, 2006). The RAPD approach was reported by Ranade and Farooqui (2002) to score genetic variations in Neem. Currently mutation analysis in this species is very limited. Hence, the present study was emphasized on mutation analysis studies on Neem. A variant of Neem (Fig. 1) was observed near our department. The leaf pattern was abnormal (crimpled or curly) phenotypically from normal. This curly leaves containing Neem tree considered as mutant against normal, and both were considered as morphological variants of neem in this study. It was considered as either environmental/chemical influence or certain variation that might lead to mutation in plant genome. To confirm mutation, in the present study, the genomic DNA was extracted from both the plants and subjected to RAPD analysis.

Neem (*Azadirachta indica* A. Juss.) morphological variants were collected from Saurashtra University Campus, Rajkot (Fig. 1). Fresh, young, and expanded leaves were collected for DNA extraction.

Genomic DNA extraction was carried out following the protocol reported by Mandaliya *et al.*, (2010a) with some modification for high yield and quality DNA extraction, this method was termed as Method-1. In brief, in initial step 0.5 g of leaves was grinded, instead of two g, in liquid nitrogen along with doubled the amount of polyvinyl pyrrolidone polymer (PVPP), each normal and mutant in four replicates. Eight ml of freshly prepared and pre-heated Triton-X-100 extraction buffer as reported by Mandaliya et al, (2010a) were added and mixed well. The tubes were incubated at 65 ^{o}C in water bath for 30 min rather than 10 min. Subsequent DNA extraction steps were carried out according to Mandaliya *et al.*, (2010a) except RNase treatment which was not given. Thereafter, some modification was done in Method-1 like Proteinase K treatment (5 □ l; stock conc. 1 mg/ml) and RNase treatment (2.5 □ l; stock conc. 10 mg/ml) given before PC, CI step to improve the DNA quality, and this method was termed as Method-2, and plant material was taken in single replicate. DNA purity was quantified according to Matasyoh *et al.*, (2008) using the Quant micro plate reader, Bio-Tek instruments incorporation, USA.

PCR amplification

The extracted DNA was subjected to RAPD analysis using selected primer enlisted in Table 1. RAPD analysis was performed according Mandaliya et al, (2010b). PCR amplification was carried out in a Veriti (96 Well Fast Thermal Cycler), Applied Biosystems, USA. Electrophoresis was carried out on 2 % agarose gel at constant voltage of 50 V in 1x TAE buffer.
The genetic variation found in the wild progenitors of plant species is very important. A molecular marker in genetic assessment provides opportunities for elucidation of genomic variation. Random amplified polymorphic DNA (RAPD) is a polymerase chain reaction (PCR) based marker technique that able to detect different types of DNA mutation in plant DNA amplification profiles (Oksanaand Sorschink, 2005). The aim of present study was to investigate the possibility of mutation changes in plant DNA using this technique.

To facilitates the molecular marker techniques high quality DNA extraction procedure is prerequisites. Most procedures for DNA isolation and purification from plant species containing high levels of secondary metabolites are modified versions of the standard protocols. Average DNA purity ratio A260/A280 was 1.23 and 1.19; and A260/230 was 1.09 and 1.02, respectively of normal and mutant variants in first method, while it has improved to 1.71 and 1.41, and 1.70 and 1.27 respective ratios in respective both the variants with modified method. Average DNA concentration was 91.33 and 91.75 □ g/gm fresh weights respectively of normal and mutant variants in first method, while it has varied to 81.93 and 76.83 □ g/gm fresh weight in respective variants with modified method. There were decrease in DNA concentration in both variants but the DNA purity was increased. The obtained DNA had a very low content of proteins. So, the DNA extraction method was optimized that yields polysaccharide and polyphenol-free high quality genomic DNA from neem plant. Genomic DNA obtained from Method-2 was the best suited for DNA amplification by using RAPD markers.

Kota *et al.*, (2006) had observed differential expression of one of the secondary metabolites i.e. Azadirachtin A, in neem and they had screened the neem with biochemical analysis. Further, the biochemical analysis was supported by molecular analysis. In his experimentation, he had observed that investigation involved molecular analysis using DNA marker studies on genomic DNA extracted from tender leaves obtained from high and low Azadirachtin A yielding neem trees, the DNA polymorphism observed between neem samples had suggested a genetic variation. The same analysis was reported by Singh *et al.*, (1999) in their studies.

The present study was designed based on the aforesaid investigation. Kota *et al.*, (2006) had emphasized first on biochemical analysis and finally confirmed by molecular analysis, while in this study, first morphological analysis were observed and confirmed by molecular analysis. In the present molecular analysis, total seventeen RAPD primers were selected which had amplified total 84 bands in them (Fig. 2a and Fig. 2b). The primer P6 and P7 both gave 12 scorable bands and P1 gave 10 scorable bands (Fig. 2a). Among the selected seventeen primers, fourteen primers had generated RAPD amplicons. The total no. of 44 amplicons was generated. The amplicons ranged between 100 bp and 3.5 kb. The morphological variants were distinguished by four primers namely P8, P9, P10 and P14 (Fig. 2a and Fig. 2b). The amplification by P8, P10 and P14 shown that one band was absent in normal and present in mutant, while in contrast P9 showed that one band was present in normal and absent in mutant. Two significant findings were noted: (i) A distinct variation in DNA pattern was observed between two morphological variants of neem trees. (ii) Furthermore, DNA amplification patterns using RAPD primers showed polymorphism within DNA from morphological variants.

Mutation studies using RAPD primers have been used to examine DNA polymorphism within selected samples (Dhakshanamoorthy and Selvaraj, 2010), to analyze particular genotypes, for cultivar identification and to study the clonal structure of several tree species (Kumar, 2009). RAPD analysis is generally considered a very good starting point for studies of species (Williams et al, 1990), has proved true in our present investigation also.
Thus, the results show that modified method was optimal for DNA isolation from morphological variants of Neem. The DNA extracted by this method was suitable for molecular analysis. RAPD profile has shown DNA polymorphism among both the variants, which proves that there were either environmental/chemical influence or certain variation that lead to mutation in plant genome. For further mutation analysis, it is necessary to add more number of primers and screen the other parameters to discriminate both the variants.

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Table 1. List of selected primers.

Sr. No.	Primer	Sequence 5' to 3'
P1	OPB-01	GTTTCGCTCC
P2	OPB-02	TGATCCCTGG
P3	OPB-03	CATCCCCCTG
P4	OPB-04	GGACTGGAGT
P5	OPB-05	TGCGCCCTTC
P6	OPB-06	TGCTCTGCCC
P7	OPB-07	GGTGACGCAG
P8	OPB-08	GTCCACACGG
P9	OPB-09	TGGGGGACTC
P10	OPB-11	GTAGACCCGT
P11	OPB-12	CCTTGACGCA
P12	OPB-13	TTCCCCCGCT
P13	OPB-14	TCCGCTCTGG
P14	OPB-15	GGAGGGTGTT
P15	OPB-16	TTTGCCCGGA
P16	OPB-17	AGGGAACGAG
P17	OPB-18	CCACAGCAGT

Figure 1. Two morphological variants (a. normal and b. mutant) of Neem.

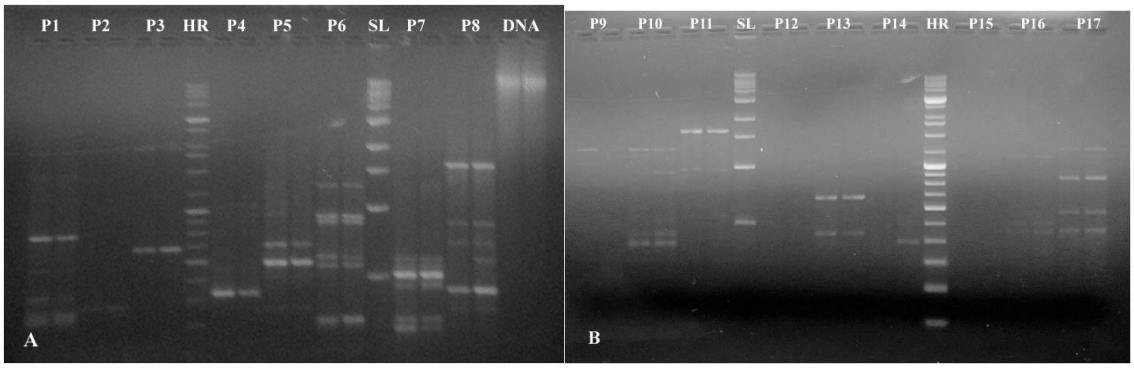

Figure 2. RAPD amplification profile and genomic DNA of two morphological variants (normal and mutant) of Neem (*Azadirachta indica* A. Juss.). In each lane, from left to right, first was normal and second was mutant. [P1 to P17 were primer enlisted in Table 1 used for RAPD amplification, HR – High Range DNA Ruller, SL – Supermix DNA ladder, DNA was extracted genomic DNA].