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INVITRO REGENERATION AND DEVELOPMENT OF RAPD- SCAR MARKER FOR THE IDENTIFICATION OF *ARTEMISIA ABSINTHIUM* L

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ABSTRACT

Experiments were carried out to determine the effect of different plant growth regulating hormones on *in vitro* propagation of *Artemisia absinthium* L. from leaf explants. Organogenic callus was observed when explants were inoculated on the MS medium supplemented with BAP, 1.0 mg/l in combination with 2,4-D, 0.5mg/l. Maximum adventitious (28) shoots were induced on MS medium supplemented with NAA, 1.0 mg/l and KIN, 2.0 mg/l after the fifth subculture. MS medium augmented with 2 mg/l gave maximum root initiation, the average number of roots per shoot were 3.9. Individual plantlets were removed from the flasks, washed and transferred to a sterilized pot having soil:sand:decomposed coffee husk at a ratio (v/v) of 1:1:2 kept at high humidity (80-90%) for 15 days. RAPD-based SCAR marker was also developed using 25 random primers for initial screening. A 475 bp polymorphic band obtained with an OPAA-8 primer which was specific to all *A. absinthium* accessions. This RAPD-amplicon was eluted, sequenced and cloned and a pair of SCAR primers was designed. A single, bright, distinct band of 225 bp was obtained which shows its specificity towards the accessions of *A. absinthium* and thus can be useful in its authentication.

Key words : *Artemisia absinthium,* authentication, in vitro propagation, medicinal plant, SCAR marker

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INTRODUCTION

The genus *Artemisia* (family- Asteraceae) consists of about 500 species of perennial herbs. *Artemisia absinthium* is commonly known as wormwood or "vilayati afsanteen" as it is native to Europe and possibly, it was imported from Europe. It also grows in the northern hilly areas of Pakistan[1]. In India, it is distributed in West Himalaya, common on slopes of Srinagar, Banihal, Tanmarg, etc. *A. absinthium* is well known for its ethnomedicinal properties which are related to anthelmintic activity[2], antifungal[3], antimicrobial activity[4]. *Artemisia absinthium* is a choleretic, antiseptic, depurative, digestive, diuretic, and is used in treating leukemia and sclerosis[5]. In China, the plant has been used in the treatment of patient infected with *Plasmodium falciparum.* Several techniques are available for the conservation of biodiversity and plant genetic resources of rare and endangered species. These techniques include seed germination, embryo rescue, cryopreservation, regeneration from callus, micrografting and micropropagation[6,7,8]. Micropropagation or plant tissue culture research has been active for many years and has now been used commercially and in research to rapidly multiply and improve a wide range of medicinal, horticultural and aromatic crops and their production systems. Indeed, micropropagation of medicinal plants is today a reliable technology applied commercially worldwide, which helps in large-scale plant multiplication, production, and their supply. Since from the dawn of civilization tradition herbs and the herbal medicines have been used in the treatment of human suffering from various diseases. Herb authentication and identification has always been a great challenge for the people using them as a source of medicine. With an increase in demand of the medicinal plants, their authentication and characterization are becoming a critical issue. For this authentication purpose, development of DNA based molecular markers is more reliable as the genetic content of each species is unique and is independent of age and physiological and environmental factors[9]. Genetic techniques that use polymerase chain reaction (PCR) and sequencing techniques provide a reliable method for the herb authentication[10, 11, 12]. Randomly Amplified Polymorphic DNA (RAPD) is easy to develop and has become one of the most effective processes for the herb authentication purpose but a lack of reproducibility[13], homoplasy, non-homology, collision, artefactual segregation[14] makes it less reliable for this. Development of Sequenced Characterized Amplified Region (SCAR) is more reliable and accurate techniques to overcome the above RAPD problems[15]. Development of SCAR marker can be used for the authentication of herbal medicines by using RAPD or AFLP derived specific PCR primers. These RAPD derived specific primers result in amplification of the given samples, and can be used in the generation of specific amplification products within closely-related samples[16, 17]. Protocol for micropropagation of *A. absinthium* has been previously established by using shoot tips[18], as an alternative, the callus culture provides an important tool for the regeneration of the whole plants. The present study aims to develop a reliable protocol for the micropropagation of *A. absinthium* by using leaf primordial tissues as explant and development of RAPD – Sequenced Characterized Amplified Region (SCAR) marker for the identification of *A. absinthium*. As the availability of *A. absinthium* in India is very less so only six accessions have taken for the RAPD analysis.

MATERIAL AND METHODS

Micropropagation of Artemisia absinthium

Young leaves of *A. absinthium* were collected from the herbal garden, Jamia Hamdard, New Delhi, India. It was then washed in tap water 4-5 times and then soaked in 5% Teepol solution and then again washed with tap water. The explants were then surface sterilized with 0.1% $HgCl_2$ for 1 min followed by several times washing with sterile water under aseptic and hygienic conditions. The leaf explants were inoculated in full /half strength MS medium[19] having different concentrations of plant growth regulators like 2,4-dichloro phenoxy acetic acid (2,4-D), α- naphthalene acetic acid (NAA), 6-benzyl adenine (BAP), indole-3-acetic acid (IAA), indole-3-butaric acid (IBA) and kinetin (KIN) etc. for the induction of callus. The pH of the medium was adjusted to 5.7 with 1N NaOH/1N HCl, before addition of 0.8% agar and autoclaved at 15 $lb/inch^2$ pressure and 121°C temperature for 20 min. The primary callus was subcultured on MS medium supplemented with BAP and NAA, in varying concentrations for shoot differentiation. Well-differentiated shoots emerging from explants were excised and rooted on half/full-strength MS plain medium supplemented with IAA and IBA, singly or in different concentrations. The cultures were maintained at 25°C ± 2°C with 8/16-h (light/dark) photoperiod. The experiments were made on six replicates with each growth regulators. A detailed statistical analysis was also carried out.

Development of RAPD-based Sequenced Characterized Amplified Region marker

Authentic plant samples of *A. absinthium* were procured from the different parts of India, (Table 1). The procured saplings were grown in the Herbal Garden of Jamia Hamdard University, New Delhi. Fresh leaf samples were collected from live plants, frozen in liquid nitrogen and stored at -80°C. Genomic DNA of fresh or frozen leaf samples of *A. absinthium* was isolated by modified CTAB (cetyltrimethyl ammonium bromide) extraction method[20]. 1 g of leaf sample was ground in liquid nitrogen to fine powder along with polyvinylpyrrolidone (PVP) . 10 ml of 2% CTAB (100 mM Tris-HCl buffer pH 8.0, 1.4 M NaCl, 20 mM, EDTA, 1% mercapto ethanol) buffer was added to the homogenate, and was incubated at 60°C for about 1 hour with shaking at the interval of 30 min. An equal volume of chloroform and isoamyl alcohol (24:1) was added to the suspension. The mixture was then centrifuged at 13,000 × g for 10 min. after centrifugation the aqueous phase was collected. 0.6 volume of cold isopropanol and 1/30 volume of sodium acetate (3 M, pH 5.2) was added to it and incubated at -20 °C for 1 h. The sample was again centrifuged at 13,000 × g for 10 min. The DNA pellet obtained was washed with 80% ethanol twice, air dried and dissolved in TE buffer (10 mM Tris buffer, pH 8.0, 1 mM Na_2EDTA). The isolated DNA was treated with RNase A (10µg/ml) at 37 °C for 30 min to remove the concentration of RNA from the pellet. Concentration of

DNA and its purity were determined by using Nanodrop spectrophotometer (Thermo scientific, USA). The quality of the DNA was also determined using agarose gel electrophoresis stained with ethidium bromide. The PCR amplification was carried out according to the method developed by McClelland *et al*[21]. PCR reactions were performed in 25 µl reaction tubes, using 25 random 10-mer primers of Operon technologies Inc., USA) and Banglore Genei (India) (Table 2). Each PCR reaction mix contained 30 ng DNA template, 1.5 mM $MgCl_2$, PCR buffer (20 mM Tris–HCl, pH8.4, 50 mM KCl), 0.5 U of Taq polymerase (Promega), 300 µM of dNTPs, and 25 pmol primers. Amplification was carried out in a thermal cycler (Master Cycler, Eppendorf, USA). A standard PCR reaction cycle was used: an initial denaturation step at 95°C for 4 min, followed by 40 cycles of 94°C for 30 sec, 34°C for 0.50 sec, and 72°C for 1 min; the final extension was held for 3 min. PCR products were resolved on 1.2% agarose gel in 1×TAE buffer along with standard 100-bp ladder stained with 0.5 µg/ml ethidium bromide, visualized and photographed under UV light on a transilluminator (UVitech, USA). RAPD fragment of 475-bp was selected from the RAPD pattern of *A. absinthium* with OPAA-08 primer. This fragment was specific to all the accessions of *A. absinthium*. These polymorphic bands were excised from gel and eluted using a Gel Extraction Kit (Qiagen, Germany). The eluted DNA was cloned into pGEM®-T easy vector (Promega, USA). The ligated plasmid was introduced into *Escherichia coli* strain DH_{10B}, following the protocols for preparing competent cells and transformation using the calcium chloride method[22]. Few distinct white colonies were picked from LB X-gal plates and grown overnight in LB medium having ampicillin. The plasmid DNA was isolated from the bacterial culture using plasmid isolation kit (Qiagen, Germany). The sequencing of the cloned DNA fragment was carried with SP6 and T7 primer out at the Center for Genomic Application, New Delhi, India. The Nucleotide sequence of 475 bp RAPD amplicon specific for all the four accessions of *A. absinthium,* was used for primer designing for the development of SCAR marker. A pair of SCAR oligonucleotide primers (Art F and Art R) based on the sequences of RAPD amplicon, which could amplify 475 bp of genomic DNA of *A. absinthium* was designed. The length of designed SCAR primers (Art F and Art R) was 20 bp for each primer. The primer sequences were custom synthesized from Eurofins Genomics India Pvt Ltd, Karnataka, India. The GC contents, melting temperature and secondary structures for each of the primers were analysed. PCR conditions for the amplification of genomic DNA of *A. absinthium* were standardized by the SCAR primers. The amplification of genomic DNA was carried out in a thermal cycler (T100, BioRad, USA) with the given reaction conditions : initial denaturation of 4 min at 95 ºC, followed by 40 cycles of 30 sec at 94 ºC, 50 sec at 60ºC, 1min at 72 ºC and final step of 72 ºC for 3 min. The annealing temperature of the SCAR primer was standardized 60ºC using gradient PCR (T100, BioRad, USA). The amplified PCR product was resolved on 1.2% agarose gel in 1× TAE buffer along with standard 100-bp ladder (Bangalore Genei, India) and visualized under UV light on a transilluminator (UVitech, USA), and a photograph was taken on gel documentation system (UVitech, USA).

RESULTS AND DISCUSSION

Effect of growth hormones on callus induction and growth

Leaf explants when inoculated on MS medium alone or MS medium supplemented with different plant growth regulators (NAA, BAP, KIN, IAA, 2,4-D) singly, in varying concentrations did not show any positive response in callusing of the explant. However, the explant when cultured on MS medium supplemented with BAP (0.25-1.0mg/l) along with 2,4-D (0.2-0.5mg/l) or NAA(0.5-1.0 mg/l) induces callus within 2 weeks of inoculation. Calli grew in size when these were subcultured on the same medium. These calli were yellowish green in colour (Fig. 1a). Among all combination tested MS medium supplemented with BA (1.25 mg/l) along with 2,4-D (0.75 mg/l) was found best for callus formation where 100 % callus occurred while, MS medium supplemented with BA (1.0 mg/l) + NAA (0.75 mg/l) induced only 75 % callus formation (Table 3). No sign of callus induction was noticed when the explants cultured on MS medium augmented with Kn + IBA or IAA + Kn at different concentration (Table 3). The present study investigated the effect of varying concentrations of different growth regulators on *in vitro* regeneration of the plant. MS medium supplemented with BAP (1.25mg/l) in combination with 2, 4-D (0.75 mg/l) was found best for the callus induction which shows 100% callus formation from the leaf explants, which is in contrast to the findings[18] in *Artemisia absinthium* (Callus formation in BA + NAA)[23] in *Artemisia pallens* (Callus formation in BA + IAA). In *Artemisia sphaerocephala*[24] best callogenesis was observed in the presence of 2,4-D and KIN.

Effect of phytohormones on in vitro shoot regeneration

The callus obtained from aforesaid composition of media was sub cultured on MS medium supplemented with IAA (0.25-1.0mg/l) in combination with KIN (1.0-2.0 mg/l) or BAP (1.0-2.0mg/l). Among the different combination tested, MS medium augmented with KIN (2.0 mg/l) along with IAA (1.0 mg/l) gave the best result where 4-5 shoots were formed (Table 4) (Fig. 1b and c). When these multiple shoots were sub cultured on the same medium, it increases number of shoot formation. After fifth subsequent subculture in the medium supplemented with IAA (1.0 mg/l) and KIN (2.0mg/l), the average number of shoots per explants increased up to 27 (Fig. 1d) (Table 4). All cytokinin and auxin (KIN + IAA or BA + IAA) were capable of shoot induction from dedifferentiated tissue. KIN (2.0 mg/l) in combination with IAA (1.0 mg/l) was found to be significantly more effective for shoot regeneration in comparison to other combination (BA + IAA). The findings are in contrast with the results obtained earlier[25, 26, 27] where high concentration of BA was best for shoot multiplication in *A. annua* and on other species of *Artemisia* such *A. Scorpia*[28], *A. ranunculus* [29] and *A. amygdalina* [30].

Effect of phytohormones on in vitro rooting

In vitro regenerated micro shoots were sub cultured on MS or ½ MS medium alone or in combination with different auxins (IAA or IBA) at different concentration. No root formation was noticed in regenerated micro

shoots on MS or ½ MS medium alone even after 4 weeks of incubation. Roots were induced when different range of IBA or IAA was added on ½ MS medium. Of the different auxin tested, the best rooting response could be observed on ½ MS medium containing IAA (2 mg/l) where maximum 75% frequency with 3.9 mean number of root were observed (Table 5). In the present context, adventitious root formation in isolated micro shoot was achieved in the presence of auxins (IAA, IBA) in 1/2 MS medium. Highest percentage of rooting (75%) was observed on half strength MS medium supplemented with 2 mg/l IAA. The promotive effect of IBA in rooting is well documented in *Artemisia annua* [31]. While in *Artemisia absinthium* [32], MS (1/2 and full) medium supplemented with IAA exhibited best rooting response. MS basal medium was found best for root initiation in *A. vulgaris*[33].

Hardening and acclimatization

Plantlets (shoots with well-developed roots) were taken for acclimation. Individual plantlets of *A. absinthium* were removed from the flasks and washed with warm water for the removal of agar from the root surface, transferred to a sterilized pot having soil : sand : decomposed coffee husk at a ratio (v/v) of 1 : 1 : 2 kept at high humidity (80-90%) to prevent desiccation for 15 days. Prior to field transplanting, after 15th day the plantlets were transferred to 70% shade net for one month and then transferred to 30% shade net for another one month.

Isolation of DNA

High molecular weight genomic DNA was isolated from all the accessions of *A. absinthium*. The yield of isolated genomic DNA from different samples, ranged from 500 µg g/l to 750 µg g/l of fresh tissue. An absorbance (A260/A280) ratio of 1.7–1.83 indicated insignificant levels of contaminating proteins and polysaccharides in the DNA samples. 25 random decamer primers used for screening, produced distinct, reproducible fingerprint of *Artemisia absinthium*. OPAA-08 consistently amplified an intense 475-bp band that was unique to *A. absinthium* samples and constant with all the four accessions of the species.

RAPD analysis and identification of specific amplicon

RAPD method was performed in search of DNA polymorphism, which can be used for generating informative SCAR marker. 20 random 10-mer primers were screened for amplification of all the four accessions of *A absinthium*. Out of the 20 random primers, (OPAA 02, OPAA 04, OPAA 07, OPAA 08, OPAA 09, OPAA 12, OPAA 15, Bg28, Bg29, Bg30) were able to produce distinct, good quality, reproducible fingerprint patterns and showed a high level of consistency. Experiments were repeated three times to confirm reproducibility of RAPD fragments under the same composition of reaction volume and same conditions for amplification and thermal cycler. A polymorphic band (475 bp) was obtained from OPAA-08 and was selected for the development of SCAR marker, considering high degree of resolution (Fig. 2). A single, reproducible, distinct and highly resolved band of 475 bp was obtained in the accessions of *A. absinthium.*

Cloning and sequence data analysis

A bright and distinct band of (475 bp), which is specific to all the four accessions of *Artemisia absinthium* was selected from the RAPD profile. This distinct band was eluted, cloned and sequenced. The eluted DNA was cloned into pGEM®-T easy vector (Promega, USA). After restriction digestion analysis using restriction enzymes EcoRI a band of ~475 bp was revealed on 1.2% agarose gel confirmed presence of insert in the vector. The recombinant was sequenced using primers SP6 and T7. The size of Art 01 marker sequence was obtained as 475 bp with 52.5 % G+C contains.

Amplification using SCAR primer

To amplify the 225 bp of genomic DNA of *A. absinthium,* a pair of SCAR oligonucleotide primers (Art F and Art R) based on the sequences of this RAPD amplicon was designed (Fig. 3). Art F (20-mer) was designed as the forward primer and Art R (20-mer) as reverse primer (Table 6). These forward and reverse primer were used for the PCR amplification of genomic DNA from *A. absinthium* with the given reaction conditions : initial denaturation of 4 min at 95 ºC, followed by 40 cycles of 30 sec at 94 ºC, 50 sec at 60 ºC, 1 min at 72 ºC and final step of 72 ºC for 3 min. A single, distinct and brightly resolved band of 225 bp was obtained with DNA isolated from *A. absinthium* (Fig. 4). It showed specificity and sensitivity of SCAR marker towards *Artemisia absinthium*. The SCAR marker can be used as a useful tool for the identification of *A. absinthium*, fresh as well as crude drug sample. Herbal medicine has been gaining popularity among the customers throughout the world. Indigenous herbs have been used for variety of ailments in traditional medicine or in herbal medicinal practice. Adulteration of market sample drugs has been a major problem in local and export markets. Drug identification and its authentication is very important to abolish illegal behavior by immoral individuals. Technique for the development of RAPD has been widely used for the characterization of the DNA of plants and other organisms[34]. It has also been used for the molecular profiling of different accessions of *Bauhinia*[35]. As the random primers are short (decamers) and the stringency of the reaction is low, the amplified RAPD patterns are sensitive to the purity of the DNA preparation, annealing temperature and the concentration of the reagents during the PCR. To overcome these problems, specific RAPD fragments are sequenced and converted into more reproducible and reliable SCAR markers. Also, the use of longer oligonucleotide primers for SCAR gives reliable and reproducible results. As SCAR markers give more prominent and reliable results than RAPD or any other molecular markers, these marker have been used for authentication of *Echinacea* sp., *Panax* sp., *Zingiber* sp., *Lycium* sp., *Phyllanthus* sp., *Apocynum* sp., *Bacopa monnieri*, *Swertia chiraita*, *and Artemisia* sp. from their adulterant species [36, 16, 37, 38, 39, 40, 17], respectively. SCAR marker has also been applied for the adulterant detection in commercially available food and spices products like ground chilli, black pepper [41, 42]. It has been developed for identification and authentication of few endangered plants like *Sinocalycanthus* [43], *Morus boninensis* [44] and *Paphiopedilum* [45]

Table 1
Plant materials of *A. absinthium* collected from different regions

Plant name	Sample code	Source	Date of collection
Artemisia absinthium	A1	Delhi	20-09-2012
Artemisia absinthium	A2	Bangalore	23-11-2012
Artemisia absinthium	A3	Banihal, Jammu	10-04-2013
Artemisia absinthium	A4	Srinagar, Kashmir	18-05-2013
Artemisia absinthium	A5	Anantnag, Kashmir	15- 08-2013
Artemisia absinthium	A6	Himachal Pradesh	23-11-2013

Table 2
Nucleotide sequence of selected primers

Primer code	Primer sequence (5' - 3')	Fragment size (bp)
OPAA- 02	TGCCGAGCTG	225-780
OPAA- 04	AATCGGGCTG	190-889
OPAA- 07	GAAACGGGTG	345-885
OPAA- 08	GTGACGTAGG	367-654
OPAA- 09	GGGTAACGCC	102-845
OPAA- 12	TCGGCGATAG	219-943
OPAA- 15	TTCCGAACCC	287-990
BG 28	GACGGATCAG	175-840
BG 29	CACACTCCAG	140-850
BG 30	TGAGTGGGTG	139-995
OPAA- 02	TGCCGAGCTG	225-780
OPAA- 04	AATCGGGCTG	190-889

Table 3
Effect of phytohormone on leaf explants of *Artemisia absinthium* for callus differentiation

Plant growth regulators	Concentrations (mg/l)	Callusing	No. of days	Percentage of explants responded (%)
MS plain medium	-	-	-	0
MS+IAA	0.10	-		
	0.25	-	-	0
	0.50	-		
	0.75	-		
	1.00	-		
MS+2,4-D	0.10	-		
	0.25	-	7days	8
	0.50	+		
	0.75	-		
	1.00	-		
MS+ BAP	0.10	-		
	0.25	-	10 days	10
	0.50	+		
	0.75	-		
	1.00	-		
MS+KIN	0.10	-		
	0.25	-	-	0
	0.50	-		
	0.75	-		
	1.00	-		
MS+IAA+BAP	0.10+0.25	-		0
	0.50+0.50	-	14 days	69.0
	0.75+0.50	-		100
MS+BAP+2,4-D	0.25+0.25	+		10
	0.50+0.50	+	18 days	75.5
	1.25+0.75	+		100
MS+BAP+NAA	0.25+0.25	+		68
	0.50+0.50	+	7days	59.5
	1.00+0.75	+		75
MS+KIN+IBA	0.25+0.50	-		
	0.50+0.50	-	-	0
	1.00+0.75	-		
MS+IAA+KIN	0.25+0.25	-	-	0
	0.50+0.50	-		
	1.0+0.50	-		

- sign shows negative response; +sign shows positive response

Table 4
Effect of phytohormones on regeneration of multiple shoots from the leaf explants of *Artemisia absinthium*

Plant growth regulators	Concentrations (mg/l)	No. of shoots after fifth subculture ±SE	No. of days	Response (%)
MS+IAA	0.10	-	7 days	00.0
	0.25	-		00.0
	0.50	2.833 ± 0.703		33.4
	0.75	0.833 ± 0.307		50.0
	1.00	-		00.0
MS+BAP	0.10	-	13 days	00.0
	0.25	-		00.0
	0.50	-		00.0
	0.75	3.407 ± 0.477		66.7
	1.00	2.889 ± 0.609		33.4
MS+KIN	0.10	-	7 days	00.0
	0.25	4.667 ± 0.506		50.0
	0.50	6.690 ± 0.645		83.3
	0.75	4.809 ± 0.358		50.0
	1.00	-		00.0
MS+IBA	0.25	-	18 days	00.0
	0.50	-		00.0
	1.00	7.787 ± 0.312		66.7
MS+BAP+IAA	1.0+0.5	14.415 ± 0.215	14 days	73.3
	2.0+1.0	21.256 ± 0.645		44.76
MS+KIN+IAA	1.0+0.5	19.413 ± 0.476	20 days	33.4
	2.0+1.0	27.896 ± 0.609		83.3

- sign shows negative response; +sign shows positive response

Table 5
Effect of auxin on *in vitro* root induction from the leaf explants of *Artemisia absinthium*

Plant growth regulators	Concentration	No. of roots/ explants	Root length (cm)	Rooting response (%)
MS medium	**-**	**-**	**-**	**-**
½ MS	**-**	**-**	**-**	**-**
½ MS + IBA	0.5	1.70±0.71	0.50±0.10	45
	1.0	1.25±0.47	0.54±0.05	62
	1.5	-	-	-
	2.0	-	-	-
	2.5	-	-	-
½ MS + IAA	0.5	-	-	-
	1.0	1.40±0.52	0.61±0.05	58
	1.5	2.60±0.50	1.56±0.08	70
	2.0	3.82±3.70	1.82±0.32	75
	2.5	1.20±0.19	0.33±0.53	52

-sign shows negative response

Table 6
Sequence of SCAR primers

SCAR primers	No. of base	Sequences (5´- 3´)	G+C content (%)	Annealing temperature (ºC)
Art F	20	CGTTCGTTTCAGGCAGACAA	50	59.07
Art R	20	GGAACCTCGGTAAGATCGCA	55	59.54

Figure 1

(a) Callus induction from the leaf explants of *Artemisia absinthium* on MS medium supplemented with BAP (1.25 mg/l) +2,4-D (0.75 mg/l),), (b) and (c) Shoot induction from callus on MS medium supplemented with KIN (2.0 mg/l) and IAA (1.0mg/l) (d) regeneration of multiple shoots from callus on MS medium supplemented with KIN (2.0 mg/l) and IAA (1.0mg/l) after fifth subculture passage.

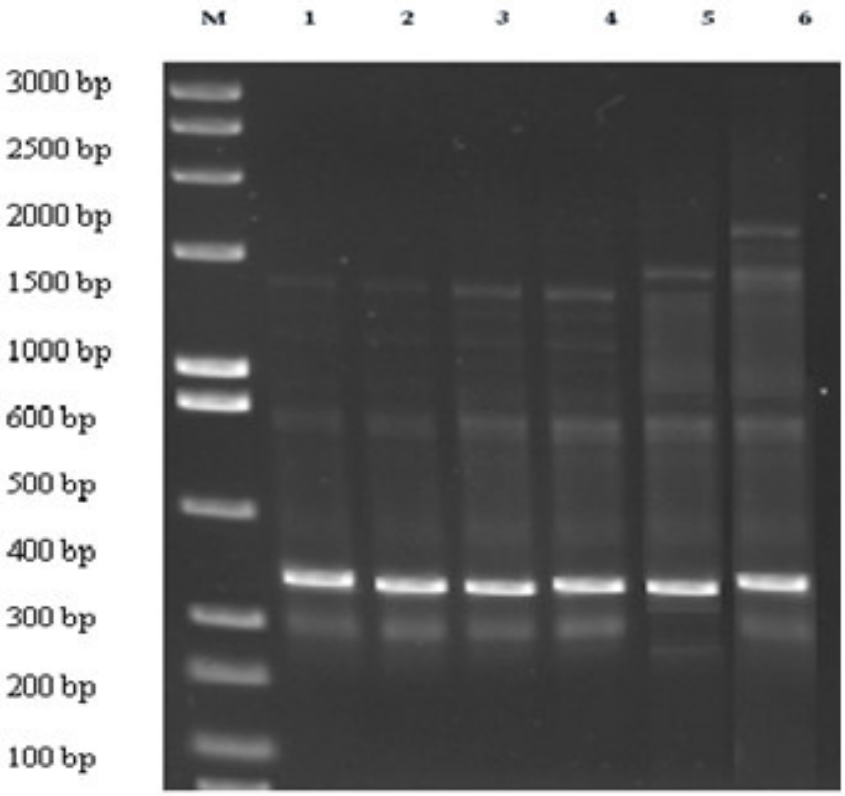

Figure 2

RAPD Profile of Artemisia absinthium with OPAA-08; Lane M=DNA Marker. Lane 1-6 are accessions of A. absinthium

1 GATTTGTGACGTAGGCAGTTAAATATTTCTTTAATAATGGGGTTTTTTCGAGTGGGTGTA

61 TTTCTTCTTCTATTGCACTTATTCCGAAAATACCTAATGCGAAGTTGGTTAAAGATTTCA

121 GGCCGATCAGTCTTATTGGCAGCCTATACAAGATTATCACTAAAATCTTGGCCAACAGAC

181 TTGCTCCTGTGATTGGAGATCATATTCACGAAGTTCAATCCGCGTTCGTTTCAGGCAGAC
>>>>>>>>>>>>>>>>>>>>>>>>

241 AAATTATGGATGGTCCTTTCATCCTTACTGAAATCATGAAATGGTGTTCGTTGAAGAAAA
>>

301 AACAAGCCCTCATTTTCAAGGTGGACTTTAAAAAAGCATGCGATTCGATAAGATGGGATT

361 TCTTGGACGAGATTCTAGTAACGTTCGGTTTTGGTGGTAAATGGCGTCAATGGATTCAAA

421 GCTGCTTGCGATCTTACCGAGGTTCCATCCTCATCAATGGTTGCCCTACGTCACA
<<<<<<<<<<<<<<<<<<<<<

Figure 3
Nucleotide Sequence of the RAPD amplicon

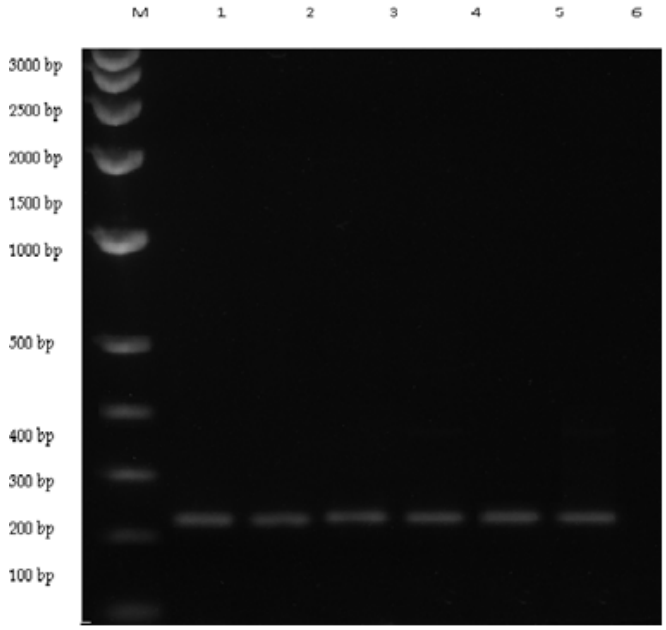

Figure 4
PCR Amplification of A. absinthium (lane 1-6) using SCAR primers (ArtF and ArtR). Lane M = DNA marker

CONCLUSION

Important medicinal plants from their close relatives or adulterants have been discriminated or authenticated using species-specific SCAR markers. In the present study, the SCAR primers designed using this sequence variation was found to be specific for *Artemisia absinthium*. It makes the technique more stringent and specific when compared with RAPD marker. A single, bright, distinct band of 225 bp was obtained in *A. absinthium* this shows its specificity towards the accessions of *A. absinthium and thus can be useful in its authentication.* These SCAR primers can be used to identify and authenticate the identity of *A. absinthium* from their other traded substitutes and adulterants or closely related species. Moreover such precise, efficient, and sensitive technique will be helpful in standardization and quality control of crude drugs imported to India as well as exported from India.

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CONFLICT OF INTEREST

Conflict of interest declared none.

REFERENCES

1. Haq I. *Medicinal Plants* Hamadard Foundation Press, Karachi, Pakistan. 1983.
2. Tariq KA, Chishti MZ, Ahmad F, Shawl AS. Anthelmintic activity of extracts of *Artemisia absinthium* against ovine nematodes. Veterinary Parasit .2009; 160: 83-88.
3. Kordali S, Cakir A, Mavi A, Kilic H, Yildirim A. Screening of chemical composition and antifungal and antioxidant activities of the essential oils from three Turkish *Artemisia* species. J Agric Food Chem. 2005; 53: 1408-1416.
4. Lopes-Lutz D, Alviano DS, Alviano CS, Kolodziejczyk PP. Screening of chemical composition, antimicrobial and antioxidant activities of *Artemisia* essential oils. Phytochemistry 2008; 69: 1732-1738.
5. Canadanovic-Brunet JM, Djilas SM, Cetkovic GS, Tumbas VT. Free-radical scavenging activity of wormwood (*Artemisia absinthium* L.) extracts. J Sci Food Agric. 2004; 85: 265-272.
6. Nitzsche W. Germplasm preservation. *In* D.A. Evans, W.R. Sharp, P.V. Ammirato, and Y. Yamada (eds.), Handbook of Plant Cell Culture. V1. Collier Macmillan Publishers, London, pp. 1983; 782-805.
7. Rick CM. Plant germplasm resources. *In* P.A. Ammirato, D.A. Evans, W.R. Sharp, and Y. Yamada (eds.), Handbook of Plant Cell Culture V2. Collier Macmillan Publishers, London, pp. 1984; 9-37
8. Stanilova MI, Ilcheva VP and Zagorska, NA. Morphogenetic potential and *in vitro* micropropagation of endangered plant species *Leucojum aestivum* L. and *Lilium*. 1994 May; 13(8): 451-453.
9. Kalpana J, Chavan P, Warude D, Bhushan P. Molecular markers in herbal drug technology. Curr Sci. 2004; 87: 159-165.
10. Zhang YB, Shaw PC, Sze CW, Wang ZT, Tong YZ. Molecular authentication of Chinese herbal materials. Food Drug Anal. 2007; 15: 1-9.
11. Shcher NJ, Carles MC. Genome-Based approaches to the authentication of medicinal Plants. Planta Med. 2008; 74: 603-623.
12. Collard BCY, Mackill DJ. Conserved DNA-Derived polymorphism (CDDP): A simple and novel method for generating DNA markers in plants. Plant mol Bio Rep. 2009; 27: 558–562.
13. Hosokawa K, Miami K, Kawahara K, Nakamura I, Shibata T. Discrimination among three species of medicinal plant *Scutellaria* plants using RAPD markers. Planta Med. 2000; 66: 270-272.
14. Bussel JD, Waycott M, Chappill JA. Arbitrarily amplified DNA markers as characters for phylogenetic inference. Persp Plant Ecol Evol Sys. 2005; 7: 3-26.
15. Paran I, Michelmore RW. Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. Theo Appl Genet. 1993; 85: 985-993.
16. Wang J, Ha WY, Ngan FN, But PPH, Shaw PC. Application of sequence characterized amplified region analysis to authenticate *Panax* species and their adulterants. Planta Medica. 2001; 67: 781-783.
17. Lee MY, Doh EJ, Park CH, Kim YH, Kim ES, Ko BS, Oh SE. Development of SCAR marker for discrimination of *Artemisia princeps* and *A. argyi* from other *Artemisia* herbs. Bio Pharm Bull. 2006; 29: 629-633.
18. Nin S, E Morosi, S Schiff, A Bennici. Callus culture of *Artemisia absinthium* L. initiation, growth optimization and organogenesis. Plant Cell Tissue Organ.Cult. 1996; 45: 67-72.
19. Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant. 1962; 15: 473-497.
20. Doyle JJ, Doyle JL. Isolation of plant DNA from fresh tissue. *Focus.* 1990; 12: 13-15.
21. McClelland M, Mathieu DF, Welsh J. RNA fingerprinting and differential display using arbitrarily primed PCR. *Trends Gene.* 1995; 11: 242-246.
22. Sambrook J, Russell DW. Molecular cloning: A Laboratory manual. Cold Spring Harbor, Laboratory Press, New York. 2001.
23. Benjamin BD, Sipahimalani AT, Heble MR. Tissue culture of *Artemisia pallens:* organogenesis, terpenoid production. Plant Cell Tissue Organ Cult. 1991; 21: 159-164.
24. Xu ZQ, Zia JF. Callus formation from protoplasts of *Artemisia sphaerocephala* Krasch and some factors influencing protoplast division. Plant Cell Tissue Organ Cult. 1996; 44: 129-134.
25. Nam-cheol K, Kim JG, Lim JH, Hahn TR. Production of secondary metabolites by tissue culture of *Artemisia annua* L. J Korean Agri Chem Soc. 1992; 35: 99-105.
26. Geng S, Chun YH, Gufeng L, Mi M, Chong K, Geng S, Ye HC, Ma M, Chong K. Flowering of *Artemisia annua* L. test tube plantlets and Artemisinin production with shoot cluster induced from flower organs explants. Chinese J App Environ. Biol. 2001; 7: 201-206.
27. Lee YM, Hisiao G, Chang JR, Yen MH. Regeneration of Egyptian medicinal plant *Artemisia judicia* L. Plant Cell Reports. 2003; 21: 525- 530.
28. Aslam N, Zia M, Chaudhary MF. Callogenesis and direct organogenesis of *Artemisia scoparia.* Pak J of Bio Sci. 2006; 9: 1783-1783.
29. Mackay WA, Kito SI. Factors affecting in vitro shoot proliferation of French Tarragon. Hortic Sci. 1988; 113: 282-287.
30. Khan M, Bashir AG, Azra NK, Kharid GM. *In vitro* plant regeneration of critically endangered medicinal plant *Artemisia amygdalina* D. Int. Res. J. Pharm. 2014; 5: 2.
31. Almaarri K, Yu X. *In vitro* direct organogenesis and micropropagation of *Artemisia annua.* J of Biotechnologie Vegetale. 2010; 26: 327-337.
32. Zia M, Mannan A, Chaudhary MF. Effect of growth regulators and amino acids on Artemisinin production in the callus of *Artemisia absinthium*. Pak J Bot. 2007; 39: 799-805.

33. Sujatha G, Kumari BD. Effect of phytohormones on micro propagation of *Artemisia vulgaris* L. Acta Physiol. Plant. 2007; 29: 189-195.
34. Williams JGK., Kubelik AR, Livak K.J, Rafalski JA, Tingey SV. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucl. Acids. Res. 1990; 18: 6531-6535.
35. Shiju M. Molecular Profiling of Bauhinia accessions using RAPD Markers. Int J Pharm Bio Sci. 2010; 2: 199-207.
36. Adinolfi BA, Chicca E, Martinotti E, Breschi MC, Nieri P. Sequence Characterized amplified region (SCAR) analysis on DNA from three medicinal *Echinacea* species. Fitoterap. 2007; 78: 43-45.
37. Chavan PD, Warude K, Joshi K, Patwardhan B. Development of SCAR (Sequence- characterized amplified region) markers as a complementary tool for identification of ginger from crude drugs and multicomponent formulations. Bio Appl Bio. 2008; 50: 61-69.
38. Sze SC, Song JX, Wong RN, Feng YB, Ng TB, Tong Y, Zhang KY. Application of SCAR analysis to authenticate *Lycium barbarum* and its adulterants. Bio Appl Bio. 2008; 51: 15-21
39. Jain N, Shasany AK, Singh S, Khanuja SPS, Kumar S. SCAR markers for correct identification of *Phyllanthus amarus*, *Phyllanthus fraternus*, *P. debilis* and *P. urinaria* used in scientific investigations and dry leaf bulk herb trade. Planta Med. 2008; 74: 1-6.
40. Yadav A, Ahmad J, Chaudhary AA, Ahmad A. Development of Sequence Characterized Amplified Region (SCAR) marker for the authentication of *Bacopa monnieri* (L.) Wettst. European J Med Plants. 2012; 2: 186-198.
41. Dhanya KS, Syamkumar, Sasikumar B. Development and application of SCAR marker for the detection of papaya seed adulteration in traded black pepper powder. Food Biotechnol. 2009; 23: 97-106.
42. Dhanya KS, Syamkumar, Siju S, Sasikumar B. SCAR markers for adulterant detection in ground chilli. British Food J. 2011; 113: 656-668.
43. Qian YQ, Ying-Xiong Q, Yan-Qi C, Jian-Xin Y, Shu-Zhen, Ming-Shui Z, Cheng-Xin F. Species specific SCAR markers for authentication of *Sinocalycnthus chinensis*.. Journal of Zhejiang University.-Science A. 2006; 7: 868-872.
44. Tani N, Kawahara T, Yoshimaru H, Hoshi Y. Development of SCAR marker distinguishing pure seedlings of the endangered species *Morus boninensis* from *M. boninensis* x *M. acidosa* hybrids for conservation in Bonin (*Ogosawara island*). *Conservation Genetics.* 2003; 4: 605-612.
45. Sun YW, Liao YJ, Hung YS, Chang JC, Sun JM. Development of ITS sequence based SCAR marker for discrimination of *Paphiopedilum armeniacum*, *Paphiopedilum micranthum*, *Paphiopedilum delenatii* and their hybrids. Scientia Horticulturae. 2011; 127: 405-410

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