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Optimization of DNA Isolation and PCR Protocol for ISSR Analysis of Species of *Spilanthes* - A Medicinal Herb from Peninsular India

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Abstract: ***Spilanthes of Asteraceae with over 140 species is distributed in the warm regions of both hemispheres. Most species of this genus are medicinally important and are widely used in folklore medicines. Genetic analysis of plants relies on high yields of pure DNA samples. The molecular characterization of Spilanthes species has not been reported in the literature. In the present study, attempts were made to optimize DNA isolation using the CTAB method and characterize six species of Spilanthes. The DNA yield from leaf tissue was 1-2 µgµl^{-1} and the purity ranged between 1.8-2.0 indicating minimal levels of contaminating metabolites. The modified technique was found to be ideal for PCR amplification of pure DNA from Spilanthes. The ISSR protocol was optimized for template DNA, dNTPs, $MgCl_2$, Taq DNA polymerase, primer concentration and annealing temperature for each species and primer. The reproducible amplifiable products were obtained in all PCR reactions. The protocol established in the present study could be employed in determining the diversity of Spilanthes species and studies relating to barcoding and phylogeny.***

Keywords: *Spilanthes*, Polyphenols, Cetyl trimethylammonium bromide (CTAB), Polymerase Chain Reaction (PCR) and Inter Simple Sequence Repeats (ISSR).

1. Introduction

Spilanthes is the genus of herbs belonging to Asteraceae, with more than 140 species distributed in the warm regions of both the hemispheres. Most species of this genus are medicinally important and are widely used in folklore medicine. The alkamides extracted from this genus are effectively used in treating toothache and throat infections. It is also used as local anaesthetic and digestive tonic (Ramsewak *et al.,* 1999). It also possess anti-inflammatory, antiseptic, analgesic, antioxidant and cytotoxic properties (Jondiko *et al*., 1986; Saritha *et al.,* 2002; Rai *et al.*, 2004; Wu *et al.*, 2008; Prachayasittikul *et al.*, 2009). The Inter Simple Sequence Repeats (ISSR) technique (Zietkiewicz *et al.,* 1994) is simple, fast and efficient that produces amplified products of 200–2000 bp length. The ISSR marker system is based on the use of 15-20 bp primers designed to be complimentary to SSR (microsatellite) sequences found throughout the eukaryotic genomes. When compared with the random amplified polymorphic DNA (RAPD method), the longer primer (16 - 20 bp) can target the template DNA and improve reliability and reproducibility (Reddy *et al.,* 2002). In addition, ISSRs are significantly cost effective than the amplified fragment length polymorphism (AFLP). Thus, ISSRs are widely used to determine the genetic variation occurring in medicinal plants (Xiao *et al.,* 2006; Qiu *et al.,* 2008).

In view of the wide utilization of *Spilanthes* in modern medicine, the systematic authentication of its species at the morphological, biochemical and in molecular levels is becoming increasingly compulsory in order to avoid confusions regarding taxonomic positions. The Cetyl trimethylammonium Bromide (CTAB) procedure detailed by Doyle and Doyle (1987) is generally carried out for the DNA extraction. The DNA isolation and Polymerase Chain Reaction (PCR) protocols for the genus *Spilanthes* rich in alkamides and polyphenols have not been documented in the literature. Preliminary study suggested that modification in CTAB protocol was required as the existing standard protocols yielded brownish yellow DNA pellet which was unsuitable for PCR reaction. Hence, in the present study attempts were made to standardize DNA isolation and establish PCR-ISSR protocol that could be of help in investigations relating to characterization of this genus.

2. Materials and Methods

2.1 Plant Material

In the present study, six species of the genus *Spilanthes*- *S. calva, S. ciliata, S. oleracea, S. paniculata, S. radicans* and *S. uliginosa* occurring in the peninsular India were selected for their characterization by using the ISSR technique. The leaf samples of the above species of *Spilanthes* to be collected for molecular profiling were grown in the net house of Division of Plant Genetic Resources, Indian Institute of Horticultural Research, Bangalore.

2.2 Reagents used in DNA Isolation

An extraction buffer consisting of 2% CTAB (w/v), Buffer A (10X: Tris HCl (pH 8.0; 0.1 M), KCl (pH 8.0; 0.5 M) with $MgCl_2$ 15 mM), EDTA (pH 8.0, 0.5 M), NaCl (5.0 M)), Poly Vinyl Propylene (PVP, 0.25 g g^{-1} of leaf tissue, added while grinding) was prepared. Sodium acetate solution (pH 5.2, 3 M), ribonuclease A (10 mg ml^{-1}), Chloroform : lsoamylalcohol (24:1), Ethanol (70% and 100%) and TE buffer (Tris HCl, 10 mM, EDTA, 1 mM, pH 8.0) were the additional solutions prepared for experimentation.

2.3 DNA Isolation by CTAB Method

Freshly harvested young and tender leaf samples (2 g) were ground in liquid Nitrogen with a relatively higher concentration of PVP (Polyvinylpyrrolidone, 25%)- for preventing the oxidation of phenolic compounds and alkamides) using a pre-chilled mortar and pestle. The ground powder was quickly transferred into a clear autoclaved 50 ml centrifuge tube and then 10 ml of pre-warmed (60˚C) extraction buffer was added and shaken gently to obtain the slurry. The tubes were incubated at 60˚C in circulating water bath for one hour with intermittent shaking for every 10 minutes with occasional inversion and brought to room temperature (RT). An equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed properly by inverting the tubes 20-25 times to obtain an emulsion and centrifuged at 8000 rpm for 20 minutes at RT to separate the phases. The chloroform:lsoamylalcohol step was repeated 2-3 times. The supernatant was carefully decanted and transferred to a new tube and was precipitated by the addition with two volumes of pre-chilled (-20˚C) absolute ethanol and Ammonium acetate (final concentration 0.3 M), and gently mixed by inverting up and down (10 minutes) to obtain fibrous DNA that was incubated at -20˚C for a minimum of one hour.

The samples were centrifuged at 6,000 rpm for 15 minutes and the pellet was washed thrice with 70% ethanol. The supernatant was decanted and DNA pellet was air-dried at RT until the white pellet turned transparent. This DNA pellet was resuspended in 300 µl of TE Buffer and 6 µl of RNAse (10 µg μl^{-1}) was added and incubated at 37˚C for one hour (RNAse treatment helped in achieving RNA-free genomic DNA). To this 600 µl of ice-chilled ethanol and 10 ml of 3M Ammonium acetate (pH 8.3) was added and incubated at -20˚C for one hour to re-precipitate DNA.

The solution was centrifuged at 10,000 rpm for 15 minutes; the pellet was dried at 37˚C and resuspended in 300 µl of TE buffer. All the centrifugation steps were carried out at RT to avoid DNA degradation and obtain good quality DNA.

2.4. Quantification of Extracted DNA and Testing for Purity

The yield of DNA per gram of leaf tissue was measured using a UV spectrophotometer at 260 nm. The purity of DNA was determined by estimating the ratio of absorbance at 260 nm to that of 280 nm. The DNA concentration and purity was also determined by running samples on 0.8% agarose gel depending on the intensities of band when compared to Hind III DNA digest (used to determine the concentration).

2.5 Optimization of Polymerase Chain Reaction (PCR)

The optimization of PCR-ISSR was carried out with the DNA extracted from all six species of *Spilanthes*. The ISSR primers obtained from the University of British Columbia (UBC), were used for amplification to standardize the PCR conditions. The reactions were carried out in a DNA Thermocycler (Eppendorf Gradient PCR Thermal cycler, Germany). Four factors including *Thermus aquaticus* (*Taq*) DNA polymerase, dNTPs, primer concentration, and annealing temperatures investigated were detailed in Table 1. Each 10 µl reaction mixture contained about 10 mM Tris-HCl (pH 8.3), 50 mM KCl with 1.5 mM $MgCl_2$, 0.4 mM dNTPs, 0.4 µM primer, 0.2 unit *Taq* DNA polymerase and approximately 60 ng template DNA (Table 1). The thermal cycler was programmed for an initial denaturation step of 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at different annealing temperature for each primer, extension at 72°C for 1 min and final extension at 72°C for 8 min and a hold temperature of 4°C at the end. 10 µl of PCR products were electrophoresed on 1.4% agarose gel, in 1X TBE Buffer at 80 V for 2 h and stained with ethidium bromide (1 µg ml^{-1}). 100 bp DNA ladder was used for determining the molecular weight. The clarity of bands were obtained by the addition of 0.01% Formamide. Gels with amplification fragments were visualized and photographed in UV light by using Gel Documentation System (Syngene, India)

3. Results and Discussion

3.1 Isolation and detection of DNA:

The modified CTAB protocol employed in the present study yielded creamish white pellet of the genomic DNA. Since the plant species contained polyphenols, routine CTAB protocol yielded brownish yellow DNA which was unsuitable for PCR amplification (Katterman and Shattuck, 1983; Angeles *et al.*, 2005). Obtaining high quality genomic DNA from plants producing polyphenolics is reported to be difficult (Weising *et al.,* 2009). The nucleic acid detection in the present study ranged from 1.8 – 2.0 (A 260/280) which indicated the absence of contaminants (Pich and Schubert, 1993). The gel picture of genomic DNA did not indicate any impurity in any lane (Figure 1). The covalently bound protein and DNA molecules with oxidized forms of polyphenols imparted colour to the DNA pellet as against the pure creamish DNA pellet which was most suitable for PCR amplifications. The increase in PVP concentration effectively removed polyphenols and this yielded pure genomic DNA. This further suggested that fresh and young leaves of *Spilanthes* could be used for DNA extraction.

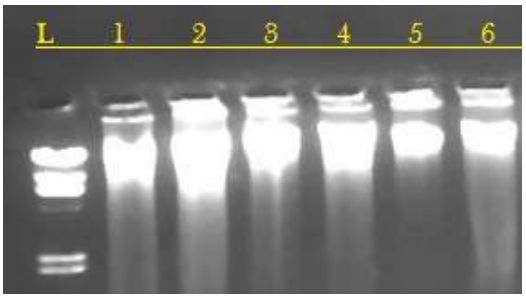

Figure 1: Genomic DNA of species of *Spilanthes* isolated by modified CTAB method: Lane 1- L- Hind III, Lane 1-6: *S. calva, S. ciliata, S. oleracea, S. paniculata, S. radicans* and *S. uliginosa,* respectively.

3.2 Optimization of PCR-ISSR and Screening of Primers

To obtain PCR amplification, all the ingredients are required in specific concentration. In the present study, the parameters were optimized for PCR-ISSR. Based on preliminary screening, few UBC ISSR primers were selected that produced clear, reproducible and relatively polymorphic bands.

Template DNA: The template DNA concentration of 60 ng per PCR reaction mixture was found ideal. The clarity of bands decreased with the increase in the DNA concentration.

Magnesium Chloride ($MgCl_2$): The Buffer A of reaction mixture containing 1.5 mM $MgCl_2$ was optimum for PCR amplification. Hence additional $MgCl_2$ was not required for the reaction.

Table 1: Optimization of PCR parameters using ISSR UBC primers for *Spilanthes* genus.

Sl. No.	*PCR parameter*	*Tested range*	*Optimum conditions*
1.	DNA template concentration (ng)	10, 20, 30, 40, 50, 60, 75, 100, 150, 175 and 200	60
2.	Magnesium chloride (mM)	0, 1.0, 1.5, 2.0, 2.5, 3.0 and 1.5	Nil
3.	Deoxynucleotide triphosphates (dNTPs) (mM)	0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.4, 0.6 and 0.8	0.4
4.	Primer concentration (µM)	0.1, 0.2, 0.3, 0.4, 0.5, 0.75, 1 and 1.5	0.4
5.	*Taq* DNA polymerase (units)	0.2, 0.3, 0.4, 0.5, 1.0, 1.5, 1.75 and 1	0.2
6.	Annealing temperature (°C)	50~58, 51, 52 or 56 for different primers.	

dNTPs: 0.4 mM dNTP concentration was optimum in the present study. All six species reproduced clear amplification of PCR products under the above optimized parameters.

Primer concentration: The primer concentration 0.4 µM was optimum in the present study; when the concentration was increased the reaction was inhibited due to hybridization of by-products of PCR.

***Taq* polymerase**: When all other parameters were optimum, the range for *Taq* polymerase was between 0.1 and 0.25 units per 10 µL reaction (Lawyer *et al*., 1989). Similarly clear bands were observed with 0.2 unit of *Taq* polymerase in the present study.

Annealing temperature: An applicable annealing temperature is 5°C below the true Tm (melting temperature) of the primers. As the *Taq* DNA *polymerase* remained active over a broad range of temperature, primer extension occurred at low temperatures, including the annealing step (Innis *et al.,* 1988). Optimization of annealing temperature was carried out in the range of -5°C to +5°C to the *Tm* of each primer. The temperature at which the clear amplification obtained was selected.
The *T*m was calculated (Wallace *et al*., 1979) as:

$Tm = 40C(G + C) + 20C(A + T)$ for primers less than 21 bases long.

Since *Tm* is affected by the individual buffer components and even the primer and DNA template concentration, this calculation could be approximation. In the present study, the annealing temperature varied from 2°C below *Tm* to 3°C above *Tm* for primers screened which could be species-dependent. Although, annealing temperatures below *Tm* often yielded weak bands or smears, in few samples, clear amplification was observed (Table 2).

Table 2: ISSR-UBC primers and optimized annealing temperatures for six species of *Spilanthes.* SC- *S. calva,* SCi- *S. ciliata,* SO- *S. oleracea,* SP- *S. paniculata,* SR- *S. radicans* and SU- *S. uliginosa,* respectively.

Sl. No.	Primer No.	Primer Sequence (5^1- 3^1)	Annealing temperature (in ° C)					
			SC	SCi	SO	SP	SR	SU
1.	841	ATG ATG ATG ATG ATG AYG	52.8	51.8	52.8	53.8	53.8	54.8
2.	844	CTC TCT CTC TCT C TC TRC	51.8	51.8	51.8	51.8	51.8	52.8
3.	845	CTC TCT CTC TCT C TC TRG	50.8	52.8	50.8	52.8	52.8	54.8
4.	847	CAC ACA CAC ACA CAC ARC	55.2	55.2	54.0	55.6	56.2	56.2
5.	851	GTG TGT GTG TGT G TG TYG	53.5	55.0	53.5	56.2	53.5	53.5
6.	852	TCT CTC TCT CTC T CT CRA	56.1	55.8	55.8	55.8	56.1	54.2

In the present study, band clarity of PCR amplicons by the addition of 0.01% Formamide. The addition of Formamide could be helpful while amplifying the GC-rich templates and templates that form strong secondary structures, which stopped DNA polymerases from proceeding. The GC-rich templates could be problematic due to an inefficient separation of two DNA strands or the tendency for the complementary GC-rich primers to form intermolecular secondary structures, which will compete with primer annealing to the template (Rees *et al*., 1993). Formamide aided in amplification in a similar manner by interfering with the formation of hydrogen bonds between two DNA strands (Geiduschek and Herskovits, 1961).

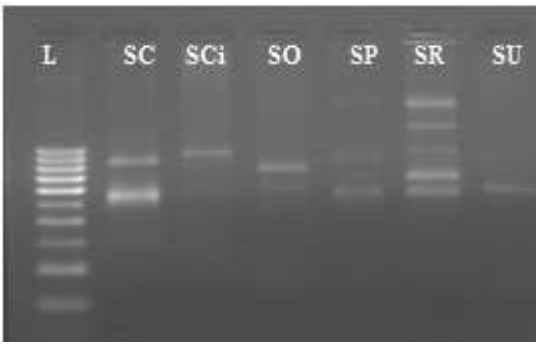

Figure 2: ISSR Primer UBC 852 banding pattern in species of *Spilanthes:* Lane 1: 100 bp DNA Ladder, Lane2-7*:* SC- *Spilanthes calva,* SCi- *S. ciliata,* SO- *S. oleracea,* SP- *S. paniculata,* SR- *S. radicans and* SU- *S. uliginosa,* respectively.

4. Conclusion

The present study reports the simple DNA isolation protocol and optimized PCR-ISSR specific for *Spilanthes* species and could be useful in studies relating to genetic diversity of plants containing phenolics and other metabolites.

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