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# RAPD studies of *Saraca asoca* by fluorescent-labeled primers and development of micropropagation protocol for its conservation

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**Abstract**

RAPD analysis of the *Saraca asoca* populations revealed highly variable identified markers. It was taken up with three fluorescent-labeled RAPD primers by a high speed automated DNA fragment analysis device based upon the automated fluorescent DNA sequencer with the molecular taxonomy software FreeTree and TreeView. The 769 amplification products produced ranged from 50.14 to 497.37 bp. Out of the 769 bands, 713 were polymorphic bands and 56 bands were monomorphic. Primer-2 produced amplification products that were monomorphic across all the plants. The average polymorphism was 356.5 and percentage polymorphism was 92.7%. The three primers generated high percent of polymorphic loci, which means that genetic polymorphism in the population, is generally high. Altogether, there is a similarity between Cp-1 and Sm-2. Therefore, there are two distinct groups or clusters: Cp-1 and Sm-2 in cluster-1 and ABC & D in cluster-2. There is a good degree of similarity between B and C implying that the accession C, procured from Karthikavanam, Dhulapally might have been raised from the same stock as the B which is a 50-year-old tree in the Osmania University Botanical garden or must have been developed from the seeds of B. Efficient and high frequency shoot regeneration was achieved from the nodal explants of the Sa-B accession with MS medium supplemented with Kinetin and Adenine sulphate, followed

by *in vitro* rooting with 9.84 µM Indole butyric acid. Regenerated plantlets were hardened and established in the field with high survival. This highly efficient micropropagation protocol directly from nodal explants through complete plant regeneration is a first report in *Saraca asoca.*

**Keywords:** *Saraca asoca*, accessions, RAPD, genetic similarity, micropropagation.

## INTRODUCTION

The Ashoka [*Saraca asoca* (Roxburgh) De Wildeman] (Caesalpiniaceae) is a rain-forest tree grown as an ornamental valued for its beautiful foliage and fragrant flowers. Ashoka grows in Pakistan, India, Bangladesh, Sri Lanka, Mayanmar and Malaysia. It is an erect, small evergreen tree, with deep green leaves growing in dense clusters and bright flowers. The bark and flowers of *S. asoca* tree are used as medicine for uterine bleeding and menorrhagia and to treat depression in women [1].

Overharvesting of *S. asoca* for medicinal use and high deforestation resulted in severe reduction in natural populations of this species [2], which is currently listed as a 'globally vulnerable' species by the IUCN (http://www.iucnredlist.org/apps/redlist/details/34623/0). Natural populations need to be preserved and successfully managed and depend on accurate assessment of genetic diversity. Information on genetic diversity within a population is important for the conservation of the species. RAPD profiling is extensively used to analyze the genetic variability in bacteria, fungi and plants and for study of genetic diversity within and between populations because of the ease of methodology and the cost-effectiveness involving a smaller number of samples [3, 4, 5, 6, 7, 8].

For propagation of *S. asoca*, nursery raising from seeds and stem grafting are generally used. However, the plant is difficult to grow and the success rate is less than 20%. Therefore, there is a need for the development of alternate methods of propagation like micropropagation through *in vitro* culture [9]. A large number of true to type plants can be propagated within a short time and space and throughout the year through tissue culture of nodal explants (micropropagation) [10].

The present study was taken up to assess genetic diversity by evaluating percentage polymorphism in *S. asoca* by using the fluorescent labeled RAPD primers method and to develop an efficient micropropagation protocol of *S. asoca* to aid in its conservation.

## MATERIAL AND METHODS

Six accessions of *Saraca asoca* (Roxb.) De Wilde. were studied presently. While one six-year-old accession Sa-A was from AP Medicinal Plants Board, Chilkur and Sa-B, was the 50 year-old tree growing in the Botanical garden of Osmania University, Hyderabad, four 6-year old accessions (Sa-C, Sa-D, Cp-1 and Sm-2) were collected from different locations [Sa-C from Karthikavanam, Dhulapally; Sa-D from Urban

forestry, Erragadda; Cp-1 from Herbal garden, Rajahmundry; Sm-2 from Herbal garden, Rajendranagar]. All the plants were authenticated by the Head, Department of Botany, Osmania University, Hyderabad and planted in the Botanical garden, Osmania University.

## RAPD analysis

RAPD was taken up to evaluate the genetic similarity/diversity in various accessions of *Saraca asoca*: Sa-A, Sa-B, Sa-C, Sa-D (referred in the figures as A, B, C and D respectively), Cp-1 and Sm-2. For the study, plant genomic DNA (Mini Prep) was isolated, Fluorescent labeled primers selected, and the DNA analyzed after PCR amplification by a high-speed automated DNA fragment analysis device based upon the automated fluorescent DNA sequencer (A.L.F., Pharmacia) [11] by using the molecular taxonomy software FreeTree and TreeView.

### *Isolation of Plant Genomic DNA (Mini Prep)*

Plant Genomic DNA from fresh young leaves was isolated with genomic DNA isolation kit (RKN 09). All centrifugation steps are carried out at 17,900xg (10,000 rpm) in a conventional table-top microcentrifuge at room temperature and the DNA pellet was dried at 37°C for 10 min. and suspended in 50 µl of glass-distilled water by placing the vial at 65°C for 15 min or at 4°C overnight. The quality of the isolated DNA was checked through electrophoresis on 1% agarose gel with ethidium bromide by using Hind III+ λ DNA as standard. The yield of DNA per gram of leaf tissue extract was measured using a UV spectrophotometer and determined by measuring the absorbance at 260 nm and 280 nm. The ratio of OD 260 / OD 280 indicates the purity of the given sample. The range of 1.7 - 1.9 ratio is considered as pure sample.

### *Primer Selection*

Twelve RAPD primers were labeled with 6-carboxyfluorescein (6-FAM) (Applied Biosystems, USA), which gives blue color in Genescan analysis and used for amplification. However, only three primers could give positive results: Primer-1: 5'AGGHCTCGATAHCMGVY3'; Primer-2: 5'CCCHGCAMCTGMTCGCACHC3' and Primer-3: 5'MTGTAMGCTCCTGGGGATTCHC3'.

### *PCR amplification*

The PCR amplification reaction was carried out with 50 µl reaction mixture comprising DNA (1 µl), RAPD fluorescent primer (4 µl), dNTPs (10mM) (2 µl), PCR assay buffer (10X)(5 µl), Taq DNA polymerase (0.5 µl) and nuclease free water (37.5 µl). PCR amplification was carried out in a ABI 3130 Genetic analyzer with the following conditions: Initial denaturation at 94ºC for 5 min, denaturation for 40 cycles at 94º C for 1 min, annealing for 1 min at 50º C, 2 min for extension at 72ºC and final extension

at 72° C for 2 min. The size marker was the 100-bp ladder. Amplification products were analyzed by a high-speed automated DNA fragment analysis device based upon the automated fluorescent DNA sequencer (A.L.F., Pharmacia) [11] by using a molecular taxonomy software.

### *DNA analysis*

RAPD-PCR amplified fragments were scored as 1 for present and 0 for absent. The binomial data generated was used to estimate the level of polymorphisms by dividing the polymer bands by the total number of scored bands. The RAPD data was analyzed using the FreeTree and TreeView software. The program computes the distance matrix, constructs the phylogenetic or phonetic tree (dendrogram) using unweighted pair group method with arithmetic averages (UPGMA) or neighbour-joining, and computes bootstrapping values for internal branches of the tree. The data was pasted into the spreadsheet of the "New analysis" of FreeTree and the Nei-Li distance [12] with 7 different similarities/distance and the method of tree construction, e.g. neighbour-joining [13] were selected to get the distance matrix. The Reference tree was checked on the basis of this distance matrix. This form of the tree was pasted into the program for drawing phylogenetic trees (dendrograms) [14]. Pairwise comparision between accessions based on the proportion of shared bands produced by the primers used, were calculated using Jaccard's similarity [15]. Polygenetic Tree (dendrogram) was constructed by UPGMA (Unweighted Pair Group Method with Arithmetic mean) method to measure the resulting phenotypic groups.

## Micropropagation studies

The nodal explants of the identified accession were thoroughly washed under running tap water and surface-sterilized on a laminar air-flow bench by washing with autoclaved distilled water containing a few drops of Tween-20, rinsing thoroughly with double distilled autoclaved water, treatment with 0.1% (w/v) mercuric chloride, rinsing with autoclaved distilled water, washing with 5% sodium hypochlorite and finally rinsing with autoclaved distilled water.

The Murashige and Skoog (MS) medium [16] was used with 3% (w/v) sucrose, solidified with 0.8% (w/v) phyta-agar (Hi-media, India), with various growth regulators [Naphthalene acetic acid (NAA), 6-benzylaminopurine (BAP), Kinetin (KN) and Adenine sulphate (AS)] either singly (BAP or KN) or in combination viz. BAP with NAA and KN with AS. The pH of the medium was adjusted to 5.8, autoclaved at 121° C for 15 lbs/cm $^2$ for 15 min and poured into sterile culture tubes (25 x 150 mm).

Thirty explants were inoculated on each culture medium with three replicates amounting to a total of ninety. The cultures were incubated in a sterile growth room at 25 ± 1°C with cool fluorescent light (irradiance 80 µmol $m^{-2}s^{-1}$) at 16 h photoperiod with 60% humidity. The regenerated shoots (multiple nodes excised into single units) were sub-cultured every 3-4 weeks on the same media for elongation. The regenerated

shoots (3-4 cm) were transferred to the root induction media for optimization [MS medium with different concentrations of Indole butyric acid (IBA)].

The frequency of regeneration of shoots / multiple shoots on the shoot induction media was determined as the percentage of the total cultured explants that responded. The frequency of rooting from the regenerated shoots was determined as the percentage of the total cultured shoots that responding by producing roots. Regenerated plantlets with well-established roots were taken out of the culture tubes carefully, washed with water and transferred to pots containing sand and manure (1:1) and were kept covered with plastic bags under controlled conditions of 70 - 80% humidity and temperature of 25 ± 1 ºC for about 10-15 days. The plantlets were later transferred to the glasshouse and slowly exposed to outer environment at 35 ± 2 ºC with 80% relative humidity. Finally, the plants were transferred to field after four weeks. Survival percentage of the plantlets was calculated after three months and presented as the percentage.

## Statistical analysis

All the experiments were set up in a completely randomized design with three replications per treatment and the assays were performed in triplicate and expressed as mean±SE.

## RESULTS AND DISCUSSION

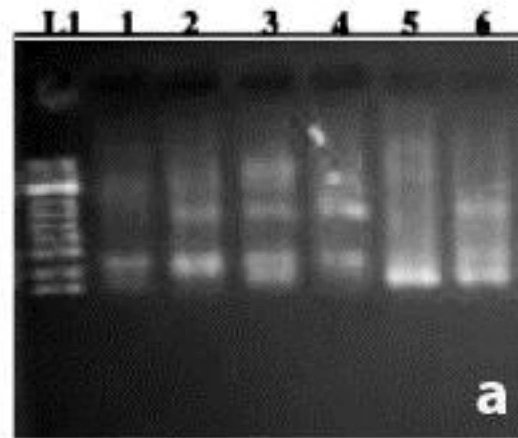
The results obtained the molecular analysis (with fluorescent labeled RAPD primers) of accessions of *S. asoca* for genetic diversity and the development of an efficient protocol for its micropropagation as an aid in its conservation are discussed.

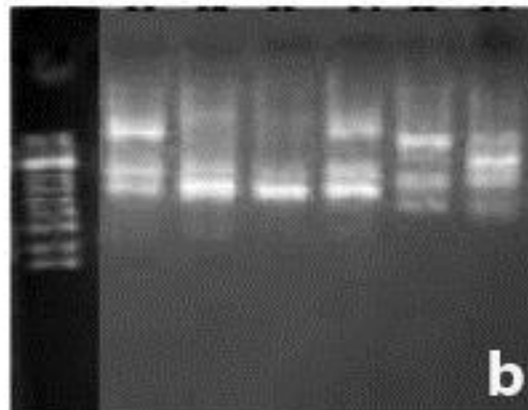
## RAPD analysis

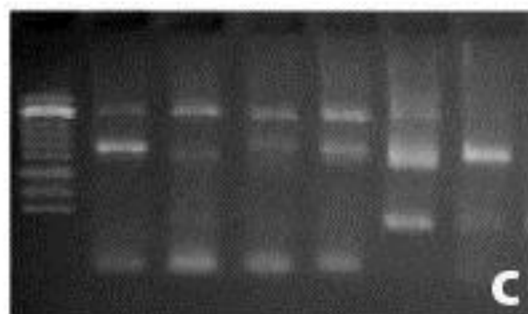
### *Polymorphism in the six accessions of Saraca asoca*

The PCR products were analyzed by scoring twelve primers initially but only 3 primers resulted in good banding patterns. However, only the primer-2: (5'AGGHCTCGATAHCMGVY3') elicited good amplification with reliable and clear band resolution (Fig. 1). The others, primer-1: 5`CCCHGCAMCTGMTCGCACHC3' and primer-3: 5'MTGTAMGCTCCTGGGGATTCHC3' elicited moderate amplification which were not as clear. The 769 amplification products produced ranged from 50.14 to 497.37 bp (Fig. 1). Out of the 769 bands, 713 were polymorphic bands and 56 bands were monomorphic. The average polymorphism was 356.5 and percentage polymorphism was 92.7%. From the 3 primers tested, only the primer-2 produced amplification products that were monomorphic across all the plants. The three primers generated high percent of polymorphic loci, which means that genetic polymorphism in the population, is generally high. An amplified product is considered polymorphic if it occurs in less than 95% of the total individuals assayed. The scale indicates the genetic distance between individuals. Despite of the small size of the

population, which generally exhibit lower levels of genetic diversity, our results based on the RAPD technique showed that the genetic diversity of this endangered plant species is not low.



***Distance Matrix and Genetic Similarity Index***

Distance matrix was calculated by Nei and Li/Dice Tree Construction Neighbor joining method and presented in Table 1. This shows the similarity index/distance matrix between the six accessions of *S. asoca*. The distance matrix calculated by the Nei's standard distance DsTree construction method of UPGMA (Unweighted Paired Group Method with Arithmetic Mean) shows the genetic diversity among the six accessions of *S. asoca* (Table 2). The similarity matrix calculated by Jaccard's Coefficient (Table 3) also showed the extent of similarity between the six accessions. The lowest similarity was between [A] and [Sm-2] (0.03593) and the highest similarity was between [B] and [C] (0.45455) (Table 3). In a summary, there is 100% similarity between Cp-1 and Sm-2, 99% similarity between B and C and 77% similarity between A and B & C. There is 100% similarity between D and all the three accessions i.e. A and B & C. Altogether, there is a similarity between the two groups of Cp-1 and Sm-2. Therefore, there are two distinct groups: Cp-1 and Sm-2 on one side and ABC & D on the other. Further, D is distinct from A, B & C. Similarly, there is a distinct grouping of B & C from A. There is a good degree of similarity between B and C implying that the accession C, procured from Karthikavanam, Dhulapally might have been raised from the same stock as the B, which is a 50-year-old tree in the Osmania University Botanical garden or must have been developed from the seeds of B. Further, the dendrogram of six accessions of *S. asoca* represented by the phylogenetic and reference trees based on the genetic distance generated by three fluorescent-labeled RAPD primers (Fig. 2) shows that there are 2 clear clusters. Cluster-1 with the accessions Cp-1 and Sm-2 and cluster-2 with A, B, C and D.

**Table-1:** Distance Matrix Table of the 6 accessions of *S. asoca*

Data input | Distance/similarity matrix | Reference tree | Trees | Resampling methods

A - Nei and Li / Dice

Tree-construction method: UPGMA / (•) Neighbor-joining

| | A | B | C | D | cp1 | sm2 |
|---|---|---|---|---|---|---|
| A | | 0.49315 | 0.51493 | 0.58156 | 0.90476 | 0.93064 |
| B | 0.49315 | | 0.37500 | 0.52448 | 0.83721 | 0.83051 |
| C | 0.51493 | 0.37500 | | 0.54198 | 0.89189 | 0.89542 |
| D | 0.58156 | 0.52448 | 0.54198 | | 0.82716 | 0.82036 |
| cp1 | 0.90476 | 0.83721 | 0.89189 | 0.82716 | | 0.58491 |
| sm2 | 0.93064 | 0.83051 | 0.89542 | 0.82036 | 0.58491 | |

Note: A, B, C, D, cp1 and sm2 refer to the accessions Sa-A, Sa-B, Sa-C, Sa-D, Cp-1 and Sm-2.

**Table-2:** Distance matrix calculated by R-Nei's standard distance Ds coefficient of the 6 accessions of *S. asoca*

Data input | Distance/similarity matrix | Reference tree | Trees | Resampling methods

R - Nei's standard distance Ds

Tree-construction method: (•) UPGMA ( ) Neighbor-joining

| | A | B | C | D | cp1 | sm2 |
|---|---|---|---|---|---|---|
| A | | 0.67945 | 0.72066 | 0.87100 | 1.99449 | 2.37680 |
| B | 0.67945 | | 0.46610 | 0.74273 | 1.44858 | 1.47420 |
| C | 0.72066 | 0.46610 | | 0.77942 | 1.91973 | 2.01435 |
| D | 0.87100 | 0.74273 | 0.77942 | | 1.41360 | 1.43922 |
| cp1 | 1.99449 | 1.44858 | 1.91973 | 1.41360 | | 0.87478 |
| sm2 | 2.37680 | 1.47420 | 2.01435 | 1.43922 | 0.87478 | |

Note: A, B, C, D, cp1 and sm2 refer to the accessions Sa-A, Sa-B, Sa-C, Sa-D, Cp-1 and Sm-2.

**Table-3:** Similarity matrix calculated by Jaccard's coefficient of the 6 accessions of *S. asoca*

Data input | Distance/similarity matrix | Reference tree | Trees | Resampling methods

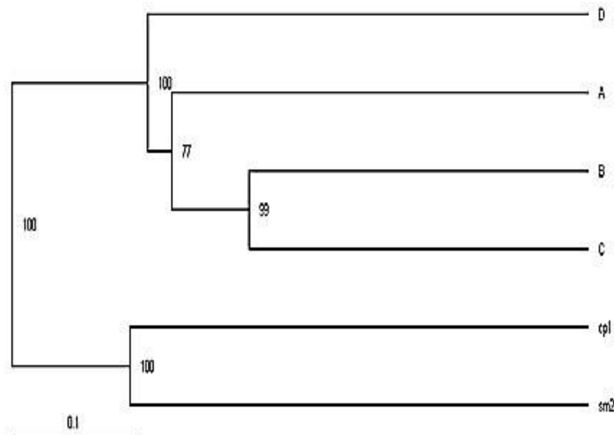
B - Jaccard

Tree-construction method: (•) UPGMA ( ) Neighbor-joining

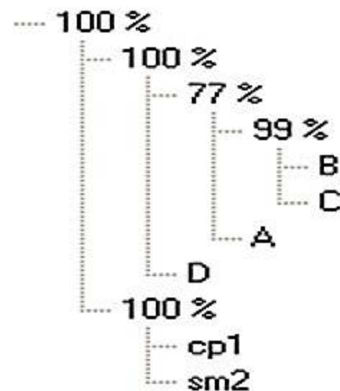
| | A | B | C | D | cp1 | sm2 |
|---|---|---|---|---|---|---|
| A | | 0.33945 | 0.32020 | 0.26457 | 0.05000 | 0.03593 |
| B | 0.33945 | | 0.45455 | 0.31193 | 0.08861 | 0.09259 |
| C | 0.32020 | 0.45455 | | 0.29703 | 0.05714 | 0.05517 |
| D | 0.26457 | 0.31193 | 0.29703 | | 0.09459 | 0.09868 |
| cp1 | 0.05000 | 0.08861 | 0.05714 | 0.09459 | | 0.26190 |
| sm2 | 0.03593 | 0.09259 | 0.05517 | 0.09868 | 0.26190 | |

Note: A, B, C, D, cp1 and sm2 refer to the accessions Sa-A, Sa-B, Sa-C, Sa-D, Cp-1 and Sm-2.

**Phylogenetic Tree:**




**Reference tree:**




**Note: A, B, C, D, cp1 and sm2 refer to the accessions Sa-A, Sa-B, Sa-C, Sa-D, cp1 and sm2.**

RAPD is one of the important molecular markers for identification of the individual species and has provided the polygenetic information of the population in a variety of taxonomic and genetic diversity studies [17, 18]. RAPD markers have the greatest advantage of its capability to scan across all regions of genome hence suited for phylogeny studies at species level [19, 20]. Sequencing based molecular techniques provide better resolution at intra-genus and above level, while frequency data from markers such as random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and microsatellites provide the means to classify individuals into nominal genotypic categories and are mostly suitable for intra-species genotypic variation study. This distinction is important to grasp for population studies, particularly when the diversity data is used as a basis for making decisions about conservation of plant resources. For instance, a recent study on Napier grass

(*Pennisetum purpureum*) has showed that RAPD is compatible with morphological data [7]. The source of polymorphism observed may be due to deletion, addition or substitution of base within the priming site sequence [21]. The present study confirms the suitability of RAPD as a reliable, simple, easy to handle and elegant tool in molecular diagnosis of 6 different accessions of *S. asoca* collected from different regions. This method can be used for identification of the original *S. asoca* plant. This method can also help distinguish *Saraca asoca* from other species or adulterants. Despite the small size of the population, which generally exhibit lower levels of genetic diversity, our results based on the RAPD technique showed that the genetic diversity of this critically endangered plant species is high.

**Micropropagation**

Micropropagation is the best tool to overcome problems in conventional propagation methods and for continuous supply of uniform genetic material, which greatly facilitates the mass propagation of plants within a short period and small area. There are several limitations for regular clonal multiplication of *S. asoca* for conservation and very few earlier reports are available with a few positive results of rooting of cuttings [22]. Multiple shoots and roots were reported from germinating seeds which could not however be processed further [25]. Apart from callusing in *S. asoca* [25], plant regeneration in *S. asoca* was met with very little success. The lone plant regeneration report [24] from several explants does not indicate comprehensive details about the regenerants. Axillary nodal explants have been chosen for investigation presently for achieving high frequency micropropagation since the axillary meristem propagates true-to-type plants. Selection of media, plant growth regulators and acclimatization process also play important roles in obtaining high frequency of plant regeneration. High frequency of regeneration was reported [24] with supplementation of BAP or KN, but use of 2, 4-D only produced callus [25].

The present study reports a rapid, reliable and reproducible protocol employing the nodal explant of the selected Sa-B accession because it contained highest amounts of medicinal components in an earlier study [26]. Shoot regeneration was recorded from adventitious shoot-buds that developed from axillary nodal meristem by the $4^{th}$ week and on subculture, the shoots elongated to about 3-4 cm in length with 1-2 internodes and fully expanded leaves (Fig. 3 A to E) (Table 4). The highest response (76%) of multiple shoot regeneration was observed on MS + KN (9.30 µM) + AS (2.47 µM) medium (Table 4). Although BAP alone could elicit moderate response, the addition of NAA helped in the increase of multiple shoots. Of all the culture media used for micropropagation of *S. asoca,* the highest efficient response of multiple shoots was recorded with the culture medium MS + KN (9.30 µM) + AS (2.47 µM) followed by MS + KN (4.65 µM) + AS (1.23 µM). Of both BAP and KN, the latter produced a better response which was improved by the addition of AS. The present efficiency of regeneration is much higher than the earlier report [24] and therefore very suitable for micropropagation of *S. asoca*.

**Table 4: Response of nodal explants of *S. asoca* in production of multiple shoots.**

| S. No | Culture medium | *Explants inoculated | Response (%) | Frequency of shoots (Mean± S.E.)* |
|---|---|---|---|---|
| 1 | MS+BAP (4.44 µM) | 30 x 3 | 46 | 13.8±0.53 |
| 2. | MS+BAP (8.88 µM) | 30 x 3 | 50 | 15.0±0.52 |
| 3. | MS+BAP (4.44 µM)+ NAA (2.68 µM) | 30 x 3 | 60 | 18.0±0.52 |
| 4. | MS+BAP (8.88 µM)+ NAA (5.37 µM) | 30 x 3 | 63 | 18.9±0.42 |
| 5. | MS+KN (4.65 µM) | 30 x 3 | 61 | 18.3±0.4 |
| 6. | MS+KN (9.30 µM) | 30 x 3 | 63 | 18.9±0.6 |
| 7. | MS+KN (4.65 µM) + AS (1.23 µM) | 30 x 3 | 70 | 21.0±0.62 |
| 8. | MS+KN (9.30 µM) + AS (2.47 µM) | 30 x 3 | 76 | 22.8±0.50 |

*All the analysis were carried out in triplicate and expressed as mean ± SE

Presently, the well-developed and healthy shoots of *S. asoca* were transferred to the root induction medium for induction of roots. The most suitable root induction medium that induced highest number of roots was MS + IBA (9.84 µM). Profuse roots were induced by the end of the third week with the highest percentage root induction (97%) recorded with the shoots regenerated on the MS+KN+AS culture medium (Fig. 3 F to G) (Table 5). Efficient root induction of 97% was presently observed with 9.84 µM of IBA, in contrast to the 19.68 µM IBA reported earlier [24]. Further, no rooting could be induced presently at the latter (higher) concentrations of IBA. However, the rooting efficiency depended on the sturdiness of the regenerated shoots, which in turn depended on the suitability of the culture medium. Healthy plantlets with well-developed roots were transferred to pots and gradually acclimatized in the glasshouse for 3 weeks and transferred to the Botanical garden (Fig. 3 H to I). A survival percentage of plants recorded in the field of 94.20%, with 390 successfully established plants out of the 414 rooted and transplanted plants. The protocols developed in the present study for shoot regeneration and rooting would be useful in micropropagation of *S. asoca* that can help conserve the plants, which are widely used in different medicinal systems of India. Further, the efficiency of micropropagation reported presently in *S. asoca* far exceeds the earlier reports.

**Table 5:** Rooting and establishment of regenerated plants of *S. asoca.*

| S. No | Shoot regeneration medium | No. of shoots transferred to rooting medium (MS+9.84 μM IBA) | No. of rooted shoots | Rooting response (%) | Survival of plantlets in the field (%) | No. of established plants in the field* |
|---|---|---|---|---|---|---|
| 1 | MS+BAP | 86 | 80 | 93.02 | 93.75 | 75 |
| 2 | MS+BAP+NAA | 110 | 102 | 92.72 | 90.19 | 92 |
| 3 | MS+KN | 110 | 104 | 94.50 | 94.23 | 98 |
| 4 | MS+KN+ AS | 131 | 128 | 97.70 | 97.65 | 125 |

* 94.20 % of the transplanted

## CONCLUSIONS

The current endeavor has accomplished the study of genetic diversity of the endangered plant *S. asoca* through molecular analysis of different accessions and the development of an efficient and repeatable protocol for *in vitro* propagation by adventitious shoot proliferation and complete plant regeneration followed by their establishment in the field with a very high survival percentage. Genetic diversity needs to be maintained for the long-term survival of endangered plant species and it helps the species to adapt to the changing environment. RAPD analysis of the *S. asoca* populations revealed highly variable identified markers, which can be used for population and genetic diversity studies within and between populations. This greatly helps in the identification of superior accessions, or for spotting adulterants and also for taking up major conservation measures of this endangered plant (through micropropagation) as it will circumvent the difficulty of acquiring the plants from seeds.

## CONFLICTS OF INTERESTS

The authors have declared that no conflict of interests exist in the paper.

## ACKNOWLEDGEMENTS

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## ABBREVIATIONS

bp Base pairs

BAP 6-Benzylaminopurine

KN Kinetin

AS Adenine sulphate

NAA Naphthalene acetic acid

IBA Indole-3-butyric acid

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