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OPTIMIZATION OF DNA ISOLATION AND ANALYSIS OF GENETIC VARIABILITY IN MEDICINAL PLANT *CISSUS* BY RAPD-PCR TECHNIQUE

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ABSTRACT

Cissus quadrangularis is the plant of *Vitaceae* family and reported to have widely applicable medicinal properties. The present study was carried out to optimize the method of genomic DNA extraction from *Cissus quadrangularis* and to determine genetic variability among four different accessions of *Cissus*. For DNA extraction, five Different procedures were tested, among which, the protocol of modified Doyle and Doyle (1990) gave good yield as well as consistent RAPD profiles with the random primer, and hence used for all further studies. The genetic variability among accessions of *Cissus* was determined using randomly amplified polymorphic DNA (RAPD) profiles. Similarities of profiles were determined using the algorithm of *Jaccard*, and UPGMA and neighbor joining trees were generated from the similarity data. Twelve RAPD primers produced a total of 79 bands, with an average of bands per primer pair, of which 62 were polymorphic. Similarity, index between all the accessions is more than 0.50, yet UPGMA cluster analyses of the SM matrix confidently separate four accessions. Optimized methods for DNA extraction and RAPD analysis for *Cissus* can be helpful in further molecular as well as genomic studies.

KEYWORDS: *Cissus,* CTAB Protocol, DNA Isolation, Optimization, RAPD, UPGMA

INTRODUCTION

Cissus quadrangle is a genus of widely distributed woody vines in the grape family (*Vitaceae*). It has been reported to have various medicinal properties. Extracts of various parts of plant in different solvents are having antioxidant and antimicrobial, antiosteoporotic (Potu, et al., 2009; Potu, et al., 2010), anti-ulcer(Jainu, et al., 2010; Rao et al., 2007), analgesic and anti-inflammatory(Srisook, et al., 2011), cancer suppressive(Vijayalakshmi, et al., 2013) and bone healing properties(Brahmkshatriya, et al., 2015). Research has been done to produce silver and gold nanoparticles from *Cissus quadrangularis* extracts, which possess antibacterial activities (Bhuvanasree, et al., 2013; Gopinath, et al., 2012). In addition to medicinal importance, research is also going on towards the use of *Cissus* at commercial level, as natural cellulose fibers has been produced from root and stem of *Cissus*(Indran, et al., 2014; Indran & Raj, 2015). Any plant having such pharmacological as well as commercial importance needs a detailed study at genetic level for the better understanding of the gene functions.

There are some accessions of *Cissus* found in Gujarat having many similarities with the morphological variability that is in the shape of stem. The study of genetic diversity among the species lying under same genus is required to determine the genetic distance and phylogenetic relationship. Molecular techniques have been found to be more useful and accurate for determination of both interspecies and intraspecies genetic variation in plants. Randomly amplified polymorphic DNA (RAPD) markers, in particular, have been successfully employed for determination of intraspecies

genetic diversity in several plants. These include *Cissus*(Jain, et al., 2015), date palm (Srivashtav et al., 2013), papaya(Madarbokus & Ranghoo-Sanmukhiya, 2012), amaranths(Štefúnová, et al., 2015) etc.

Genetic analysis of plants relies on high yields of pure DNA samples. Different plant species often may not allow optimal DNA production from one extraction protocol. So, for each plant species, an efficient protocol for extraction of DNA as well as the optimization of the PCR conditions is required. Many protocols for DNA extraction from plant cells have been reported.(Dellaporta, et al., 1983; Doyle, 1990; Ghaffariyan, et al., 2012; Sharma, et al., 2010) However, due to presence of high amount of different secondary metabolites such as essential oils, flavonoids, alkaloids, terpenoids, ketosteroids etc., it is difficult to extract and purify better quality DNA from plant tissues of *Cissus*. In order to overcome these problems we optimize the DNA isolation and Polymerase chain reaction (PCR) conditions for Random Amplified Polymorphic DNA (RAPD) analysis of four different *Cissus* accessions found in Gujarat.

In the present work, DNA extraction method was optimized using five different protocols and genetic variation as well as genetic distance among various accessions of *Cissus quadrangularis* were determined by using RAPD markers and phylogenetic analysis.

MATERIALS AND METHODS

Plant Material

For the DNA extraction, plant samples of four accessions of *Cissus* including *Cissus quadrangularis* and one plant of bougainvillea as an out group were collected from the Bapalal Vaidya botanical garden, Veer Narmad South Gujarat University in Surat, Gujarat. Four accessions in the species were identified based on stem morphological variations and those were Square winged stem, Round smooth stem, quadrangular flat stem, and two winged flattened stem mentioned as accessions 1, 2, 3 and 4. For the DNA extraction, fresh stems of collecting plants were used after washing thoroughly for the removal of any source of foreign DNA.

DNA Extraction Protocols Tested

Five published DNA Extraction protocols viz. Modified CTAB extraction protocol (Doyle, 1990), Doyle and Doyle, as a “classical” protocol(Doyle J. J. & Doyle, J. L., 1987), Oard & Dronovalli plant genomic DNA extraction protocol(Oard & Dronavalli, 1992), SDS method for DNA extraction(De Kochko & Hamon, 1990) and Modified CTAB method with difference in incubation period (Rogstad, 1992) were compared for their ability to produce good quality DNA from fresh tissues of the selected plants.

Modified CTAB extraction protocol (Doyle, 1990) used the CTAB buffer with additional sodium thiosulphate. Doyle and Doyle, as a “classical” protocol(Doyle J. J. & Doyle J. L., 1987) was the classical protocol with use of CTAB buffer. Oard & Dronovalli plant genomic DNA extraction protocol(Oard & Dronavalli, 1992) was the method which used no detergent for lysis. SDS method for DNA extraction(De Kochko & Hamon, 1990) used SDS buffer as a detergent for lysis and Modified CTAB method by Rogsted(Rogstad, 1992) used simple CTAB buffer for extraction but differ from Doyle and Doyle protocol(Doyle J. J. & Doyle J. L., 1987) there is longer incubation period of about 6 hours to overnight and the use of PVP (Polyvinyl Pyrolydone).

Extracted DNA samples were visualized on 0.8% agarose gel.

Agarose Gel Electrophoresis

Amplification products were separated by electrophoresis at a constant current of 5A through 0.8 % Agarose gels in 0.5 × TBE buffer according to Sambrook et al.(Sambrook, et al., 1989), visualized and imaged after staining with ethidium bromide.

Polymerization Chain Reaction Using RAPD Primers

Twelve Decamers were used as primers. DNA was amplified essentially following Williams et al.(Williams, et al., 1990). Initially, pilot experiment was carried out varying primer, template DNA and Mg^{2+} ion concentrations. The final amplification reaction mixture contained 10 mM N-tris(hydroxymethyl)methyl-3-amino-propanesulfonic acid (TAPS) (pH 8.8), 3 mM $MgCl_2$, 0.2 mM dNTPs (Bangalore Genei), 10 pmoles primers, 0.6 U Taq DNA polymerase (Bangalore Genei) and 2 µl *Cissus* DNA template in 15 µl reaction volume. Amplification of the DNA was performed in a thermocycler (Eppendrof) in following manner: initial denaturation at 96˚C for 180 seconds, followed by denaturation at 94˚C for 45 seconds, primer annealing at 35˚C for 45 seconds and extension at 72˚C for 1 min with a final extension for 5 min at 72˚C.

Final PCR products were fractionated on 0.8% agarose gel using 1×TAE buffer containing 10mg/ml ethidium bromide and were visualized under UV light. The gel was photographed using the UV gel documentation system.

DATA ANALYSIS

Data (Fragment sizes of all the amplification products, estimated from the gel by comparison with standard molecular mass marker) were scored as discrete variables, using ″1″ to indicate presence and ″0″ to indicate absence of a band. From the band data, monomorphic and polymorphic bands were identified for each accession. Jaccard's similarity matrix was used to calculate genetic similarity. From the pairwise similarity data, the UPGMA phylogram was generated.

RESULTS

DNA extraction protocols gave different results for the amount of DNA obtained and its purity. Among all the five methods used in this study, modified Doyle and Doyle methods showed the best results in *Cissus*. The quantity and quality of DNA extracted by this procedure were higher than other methods (Figure 1).

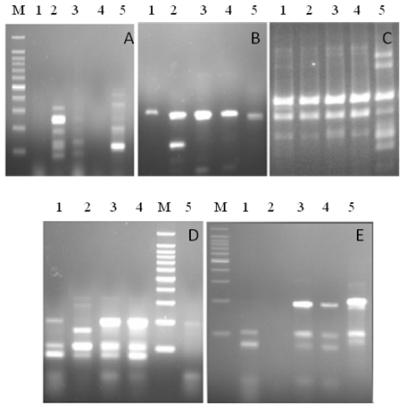

Figure 1: The Gel Images Show the Profile for Cissus Accessions using Primer (A) RPL-5, (B) RPL-6 (C) RPL-9. (D) APS-1 AND (E) APS-3. The Reactions were Resolved on 1.2% Agarose Gel in TAE (1X). Lane M has 200 bp DNA Ladder used as Molecular Weight Marker

The entire set of *Cissus* DNAs extracted from selected method were tested in RAPD reactions triplicate. All the DNAs were tested with fifteen RAPD Decamer primers. Of these, 12 primers (Table 1) resulted in a reproducible RAPD profile in all the four *Cissus* accessions (Figure1). The data from the twelve primers considered cumulatively resulted in a matrix of 79 distinct bands, of these 17 were polymorphic which revealed 21.51% polymorphism.

Table 1: Details of RAPD Markers used for Molecular Characterization of Cissus Accessions

Primer Name	Primer Sequence (5' – 3')	Annealing Temp.(^{o}C)
RPL-2	AACGCGTCGG	35
RPL-3	AAGCGACCTG	35
RPL-5	AATCGGGCTG	35
RPL-6	ACACACGCTG	35
RPL-7	ACATCGCCCA	35
RPL-8	ACCACCCACC	35
RPL-9	ACCGCCTATG	35
RPL-10	ACGATGAGCG	35
APS-1	CAGCTCACGA	35
APS-2	GGCTCATGTG	35
APS-3	AGCGTCCTCC	35
APS-4	GTCAGGGCAA	35

Table 2: Similarity Matrix for Four *Cissus* Accessions, RAPD Data, Jaccard's Coefficient based Distance were Calculated using Program FREETREE

	1	2	3	4	5
1	1.00000				
2	0.56604	1.00000			
3	0.59615	0.59322	1.00000		
4	0.61224	0.57895	0.76471	1.00000	
5	0.37500	0.31343	0.35385	0.37705	1.00000

From this matrix, similarity indices were calculated among *Cissus* accessions (Table 2). According to that, 3 and 4 were found to have the highest similarity (SI= 0.76471, Table 2) while 1 and 2 exhibits least similar (SI= 0.56604 Table 2). Accession 1 (*Cissus quadrangularis*) has the highest similarity with accession 3 (SI= 0.61224 Table 2). Out group is clearly distinguished from *Cissus* accessions in the phylogram (Figure 2). Phylogram was divided into a cluster where the out group is out of the cluster. That cluster divided in two sub-clusters. One sub-cluster posses 1 and 3 accessions while the other possesses 2 and 4.

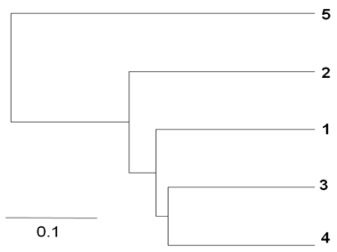

Figure 2: Cluster Analysis of Cumulative RAPD Data in the *Cissus* Accessions. The Phylogram Generated by the UPGMA Method

Table 3: The Average Pair Wise Similarity Indices for the RAPD Band Data in Case of *Cissus*

Type	No. of Bands Total	Monomorphic	Polymorphic	Polymorphic Fraction Of Bands
Cissus	79	62	17	0.78

DISCUSSIONS

The objective of the present work was to determine the genetic variability in the available accessions of *Cissus*. In spite of a variety of medicinal uses, there is very little molecular work has been reported and there was no report of any particular procedure for the isolation of DNA from *Cissus* specifically. Therefore, it was essential to optimize the DNA isolation method first. The results showed that among the five methods used for DNA extraction in *Cissus*, modified Doyle & Doyle, 1990, with addition of $Na_2S_2O_5$ in comparison with other methods had the best result which can subsequently be used for PCR extension.

In Dekochko & hamon DNA Extraction method, potassium acetate was used for protein removal. This protocol uses SDS as detergent and the addition of potassium acetate resulted in the removal of some polysaccharides and proteins as a complication with the potassium-SDS precipitate (Ribeiro & Lovato, 2007). This extraction method for *Cissus* did not show acceptable results may be because of SDS buffer that used in this method attached to the secondary metabolites and prevented extraction DNA with high quality. In case of the protocol of Oard & Dronovalli, 1992, due to the use of Ammonium Acetate, proteins and phenolic compounds bind to genomic DNA were separated well and DNA had good quality but quantity was very low. DNA extracted with these methods had resulted inconsistent or no profile when tested with RAPD primers. The protocol of Doyle and Doyle is based on lyses and purification with CTAB that selectively precipitates DNA while maintaining the solubility of many polysaccharides (Ribeiro & Lovato, 2007). In the protocol of Doyle and Doyle modified by Rogsted, PVP was added along with CTAB which may bind to the polyphenolic compounds by forming a complex with hydrogen bonds and may help in the removal of impurities to some extent. But this method in *Cissus* also had not much success. And the extracted DNA obtained from this protocol had good quality, but not considerably higher yield. The reason may be, DNA extracted with this method would surrounded by Ammonium acetate which causes formed gelatinous deposit(Padmalatha & Prasad, 2006). The protocol of modified Doyle and Doyle (1990), which gave good yield as well as consistent RAPD profiles with the random primer tested in our hands, was thus used for all further studies.

The RAPD analysis revealed the genetic similarity coefficients among the accessions ranging from 0.56 to 0.76. Genetic variability analysis, among the available accessions was important to know whether the morphological differences among the accessions is only a phenotypic differences or it is the result of genetic differences, and to determine whether all the four accessions are different species. According to the similarity found by similarity matrix, accession 1 is highly similar to accession 4 (SI=0.61224, Table 2) and average similarity with accession 3 (SI=0.59615, Table 2). There is very less similarity between accession 1 and 2 as per the phylogenetic analysis of data obtained by RAPD.

CONCLUSIONS

Genetically, characterized RAPD markers have been used to distinguish among the four accessions. UPGMA cluster analyses of the SM matrix confidently separate the accessions. Results indicate that as being quick and reliable, RAPD markers could be effectively used for genetic variability assessment among various species. However, there are serious caveats associated with the use of RAPD markers. Before any application, it is crucial that RAPD markers be characterized, either by segregation analysis or band sequence homology. There is a possibility of detailed study of genetic variations between these accessions by using other methods such as use of ISSR markers, SSR markers, SCARs, RFLPs etc.

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