Molecular approach for sex identification and *in vitro* propagation of *Simarouba glauca* DC.

Thesis submitted to the University of Hyderabad for the award of

Doctor of Philosophy

by

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CERTIFICATE

This is to certify that **Mrs. Bramhanapalli Madhavi** has carried out the research work embodied in the present thesis under the supervision and guidance of Prof. G. Padmaja for a full period prescribed under Ph.D. ordinances of this University. We recommend her thesis entitled **"Molecular approach for sex identification and *in vitro* propagation of *Simarouba glauca* DC."** for submission for the degree of Doctor of Philosophy of the University.

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DECLARATION

I, **Bramhanapalli Madhavi**, hereby declare that this thesis entitled **"Molecular approach for sex identification and *in vitro* propagation of *Simarouba glauca* DC."** submitted by me under the guidance and supervision of **Prof. G. Padmaja** is an original and independent research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

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Sl. No.	Course Code	Name	Credits	Pass/Fail
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2.	PL 802	Research ethics and management	2	Pass
3.	PL 803	Biostatistics	2	Pass
4.	PL 804	Analytical techniques	3	Pass
5.	PL 805	Lab work	4	Pass

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Dedicated to

My husband, daughter

& parents

CONTENTS

ABBREVIATIONS AND SYMBOLS

AFLP	:	Amplified fragment length polymorphism
ANOVA	:	Analysis of variance
amu	:	Atomic mass unit
BAP	:	6-benzylaminopurine
BLAST	:	Basic local alignment search tool
bp	:	Base pair
BSA	:	Bulk segregant analysis
$CaCl_2$	:	Calcium chloride
CE	:	Catechin equivalents
cm	:	Centimeter
CTAB	:	Cetyltrimethylammonium bromide
°C	:	Degree Celsius
DARwin	:	Dissimilarity analysis and representation for windows
DNA	:	Deoxyribonucleic acid
dNTPs	:	Deoxynucleotide triphosphates
DW	:	Dry weight
EB	:	Elution buffer
EtBr	:	Ethidium bromide
EDTA	:	Ethylenediaminetetraacetic acid
Na_2EDTA	:	Disodium ethylenediaminetetraacetate dehydrate
eV	:	Electron volt
Fig	:	Figure(s)
gm	:	Gram
GA_3	:	Gibberellic acid
GAE	:	Gallic acid equivalents
GC-MS	:	Gas chromatography-Mass spectrometry
$HgCl_2$	:	Mercuric chloride
HC	:	Hierarchical clustering
HCl	:	Hydrochloric acid
h	:	Nei's gene diversity
hr	:	Hour

I	:	Shannon's information index
IBA	:	Indole-3-butyric acid
ISSR	:	Inter simple sequence repeat
IPTG	:	Isopropyl-beta-D-thiogalactoside
Kb	:	Kilobase pair
L	:	Litre
LB	:	Luria-Bertani
$MgCl_2$	:	Magnesium chloride
$MnCl_2$	:	Manganous chloride
M	:	Molar
mg	:	Milligram
min	:	Minute
ml	:	Milliliter
mM	:	Millimolar
µg	:	Microgram
µM	:	Micromolar
µl	:	Microlitre
MS	:	Murashige and Skoog's
m/z	:	Mass by charge number
ng	:	Nanogram
NCBI	:	National Centre for Biotechnology Information
NJ	:	Neighbor-joining
NAA	:	α-Naphthaleneacetic acid
NaCl	:	Sodium chloride
NaOH	:	Sodium hydroxide
na	:	Observed number of alleles
ne	:	Effective number of alleles
ORF	:	Open Reading Frame
PIC	:	Polymorphic information content
PVP	:	Polyvinylpyrrolidone
PCR	:	Polymerase Chain Reaction
PCoA	:	Principal Coordinates Analysis
RNA	:	Ribonucleic acid

RAPD	:	Random amplified polymorphic DNA
RFLP	:	Restriction fragment length polymorphism
RE	:	Rutin equivalents
RNase	:	Ribonuclease
ROS	:	Reactive oxygen species
rpm	:	Revolutions per minute
sec	:	Second
SSR	:	Simple sequence repeats
SNP	:	Single nucleotide polymorphism
SCAR	:	Sequence characterized amplified region
SE	:	Standard error
SCoT	:	Start codon targeted polymorphism
Taq	:	*Thermus aquaticus*
TAE	:	Tris–acetate–EDTA
TE	:	Tris- EDTA
Tris-HCl	:	Tris hydrochloride
TDZ	:	Thidiazuron
UV	:	Ultraviolet
UPGMA	:	Unweighted pair group method based on arithmetic average
V	:	Volts
v/v	:	Volume by volume
w/v	:	Weight by volume
WP	:	Woody plant
X-gal	:	5-Bromo-4-chloro-3-indolyl-beta-D-galactoside

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ABSTRACT

Simarouba glauca DC. is a multipurpose, eco-friendly, fast growing valuable tree species known for oil, medicinal properties, and timber. It is a polygamodioecious tree species that belongs to family Simaroubaceae. The seeds are rich in oil with the potential to be used as biodiesel. For commercial purpose, plantations with females and males in the ratio of 25:1 are desired for realizing economic benefits. In the present study, 61 random amplified polymorphic DNA (RAPD) and 24 inter simple sequence repeat (ISSR) primers that produced clear amplification were used to assess the molecular genetic variability among female, male and andromonoecious genotypes of *S. glauca*. The ISSR markers revealed higher average number of polymorphic loci (4.92), polymorphic percentage (61.48%), polymorphic information content (PIC) value (0.13), observed number of alleles (na: 1.62), effective number of alleles (ne: 1.36), Nei's gene diversity (h: 0.21), and Shannon's information index (I: 0.31) than RAPD primers (average number of polymorphic loci: 3.43, polymorphic percentage: 46.8%, PIC: 0.09, na: 1.45, ne: 1.27, h: 0.16, and I: 0.24). The mean PIC values for female, male and andromonoecious sexes using RAPD (0.09, 0.07, and 0.10, respectively) and ISSR markers (0.16, 0.08 and 0.13, respectively) revealed less molecular genetic variation among male genotypes than female and andromonoecious genotypes. Thus, the RAPD and ISSR primers were informative in revealing the molecular genetic variability in different sexes of *S. glauca*, and this information can be utilized in its breeding, selection and conservation programmes.

The sex differentiation mechanism is unknown in *S. glauca* and the sex of this tree species cannot be distinguished prior to flowering based on morphological characteristics. Thus, there is a need to identify the molecular markers for identification of sex at seedling stage in *S. glauca* as it exhibits sex-specific commercial value. Out of 61 RAPD and 24 ISSR primers screened on different sexes of *Simarouba,* only 3 RAPD primers *i.e.,* OPA-12, OPK-10, and OPA-08 generated sex-specific fragments of ~2900 bp, ~1250 bp, and ~350 bp, respectively. The sex-specific bands were cloned, sequenced and converted into more reliable SCAR (Sequence Characterized Amplified Region) markers. Protein BLAST analysis of translated sequence for 1239 bp fragment revealed the presence of conserved putative domain for retrotran_gag_2. The SCAR markers for only two primers, OPK-10, and OPA-08 resulted in sex-specific

amplification when tested on all *Simarouba* genotypes. The SCAR marker Sg SCAR1 differentiated female and andromonoecious genotypes from males by producing 1063 bp band whereas SCAR marker Sg SCAR2 differentiated male and andromonoecious genotypes from females by exhibiting sex-specific amplification of 341 bp band. Additionally, the SCAR markers developed were validated on trees of known sex types of a different location (IIOR, Narkhoda) and the sexes of seedlings and plants of unknown sexes growing in the campus of University of Hyderabad were determined successfully in the study. Thus, for the first time, a two-level screening strategy was developed in the study that has successfully identified the sex type of young plants of *Simarouba* prior to flowering. The SCAR markers developed can be exploited for raising plantations in the desired ratio for achieving economic gains.

Here, we evaluated the phytochemical differences in relation to sex in leaf and shoot apex samples of *S. glauca*. The methanolic extracts of leaves and shoot apex of male plants showed higher total phenolic contents in males than female and andromonoecious genotypes of *S. glauca*. The total flavonoid and tannin content varied depending on the extract and sex. The highest total antioxidant activity and DPPH free radical scavenging activity was observed for shoot apex methanolic extracts of males as compared to other genders which correlated with higher total phenolic contents in the methanolic extracts. The GC-MS analysis also revealed differences in sex-related metabolites like testosterone (testosterone, 17-O-(t-butyldimethylsilyl)), estrogens (estra-1,3,5(10)-trien-4-amine, 3,16,17-tris[(trimethylsilyl)oxy]-, (16à,17á)-), and epiandrosterone (o-tetramethylen (tert-butyl) silylepiandrosterone) among different sexes of *S. glauca*. The differences in primary and secondary metabolites in leaf and shoot apex samples identified in different sexes pave way for further studies related to sex and medicinal properties of this tree species.

The present study led to development of an efficient *in vitro* regeneration system using explants derived from *in vitro* germinated seedlings. A two-step procedure for achieving high shoot bud induction and elongation was developed in the study with the best response observed from cotyledonary explants followed by epicotyl and hypocotyl explants. The *in vitro* regenerated shoots were rooted *in vitro* and established in soil with high success rate. The genetic similarity among regenerated plants was evaluated using 14 RAPD and 15 ISSR primers which revealed a high number of monomorphic bands, with only 1.6-2.6% of the bands being polymorphic. The *in vitro* regeneration

system established in the study has the potential to be used for rapid multiplication, conservation, genetic transformation and secondary metabolite production of this species.

Key words: Genetic variability, Sex identification, Molecular markers, RAPD, ISSR, SCAR, Metabolites, Polyphenols, Flavonoids, Tannins, GC-MS, Animal sex hormones, *In vitro* plant regeneration, Genetic stability, *Simarouba glauca.*

Introduction

1. INTRODUCTION:

1.1. Inevitability for oil-yielding trees in India:

The economic development in countries like India with a vibrant economy depends on the self-dependence of energy resources, which devours an enormous amount of fossil fuels. In India, oil is considered as the primary source of energy for 95% of transportation and therefore the demand for fossil fuels is increasing in day-to-day life. According to IPCC (Intergovernmental Panel on Climate Change), the demand for oil will rise from 75 MB/day for the year 2002 to 120 MB/day for 2030 globally (Dhyani *et al*. 2015). The fossil fuels cause deteriorating effects on the atmosphere and leads to global warming. Moreover, due to the uncertainty in the supply and increased prices, fossil fuels in the international market are imposing severe problems to the economy of developing countries (Wani and Sreedevi 2005). To overcome the problem of shortage of oil, different forms of renewable energy sources like hydrothermal energy, solar energy, bioenergy, windmills, etc. are being exploited commercially.

A committee constituted in 2003 reported to Planning Commission of India for the utilization of biofuel species, which are of global importance and serve as an alternate source of energy with less air pollution and a lower price (Okoro *et al*. 2011) compared to fossil fuels. The funding agencies like NOVOD (National Oilseeds & Vegetable Oils Development) Board, DBT, DST, ICAR, CSIR, and DRDO have funded several research organizations for R & D development and commercial exploitation of plant species that are potential sources of biodiesel. During the period of 10^{th} plan, a scheme called 'Integrated Development of Tree Borne Oilseeds' was introduced to couple the present and future potential of TBOs like Jatropha, Karanja, Mahua, Neem, *Simarouba*, Jojoba, Wild Apricot, Cheura, Kokum, etc. Different states of India have promoted programs for R & D on biofuels.

The utmost importance in India is given to agriculture, where land more than 50% is cultivable and therefore it is essential to promote systems based on bioenergy. At present, the demand for alternatives to petroleum-based fuels is increasing due to the depletion of natural resources for these fuels. Due to this demand for alternate bioenergy sources, replacing the petrol with agriculturally produced oil in engines attained significance (Energy Information Administration). The use of edible oils for biofuel production increases the competition for oils at a global level and further leads to

scarcity for edible oil (Gui *et al*. 2008). More than 90% of the oil used for biodiesel production exploited edible oils in 2007. With respect to edible oil, India is a net importer, thus there is a need to identify plants such as Karanja, Jatropha, *Simarouba*, etc. which produce non-edible oils as a source for biofuel production. In order to achieve self-sufficiency concerning edible oil and biodiesel, tree-borne oilseeds play an important role in this direction. Among different TBOs (Tree Borne Oilseeds), *Simarouba glauca* DC. is promoted as an important tree for oil production, and interestingly this tree can be cultivated even on wastelands. *S. glauca*, as a source of vegetable oil and biodiesel, offers scope to overcome the problem of oil shortage for the next generations.

1.2. Economic importance of *S. glauca*:

S. glauca DC. commonly known as a Paradise tree is a fast-growing tree species having multiple uses. The seeds of *Simarouba* have high oil content (60-75%) rich in both saturated and unsaturated fatty acids, making it suitable for human intake as well as for industrial purposes (Joshi and Hiremath 2000). The refined oil of *Simarouba* can be used in the manufacture of vegetable fat or margarine (Armour 1959; Joshi and Hiremath 2001). The seed oil of *S. glauca* is used for industrial purposes in the manufacture of soaps, lubricants, paints, polishes, and pharmaceuticals.

Apart from oil bearing seeds, *S. glauca* has several medicinal properties attributed to different parts such as bark, wood, leaves, roots, and seeds. Traditionally, it is used to cure diarrhea, fever, and amoebic dysentery and decoction prepared from bark has been externally applied to treat skin infections and wounds. The leading group of active phytochemicals of *Simarouba* are quassinoids which are known to have medicinal properties (Govindaraju *et al*. 2009; Patil and Gaikwad 2011). The wood of *S. glauca* is light in weight and mostly resistant to insects, therefore used in the manufacture of paper pulp, match sticks, furniture, toys, etc. Overall, *Simarouba* has a well-developed root system that favours the microbial life of the soil, increases the underground water availability, effectively checks soil erosion, and also global warming throughout the year, especially in summer.

1.3. Research problem statement:

S. glauca is a polygamodioecious tree that bears yellow flowers and shows variations in floral characteristics where 5% segregates into males, 40-50% segregates into females, and 40-50% segregates into andromonoecious sexes having few bisexual and mainly male flowers. It starts flowering in 4-6 years with females having commercial value for seeds. In total, only 50% of trees are productive and thus for plantation purposes, the ratio of females and males in 25:1 is recommended as it enables effective utilization of space and resources. The sex of this tree species is known only at the time of flowering as there are no morphological differences between the different sexes that can be used for identifying the sex in young plants. Therefore, sex identification at early seedling stage is essential for plant breeders to raise the plantations in the desired sex ratio. Hence, it is highly desirable to develop molecular marker(s) for early identification of the sex in *S. glauca*. The analysis of genetic variation provides an important input in the breeding and conservation programmes aimed at improvement of this tree species. Consequently, molecular and biochemical approaches will be useful to analyze genetic variations among different sex genotypes and identify sex-specific marker(s). Although this tree species is known to have many medicinal properties which are due to the presence of phytocompounds, sex-related phytochemical studies have not received attention in this tree species. Analysis of metabolites among genders of *S. glauca* would provide more insights into sex determination process at metabolite level. The seeds of *S. glauca* have been reported to have short viability for 2-3 months at ambient temperature which restricts its large-scale plantation throughout the year. *In vitro* methods hold promise in rapid multiplication of elite and desired sex types; facilitate genetic transformation of this tree species for the traits of interest and for secondary metabolite production and their enhancement. These research gaps should be addressed to encash the advantages of this multipurpose tree species.

1.4. Molecular markers for detection of genetic variability in different sexes:

DNA markers are known to be highly accurate and efficient for studying genetic variation as they are not influenced by environmental factors, type of tissue, stage of ontogenic development in individuals, gene regulation and post transcriptional and translational modifications. PCR-based DNA markers have been used for analysis of

genetic diversity, identifying and selecting the superior genotypes with desirable variations, mapping or tagging desirable genes, and also to identify the gene of interest before its phenotypic expression (Joshi *et al.* 1999; Lübberstedt and Varshney 2013). Thus, molecular markers are widely used in different plant species for genetic studies in breeding programmes by uncovering the extent of genetic variability through increased coverage of the genome. Previous studies indicated that employing DNA markers in crop improvement can economize both time and resources. Recently, for genetic diversity analysis various DNA based markers like Random Amplification of Polymorphic DNA (RAPD), Inter Simple Sequence Repeats (ISSR), Sequence Characterized Amplified Region (SCAR), Amplified Fragment Length Polymorphism (AFLP) have been widely used in different species (Tian *et al*. 2015; Verma *et al*. 2017; Zreikah *et al*. 2019; Louati *et al*. 2019; Khankahdani and Bagheri 2019). The knowledge of genetic polymorphism existing in different sexes of dioecious tree species would provide essential information for its improvement, management and conservation. Kumar and Agrawal (2017) have analyzed genetic diversity and structure of populations of *S. glauca* using ISSR and SRAP markers. As there are relatively very few papers on analysis of genetic polymorphism in different sexes in *S. glauca*, there is a need to understand the genetic variations and relationships as it increases the chances to get new genetic combination and would help in knowing the adaptation and evolutionary potential of the species.

1.5. Sex identification and use of molecular markers:

Around 7% of genera and 6% of species of angiosperms exhibits dioecism which is a condition in flowering plants during the process of evolution to avoid inbreeding (Renner and Ricklefs 1995; Barrett 2002). In angiosperms, most of the flowering plants are hermaphrodites with bisexual flowers where outcrossing is enhanced by genetic mechanism such as heterostyly *i.e.* stamen and stigma vary in length to prevent selfing (Irish and Nelson 1989). The dioecious species are presumed to be evolved from individuals that are monoecious or hermaphrodites because of two mutations that are independent and resulted in functional dioecy in plants (Charlesworth 1991). Thus, in advanced dioecious plants, flowers are present separately on male and female individuals and possess poorly developed gynoecium and androecium, respectively and thus dioecy results in outcrossing in these plants.

Sex determination in most of the dioecious plants is determined genetically due to the presence of distinct sex chromosomes or no distinguishable chromosomes with genetic control of one or several loci for sex determination. Surprisingly, in most of the dioecious plants, only a very few species have well known heteromorphic sex chromosomes (Vyskot and Hobza 2004) and cytologically the sex of these plants can be determined. Conversely, in most of the dioecious plants, the sex of the plants is not revealed morphologically due to lack of distinct sex chromosomes as in *Trichosanthes dioica* Roxb (Sinha *et al*. 2007) and therefore the female and male plants cannot be distinguished at the seedling stage using cytological techniques. However, for plant breeders especially for those who cultivate dioecious plants of unknown sex, it is important to determine the sex of plant at an early stage, as male and female plants have distinct uses. Generally, female species are considered to have more economic value with respect to their capability to produce fruits and seeds compared to the males.

In breeding programmes for early identification of sex in dioecious plants, molecular markers are given utmost priority to understand the sexual dimorphism and to raise the economic potential. Mwase *et al*. (2007) have reported the possibility for identification of sex by using DNA markers when sex is determined by a distinct gene or closely linked group of genes. A marker is effective when it exhibits reproducibility and high polymorphism. Molecular markers like RAPD, ISSR, AFLP, SCAR, etc. are used to identify sex in dioecious species with long juvenile and vegetative phase like *Pistacia vera* (Esfandiyari *et al*. 2011), *Phoenix dactylifera* (Dhawan *et al*. 2013; Al-Ameri *et al*. 2016; Al-Qurainy *et al*. 2018*), Hippophae rhamnoides* (Korekar *et al*. 2012), *Piper betle* (Sheeja *et al*. 2013), *Piper longum* (Manoj *et al*. 2005), *Simmondsia chinensis* (Hosseini *et al*. 2011; Heikrujam *et al*. 2014a & 2014b), and *Carica papaya* (Reddy *et al*. 2012).

Baratakke and Patil (2010) have used cytological methods to study the molecular mechanism for identifying sex by viewing the karyotype and morphological characters in different sexes of *S. glauca* and could not find heteromorphic sex chromosomes. Due to lack of information of sex chromosomes and the underlying genetic basis of sex determination in *S. glauca*, there is a need to develop molecular markers to identify different sexes at seedling stage that could help in raising of plantations in suitable ratio of males and females. There are few studies related to identification of sex in *S. glauca* using different molecular markers like RAPD, ISSR

and SCAR (Savitha *et al*. 2008; Simon *et al*. 2009; Baratakke and Patil 2014; Ghumatkar *et al.* 2015; Kisan *et al*. 2016).

1.6. Phytochemical investigations in dioecious plants:

Currently, traditional usage of these medicinal plants is playing an important role in human life because of their inherent therapeutical properties that are helping to prevent several diseases. The medicinal plant research is helping to find and validate many herbal products (Saxena *et al*. 2012). These medicinal plants were found to contain many secondary metabolites like flavonoids, alkaloids, steroids, terpenoids, saponins, tannins and polysaccharides which are having a profound biological importance in both traditional as well as modern therapeutics (Doughari *et al*. 2012; Mahboubi *et al*. 2013).

Several studies were carried out on preliminary phytochemical screening, metabolites and their antioxidant assays in different dioecious plants. The different species of *Pistacia* exhibited variations in anatomical, morphological, molecular and chemical traits based on their geographical distributions and genders (Gratani *et al*. 2013; Werner *et al*. 2013). Various studies have focused on knowing the impact of dioecy in pharmacological and chemical properties of plants (Bajpai *et al*. 2012; Simpson 2013). Phytochemical assays have revealed the chemical differences between female and male plants in different plant parts in *Simmondsia chinensis* (Prasad and Iyengar 1985), *Carica papaya* L. (Dutta and Mazumdar 1989; Helail and Atawia 1990), *Tinospora cordifolia* (Bajpai *et al*. 2015), *Piper betle* L. (Bajpai *et al*. 2012), etc. Sex related differences in phenol contents, metabolites and antioxidant activities have been reported previously in dioecious plant, *Pistacia lentiscus* (Yosr *et al*. 2018).

Studies have been previously conducted on phytochemicals, antioxidant assays, antibacterial and cytotoxic activities using extracts of different plant parts of *S. glauca* (Lakshmi *et al*. 2014; Umesh 2015; Sajeeda *et al*. 2019). However, these studies in *S. glauca* have not considered the impact of gender on phytochemical and pharmacological activities. As the major phytoconstituents in *S. glauca* with respect to gender remained unexplored, there is a need to analyze the gender-based metabolite differences if any, in this polygamodioecious tree where the sex of the plant is known only after flowering.

1.7. Rapid *in vitro* multiplication of *S. glauca*:

Conventionally, *S. glauca* is propagated mainly through seeds. However, the loss of seed viability within 2–3 months at ambient conditions, physical dormancy imposed by its hard seed coat, and germination problems are the major limiting factors for mass propagation (Orwa *et al.* 2009; Patil and Awate 2014). Seeds are stored in paper or cloth bags at room temperature for 9-12 months or at low temperature to retain viability for long periods (Kureel *et al.* 2009). Previous studies showed that mechanically broken seeds germinated faster than intact seeds and that plant growth regulators such as gibberellic acid (GA_3), 6-benzylaminopurine (BAP), and salicylic acid (SA) are involved in breaking physical dormancy in *Simarouba* (Patil and Gaikwad 2011). Vegetative propagation through grafting, air layering, and cutting has been reported previously (Joshi and Joshi 2002) but has practical limitations. *In vitro* propagation offers several advantages over conventional propagation methods such as rapid multiplication, season-independent production of plants, production of disease-free plants, and germplasm conservation (George and Debergh 2008). Regeneration of plants is achieved *in vitro* through organogenesis or somatic embryogenesis by direct or indirect means. The indirect regeneration method intermediates callus formation which leads to somaclonal variations, therefore the direct regeneration without callus stage is majorly preferred for micropropagation of plants as it exhibits low risk of genetic instability (Evans and Bravo 1986; Giri *et al*. 2004; Jani *et al*. 2015).

The best source of explants for micropropagation of tree species are nodal explants or shoot tips obtained from mature trees, as they are expected to produce true-to-type plants. However, micropropagation using explants from mature trees is generally difficult due to problems of phenolic exudation, microbial contamination, browning of media and explants, and recalcitrance to *in vitro* regeneration (Amin and Jaiswal 1987). Alternatively, micropropagation of plant species can be carried out using explants of germinated seedlings (Giri *et al*. 2004; Sarker *et al*. 2009), which are easily available and exhibit high regeneration capacity. However, the seedlings of outcrossed plant species are genetically heterogeneous and heterozygous and might not retain the desirable characteristics of the donor plant, thus limiting their use for micropropagation and genetic transformation. The disadvantages of using seedlings of unknown genotypes for micropropagation and genetic transformation can be minimized by using seeds produced by controlled pollination of plants of known genetic background or

apomictic seeds (Yıldırım 2012). The regenerated plants derived from heterozygous seedlings, however, could be used for purposes of reforestation and plantation programmes where genetic variation is required.

There are relatively few published papers on *in vitro* propagation of *S. glauca* using juvenile or mature explants. Regeneration of plants has been achieved in *S. glauca* by different research groups using different explants like immature cotyledons, immature zygotic embryos and leaves (Rout and Das 1994a & 1994b; Das 2011; Hadke *et al*. 2008; Jyothi *et al.* 2014). Others have reported regeneration through organogenesis from explants like axillary buds, nodes and shoot tips (Rout and Das 1995; Shukla and Padmaja 2012; Dudhare *et al.* 2014). As the shoot multiplication response from the explants used has been low, there is a need to enhance the rate of multiplication that can be utilized for large-scale plant propagation of elite trees, genetic transformation, conservation, and phytochemical production in this tree species. The performance of micropropagated plants in the soil needs to be determined before practical exploitation.

In vitro cultures are subjected to various degrees of stress due to exposure to different culture media, growth regulators and conditions. Tissue culture induced variation (somaclonal variation) is quite common and can limit the utility of an *in vitro* regeneration system for various applications (Salvi *et al.* 2001). In tree species with long generation times, assessing genetic variation by morphological, karyotypic and isozyme analysis has several limitations (Gupta *et al.* 2009), and therefore DNA-based markers are being increasingly used for this purpose. Among DNA markers, RAPD (Williams *et al.* 1990) and ISSR (Zietkiewicz *et al.* 1994) markers have been widely used in the genetic analysis of micropropagated plants in many plant species. Several researchers reported that regenerated plants were found to be genetically stable using RAPD and ISSR analysis in different plant species such as *Sapindus trifoliatus* (Asthana *et al.* 2011), *Dendrocalamus strictus* (Goyal *et al.* 2015), *Magnolia sirindhorniae* Noot. & Chalermglin (Cui *et al*. 2019). In contrast, genetic variations have been reported in regenerated plants in comparison to the donor seedling using RAPD and ISSR markers in *Prunus dulcis* (Martins *et al.* 2004) and *Saussurea involucrata* (Yuan *et al.* 2009). It has been suggested that the presence or absence of genetic variation depends on several factors such as the genotype, type of explant, mode of regeneration, growth regulators, number and duration of subcultures (Goto *et al.*1998; Martins *et al.* 2004). Thus, it is

essential to ascertain the genetic uniformity of *in vitro* regenerants of any plant species before commercial exploitation.

Based on this background, the present study was aimed to assess the genetic variability using RAPD and ISSR markers among genotypes of different sexes of *S. glauca*. The study also attempted to identify the sex specific markers using RAPD, ISSR and SCAR for differentiating the sex at the seedling stage. The phytochemical constituents were also investigated in different sex types using biochemical assays and GC-MS analysis, to examine the gender-based differences of phytochemicals and metabolites, if any. *In vitro* studies were performed with mature and juvenile explants to achieve high rates of plant regeneration and the RAPD and ISSR markers were used to study genetic similarity of regenerated plants.

1.8. The objectives framed to carry out the work are as follows:

- Assessment of genetic variation among different sexes of *S. glauca* using RAPD and ISSR molecular markers.
- Identification of sex-associated RAPD and ISSR markers, SCAR marker development and their validation in *S. glauca*.
- Analysis of metabolites and antioxidant activities in different genders of *S. glauca* for understanding sex-associated differences.
- Development of efficient *in vitro* regeneration method from different explants and genetic stability analysis of *in vitro* regenerated plantlets.

Review of Literature

2. REVIEW OF LITERATURE:

Simarouba glauca DC. is an evergreen eco-friendly oilseed tree having a wide range of economic values beneficial to society. All parts of the tree are believed to be useful with different parts being tapped as a source of oil, biofuel, medicine, food, manure, timber, thermal power, ethanol production etc. *S. glauca* is well established in wastelands of different states of India like Gujarat, Karnataka, Orissa, Tamil Nadu, Maharashtra, Bihar, Chhattisgarh, Andhra Pradesh and Telangana. Like other tree-borne oilseeds, *S. glauca* is not well exploited for production of edible oil, drugs, and other industrial purposes and therefore its cultivation in large scale should be encouraged in India. The cultivation of *S. glauca* provides rural employment opportunities at a microeconomic level thus allowing the nation to gain self-sufficiency in terms of biofuels, edible oil, industrial oils, pharmaceuticals and timber on a long-standing basis. It also overcomes the energy crisis, reduces greenhouse gas emissions while reducing the need to import oil globally (Dash *et al*. 2008; Arivu *et al*. 2017). The present study mainly aimed at analyzing the molecular genetic variation in different sexes and identification of sex-linked molecular marker(s) in *S. glauca*. Phytochemical investigations in relation to sex as well as *in vitro* regeneration studies were conducted to establish efficient methods for rapid multiplication of *S. glauca*.

The literature pertaining to the general features, distribution, importance of *S. glauca*, uses of molecular markers such as RAPD, ISSR and SCAR markers for genetic variation analysis and sex identification, metabolite analysis in different genders and *in vitro* regeneration for mass propagation of plants that formed the basis of the present study are presented here.

2.1. General features of *Simarouba glauca* DC. and its impact on the environment:

S. glauca, commonly known as a Paradise tree, King Oilseed tree, Lakshmi taru, Aceituno, or Bitterwood is a fast-growing multipurpose tree valued for oil, wood, and medicinal properties. Simaroubaceae is branded by the presence of quassinoids which are secondary metabolites possessing biological activities with a broad range such as antitumor, antiviral, antimalarial, antiparasitic, amebicide, insecticide, herbicidal, and feeding deterrent. A few of the species that belong to Simaroubaceae *viz*., *S. glauca*, *S. amara*, *S. excelsa* and *S. versicolor* are known to have analogous properties. *S. glauca* is a polygamodioecious tree species that segregates into 3 sex types, females (40-50%),

andromonoecious (40-50%) and males (5%). The andromonoecious plants exhibits a high proportion of male flowers with few bisexual flowers.

The planting of *S. glauca* is known to have a great impact on environment. The root system in *S. glauca* is well developed and helps in prevention of soil erosion and enhances ground water levels, promotes soil microbial life and its intense evergreen canopy helps in preventing extensive heating of soil especially in summer and increases conversion of abundant solar energy to biochemical energy (Patil and Gaikwad 2011). *S. glauca* cultivation requires less water and withstands high temperatures and therefore is best suited for both semi-arid and arid lands where no other plants of economic value can grow (Joshi and Hiremath 2000). The cultivation of this tree species does not require any form of pesticides as they are less prone to pests and diseases.

2.2. Distribution of *S. glauca*:

S. glauca is an evergreen tree that is native to the forests of Central and South America. Simaroubaceae, a family of flowering plants comprises of 32 genera and around 170 species of bushes and trees of pantropical distribution which is ecologically more diverse. National Bureau of Plant Genetic Resources (NBPGR) first introduced it in the 1960s in the Research Station at Amravathi, Maharashtra, for retrieval of wastelands and conservation of soil. The seeds were used as starting material for *S. glauca* plantation in India. In 1986, *S. glauca* was brought to the University of Agricultural Sciences, Bangalore, and from 1992 onwards, the developmental activities and the regular research was started. Subsequently, its cultivation was extended into the states of Gujarat, Orissa, Rajasthan, Tamil Nadu, Karnataka, Andhra Pradesh, Telangana, West Bengal and Haryana. *S. glauca* withstands arid and semi-arid conditions and thus can be cultivated in areas where no other plants of commercial value can be grown. It occurs as an associate with moist plants in subtropical forests (ICRAF Agroforestry Tree Database, http:// ecocrop. fao. Org /ecocrop/srv/en/cropView?i d=9785 2007). It is a shade-tolerant tree that grows under the canopy of large fruit trees. It can grow in degraded soils that are poor in nutrients and too dry and unsuitable for the cultivation of other crops. Thus, these trees were successfully cultivated in different regions with different climatic conditions in India.

2.3. *S. glauca,* a multipurpose tree species*:*

2.3.1. *Simarouba* seed oil and its potential as a source of biodiesel:

S. glauca is a promising oilseed tree having multiple uses. It offers considerable scope and potential for overcoming the oil shortage problem as it grows reasonably well even in marginal lands and has high yield potential. The *S. glauca* can produce 2000-2500 kg seed/ha per year (Joshi and Hiremath 2000). The seed oil of *S. glauca* is used in industry for manufacture of pharmaceuticals, paints, polishes, soaps and lubricants. The dry seeds of *S. glauca* have 59-62% of unsaturated fatty acids and about 30-40% protein (Armour 1959; Kumar and Agrawal 2015). The refined fat of seeds does not require blending or hydrogenation with other fats for its use in baking and cooking purposes and as natural vegetable oil. The chemical composition of *Simarouba* oil is like olive oil, which contains about 85% oleic acid. Rout *et al*. (2014) reported that fatty acid composition of *S. glauca* oil was comparable to groundnut oil with respect to all parameters studied. In the bakery, chocolate, and confectionery industries, oil can be used as extenders and as an alternative for cocoa butter as it is a source of 30% monosaturated triacylglycerols (Jeyarani and Reddy 2001; Kumar *et al*. 2014). Due to its high NPK values and high protein amount of 48%, oil cake is used in the production of green organic manure for fields.

Various studies have revealed higher oil content in the kernels of *Simarouba* in comparison to Jatropha, Rapeseed, Neem and Karanja (Pradhan *et al*. 2009; Duhan *et al*. 2011; Nde *et al*. 2013). Among the oil-bearing trees, *S. glauca* appears to be a potential source of biodiesel, which has evoked worldwide interest (Armour 1959; Shantha *et al*. 1996; Joshi and Hiremath 2000). Different proportions of methyl esters of eucalyptus and paradise oils were used to replace biodiesel, and the characteristics of this biodiesel are like that of diesel (Devan and Mahalakshmi 2009). Jena *et al*. (2010) produced biofuels by blending *Simarouba* and Mahua oils. Sivamani *et al*. (2018) have optimized different parameters for the production of biodiesel using RSM (Response surface methodology) and ANN (Artificial neural network) models and reported that *S. glauca* oil has the potential to be used as biodiesel. The biodiesel obtained from *S. glauca* is biodegradable, sulfur, and aromatics free, non-toxic, and can be easily blended with fossil fuels with ignition and compression value (Bart-Plange and Baryeh 2003). Duhan *et al.* (2011) used a series of procedures such as degumming, alkali

refining, and bleaching to refine the oil. The viscosity of *Simarouba* oil is closer to that of diesel, and the fatty acid composition has pure triglyceride esters of 96.11% which as per Bureau of Indian Standards (BIS) norms suggested the suitability of *Simarouba* oil methyl esters as biofuel (Mishra *et al.* 2012).

2.3.2. Medicinal properties and other uses of *S. glauca*:

S. glauca is often used in traditional medicine for treating various diseases. The fruit pulp, seeds, bark and leaves were also found to have medicinal properties and used as an astringent, emmenagogue, analgesic, antiviral, antimicrobial, anticancer, vermifuge and stomach tonic (Roig 1974; Caceres *et al.* 1990; Franssen *et al.* 1997; Rivero-Cruz *et al.* 2005; Rodriguez *et al.* 2006; Valdez *et al*. 2008). The crushed seeds have been used as anti-venom against snake bite. Lele (2010) created an online portal for *Simarouba* medicinal properties.

The medicinal properties of extracts, fractions, and isolated constituents of *S. glauca* have been investigated. The pharmacologically important compounds identified include ailanthinone, glaucarubin, benzoquinone, quassinoids, melianone, holacanthone, sistosterol, simarolide, simaroubidin and simarubolide. The extract of *S. glauca* was used to treat gastrointestinal disorders (Caceres *et al*. 1990; Lidia *et al*. 1991). In a US patent, Bonte *et al.* (1997) showed the use of *Simarouba* extract in the promotion of keratinocytes differentiation, manufacturing cosmetics, reduction of patchy skin pigmentation particularly vitiligo, liver spots, and improvement of the protective function of skin and hair. Jose *et al*. (2018) have purified and characterized tricaproin which is a lipid-based inhibitor of HDAC, from the leaves of *S. glauca* that exhibited *in vitro* anti-cancer properties. The anti-proliferative effects and anti-cancer potency of different extracts of *S. glauca* against the cancer cell lines have been recently reported by Jose *et al*. (2020).

Govindaraju *et al*. (2009) suggested that the livestock meal obtained from *S. glauca* is a rich source of protein with 92% solubility, and 88% of *in vitro* protein digestibility, which is higher than the *Jatropha curcas* (75%). The excellent quality vermicompost is produced from fruit pulp, whereas good manure is obtained from leaf litters (Govindaraju *et al*. 2009).

2.4. Molecular markers:

Markers are classified into three types based on their characteristics, namely morphological, biochemical, and molecular markers. The morphological markers represent the physical traits like the plant height, leaf shape, color of flowers, etc. that are used to assess the genetic variation (Tanksley 1983; Emami and Sharma 1999). Biochemical markers comprise isozymes, and secondary metabolites. Molecular markers include hybridization-based DNA markers like RFLP, and PCR based DNA markers like AFLP, RAPD, SSR, ISSR, SNP etc. Both morphological and biochemical markers are centered on the expression of genes, dependent on developmental stage and environment, influenced by pleiotropic and epistatic interactions, and their limited number restricts their use in large scale applications for germplasm analysis (Sarwat *et al*. 2011). In contrast, molecular markers are abundant in plant genomes, and most importantly, they are neutral and not dependent on the environment and developmental stages. Molecular markers analyze the variations among individuals, regardless of their development stage (Sztuba-Solińska 2005) They are particularly useful in identification of sex, formulation of strategies for conservation, for improving and managing the existing germplasm collections by MAS (Marker assisted selection), genetic diversity analysis, identification of genes linked to desirable traits, DNA fingerprinting and phylogenetic studies (Sarwat *et al*. 2011). Currently, molecular markers have been used widely in different applications due to the advancement in automated technologies for marker detection and availability of a large amount of information related to sequences.

PCR based markers are co-dominant or dominant markers that are targeted to a locus that is specific or to multiple loci that are randomly distributed. These markers are comparatively easy to use in studies like mapping and for analysis of genetic variation/diversity.

2.4.1. Randomly amplified polymorphic DNA (RAPD):

The RAPD (Welsh and McClelland 1990; Williams *et al*. 1990) is a technique that is based on PCR and used for the analysis of genome using short primers of random sequence. In this procedure, usually, the RAPD primers bind at 3-15 sites/loci of the genome simultaneously and results in amplified products of different sizes only when they bind within the amplifiable range (Tingey and del Tufo 1993). Even a mismatch of a single nucleotide prevents the binding of RAPD primers which are short mers

sequences of 10 bps. RAPD markers detect the nucleotide sequence polymorphism in DNA, and hence used in different plant species for analyzing genetic variation, phylogenetic studies within and among species/populations, early identification of sex, genetic mapping, fingerprinting, gene tagging, marker-assisted selection and assessment of genetic uniformity among micropropagated plants (Mallaya and Ravishankar 2013; Verma *et al*. 2017; Zhou *et al*. 2018). Polymorphisms detected by RAPD are based on the presence or absence of amplified bands on an agarose gel, and thus, they are dominant. The use of RAPD markers is limited to some extent as they cannot differentiate homozygous and heterozygous individuals. The amplification patterns are not reproducible as they vary with conditions of experiments (Heun and Helentjaris 1993; Ellsworth *et al*. 1993). Ulloa *et al*. (2003) reported that the problems associated with reproducibility could be overcome by strictly maintaining the quality of DNA and the experimental conditions for all the sets of reactions.

2.4.2. Inter Simple Sequence Repeats (ISSR):

Zietkiewicz *et al*. (1994) were the first to introduce ISSR**,** which is also known as Inter SSR amplification (ISA). The primers which are based on microsatellite DNA sequences are utilized to amplify ISSR DNA sequences. The ISSR markers are used to detect levels of variation in the regions of microsatellites scattered mostly in nuclear genomes (Bornet and Branchard 2001). The ISSRs are mostly dominant markers, requires no sequence information for primer designing and are distributed throughout the genome. ISSRs are more reproducible because of lengthier primers and higher annealing temperatures used for PCR amplification. They are used for strain and clone identification, understanding taxonomic relationships in closely related species, and genetic mapping (Zheng *et al*. 2009; Kumar and Agrawal 2017; dos Santos *et al*. 2019).

2.4.3. Sequence characterized amplified region (SCAR):

SCAR is a technique where the terminal regions of RAPD markers are sequenced, and pair of longer primers (22-24 bases long) are designed for amplifying a specific locus (Paran and Michelmore 1993). Thus, SCARs are regions of genome flanked by known specific markers. SCARs are more reproducible and reliable compared to other markers like RAPD and ISSR markers, as they are amplified at high annealing temperatures. SCARs are locus specific, co-dominant, unaffected by reaction conditions and by the presence of introns that could remove the binding sites for

primers. Other markers like ISSR, AFLP, SCoT (start codon targeted polymorphism), etc. are converted to SCAR markers through molecular cloning (Cheng *et al*. 2015). The development of SCAR markers from RAPD is easy compared to other primers as they are cost-effective, easy to use and less time consuming compared to other conventional markers. SCARs are effectively used for genotype identification, genetic mapping, marker assisted selection, detection of sub-species, map-based cloning and sex identification (Yuskianti and Shiraishi 2010; Esfandiyari *et al*. 2011; Sairkar *et al*. 2016; Feng *et al*. 2018; Zhou *et al*. 2018).

2.5. Assessment of genetic variation using molecular markers:

The genetic variability that exists among individuals of populations of a species is referred to as genetic diversity (Brown 1983). In all the species, the genetic variability is sustained by the environment. The genetic variation and diversity are important for a species as they allow the individuals or populations to adjust to fluctuations in the environment and increase their capacity to endure the stress in any form (Eriksson *et al*. 1993). The knowledge of genetic variation in a species is important for devising strategies for plant breeders as it exploits the potential for selection of genomes in breeding programmes (Lübberstedt and Varshney 2013). Therefore, it is essential to measure the magnitude of variability at the molecular level using morphological, biochemical, and molecular markers. Among all markers, molecular markers are found to be extremely useful at DNA level in analyzing genetic variation owing to high discriminatory power to detect variations at the DNA level.

2.5.1. Assessment of genetic variation among different genders by RAPD and ISSR markers in *S. glauca*:

There are relatively few published reports on the assessment of genetic diversity or variation in different genotypes/accessions of *S. glauca*. In the recent study, Kumar and Agrawal (2017) employed 14 ISSR and 11 SRAP (sequence-related amplified polymorphism) markers to assess the genetic diversity in female and male accessions of *S. glauca* from diverse regions of India. For genetic diversity analysis, SRAP markers were found to be more useful with higher mean values for polymorphism percentage (26.54%), ne (1.45), I (0.39), h (0.26) and PIC (0.14) compared to ISSR marker values (polymorphism percentage: 14.43%, ne: 1.27, I: 0.23, h: 0.16 and PIC: 0.10). In overall accessions of *S. glauca* from different locations, the mean values for genetic diversity

analysis were found to be very less (ne: 1.30, I: 0.22, h: 0.16). Similar values of mean genetic diversity (h: 0.17) were noticed among female, male, and bisexual plants. Choudhary (2014) used 67 RAPD and 22 ISSR primers to screen 96 genotypes of *S. glauca* that produced average polymorphism percentage of 42.3% and 39.3%, for RAPD and ISSRs, respectively.

2.5.2. Assessment of genetic variation among different genders by RAPD and ISSR markers in other dioecious plants:

Khankahdani and Bagheri (2019) analyzed the genetic relations between males and females of 34 cultivars of *Phoenix dactylifera* L. employing 12 ISSR markers and morphological markers. The ISSR markers were able to distinguish male and female cultivars, whereas no clear-cut separation was seen among male and female cultivars by morphological markers. The grouping of male and female cultivars using ISSR markers was confirmed by PCA analysis, which confirmed their utility to separate the cultivars. Mitra *et al*. (2011) conducted a comparative study in 45 date palm genotypes, including 30 female and 15 male plants to assess genetic diversity using 37 RAPD and 53 ISSR primers which produced 95.0% and 90.9% polymorphic bands respectively and thus were informative to evaluate the genetic diversity.

Srivashtav *et al*. (2013) investigated 8 date palm genotypes using 13 RAPD and 2 ISSR primers. RAPD primers revealed 39.77% polymorphism and were more effective compared to ISSR primers (23.07%). Kumar and Agrawal (2019) used 20 ISSR and 22 SCoT primers to analyze population structure and genetic diversity among 52 *Trichosanthes dioica* Roxb. accessions from different locations of India. They reported that ISSR markers are more informative for genetic diversity analyzes with a high percentage of polymorphism (95.96%) and PIC (polymorphic information content) value (0.47) than SCoT primers (with 92.20% polymorphism and 0.45 PIC value). Female plants of *T. dioica* were found to be less variable compared to male plants.

Bhardwaj *et al*. (2010) assessed genetic diversity among female and male plants of 10 jojoba genotypes [*Simmondsia chinensis* (Link) Schneider] employing 50 RAPD and 55 ISSR primers which resulted in 60.7 and 69.3% polymorphism, respectively. Nimisha *et al*. (2019) analyzed 10 genotypes of each sex (10 males and 10 females) of Spine guard (*Momordica dioica* L.) using 50 RAPD primers out of which 28 primers generated 182 fragments. RAPD analysis resulted in 90.66% polymorphism and PIC

ranging from 0.3598 to 0.9011, and an average value of 0.7555 per primer. The study suggested the use of RAPD primers for genetic diversity analysis but was unable to identify male and female sex-specific markers. Amirebrahimi *et al*. (2017) employed 12 ISSR primers for studying the genetic diversity among 20 male and 36 female pistachio genotypes. The ISSR primers generated a total of 178 bands, out of which 169 were found to be polymorphic with average PIC values varying from 16% to 35%. The results demonstrated the usefulness of ISSR markers for studying genetic variation among male and female pistachio genotypes.

2.6. Sex identification in dioecious plants using molecular markers:

Molecular markers such as RAPD, ISSR, and SCARs have been used to determine sex in various dioecious plants. The early identification of sex of dioecious plants by using molecular strategies has been given importance as most of them have sex-specific economic value and thereby aids in breeding programmes and understanding the evolutionary and developmental pathways of dimorphism (Shibu *et al*. 2000; Sharma *et al*. 2008). The mechanisms for sex determination in monoecious and dioecious species play a significant role in classification, identification and sex-specific evolution. The sex-determining mechanisms are well studied in model herb species (Kumar *et al*. 2012; Huang *et al*. 2013) compared to woody plants, which are highly heterozygous, having large genomes with less genome information, complex processes of development (Niu *et al*. 2016), polymorphic genetic background (Song *et al*. 2013) and absence of a suitable model system.

In dioecious plants, there are varieties of ways involved in the determination of sex. They are genetic control of sex determination by single or multiple loci (Heikrujam *et al*. 2015), hormonal control of sex determination where phytohormones like cytokinins, auxins, gibberellins, and ethylene control the sex expression (Ming *et al*. 2007 & 2011; Milewicz and Sawicki 2012) and epigenetic control of sex determination (Soldatova and Khryanin 2010; Milewicz and Sawicki 2012) in dioecious plants (Aryal and Ming 2014).

2.6.1. Identification of sex-linked molecular markers in *S. glauca* by RAPD, ISSR and SCARs:

There are few published reports related to identification of gender in *S. glauca* using molecular markers especially involving reliable SCAR markers. The early identification of sex of *S. glauca* at seedling stage is advantageous as the desired sex with commercial value can be identified for raising large scale plantations comprising females and males in appropriate ratio.

Savitha *et al*. (2008) reported that out of 150 RAPD primers screened on different sexes of *S. glauca,* 17 primers resulted in polymorphic bands. Among these polymorphic primers, only OPS-6 produced amplification specific to males and absent in other plants. Simon *et al*. (2009) screened 70 RAPD primers on male and female bulk DNA samples, of which OPB-20, OPC-01, OPD-20, and OPF-07 produced bands that were distinct for male and female bulk DNA samples. These primers when tested on individual samples, only OPD-20 primer produced reproducible band of 900 bp present in females and absent in male plants indicating that OPD-20 marker could be linked to sex controlling genes in *S. glauca*. Prasanthi *et al*. (2010) screened 250 random primers on 27 female and 32 male plants of known sex to identify marker linked to sex of *S. glauca*. Out of these, primer OPA-08 produced male and andromonoecious specific 900 bp band. SCAR primers (SCAR 12f/r) were designed which produced specific amplification of 915 bp band only in male and andromonoecious plants whereas absent in females.

Vaidya and Naik (2014) screened 85 RAPD primers on 2 females, 2 hermaphrodites and a male accession, among which 5 primers showed association with sex. The female, male, and hermaphrodite specific bands were produced by OPD-19, OPU-10 and OPU-19 primers respectively. The female and hermaphrodite specific band was produced by the primer OPS-05 and the male and hermaphrodite specific band was produced by the primer OPW-03. Baratakke and Patil (2014) tested 50 random primers on male and female populations, of which only OPA-18 resulted in differences among male and female plants. The SCAR primers (MSSMS-01F and MSSMS-01R) resulted in 1110 bp band specific to male plants. Ghumatkar *et al*. (2015) used 69 RAPD and 12 ISSR primers for screening bulk DNA of 15 female and 5 male genotypes. Among all the RAPD and ISSRs used, the primers ISSR 864, ISSR 824, OPE 05, OPC 06, OPD

15, OPD 12, and OPC 16 resulted in bands specific to female and male plants. These 7 primers when tested on individual samples, only one RAPD primer OPE 05 resulted in sex-specific amplification of 521 bp in males and absent in female plants.

Choudhary (2014) screened male, female and bisexual plants using 67 RAPD and 22 ISSR primers that produced good amplification, of which two RAPD primers (RP-07, RP-49) and two ISSR primers (UBC-814, UBC-815) resulted in sex-specific amplification. The SCAR markers were developed for RP-49 (sgF1/sgR1) and UBC-815 (sgF2/sgR2). The SCAR primers sgF1/sgR1 resulted in female and bisexual plants sex-specific amplification of 353 bp band, and the SCAR primers sgF2/sgR2 SCAR primers produced male and bisexual plants sex-specific amplification of 721 bp band.

2.6.2. Identification of sex-specific molecular markers in dioecious plants by RAPD, ISSR and SCAR markers:

Many researchers have made several efforts in dioecious plants to identify the gender at early seedling stage as they possess gender-specific commercial value and thereby avoid the maintenance of excess unwanted sexes in the plantations.

Adhikari *et al*. (2014) have screened 42 ISSR primers for identification of sex, out of which one primer ISSR-6 resulted in 500 bp fragment only in male plants of the pointed guard. Thogatabalija and Gudipalli (2012) used 24 RAPD primers to test individual DNA of female and male plants (5 each) for sex-identification in *Givotia rottleriformis* Griff., of which single primer OPT-17 exhibited partial sex association as it amplified 1000 bp band in all females along with 1 male plant. Gangopadhyay *et al*. (2007) carried out sex identification studies in *Carica papaya*, and *Cycas circinalis* using RAPD and ISSR markers. The ISSR primer $(GACA)_4$ resulted in a band specific to female and hermaphrodite plants except for male plants of papaya. The RAPD primer OPB 01 gave a male-specific amplified product in *C. circinalis* and the sequencing result of the amplified band showed homology with putative retroelements, suggesting that it might be playing a role in sexual development of *C. circinalis*.

Al-Qurainy *et al*. (2018) tested 300 RAPD primers for identification of genders at the seedling stage in *Phoenix dactylifera* L., out of which the primer OPC-06 amplified sex-specific band of 294 bp in males. The band was cloned; sequenced and specific primers were designed resulting in 186 bp product. The SCAR marker was

found to be effective as it showed amplification only in males and absent in female date palm plants. Joseph *et al*. (2014) employed RAPD primers to identify a sex-linked marker in dioecious fruit yielding plant *Garcinia gummi-gutta* (L.) Robs. Out of 150 primers tested, only one random primer (OPBD 20) showed band specific to male plants and thus male-specific SCAR marker (CAM-566) was developed. The SCAR primers OPBD20F and OPBD20R were validated on 12 female and 12 male plants. The primers resulted in 556 bp band only in males and absent in females at higher annealing temperatures whereas bands were observed in both males and females at low annealing temperatures. Additionally, the marker developed was also able to identify sexes of 10 unknown samples. In *Coccinia grandis*, Bhowmick *et al*. (2014) tested 19 RAPD primers of which only OPC 08 primer produced a 830 bp band specific to male which was converted into SCAR marker (CgY1) and was amplified only in male plants.

Patil *et al*. (2012) screened 50 RAPD primers for the identification of sex at an early stage in *Momordica dioica* Roxb. Among them, only OPA-15 resulted in 1500 bp band, which is male-specific and based on the sequence a pair of forward and reverse SCAR primers were developed for SCAR marker (MSSM-01) and resulted in male-specific amplification of 1501 bp band. Gao *et al*. (2007) used 100 RAPD primers to identify sex-specific marker in *Asparagus officinalis* L., of which the primer S368 amplified two bands (S368-928 bp and S368-1178 bp) specific to female plants. Out of these two markers, only S368-928 bp sex-specific band was amplified when tested on 30 males and females and SCAR marker was developed for identification of females. However, Southern blot analysis for male and female plants resulted in the hybridization of S368-928 in both of them. Jangra *et al*. (2014) reported that the ISSR primer UBC807 resulted in 1100 bp band in all males and absent in females of *Simmondsia chinensis* (Link) Schneider. Based on sequence information, SCAR primers were designed that amplified approx. 1000 bp band only in male plants.

Sun *et al*. (2014) used 21 RAPD and 20 ISSR primers to determine the sex at the seedling stage in *Pistacia chinensis* Bunge. Among the tested primers, only two primers (S1 and S281) amplified female-specific bands of sizes 473 and 1242 bp, respectively. Among the two sex-specific bands, only FS281 was successfully converted into the SCAR marker using specific primers (S281-1 and S281-2) and resulted in a 636 bp band in all females and absent in male plants. Esfandiyari *et al*. (2011) used 30 RAPD primers for identifying sex-linked marker in various *Pistachio* species. Only one RAPD

primer (BC1200) amplified a female-specific band of 1200 bp. The SCAR markers developed resulted in amplification of 300 bp band only in females and absent in all male plants.

Sheeja *et al*. (2013) identified sex in betel vine plants by screening bulk DNA of female and male plants using 82 RAPD primers. Out of these, 2 primers OPE-1 and OPB-20 amplified 400 and 488 bp bands, respectively specific to female plants and one primer OPE-11 amplified ~600 bp band specific to male plants. Later, these 3 markers were screened on 19 individual genotypes (10 females and 9 males). SCAR marker was developed using 488 bp sequence information obtained for OPB-20 primer and differentiated females from males. Samantaray *et al*. (2012) used 50 RAPD primers to screen betel vine bulk samples, of which 4 primers resulted in sex-specific amplification. These 4 primers were screened on individual plants resulting in markers that can identify males and females. The specific markers for males were $OPA04_{1400}$, $OPA08_{650}$, and $OPN02_{850}$, and for females are $OPA08_{1200}$ and $OPC06_{980}$. Khadke *et al*. (2012) tested 35 ISSR primers on 20 germlines of a *Piper* and observed that only 3 primers produced sex-specific amplification in bulk samples. The primers UBC-852 and ISSR-10 amplified 459 and 1250 bp bands in male plants, and the primer ISSR-23 amplified 636 bp band in all female plants tested. SCAR marker developed from ISSR-23 resulted in amplification only in females and absent in all male plants.

2.7. Phytochemicals, antioxidant assays and metabolite analysis in dioecious plants:

Plant-based medicines have gained worldwide popularity due to their potential to cure several diseases. Over the last few years, several effective chemotherapeutic drugs and bioactive metabolites with antibacterial and antifungal biological activities have been derived from plants for human health (Ezzatzadeh *et al*. 2012; Prasannabalaji *et al*. 2012). So far only 6% plant species out of 20% scientifically studied plants have been screened for potent pharmaceutical properties in the world (Lahlou 2013; Cragg and Newman 2013).

For a long time, *S. glauca,* an important medicinal tree species from the Simaroubaceae family, has been used as a herbal drug. Simaroubaceae family is well known for its diverse range of chemical compounds in different parts of the plant like stem, root, leaves and seeds (Vikas *et al*. 2017). Phytochemicals isolated from different parts of the plants of the Simaroubaceae family include alkaloids, steroids, quassinoids,

triterpenes, anthraquinones, coumarins, flavonoids, anthraquinones, sesquiterpenes, limonoids, fatty acids, saponins, lignans, vitamins, coumarins etc. (Barbosa *et al*. 2011). Quassinoids (triterpenes) were found to be the taxonomic marker in the Simaroubaceae family (Fernando *et al*. 1995; Muhammad *et al*. 2004; Saraiva *et al*. 2006; Almeida *et al*. 2007). Bhatnagar *et al*. (1984) reported the presence of the two quassinoids namely glaucarubolol and glaucarubolone in the seeds. Most of the Simaroubaceae members are used in treating cancer, viruses, inflammation, malaria, worms, gastritis, ulcer, diarrhea and diabetes. The metabolites were found to have antitumor activity (Rivero-Cruz *et al*. 2005), tonic and healing activities and also exhibited antileishmanial activity (Bhattacharjee *et al*. 2009). In recent years, metabolite profiling is done for the identification of primary and secondary metabolites, which have biological and nutritional importance. Among several techniques for analyzes of plant metabolome, GC or LC coupled with mass spectroscopy are gaining importance (Steinhauser and Kopka 2007; Bedair and Sumner 2008; Dhuli *et al*. 2014; Akter *et al*. 2016).

ROS (reactive oxygen species) and free radicals are the end products of cellular mechanisms and biochemical pathways in humans as a byproduct (Young and Woodside 2001; Nantitanon *et al*. 2007). In human body, the oxidative stress results in production of a higher level of free radicals like H_2O_2, $^{\bullet}OH$, $^1O_2$, $O_2^{-\bullet}$ (Hossain *et al*. 2017). Further, these unstable free radicles in the cells may cause mutations, Alzheimer's, myocardial infarction and several other clinical disorders (Adiguzel *et al*. 2009; Sukumaran and Raj 2010). Antioxidants can be either synthetic or natural compounds that are used to control the adverse effects of free radicals by inhibiting or delaying the oxidation of molecules. Propyl gallate, BHA, tertbutyl hydro quinine and BHT are the commonly used synthetic antioxidants. Several studies have reported that these synthetic antioxidants are resulting in several side effects on human health causing liver damage due to carcinogenicity and toxicity. Hence, scientists are concerned about the safety of these synthetic antioxidants that are the major limitation. To overcome these limitations, plant-derived natural antioxidants were used for better human health (Rajasekar *et al*. 2011). It is well known that plants are the richest source of antioxidants and have traditional medicinal values as they are used to treat several diseases (Altemimi *et al*. 2017). Tocopherols, lignins, carotenoids, anthocyanins, flavonoids, phenolics, tannins, and vitamins A, C, E found in plants act as natural antioxidants and are beneficial for promoting human health (Altemimi *et al*. 2017). Antioxidant activity

measured by FRAP (Ferric reducing antioxidant power) assay, DPPH (2,2-diphenyl-1-picrylhydrazyl) assay, lipid peroxidation, superoxide, and hydroxyl radical scavenging activity in several studies have shown significant correlation between the concentration of the extract and the percentage of inhibition of free radicals. Kähkönen *et al*. (1999) reported that antioxidant activity does not necessarily correlate with high amounts of phenolics. Therefore, for evaluating the antioxidant potential of extracts, both phenolic content and antioxidant activity need to be determined.

The studies on metabolite analysis in relation to sex are relatively less in dioecious species. In both male and female plants, the chemical compounds like secondary metabolites are used in attracting pollinators, tolerance against stress conditions (biotic and abiotic), and growth and development of plants; and vary with seasons, environmental factors and phenological stages of plant (Vinod *et al*. 2007; Carvalho *et al*. 2014). There are studies that showed gender-specific reaction to stress conditions like drought, low temperature, CO_2 increase in atmosphere, etc. caused by environmental factors (Wang and Curtis 2001; Chen *et al.* 2010; Zhang *et al*. 2011) and concluded that females are sensitive to environmental stress. The reports for other species showed that females are exhibiting equal or more resistance than males to stress conditions (Juvany *et al*. 2014). As the studies related to sex-related chemical differences are less, there is need to focus on this topic in dioecious species like *S. glauca*.

2.7.1. Phytochemical and antioxidant studies in *S. glauca*:

There are published papers related to phytochemical and antioxidant assays and GC-MS profiles for leaves, bark, and flowers with respect to medicinal value of *S. glauca.* However, the phytochemical differences among different genders of this tree species have not yet been investigated.

Kumar *et al*. (2016) reported the presence of secondary metabolites like phenols, flavonoids, tannin, alkaloids, saponins, steroids, glycosides, lignin and terpenoid in methanol and ethanol extracts of *Simarouba* leaves. The methanolic extracts yielded higher phytochemical content compared to the ethanolic extracts tested. Lakshmi *et al*. (2014) reported antimicrobial, antioxidant, thrombolytic and haemolytic activities in methanolic, chloroform and ethyl acetate leaf extracts of *S. glauca*.

Umesh *et al*. (2015) reported high phenolic content in water extracts of leaves, high tannin content in ethanol extract and low flavonoid content in all extracts (methanol, ethanol and water). Methanolic extract showed highest antioxidant activity for reducing power, FRAP and phosphomolybdenum assays whereas ferric-ferrozine assay resulted in more antioxidant activity with aqueous extracts. Sajeeda *et al*. (2019) reported that leaves and bark extracts have maximum number of phytochemicals compared to flowers. The content of phenols, alkaloids, flavonoids, and carbohydrates varied among different extracts (water, methanol, chloroform, and ethanol) and samples used. The water and methanol extracts of bark and leaves showed DPPH radical scavenging and reducing activities whereas NO radical scavenging was exhibited by water extract of bark.

Ramya *et al*. (2019) reported that methanolic and chloroform extracts exhibited good antibacterial activity whereas ethyl acetate extract resulted in good antifungal activity. Ramesh *et al*. (2017) observed the presence of Vitamins (A, B1, B2, B3 and C) with high concentration of Vitamin-C in the ethanolic extracts of leaves. The DPPH assay revealed the antioxidant activity of *S. glauca* leaves with IC_{50} value of 51.50 (µg/ml). Varghese *et al*. (2016) carried out the GC-MS analysis of different extracts (ethanol, ethyl acetate, petroleum ether, water and acetone) of leaves of *S. glauca*. The study revealed the presence of bioactive compounds with antioxidant and antimicrobial activities against the opportunistic pathogens causing infections in HIV/AIDS patients. Kaushik *et al*. (2014) and Mikawlrawng *et al*. (2014) showed that the ethanolic extracts of leaves exhibited more antifungal activity against *Aspergillus parasiticus* compared to the methanolic extracts tested and therefore *S. glauca* leaves were considered as antifungal agents.

2.7.2. Differences in chemical constituents among sexes in dioecious plants:

Prasad and Iyengar (1985) observed differences in proteins, carbohydrates and nuclei acids among sexes of *Jojoba* during different seasons where females showed higher metabolic activities, more RNA and DNA compared with the male plants. Bajpai *et al*. (2012) have analyzed the metabolite-based differences in male and female plants of *Piper betle* using Direct Analysis in Real Time Mass Spectroscopy (DARTMS). Characteristic differences between female and male leaf samples were noticed in the spectra and the identified peaks showed gender specific metabolites and the analysis of

peaks showed 2 distinct clusters with male and female landraces. The study of Bajpai *et al*. (2015) revealed significant variations in metabolomics (chemical profiles) and quantity of bioactive alkaloids in ethanolic samples of male and female *Tinospora cordifolia*.

El-Yazal (2018) observed that female trees of date palm (*Phoenix dactylifera* L.) showed high anabolic capacity and accumulation of chemical constituents like total phenols, total free amino acids, total carotenoids, chlorophyll a & b, total anthocyanin, total indoles, total carbohydrates & reducing sugars, crude protein, nitrogen, silicon and dry matter whereas male trees had increased concentrations of proline and ash in the pinnae. These differences were used to predict the sex of nine-year-old date palm trees. Yosr *et al*. (2018) reported marked quantitative and qualitative differences in phenolic and terpenoid compositions with regard to sex, part of plant material, and collecting periods in *Pistacia lentiscus* L. The leaves and flowers of males showed highest content of total flavonoids, phenolic and condensed tannins than females and decreased in both the sexes from vegetative stage to ripening stage.

Hajaji *et al*. (2011) observed no differences in preliminary phytochemical screening of flavonoids, alkaloids, tannins and saponins among the three genders and extracts (ethyl acetate and methanol) in carob tree barks. The total phenol content and antioxidant activity by DPPH assay revealed high polyphenols and DPPH activity in males compared to spontaneous and grafted females. Among the extracts used, methanol's bark extract (crude) had greater amounts of phenols and antioxidant activity for all three genders than the ethyl acetate bark extract. Sharma *et al*. (2015) differentiated male and female guduchi based on total starch, total alkaloid and total mucilage contents that were high in female plants than males. Amri *et al*. (2018) studied the methanolic extracts of leaves of *P. atlantica* for antioxidant and anti-inflammatory activities and reported that total flavonoids and phenolic contents and antioxidant activity by DPPH and ABTS (2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid) showed no significant differences among sexes. The FRAP assay resulted in significant antioxidant activity in extracts of male plants compared to female plants.

2.7.3. Occurrence of sex hormones in plant species:

The presence of steroids is well-known in animals, but they were also known to occur naturally as plant integral compounds and act as chemical messengers that

regulate cell-cell communication and physiological processes associated with development, growth, and reproduction (Heftman 1975; Geuns 1978; Janeczko and Skoczowski 2005; Janeczko 2012). Simon and Grinwich (1989) screened over 128 species of plants of 50 families by radioimmunoassay to demonstrate the widespread occurrence of progesterone, androgens and estrogens. They found that 80% of investigated plant species have progesterone, 70% of species showed testosterone and its derivatives, 50% species were found to possess estrogens (estrone and 17β-estradiol) and especially androgens were identified in the seeds of all tested species. The signaling molecules like phytoecdysteroids and steroid hormones of plants like brassinosteroids (Tarkowská and Strnad 2018), progesterone, testosterone, and its derivatives have phytosterols as starting material for their biosynthesis. The squalene, a C30 linear hydrocarbon, is an essential intermediate for the synthesis of plant sterols (Huang *et al*. 2009; Ali *et al*. 2016; Casuga *et al*. 2016; Zimila *et al*. 2020) which results in Δ^{24} sterol cycloartenol, a C30 compound by undergoing cyclization process in photosynthetic plants (Janeczko and Skoczowski 2005). The squalene was obtained from two molecules of farnesyl diphosphate by condensation (Zhao *et al*. 2010), and the squalene produced in eukaryotes play a prominent role in the sterol pathway.

The molecular studies carried out in *Arabidopsis* related to the perception of steroids in plant cells have resulted in the existence of plasma membrane-localized binding protein for steroids and have the abilities to bind with different affinities to several steroid molecules (Yang *et al*. 2005). Janeczko *et al*. (2008) have employed radio ligand binding technique and observed the presence of putative receptors for steroids that act as binding sites specific to progesterone and 17beta-estradiol located in the cell membrane and cytoplasm of wheat. Simerský *et al*. (2009) and Iino *et al*. (2007) have provided evidence-based on mass spectrometry for the presence of the plant hormones progesterone, testosterone, and their derivatives in minimal concentrations in plant tissues.

Hirotani and Furuya (1974) for the first time found the testosterone metabolites in plant systems such as 5α-androstane-3β, 17β-diol dipalmitate, and 3- and 17-monoglucosides, testosterone glucoside, and epiandrosterone palmitate and glucoside in suspension cultures of tobacco callus. The testosterone, epitestosterone and Δ^4-androstene-3, 17-dione were found to co-occur in the pollen of *Pinus silvestris* and later in *Pinus nigra* (Šaden-Krehula *et al*. 1971 & 1979). In Arabidopsis and winter wheat

(*Triticum aestivum*), androst-4-ene-3, 17-dione (AED), the precursor of testosterone stimulated the callus proliferation and affected plant growth by promoting germination and immature embryos growth, respectively (Janeczko 2000; Janeczko *et al*. 2002). The AED in Arabidopsis significantly increased the plant's ability to attain the reproductive stage (Janeczko *et al*. 2003).

The existence of estrogens in various plant species has been reported by many authors in species that belong to the *Prunus* genus, bean, date palm, and pomegranate (Hassan and Wafa 1947; Bennett *et al*. 1966; Kopcewicz 1971a; Dean *et al*. 1971; Awad 1974; Young *et al*. 1977) and the receptor-like proteins for estrogens in *Solanum glaucophyllum* were also isolated from several plant organs which were confined to the nucleus (Milanesi *et al*. 2001). The endogenous levels of estrogens were found to be high in reproductive plant parts such as pollen grains, flowers, seeds, and fruits whereas less in vegetative plant parts like leaves, stem, and roots (Sláma 1980).

In plants, prostaglandins were first discovered by Attrep *et al*. (1973) in *Allium cepa*. Attrep *et al*. (1980) employed GC-MS technique to re-examine their findings done on the onion in 1973 and proved the presence of prostaglandin (PGA_1) presence in higher plants. Janistyn (1982) employed GC-MS analysis in *Kalanchoe blossfeldiana* and detected prostaglandins specifically in flowering plants compared to vegetative plants. The precursor for prostaglandin *i.e.,* arachidonic acid occurred in buds of *Populus balsamifera*, wheat germ oil, *Aloe vera* and garlic (Groenewald and van der Westhuizen 1997). The enzymes responsible for prostaglandin synthesis were studied in plants and the enzyme (lipoxygenase 2) extracted from soybean catalyzed the synthesis of prostaglandin PGF2α from arachidonic acid (Bild *et al*. 1978). Thus, the presence and effect of animal sex hormones in many plants as well as few metabolic pathways were revealed from various studies.

2.8. *In vitro* regeneration potential of explants:

The establishment of an efficient *in vitro* plant regeneration system is of great importance for rapid multiplication of desired genotype as well as conservation of the germplasm in any plant species. The availability of regeneration procedures offers considerable scope for application of biotechnological tools for the genetic improvement of the species. The source of explants, the composition of media, type and concentrations of hormones, and culture conditions play an important role in *in vitro*

regeneration of plant species (Kalia *et al*. 2014). The nutritional requirements for *in vitro* growth vary among the species and different explants obtained from the plant. The nodal explants or shoot tip explants obtained from mature trees are preferred for micropropagation as the regenerated plants are less prone for genetic variations. The age of the donor tree, type of explant, and season-dependent explant collection, restricts the tissue culture response of explants collected from mature trees. Moreover, the high contamination rates, browning of explants, lower regeneration efficiency would further limit their utilization for rapid propagation of plants. The meristematic tissue from embryo and seedlings were found to be highly responsive when compared to explants obtained from mature plants (Novak and Konecna 1982; Lu *et al*. 1982; Ahuja *et al*. 1983).

2.8.1. *In vitro* regeneration studies in *S. glauca*:

In vitro regeneration has been previously reported by various researchers from different explants in *S. glauca*. Rout and Das (1994a) first reported somatic embryogenesis in callus cultures derived from immature cotyledons of *S. glauca* using 6-benzylaminopurine (BAP) and α-naphthaleneacetic acid (NAA). From those cultures, 20–25% of the somatic embryos germinated and regenerated into plants. Subsequently, Rout and Das (1994b) showed an enhancement in the frequency of somatic embryogenesis to 80% from immature-cotyledon-derived callus cultures using BAP, NAA, and ascorbic acid in Murashige and Skoog's (MS) medium. In recent studies, somatic embryos were also produced from immature zygotic embryos using BAP and NAA, of which 25% germinated and developed into plants (Das 2011). Jyothi *et al*. (2014) reported that a combination of NAA and 2,4-dichlorophenoxyacetic acid (2,4-D) was effective for induction of friable embryogenic callus from basal leaf segment than apical and middle segments of leaf explants in *S. glauca.* The effect of orientation of explants on efficiency of regeneration was studied, where the horizontal position of explants resulted in higher efficiency for regeneration compared to vertical position of explants in the medium. Hadke *et al*. (2008) observed initiation of callus from nodal explants in medium with different concentrations for BAP and 2,4-D which improved with increase in concentration of BAP and 2,4-D.

In vitro shoot multiplication using nodal explants of *S. glauca* has been reported by Rout and Das (1995), with induction of 5.83 shoots per explant on MS medium with

BAP (2.5 mg /l) and NAA (0.1 mg/l). MS medium amended with IBA (1.0 mg/l to 1.5 mg/l) was suitable for root induction. Rout *et al*. (1999) showed that microshoots produced from nodal explants could be rooted on MS medium with IBA (1.0 mg/l) and sucrose. Shrivastava and Banerjee (2008) observed that the axillary nodes collected from plants before flowering resulted in best regeneration. Shukla and Padmaja (2012) reported better regeneration capacity from nodal explants as compared to shoot tip explants on MS medium with BAP and NAA. Recently, Dudhare *et al*. (2014) attempted *in vitro* propagation of *S. glauca* using shoot tips and axillary buds and observed that the shoot multiplication response from both explants was low on MS medium with BAP and indole-3-acetic acid (IAA). Kala *et al*. (2017) studied the effect of IBA concentrations with respect to rooting and survival of the cuttings obtained from stems of field-grown plants. Of all the concentrations of IBA tested, 4000 ppm resulted in efficient rooting (45.16%), sprouting (52.31%), and survival (42.56%). Despite the attempts made by various investigators to regenerate the plants using different explants, an effective regeneration system is yet to evolve for exploitation for different applications.

2.8.2. Genetic stability analysis of *in vitro* regenerated plants:

The assessment of the genetic stability of *in vitro* regenerated plants with respect to mother plants is essential before their utilization for commercial purpose, as there is a chance of the occurrence of somaclonal variations in micropropagated plants (Rani and Raina 2000; Rahman and Rajora 2001; Devarumath *et al.* 2002). During cultures and sub-cultures, changing the concentrations of phytohormones intensifies the formation of free radicals and induces mutations in regenerated plants (Mohammed *et al*. 2011). The somatic recombination, genetic rearrangements, chromosomal mutations, sister chromatid exchanges, instability of tandem repeat sequences, and activity of transposable elements and epigenetic changes like DNA methylation are proposed mechanisms only, as the actual reasons are not known for the incidence of somaclonal variations (Phillips *et al*. 1994; Mohan 2001). Even though the possibility of occurrence of somaclonal variations through direct regeneration is very less, it might result in off-types that limit the economic value of regenerated plants (Oh *et al*. 2007; Nookaraju and Agrawal 2012). Hence, the genetic stability of micropropagated plants should be ascertained before commercial exploitation. The genetic stability of micropropagated plants can be assessed by using phenotypic, allozyme and molecular techniques. The

absence of changes in the morphology of plants, does not rule out the possibility of variations at DNA level. The assessment of genetic stability using morphological characters is limited in tree species due to long generation cycles. These limitations can be overcome by using molecular markers like RAPD, ISSR, AFLP, etc., which detect DNA polymorphism at different loci present in the genome and facilitates genetic stability analysis of regenerated plants.

Yuan *et al*. (2009) investigated genetic variations in calli, adventitious shoots, regenerated plantlets, two-year-old regenerated plants along with one and two-year-old seedlings of *Saussurea involucrate* by RAPD and ISSR markers. Among 11 types of plant material examined, genetic variations were detected by RAPD and ISSR with 35% and 33% polymorphism, respectively. Rady (2006) studied the regenerated plants from shoot tip explants using 9 RAPD primers and observed 7.74% polymorphism. Lata *et al*. (2010) confirmed the genetic stability of *Cannabis sativa* micropropagated plants obtained from nodal explants with axillary buds after subculturing, using ISSR markers.

Cui *et al*. (2019) compared the plants regenerated from lateral buds of *Magnolia sirindhorniae* Noot. & Chalermglin with the mother plant using RAPD and ISSR markers, and observed monomorphic bands confirming the genetic stability and uniformity. Goyal *et al*. (2015) studied the genetic fidelity of micropropagated plants of *Dendrocalamus strictus* using RAPD and ISSR markers which revealed all monomorphic bands indicating that nodal explant are suitable explants to produce clones in *Dendrocalamus strictus* (Roxb.). Sebastiani and Ficcadenti (2016) employed 20 RAPD primers for genetic stability analysis and revealed the genetic uniformity among regenerated plants. Pathak and Dhawan (2012) studied clonal fidelity of *in vitro* regenerated apple rootstock Merton from axillary buds using 24 ISSR primers and 15 ISSR primers. The comparison of banding profile of tissue culture-raised plants with donor plant demonstrated the utility of the protocol for multiplication by axillary buds as the suitable method to obtain true-to-type plants.

Materials and Methods

3. MATERIALS AND METHODS

3.1. Plant material:

The leaves of *Simarouba glauca* were collected from 6-10-year-old trees (Fig. 1) growing at Professor Jayashankar Telangana State Agricultural University (PJTSAU), Rajendranagar, Hyderabad every year during February 2014-2018. The sex of individual trees was ascertained by observing the sex expression pattern of flowers (Fig. 2a-c) during flowering time and labeled as females, males, and andromonoecious genotypes. The leaf samples collected separately from 36 genotypes of different sexes (15 females, 6 males and 15 andromonoecious trees) were employed for genetic variation and sex-identification studies using molecular markers (RAPD, ISSR and SCAR markers). In the study, the females were designated as F1-F15, males as M1-M6 and andromonoecious as A1-A15. The leaves were collected from trees growing at Indian Institute of Oilseed Research (IIOR), Narkhoda, Shamshabad, Ranga Reddy district, whose sex was ascribed, based on observations of flowers of the trees during February 2019 and the females were designated as NF, males as NM and andromonoecious as NA. The leaf samples collected from trees of different sexes were immediately placed in polythene covers or aluminum foils and brought to the laboratory in a box with ice. The leaves were stored in a deep-freezer at -80°C until DNA extraction. Leaves were also collected from ten *in vitro* raised seedlings and twelve 2-3-year-old plants growing at University of Hyderabad campus.

Leaf samples were collected from 15 trees of different sexes (5 each of females, males and andromonoecious) growing at PJTSAU campus in 2018. The collected plant material was washed thoroughly under tap water, then dried under shade for 20 days, powdered and used for phytochemical assays.

The shoot tips, nodal explants and seeds obtained from 6-7-year-old mature trees were used for *in vitro* shoot regeneration and *in vitro* germination studies. The seeds collected from PJTSAU were stored in paper bags at ambient temperature (24 ± 2°C) until further use for germination. The explants obtained from 25-day-old *in vitro* germinated seedlings were further used for regeneration studies. The leaf samples collected from 25-day-old *in vitro* germinated donor seedling of *S. glauca* and ten *in vitro* regenerated plants produced from seedling-derived explants were used for genetic stability analysis using molecular markers (RAPD and ISSRs).

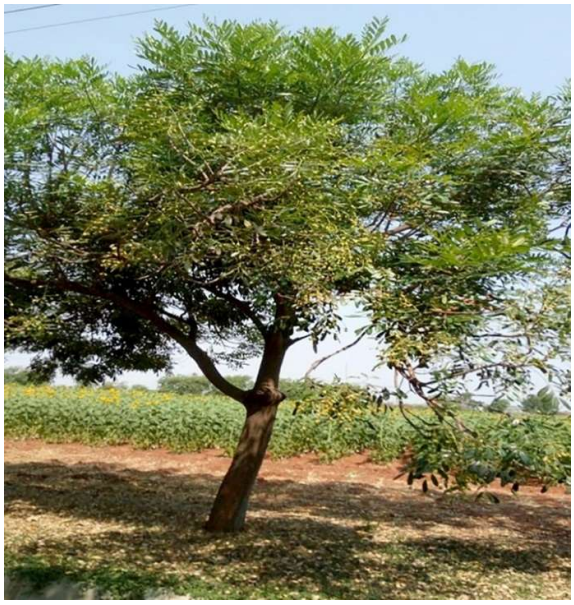

Fig. 1. Nine-year-old tree of *Simarouba glauca* DC. with fruits.

Fig. 2. Flowers of different sex types in *S. glauca.* (*a*) Female flower, (*b*) Male flower, (*c*) Bisexual flower.

3.2. Genomic DNA extraction and quantification:

3.2.1. Genomic DNA isolation using the CTAB method:

The total genomic DNA was extracted from leaves of different sexes (female, male and andromonoecious genotypes) collected from PJTSAU, Rajendranagar and IIOR, Narkhoda using the modified cetyltrimethylammonium bromide (CTAB) method adapted from Doyle and Doyle (1990). The leaf samples (1 gm) were homogenized in liquid nitrogen using mortar and pestle. The ground tissue powder was suspended in 3 ml of extraction buffer consisting of 2.5% CTAB (Sigma-Aldrich®, St. Louis, USA), 2% PVP (polyvinylpyrrolidone, Himedia, Mumbai, India), 0.5 M Na_2EDTA (pH 8.0) (disodium ethylenediaminetetraacetate dehydrate, SRL [Sisco Research Laboratories], Mumbai, India), 1M Tris-HCl (pH 8.0, Tris hydrochloride, SRL), 5M NaCl (sodium chloride, Himedia) and 0.2% (v/v) β-mercaptoethanol, Himedia. The extraction buffer was prepared each time freshly before use. The mixture was collected in 2 ml tubes with activated charcoal and vortexed thoroughly and incubated at 65°C for half-an-hour. After incubation, the above mixture was centrifuged at 12,000 rpm for 10 min and then the supernatant was collected and taken into a separate tube. An equal volume of chloroform and isoamyl alcohol (24:1, v/v) was added and mixed for 10-15 min. The mixture was centrifuged at 12,000 rpm for 10 min, and the aqueous (upper) phase was collected, and then an equal volume of chilled isopropanol was added and incubated at -20°C for 30-60 min to precipitate the DNA. After incubation, the mixture was centrifuged at 10,000 rpm for 10 min, and the supernatant was discarded, and the pellet was collected into a microfuge tube. Then, 1 ml 70% ethanol was added to the pellet, mixed for 10 min and centrifuged at 10,000 rpm for 10 min. The ethanol was pipette out, and the pellet was dried at 35°C for 20-30 min. The pellet was resuspended in 1 ml of RNase free water. The suspended pellet was then given RNase treatment by adding 2 µl of 10 mg/ml RNase (Sigma-Aldrich®) and mixed by inverting the tubes. The tubes were incubated at 37°C for 1½ hr or left overnight at room temperature. After incubation, an equal volume of chloroform and isoamyl alcohol (24:1, v/v) was added and mixed for 10-15 min and then centrifuged at 12,000 rpm for 10 min. The aqueous (upper) phase was collected in a separate tube into which 1/10 volume of 3M sodium acetate (pH 4.8) and an equal volume of chilled isopropanol was added and incubated at -20°C for 30 min to precipitate DNA. The mixture was centrifuged at 10,000 rpm for 10 min, and the supernatant was discarded. The pellet was collected in 1 ml of 70%

ethanol and centrifuged at 10,000 rpm for 10 min. The ethanol was pipetting out, and the pellet was placed at 35°C until it dried completely. The pellet was dissolved in 500 µl of RNase free water or TE buffer (10 mM Tris, SRL, Mumbai, India], 1 mM EDTA (SRL, Mumbai, India) buffer, pH 8.0 for long term storage.

3.2.2. Genomic DNA isolation using the Kit method:

The genomic DNA was isolated from young leaf samples of regenerated plants and greenhouse established plants (300 mg) using a Plant DNA extraction kit (Bioserve Biotechnologies, Hyderabad, Andhra Pradesh, India). The leaf samples were blended with the help of mortar and pestle in the liquid nitrogen. Then, 1 ml of lysis buffer PL1 (Bioserve Code) and 50 mg of activated charcoal were added, and the resulting mixture was transferred into a 1.5 ml microfuge tube and vortexed thoroughly. Then, 25 µl of Proteinase K (Bioserve Biotechnologies) and 1 µl of RNAse (10 mg/ml, Sigma-Aldrich®) were added, followed by incubation at 60°C for 30 min. After incubation, the above lysate was centrifuged at 12,000 rpm for 10 min, and the supernatant was collected into a separate tube, and an equal volume of buffer PLB (DNA binding buffer) was added. For each preparation, one Bioserve column was taken and placed in a 2 ml collection tube. The above lysate was then transferred to the column and centrifuged at 6500 rpm for 2 min. The flow-through was discarded, and 500 µl of Wash buffer-1 was added to the column and centrifuged for 1 min at 10,000 rpm. The flow-through was discarded and the Bioserve column was placed back into a 2 ml collection tube. Then, 600 µl of Wash buffer-II was added to the column and centrifuged at 10,000 rpm for 1 min. The flow-through was discarded and the columns were centrifuged with caps open at 11,000 rpm for 3 min for drying. The columns were left open for 2 min before proceeding to the elution step. The Bioserve columns were placed in a fresh 1.5 ml microfuge tube, and 50-80 µl of elution buffer PLE (DNA elution buffer) preheated at 70°C was added to the column and then left at room temperature for 10 min. The bound DNA to the column was eluted by centrifugation at 10,000 rpm for 2 min.

3.2.3. DNA quantification:

Qualitative and quantitative assessment of genomic DNA isolated by CTAB and Kit methods was carried out with a NanoDrop™ ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) at 260 nm, and the quality of DNA was determined by the A_{260}/A_{280} ratio. The qualitative assessment was done by running the dissolved

DNA along with 6X DNA loading dye (Thermo Scientific™, Waltham, MA, USA) on 1% (w/v) agarose (Sigma-Aldrich®) gel electrophoresis. The 1X TAE (Tris–acetate–EDTA, SRL) was used as running buffer, and the gel was run for 1 hr at 100 V, and then stained with ethidium bromide (EtBr; 0.8 mg/l Sigma-Aldrich®). The purity and integrity of DNA was visualized under UV transilluminator (Uvitec, Cambridge, UK). Based on the nanodrop readings, the concentrated DNA stock was diluted to 6 ng/µl Milli-Q and the diluted aliquots that were stored at -20°C were used for DNA amplification for genetic variation and sex-identification studies.

3.3. Analysis of genetic variation and identification of sex-specific markers using molecular markers:

3.3.1. Screening of female, male and andromonoecious genotypes for analysis of genetic variation by RAPD and ISSR primers:

The RAPD and ISSR primers were screened on female, male and andromonoecious genotypes for PCR (Polymerase chain reaction) amplification using bulk-segregant analysis (BSA, Michelmore *et al*. 1991). The bulk DNA samples of female, male and andromonoecious genotypes were made by pooling equal volumes of genomic DNA from the diluted aliquots (6 ng/µl) of 5 individual genotypes of three sexes of *S. glauca* collected from PJTSAU. Initially, 64 RAPD and 33 ISSR primers (including previously reported primers) were used to screen 3 bulked DNA samples (Table 1, 2 & 3; Fig. 3). Later, 61 RAPD and 24 ISSR primers, which resulted in precise and reproducible PCR amplification, were used to test all individual genotypes to analyze genetic variation between female, male and andromonoecious genotypes.

3.3.2. Screening of female, male and andromonoecious genotypes for sex identification by RAPD and ISSR primers:

The polymorphism among bulk DNA samples of female, male and andromonoecious genotypes of *S. glauca* was assessed using 61 RAPD and 24 ISSR primers (Table 1, 2 & 3; Fig. 3) to identify primers that resulted in sex-specific amplification. The primers that detected polymorphism in bulk DNA samples were screened on the DNA isolated from individual genotypes of all three sexes to identify sex-linked markers. The PCR amplified DNA bands that could differentiate sexes in

Table 1. List of RAPD primers used for genetic variation analysis and sex identification studies of *Simarouba glauca* DC.

S. No.	Primer	Primer sequence (5'-3')	S. No.	Primer	Primer sequence (5'-3')
1	OPF-03	CCTGATCACC	33	OPA-06	GGTCCCTGAC
2	OPF-11	TTGGTACCCC	34	OPB-05	TGCGCCCTTC
3	OPA-04	AATCGGGCTG	35	OPB-20	GGACCCTTAC
4	OPD-14	CTTCCCCAAG	36	OPO-08	CCTCCAGTGT
5	OPS-05	TTTGGGGCCT	37	OPS-06	GATACCTCGG
6	OPAW-7	AGCCCCCAAG	38	OPT-07	GGCAGGCTGT
7	OPT-18	CATGCCAGAC	39	OPW-03	GTCCGGAGTG
8	OPAB-06	GTGGCTTGGA	40	OPG-16	AGCGTCCTCC
9	OPAL-08	GTCGCCCTCA	41	OPG-08	TCACGTCCAC
10	OPAK-14	CTGTCATGCC	42	OPG-06	GTGCCTAACC
11	OPT-17	CCAACGTCGT	43	OPG-17	ACGACCGACA
12	OPZ-01	TCTGTGCCAC	44	OPK-01	CATTCGAGCC
13	OPZ-10	CCGACAAACC	45	OPK-09	CCCTACCGAC
14	OPZ-06	GTGCCGTTCA	46	OPK-07	AGCGAGCAAG
15	OPB-12	CCT TGA CGCA	47	OPC-09	CTCACCGTCC
16	OPA-18	AGGTGACCGT	48	OPC-06	GAACGGACTC
17	OPA-17	GACCGCTTGT	49	OPC-10	TGTCTGGGTG
18	OPA-16	AGCCAGCGAA	50	OPK-10	GTGCAACGTG
19	OPA-11	CAATCGCCGT	51	OPK-16	GAGCGTGCAA
20	OPV-17	ACCGGCTTGT	52	OPC-08	TGGACCGGTG
21	OPC-05	GATGACCGCC	53	OPC-07	GTCCCGACGA
22	OPC-18	TGAGTGGGTG	54	OPH-03	AGACGTCCAC
23	OPP-03	CTGATACGCC	55	OPA-08	GTGACGTAGG
24	OPV-08	GGACGGCGTT	56	OPU-10	ACCTCGGCAC
25	OPW-17	GTCCTGGGTT	57	OPD-19	CTGGGGACTT
26	OPA-12	GACGCGAACC	58	OPU-19	GTCAGTGCGG
27	OPAL-20	AGGAGTCGGA	59	OPD-20	ACCCGGTCAC
28	OPE-20	AACGGTGACC	60	OPA-05	AGGGGTCTTG
29	OPB-07	GGTGACGCAG	61	OPC-16	CACACTCCAG
30	OPF-08	GGGATATCGG	62	OPD-15	CATCCGTGCT
31	OPG-14	GGATGAGACC	63	OPE-05	TCAGGGAGGT
32	OPK-03	CCAGCTTAGG	64	OPD-12	CACCGTATCC

Table 2. List of ISSR primers used for genetic variation analysis and sex identification of *S. glauca.*

S. No.	Primer	Primer sequence (5'-3')	S. No.	Primer	Primer sequence (5'-3')
1	ISSR-12	GAGAGAGAGAGAGAGAGAT	18	UBC-842	GAGAGAGAGAGAGAGAYG
2	ISSR-13	GAGAGAGAGAGAGAGAGAA	19	UBC-844	CTCTCTCTCTCTCTCTRC
3	ISSR-14	AGAGAGAGAGAGAGAGC	20	UBC-851	GTGTGTGTGTGTGTGTYG
4	ISSR-17	CTCTCTCTCTCTCTCTG	21	UBC-862	AGCAGCAGCAGCAGCAGC
5	ISSR-18	ACACACACACACACACT	22	UBC-867	GGCGGCGGCGGCGGCGGC
6	ISSR-19	ACACACACACACACACC	23	UBC-866	CTCCTCCTCCTCCTCCTC
7	UBC-813	CTCTCTCTCTCTCTCTT	24	UBC-865	CCGCCGCCGCCGCCGCCG
8	UBC-820	GTGTGTGTGTGTGTGTC	25	UBC-873	GACAGACAGACAGACA
9	UBC-823	TCTCTCTCT CTCTCTCC	26	UBC-875	CTAGCTAGCTAGCTAG
10	UBC-824	TCTCTCTCTCTCTCTCG	27	UBC-876	GATAGATAGACAGACA
11	UBC-834	AGAGAGAGAGAGAGAGTT	28	UBC-874	CCCTCCCTCCCTCCCT
12	UBC-835	AGAGAGAGAGAGAGAGCC	29	UBC-879	CTTCACTTCACTTCA
13	UBC-846	CACACACACACACACAAT	30	UBC-881	GGGTGGGGTGGGGTG
14	UBC-880	GGAGAGGAGAGGAGA	31	UBC-814	CTCTCTCTCTCTCTCTA
15	UBC-899	CATGGTGTTGGTCATTGTTCCA	32	ISSR-864	ATGATGATGATGATGATG
16	UBC-818	CACACACACACACACAG	33	ISSR-06	GACAGACAGACAGACA
17	UBC-819	GTGTGTGTGTGTGTGTA			

Table 3. List of previously reported RAPD and ISSR primers tested in the present study to determine the sex of *S. glauca.*

S. No.	Primer	Primer sequence (5'-3')	Size of fragment (bp)	Presence of sex-specific band	Reference
1	OPU-10	ACCTCGGCAC	>1kb	Male specific	Vaidya and Naik (2014)
2	OPD-19	CTGGGGACTT	350 bp	Female specific	
3	OPU-19	GTCAGTGCGG	500 bp	Hermaphrodite specific	
4	OPS-05	TTTGGGGCCT	>1kb	Female and Hermaphrodite specific	
5	OPW-03	GTCCGGAGTG	600 bp	Male and Hermaphrodite specific	
6	OPE-05	TCAGGGAGGT	521	Male specific	Ghumatkar *et al*. (2015)
7	OPA-18	AGG TGA CCGT	1110 bp	Male specific	Baratakke and Patil (2014)
8	OPD-20	ACCCGGTCAC	900bp	Female specific	Simon *et al*. (2009)
9	OPS-06	GATACCTCGG	-	Male-specific	Savitha *et al*. (2008)
10	RP-49 (OPD-20)	ACCCGGTCAC	950bp	Females and bisexual specific	Choudhary (2014)
11	RP-07 (OPA-08)	GTGACGTAGG	900bp	Male and bisexual specific	
12	UBC-814	CTCTCTCTCTCTCTCTA	1000bp	Male specific	
13	UBC-815	CTCTCTCTCTCTCTCTG	1500bp	Male and bisexual specific	

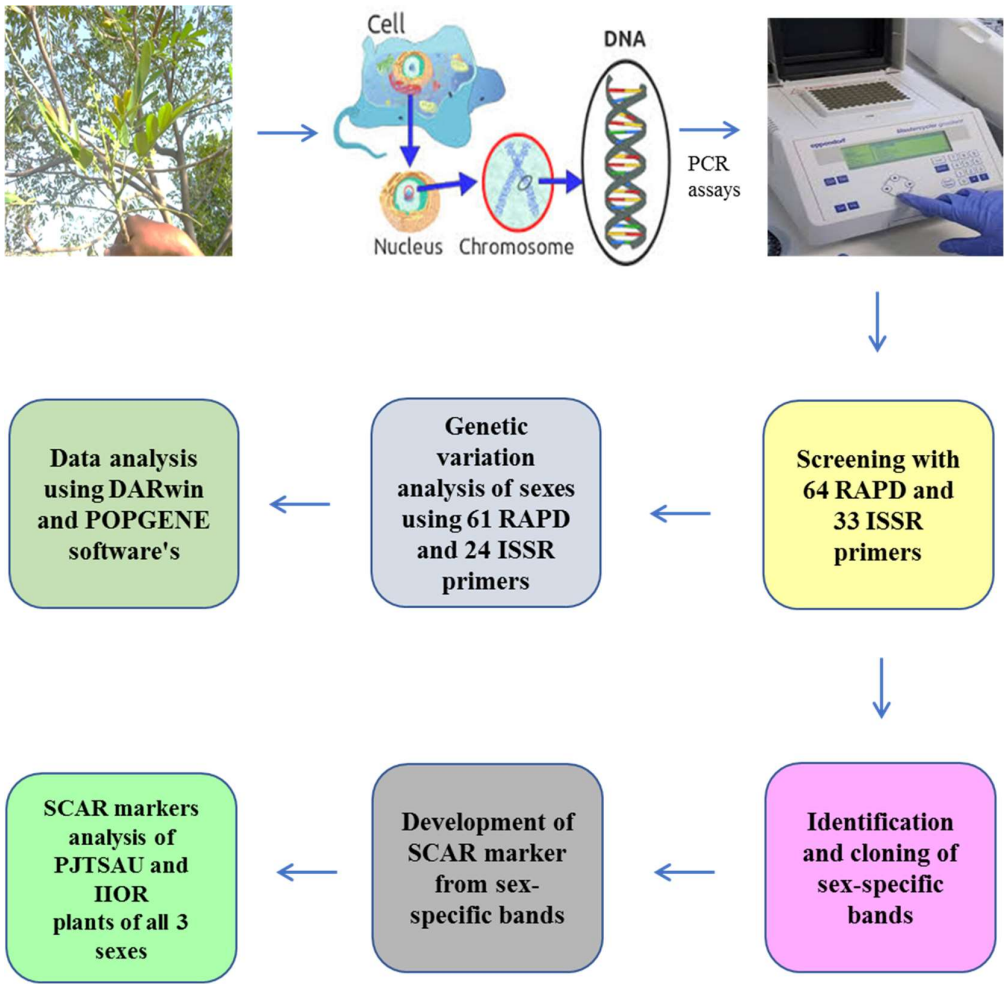

Fig. 3. Schematic representation for analysis of genetic variation and determination of sex-types of *S. glauca.*

both bulk and individual DNA samples were identified as putative markers for sex identification in *S. glauca*.

3.3.3. Testing of previously reported SCAR markers for differentiation of female, male and andromonoecious genotypes of *S. glauca*:

The SCAR markers previously reported by other researchers in *S. glauca* were synthesized commercially (Table 4). The SCAR primers were initially used to screen the bulk DNA of all three sexes of *S. glauca*. The SCAR primers that resulted in amplification of appropriate size as reported were then screened on individual DNA samples of all 36 genotypes. The annealing temperatures used were as reported for SCAR markers, and different annealing temperatures were also tried to obtain proper amplification. The PCR conditions and programs were used are mentioned in section 3.5.1. The amplified PCR products were separated on 1.2% agarose gel electrophoresis, stained with EtBr, and visualized in UV light. The gels obtained were further analyzed for the presence of sex-specific bands of appropriate size as reported for SCAR markers (Table 4).

3.3.4. Optimization of PCR conditions for RAPD and ISSR markers:

Polymerase Chain Reaction (Sambrook *et al*. 1989) was performed for RAPD (Williams *et al*. 1990) and ISSR (Zietkiewicz *et al*. 1994) analysis in an Eppendorf™ Mastercycler™ (Hamburg, Germany). Initially, the conditions for PCR amplification were optimized using different annealing temperatures for RAPD analysis (35ºC, 37ºC and 40ºC) and ISSR analysis (46ºC, 50ºC and 54ºC). The different concentrations of Taq DNA polymerase (Thermo Scientific, Waltham, MA, USA) (0.5, 1.0, 1.5 and 2.0 U), $MgCl_2$ (magnesium chloride, Thermo Scientific™) (0.5, 1.0, 1.5, 2.0 and 2.5 mM) and DNA template (15, 30 and 50 ng) were studied to standardize the conditions and the best concentrations and conditions suitable for reproducible PCR amplification of RAPD and ISSR markers were determined.

The PCR conditions used for RAPD reactions consisted of an initial denaturation step at 94°C for 3 min, with 42 cycles of amplification and each cycle comprising of denaturation at 94°C for 30 sec, annealing of primers at 37°C for 1 min, and primer extension at 72°C for 2 min; followed by final extension for 5 min at 72°C. The amplified PCR products were held at 4°C in the thermocycler. The reaction mixture

Table 4. List of previously reported SCAR markers tested in the present study to determine the sex of *S. glauca*.

S. No.	SCAR Primer	SCAR primer sequence (5'-3')	Primer length (bp)	Annealing temperature (Ta)	Sex-specific band (bp)	Presence of sex-specific band	Reference
1	RP-49 SCAR 353 FP	AAAAAGTTTGCTTCCCAATTGTA	23	60°C	353	Females and Andromonoecious	Choudhary (2014)
	RP-49 SCAR 353 FP	GTAGTGGTGCCGGTGCTAAT	20				
2	UBC-815 SCAR 721 FP	TTCCACCTACCCCAGTCTTG	20	59°C	721	Males and Andromonoecious	
	UBC-815 SCAR 721 RP	CTTGTTGTGTTGGGCAAATG	20				
3	OPA-18 (MSSMS-01F)	GTTAGCAGGTGACCGTAG	18	60°C	1100	Males	Baratakke and Patil (2014)
	OPA-18 (MSSMS-01R)	TAGGTGACCGTCCATGAATG	20				
4	OPA-08 SCAR1-F	GTGACGTAGGTATGG	15	50°C	91	Males and Andromonoecious	Prasanthi *et al*. (2010)
	OPA-08 SCAR1-R	GTGACGTAGGGAGA	14				
5	OPA-08 SCAR2-F	GTGACGTAGGTA	12	51°C	915		
	OPA-08 SCAR2-R	GTGACGTAGGGA	12				

consisted of a total volume of 25 µl containing 2.5 µl of 10X PCR buffer (Thermo Scientific™), 2 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTP (Bioserve Biotechnologies, Hyderabad, India), 2 µl of 10 pmol/µl random primers (Bioserve Biotechnologies), 15 ng of template DNA, 0.2 µl of Taq polymerase (5 U/l) and sterile Milli-Q water.

The ISSR reactions were carried out with an initial denaturation step of 94°C for 5 min; 42 cycles of amplification, with each cycle comprising a denaturation step for 1 min at 94°C, primer annealing at 50°C for 1 min, and primer extension at 72°C for 2 min; followed by terminal extension at 72°C for 10 min. PCR products amplified were held at 4°C in the thermocycler. The reaction mixture had a total volume of 25 µl containing 2.5 µl of 10X PCR buffer (Thermo Scientific™), 2 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTP (Bioserve), 2 µl of 10 pmol/µl ISSR primer (Bioserve), 30 ng of template DNA, 0.2 µl of Taq polymerase (5 U/µl) and Milli-Q water. These PCR conditions were used in RAPD and ISSR analysis to study the genetic variability among the sexes at the molecular level and for sex-specific identification in *S. glauca*.

The amplified products of RAPD and ISSR reactions were mixed with the 6X DNA loading dye (Thermo Scientific™) to maintain the final 1X concentration. They were separated on 1.2% (w/v) agarose gels in 1X TAE buffer for 2 hr at 100 V. The ethidium bromide (0.8 mg/l Sigma-Aldrich®) was added to stain the DNA and photographed in a gel documentation system (Syngene, Frederick, MD). The size of the amplified PCR products was determined with a 100-3000 bp DNA ladder (GeneRuler 100 bp Plus DNA Ladder, Thermo Scientific). All PCR reactions for RAPDs and ISSRs were performed at least thrice to check for reproducibility, and only the precise and reproducible bands were scored.

3.3.5. Data scoring and analysis of data by RAPD and ISSR markers:

The data were scored as “1” for the presence and “0” for the absence of a band for each genotype analyzed by observing the gel images, and a binary data matrix was generated manually, which is used for further analysis. Bands that were present in all plants were considered as monomorphic, whereas those that were absent in one or more plants were considered as polymorphic. All monomorphic and polymorphic bands were considered for data analysis. The percentage of polymorphic bands was calculated based on the total number of polymorphic PCR products and the number of amplified PCR products produced by all primers across the genotypes. The binary data matrix

generated for RAPD and ISSR markers for female, male and andromonoecious genotypes were analyzed using DARwin (Dissimilarity Analysis and Representation for windows) software version 6 (Perrier and Jacquemoud-Collet 2006). The Jaccard dissimilarity index (Jaccard's coefficient) of DARwin was used to generate the genetic distance matrix based on binary data matrix, and dissimilarity between the variables was estimated using 1000 bootstraps. The formula for Jaccard's coefficient is dij = (b + c)/ [a+ (b + c)] where dij represents the dissimilarity between the i and j units, 'a' represents the number of variables where both 'Xi' and 'Xj' were present, 'b' represents the number of variables of which 'Xi' is present and 'Xj' is absent, and 'c' represents the number of variables of which 'Xi' is absent and 'Xj' is present. The factorial analysis was done to analyze the genetic relationships between the genotypes based on the dissimilarity matrix generated by Jaccard's coefficient to display the Principal coordinates analysis (PCoA) plot. Thus, PCoA is a model based on distance that uses both dissimilarity matrix and a factorial analysis. By using the dissimilarity matrix, the Neighbor-Joining (NJ) tree or dendrogram was constructed based on the Unweighted Neighbor-Joining method under 1000 bootstraps (replicates), and hierarchical clustering (HC) was done by Un-weighted pair group method with arithmetic averages (UPGMA) module to know the genetic relationship between genotypes.

Polymorphic information content (PIC) was calculated based on the formula PIC = 1- Σpi^2, where pi represents the i^{th} allele frequency for the presence of band (Smith *et al*. 1997). POPGENE software version 1.32 (Yeh *et al*. 1999) was used to study the genetic diversity parameters such as the observed number of alleles (na), an effective number of alleles (ne), Shannon's information index (I) and Nei's gene diversity (h) to evaluate genetic variation among different sexes of *S. glauca* based on RAPD and ISSR data matrix.

3.3.6. Sex-specific RAPD bands among all genotypes of *S. glauca*:

The specific bands ~1250 bp, ~2900 bp and ~350 bp produced from RAPD primers associated with sex were identified in bulked and individual DNA samples of all the three sexes. These selected bands were excised from the gels for cloning and sequencing to develop sex-linked SCAR markers (Table 5).

3.3.7. Polymorphic RAPD and ISSR bands among all genotypes of *S. glauca*:

The polymorphic bands [~950 bp (OPD-20), ~1200 bp (OPD-12), ~1100 bp (OPC-06), ~400 bp (ISSR-19 and UBC-873)] generated by RAPD and ISSR primers, which were not sex-specific, were identified and selected for cloning and sequencing (Table 5). These polymorphic bands were excised from the gels, cloned, and sequenced for further analysis of sequence homology with a non-redundant database of the National Centre for Biotechnology Information (NCBI).

3.4. Cloning and confirmation of the sex-specific and polymorphic RAPD and ISSR bands:

3.4.1. Gel elution of sex-specific and polymorphic RAPD and ISSR bands:

The sex-specific bands for RAPD primers namely OPK-10 (~1250 bp), OPA-12 (~2900 bp) and OPA-08 (~350 bp), and non-sex-specific polymorphic PCR products obtained from RAPD and ISSR primers, *i.e.* OPC-06 (~1100 bp), OPD-20 (~950 bp), OPD-12 (~1200 bp), ISSR-19 (~400 bp) and UBC-873 (~400 bp) as shown in Table 5 were excised from the agarose gel with a sharp, clean scalpel blade as per the procedure described in QIAquick Gel Extraction kit**.** The excised gel was weighed, and 3 volumes of buffer QG (solubilization buffer) was added (approx. 300 µl for 100 mg gel) to one volume of gel, and incubated at 50°C for 10 min or until the gel piece had dissolved completely by vortexing the tubes every 2-3 min. After dissolving the gel, one gel volume of isopropanol was added to the sample and mixed. The spin column was placed in a 2 ml collection tube provided with the kit. The mixture was added to the column and centrifuged at 13,000 rpm for 1 min. The flow-through was discarded, and the column was placed back into the collection tube and repeated until the entire sample is passed through the column. Then, 0.5 ml of buffer QG was added to the column and centrifuged at 13,000 rpm for 1 min, and the flow-through was discarded. The column was washed with 0.75 ml PE buffer (wash buffer) by adding to the column and centrifuged at 13,000 rpm for 1 min. The flow-through was discarded, and the column was centrifuged at 13,000 rpm for 1 min to remove the excess buffer. The column was placed in a 1.5 ml microcentrifuge tube, and elution buffer (EB) of 50 µl was added to the column and allowed to stand for 4 min. The microfuge tubes were centrifuged at 13,000 rpm for 1 min. The eluted DNA obtained was mixed with loading dye and

Table 5. Amplified bands (both sex-specific and polymorphic) produced with RAPD and ISSR primers which were selected for cloning and sequencing.

S. No.	Primer	Unique band size (bp) approx.	Presence of polymorphic band	Size of sequenced bands (bp)
1	OPK-10	~1250	Female and Andromonoecious specific	1239
2	OPA-12	~2900	Female and Andromonoecious specific	1856
3	OPA-08	~350	Andromonoecious specific	341
4	OPD-20	~950	2 Males, 5 Females and 5 Andromonoecious	972
5	OPD-12	~1200	3 Males, 6 Females and 5 Andromonoecious	1201
6	OPC-06	~1100	3 Females and 4 Andromonoecious	1130
7	ISSR-19	~400	4 Males, 8 Females and 14 Andromonoecious	409
8	UBC-873	~400	2 Females, 1 Andromonoecious	414

analyzed on agarose gel (0.8%) stained with EtBr and stored at -20°C until used. The eluted products of polymorphic bands *viz*., ~1250 bp (OPK-10), ~350 bp (OPA-08), ~2900 bp (OPA-12), ~1100 bp (OPC-06), ~950 bp (OPD-20), ~1200 bp (OPD-12), ~400 bp (ISSR-19) and ~400 bp (UBC-873) bands were directly used for cloning into pTZ57R/T vector.

3.4.2. Ligation of PCR product in pTZ57R/T cloning vector:

For ligation of eluted DNA bands, the InsTAclone PCR Cloning Kit (Thermo Scientific) was used. In the cloning vector pTZ57R/T (2868 bp), the *lacZα* gene contains the multiple cloning site (MCS) where the desired DNA band gets inserted to inactivate the gene and gives blue-white colonies on LB agar medium with IPTG and X-gal. The vector also possesses a T7 promoter, having 3'-ddT overhangs at the end regions of the cloning site and M13 primer sequence for easy sequencing of the desired DNA band. The map of the vector and sequences around the cloning site are represented in Fig. 4. The ligation was carried out for each PCR product of sex-specific and polymorphic bands at 4°C overnight. The 3:1 ratio of a gene of interest to vector was used. For the ligation of PCR product, 1 µl of the vector (55 ng/µl), 1 µl of T4 DNA ligase (5 U/µl), 3 µl of 5X buffer with the volume of the eluted PCR product, and Milli-Q water depending on the concentration of eluted PCR product in a total reaction volume of 15 µl was used.

3.4.3. Competent cells (DH5α) preparation:

DH5α (*Escherichia coli* strain) cells were used for competent cell preparation. The single well-isolated colony of DH5α from the freshly streaked plate was taken and the culture was initiated and allowed to grow in LB (Luria broth) medium of pH 7.4 for overnight. To 200 ml of LB broth, 2 ml inoculum from overnight culture was added and incubated at 37ºC in a shaker until it reached 0.4-0.6 OD (Optical Density). The culture was collected and kept in ice for 30 min. The culture was pelleted at 4000 rpm for 15 min at 4ºC. The pellet was suspended in 200 ml of filter-sterilized buffer-A, which is acid salt buffer having $CaCl_2$ (100 mM), $MnCl_2$ (70 mM) and sodium acetate (70 mM) with pH 5.5 and centrifuged for 30 min at 3700 rpm. The resulting pellet was dissolved in buffer-B (3-5 ml), which consisted 15% glycerol in buffer-A and aliquots of 100 µl

each in 1.5 ml eppendorf tubes were made. The eppendorf tubes were then frozen in liquid nitrogen and stored at -80ºC for future use.

3.4.4. Transformation and selection of recombinant clones:

The transformation procedure involved adding a whole ligated mixture (15 µl) of desired RAPD and ISSR bands to 100 µl of DH5α competent cell suspension. The mixture of recombinant vectors and competent cells was incubated for 30 min on ice. After that, the entire mixture was given heat shock treatment at 42ºC for 90 sec and immediately placed on ice for 30 min. After transformation, 1 ml LB broth was added to the bacterial cell suspension and incubated for 1 hr at 37ºC in a rotary shaker maintained at 200 rpm. After incubation, the culture was centrifuged at 10000 rpm for 1 min, and the pellet was resuspended in leftover 100 µl of LB medium. The resuspended culture was plated on LB agar plate containing 100 µg/ml ampicillin (Thermo Scientific), 40 µl 5-bromo-4-chloro-3-indolyl-beta-D-galactoside (X-gal; 24 mg/ml dissolved in DMSO, Thermo Scientific), and 40 µl isopropyl-beta-D-thiogalactopyranoside (IPTG; 40 mg/ml dissolved in Milli-Q, Thermo Scientific) and incubated at 37ºC for overnight. The positive white colonies were selected by blue-white selection (Fig. 5) procedure.

3.4.5. Confirmation of recombinant clones by colony PCR:

The positive white colonies obtained after transformation were confirmed by using them as template DNA for colony PCR. Five to seven colonies from each sample were picked and used for colony PCR and also streaked on LB agar master plate with ampicillin for further plasmid isolation from confirmed white colonies. For colony PCR, the selected colonies were picked with tip and diluted with 10 µl Milli-Q. Then the tubes were incubated for 10 min in boiling water to disintegrate the cell wall. A short spin was given to pellet down the cell wall debris. One µl supernatant was used as a template for colony PCR. The colony PCR reaction volume consisted of 25 µl with 2.5 µl of 10X buffer, 1.5 µl of Taq polymerase (1U/µl), 0.5 µl of 10 mM dNTP mix, 1 µl of each forward and reverse M13 primer (10 picomoles/µl; Bioserve Biotechnologies). The PCR conditions used for colony PCR were initial denaturation step of 94°C for 2 min and 30 cycles each of denaturation at 94°C for 30 sec, with primer annealing at 50°C for 30 sec and 2 min primer extension at 72°C, followed by a terminal extension at 72°C of 10 min. The amplified products were separated on 1% agarose gels, stained

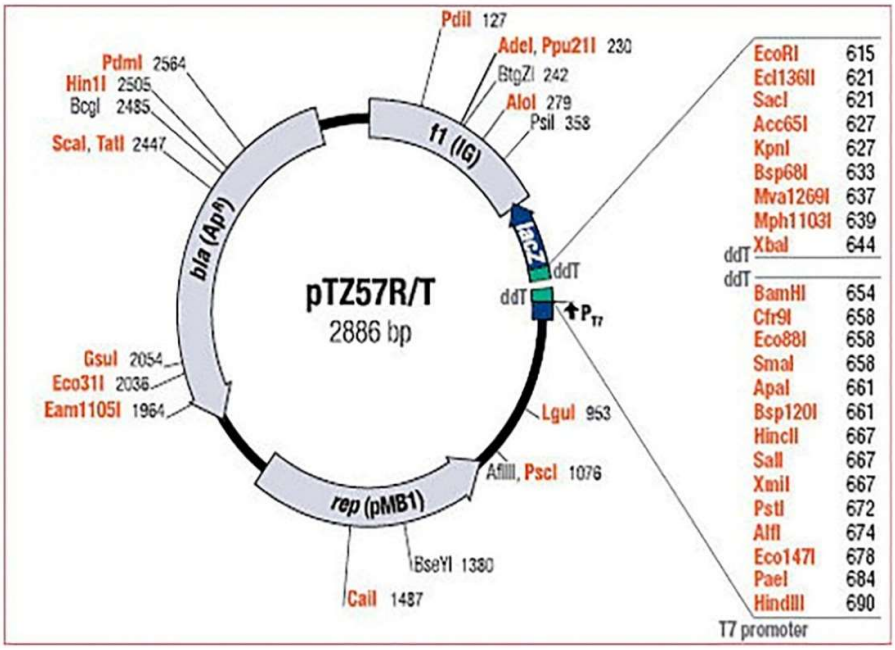

Fig. 4. Map of pTZ57R/T cloning vector used for cloning the desired amplified DNA bands.

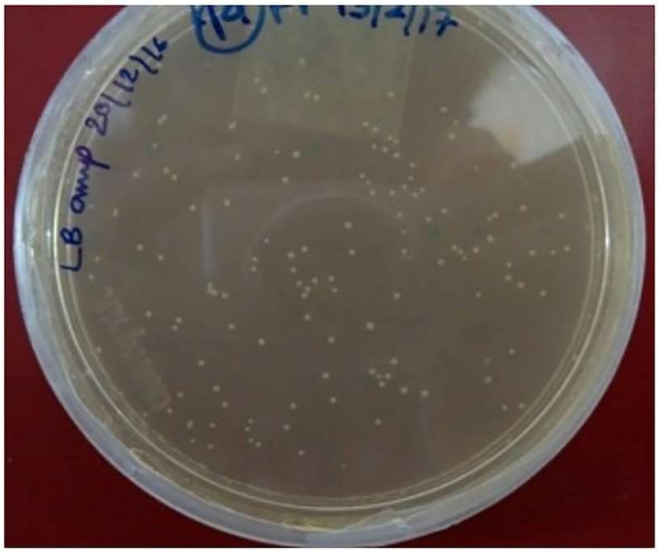

Fig. 5. Blue-white screening of transformed DH5α cells containing pTZ57R/T cloning vector with desired DNA bands.

with ethidium bromide, and photographed in a gel documentation system (Syngene). The amplified PCR product sizes were determined with a 100 bp DNA ladder (Thermo Scientific). The positive colonies for the desired DNA insert were picked for plasmid isolation, followed by restriction digestion.

3.4.6. Plasmid isolation from transformed colonies with desired RAPD and ISSR fragments:

The positive colonies which showed amplification of the desired PCR product were subjected to plasmid isolation (10 ml culture) using QIAprep Spin Miniprep kit (Qiagen, Maryland, USA). The selected colonies were grown overnight with a particular antibiotic (ampicillin). The overnight culture is centrifuged at 10000 rpm for 3 min at room temperature. The supernatant was discarded, and the cells were resuspended in P1 buffer (500 µl). To the resuspended cells, 500 µl of P2 buffer was added, and the tubes were thoroughly mixed by inverting them for 4-6 times until a clear solution was observed. This lysis reaction was not allowed for more than 5 min. After lysis, 700 µl of N3 buffer was added and mixed immediately and thoroughly by inverting the tubes 4-6 times and centrifuged for 10 min at 13,000 rpm. The supernatant was collected, and 800 µl was added to QIAprep 2.0 spin column and centrifuged for 1 min; this flow-through was discarded, and the same was repeated. The column was washed with buffer PB by centrifuging at 13,000 rpm for 1 min. The column was again washed by adding 750 µl of buffer PE by centrifuging at 13,000 rpm for 1 min. Then the column is placed in a clean 1.5 ml microfuge tube. To the column, 50 µl of elution buffer EB was added and allowed to stand for 1 min, centrifuged for 1 min at 13,000 rpm, and plasmid DNA was eluted and checked on agarose gel (1%) by electrophoresis and stored at -20ºC for further analysis.

3.4.7. Restriction digestion confirmation of plasmid DNA with desired RAPD and ISSR fragments:

Restriction digestion of plasmid DNA was done as per the standard procedures (Sambrook *et al*. 1989) using restriction endonuclease enzymes *EcoR*I and *Hind*III (Thermo Scientific) in compatible buffer at 37ºC for 2 hrs. The digested product was analyzed in the 1% agarose gel electrophoresis. The recombinant plasmid DNA was diluted to 100 ng/µl and further used for restriction digestion analysis. Double digestion

of plasmid DNA containing desired DNA inserts of female and male plants was carried out in a total reaction volume of 10 µl consisting of 1 µl 10X buffer R, 0.5 µl each of *ECoR*I (10U/µl), and *Hind*III (10U/µl) restriction enzymes, 5 µl of plasmid DNA with desired insert (approx. 100 ng/µl) and 3 µl of MilliQ water.

3.4.8. Sequencing of recombinant plasmids and analysis of sequence homology of cloned RAPD and ISSR fragments:

The purified plasmids confirmed by double digestion were sent for sequencing to Eurofins Genomics India Pvt. Ltd (Whitefield, Bengaluru, India) using M13 universal primers for both forward and reverse directions. The sequencing result obtained in the FASTA format for recombinant plasmids was analyzed for sequence homology with the existing Gene bank non-redundant database of NCBI. The sequence was analyzed by standard Nucleotide BLAST (BLASTN) for nucleotide sequence analysis, whereas addgene (sequence analyzer, https://www.addgene.org/) and ORF finder were used to detect and identify open reading frames (ORF), restriction sites, and map of the DNA band analyzed. VecScreen (https://www.ncbi.nlm.nih.gov/tools/vecscreen/) was used for eliminating the vector contamination of desired insert sequence. Then, the BLASTP was used to compare the translated nucleotide sequence with proteins in the database.

3.5. SCAR markers development and their validation on known and unknown sexes:

3.5.1. Designing SCAR primers and their sex-specific SCAR-PCR amplification:

The SCAR primers were designed using the Primer-BLAST tool (NCBI) based on sequence obtained for sex-specific bands (1239 bp, ~2900 bp and 341 bp) amplified by RAPD primers. The primers for ~2900 bp band were designed based on forward sequence obtained by sequencing analysis. The formation of the intra-molecular hairpin loop and inter-molecular self-complementarity of primers were checked using oligocalc (http://biotools.nubic. northwestern. edu/OligoCalc.html). The forward (Sg SCAR FP) and reverse (Sg SCAR RP) primers for 3 SCAR markers of sex-specific RAPD cloned bands as shown in Table 6 were synthesized by Bioserve Biotechnologies.

The bulked DNA samples were screened with sex-specific SCAR markers (Sg SCAR1, Sg SCAR2 and Sg SCAR3), and those SCAR markers that resulted in

Table 6. List of SCAR primers designed for sex identification and validation in *S. glauca*.

S. No.	Primer name	Primer sequence (5'->3')	Length of primers (bp's)	PCR Product size (bp)
1	Sg SCAR1 (1239 bp, OPK-10)	FP TGGTCCAGTGTTTTTGGGTCC	21	1063
		RP GCAACGTGCTAAAAGAGAGCC	21	
2	Sg SCAR2 (341bp, OPA-08)	FP GTGACGTAGGTGTTCGTGGAAG	22	341
		RP GTGACGTAGGCTTGTTGATCCCTT	24	
3	Sg SCAR3 (~2900 bp OPA-12)	FP GCTGCTACATTGGGAGTGGA	20	762
		RP CGCTGAGGAGTTGGGATCTT	20	

Sg- *Simarouba glauca*, FP- Forward primer, RP- Reverse primer

amplification were used to test all individual genotypes of PJTSAU. The SCAR-PCR amplification was carried out with an initial denaturation step of 94°C for 4 min; 35 cycles of amplification with each cycle comprising denaturation for 1 min at 94°C, primer annealing at different temperatures based on Tm for specific primer pair for 50 sec and primer extension at 72°C for 1.30 min; followed by final extension for 10 min at 72°C. The amplified PCR products were held at 4°C in the thermocycler. For reported SCAR markers, the conditions as mentioned in respective papers were used along with different annealing temperatures and conditions. The total volume of the reaction mixture was 25 µl, which contained 2.5 µl of 10X PCR buffer (Thermo Scientific™), 2 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTP (Bioserve), 1 µl of 10 pmol/µl for each forward and reverse primer (Bioserve), 30 ng of template DNA, 0.4 µl of Taq polymerase (5 U/µl) and sterile Milli-Q water. The amplified DNA fragments were resolved on 1% TAE agarose gel, stained with ethidium bromide and visualized under ultraviolet (UV) light.

3.5.2. Validation of SCAR markers for sex-specific amplification:

The SCAR primers designed for sex-specific RAPD bands were tested on genomic DNA samples of 15 (5 each) females, males and andromonoecious trees growing at IIOR, Narkhoda. Ten *in vitro* raised seedlings and twelve 2-3-year-old plants growing at University of Hyderabad campus were also screened for validating SCAR markers. The PCR amplification was carried out using conditions as mentioned for SCAR-PCR amplification. The amplified DNA fragments were resolved on 1% agarose gel, stained with ethidium bromide and visualized under ultraviolet (UV) light. The amplified bands for successful SCAR markers, Sg SCAR1 and SCAR2 of sizes 1063 bp and 341 bp, respectively were eluted, cloned, confirmed and sequenced as mentioned in above section (3.4.).

3.6. Analysis of phytochemicals and antioxidant activity among different genders of *S. glauca*:

3.6.1. Plant material and preparation of extracts:

The fresh disease-free, healthy mature leaves and shoot apex samples (approx. 10 cm in size) were collected from 15 trees (5 each of female, male and andromonoecious) of *S. glauca* in October 2018 at PJTSAU campus, Hyderabad. The

leaf and shoot apex samples for each gender were pooled to form three bulks of female, male and andromonoecious genotypes and 3 technical replicates were used in each experiment. The plant material collected was washed twice thoroughly with tap water and shade dried at room temperature for 20 days or until they were dried completely. The dried leaves and shoot apices were blended in an electric mixer and stored in an air-tight container at ambient temperature and used for further analysis. All the chemicals and solvents used for phytochemical analysis were procured from Hi-Media, SRL, and Merck (Germany). The solvents used in this study were of analytical grade. The standards used were obtained from Sigma-Aldrich®.

The leaf and shoot apex samples of three genders were initially extracted using 3 solvents *i.e.* methanol, acetone and hexane, with different polarities. The crude extracts of leaf and shoot apex samples were prepared by adding 5 gm of fine powder to 25 ml of methanol, acetone and hexane separately and stirred for 24 hr at room temperature on a magnetic stirrer. After 24 hrs, all the extracts of leaves and shoot apices were filtered using Whatman filter paper. The residue was collected separately and re-extracted by repeating the process for another time by adding another 25 ml of solvents to each filtered residue. The extracts were then filtered, and the resulted filtrates for each solution were combined and stored at -20°C until use. The methanol, acetone, and hexane extract of leaf and shoot apex was screened for preliminary qualitative phytochemical analysis. The solvents that produced positive results for the presence of of the phytochemicals in the preliminary analysis were used for further antioxidant activity studies. The methanol and acetone extracts of both samples were aliquoted into separate tubes and were subjected to a rotary evaporator, which resulted in dry mass. The concentrated dried extracts of leaf and shoot apex samples were stored in tubes covered with aluminum foil at -20°C. The stock solutions of 20 mg/ml were prepared for dried extracts of methanol and acetone. The stock solutions for standards used in the study were made for 5 mg/ml. Different concentrations from the stock solutions were used to determine total phenolic, flavonoid and tannin contents, and to analyze antioxidant activities. The fine powder of leaf and shoot apex was directly used to analyze metabolite differences among the three genders of *S. glauca*.

3.6.2. Qualitative preliminary phytochemical screening among different genders:

The preliminary phytochemical investigations were carried out by using the methanol, acetone and hexane crude extracts of leaves and shoot apices of three genders for the qualitative detection of major secondary metabolites like phenols, flavonoids and tannins. The detection of phytochemicals was carried out using standard methods as per Chaitanya and Bhavani (2013) for phenols and flavonoids, and Batool *et al*. (2019) for tannins.

3.6.3. Quantitative determination of phytochemicals among different genders:

The rotary evaporated methanolic and acetone extracts of leaves and shoot apices of three genders of *S. glauca* were analyzed for total contents of phenols, flavonoids and tannins. All the extracts were diluted to 1 mg/ml in respective solvents and used for quantitative analysis. The standard compounds 5 mg/ml stocks and 0.5 µg/ml working solution were prepared and stored at -20°C.

3.6.3.1. Determination of total phenolic contents:

The total phenolic content in leaf and shoot apex extracts was determined using the Folin-Ciocalteu method (Gul *et al*. 2011) with slight modifications. The sample of 20 µl was taken from each diluted extract (1 mg/ml), and mixed with 1 ml of 0.2N Folin-Ciocalteu (FC) reagent and incubated at room temperature for 10 min. After incubation, 400 µl of 20% sodium carbonate (Na_2CO_3) was added, and the volume was made up to 1.5 ml with distilled water. The reaction was incubated in the dark for 30 min and the absorbance was measured at 765 nm against the blank using a UV-Visible spectrophotometer (UV-1800, Shimadzu Corporation, Kyoto, Japan). The total phenolic content of leaf and shoot apex methanol and acetone extracts was measured as milligrams of gallic acid equivalents (mg GAE) per gram dry weight (g DW) through the standard calibration curve obtained for gallic acid (4-40 µg). The analysis was done in triplicates, and the values represent the means of the triplicates performed in the study.

3.6.3.2. Determination of total flavonoid contents:

The total flavonoid content was assessed by a colorimetric method (Gul *et al*. 2011), with a slight variation where the rutin was used as standard. To 10 µl of each

extract of leaf and shoot apex samples, 500 µl Milli-Q water was added and mixed. To this mixture, 30 µl of 5% $NaNO_2$ solution was added and incubated for 5 min at room temperature. Subsequently, a 10% $AlCl_3$ solution of 60 µl, 1M NaOH of 350 µl was added, and the final volume was made up to 1 ml by adding Milli-Q water. The sample tubes were then incubated at room temperature for 15 min, and the absorbance of the tested samples was recorded at 510 nm against the blank. The total flavonoid content of different extracts was evaluated as milligrams of rutin equivalent per gram dry weight (mg RE/g DW) over the calibration curve for rutin standard (5-50 µg), and the values were expressed as mean of triplicates.

3.6.3.3. Determination of total condensed tannin contents:

The modified vanillin assay described by Sun *et al*. (1998) and Yosr *et al*. (2018) were used to estimate the total condensed tannins in the extracts of leaf and shoot apex samples. To 40 µl of the sample of each extract taken from stock solution, 1 ml of 2% vanillin in methanol solution was added. Subsequently, 0.5 ml of 25% concentrated H_2SO_4 in methanol was added and incubated at room temperature for 15 min. For each sample, the absorbance was measured at 500 nm against a blank. The total condensed tannins content was expressed as milligrams of catechin equivalent per gram dry weight (mg CE/g DW) through the calibration curve for catechin standard (5-40 µg). All the values in the study were presented as means of triplicates.

3.6.4. Evaluation of antioxidant activity among three genders of *S. glauca*:

The antioxidant activities of leaves and shoot apices of methanol and acetone extracts were carried out to analyze the differences between the three genders, if any. The total antioxidant and the free radical scavenging activity assays were performed to know the antioxidant potential of samples.

3.6.4.1. Evaluation of total antioxidant activity (TAA):

The extracts of leaves and shoot apices of three genders were evaluated for total antioxidant potential using phosphomolybdenum assay (Gul *et al*. 2011). The method depends on the capacity of antioxidant compounds in the sample to reduce Mo (VI) to Mo (V) and at acidic pH results in the development of green color phosphate/Mo (V) complex. To 10 µl of each extract obtained from stock (20 mg/ml), 90 µl of distilled

water was added and mixed. One milliliter of the reagent solution [sulphuric acid (0.6 M), sodium phosphate (28 mM) and ammonium molybdate (4 mM)] was added to the 1.5 ml tubes, and the tubes were incubated with caps closed at 95°C in a thermal block (Eppendorf, Hamburg, Germany). After incubation, the tubes were allowed to cool to room temperature and given a short spin at 12000 rpm. The absorbance of each extract was measured against the blank at 695 nm. The standard solution of ascorbic acid was used, and the total antioxidant activity was presented as milligrams of ascorbic acid equivalents per gram dry weight (mg AAE/g DW).

3.6.4.2. Evaluation of DPPH radical scavenging activity:

The antioxidant activity of extracts of leaves and shoot apices were analyzed by a method described by Gul *et al*. (2011), where the scavenging activity of stable and free radicals of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) by antioxidant molecules of the samples results in a colorless solution. The DPPH solution of 0.004% in 95% methanol was prepared, and the extracts were serially diluted from the working solutions (1 mg/ml) to obtain a series of concentrations for each extract. The methanol and acetone extracts were serially diluted to obtain 5-50 µg concentrations for DPPH assay. These different concentrations of methanol and acetone extracts were mixed with 900 µl DPPH and incubated for 30 min in the dark. The absorbance of the serially diluted samples of each extract was measured at 517 nm against 95% methanol as blank, DPPH solution without extract served as control, and ascorbic acid as a reference standard (5-50 µg). The inhibition percentage of DPPH free radicals was calculated for both leaves and shoot apices using the formula:

$$\% \text{ Inhibition} = \left(\frac{\text{Control absorbance} - \text{Sample absorbance}}{\text{Control absorbance}} \right) \times 100$$

Each extract concentration required to scavenge DPPH free radicals of 50% (IC_{50}) was calculated based on a percentage inhibition graph for leaves and shoot apices. The concentrations of extracts used to scavenge half of the free radicals were presented as IC_{50} values for each extract.

3.6.5. Statistical analysis:

The raw data of total phenols, flavonoids, condensed tannins and antioxidant assays were analyzed using software Microsoft EXCEL (Microsoft Corporation, Seattle, WA, USA). The assays for all the experiments were performed in triplicates, and the values represent means ± standard deviation of three replicates (n=3). The results were presented graphically using SigmaPlot for Windows (Systat Software, Inc., Bangalore, India). The means obtained for different extracts were analyzed statistically by one-way analysis of variance (ANOVA), and mean comparisons were made by the Student-Newman–Keul's multiple comparison test at the 5% probability level using SigmaPlot. The values of experimental results with $P<0.050$ were considered to be significant statistically.

3.6.6. Qualitative metabolite analysis by GC-MS among different genders of *S. glauca*:

3.6.6.1. Extraction and derivatization for GC-MS analysis:

A modified protocol by Roessner *et al.* (2000) was used for the identification and metabolite profiling using GC-MS. To one mg of fine leaf powder of different genders of *S. glauca*, 50 µl of 100% methanol and 6 µl of internal standard (0.2 mg/ml ribitol in Milli-Q water) was added and extracted at 70°C until it was dried completely. To the dried leaf and shoot apex residue, 20 µl of pyridine containing methoxyamine hydrochloride (20 mg/ml) was added and incubated at 70°C for 90 min. After incubation, 40 µl of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) was added and incubated at 90°C for 30 min. The mixture was centrifuged at 12,000 rpm for 15 min and the supernatant was collected. The derivatized samples were analyzed for metabolite profiling by GC-MS.

3.6.6.2. GC-MS analysis:

One microliter of each derivatized leaf and shoot apex sample was injected into the GC-MS instrument. The metabolite profile of different genders was analyzed by LECO-PEGASUS Gas Chromatography-coupled to Mass Spectrometry (GC–MS). The system is equipped with an Agilent 7890A mass spectrometer (Agilent Technologies, California, USA). The column DB-1HT, which is 29.30 m in length, 0.25 mm internal

diameter, and 0.10 µm film thickness (Restek, USA) was used. The GC-MS possesses an electron ionization system which uses the electrons of high energy (70 eV) for spectroscopic detection. The helium (He) gas was utilized as a carrier gas with a flow rate set to 1.5 ml/min. The injection temperature and ion source were set at 250°C and 230°C. The program was set with isothermal heating for 5 min at 70°C, followed by 70-300°C, 300°C for 4 min, with m/z range of 35 to 1000 amu, and data acquisition rate was five spectra per second.

The Chroma TOF software 4.44.0.0 version (LECO Corporation, USA) of chromatography was used to generate the raw SMP files (Samplevision format file). The ratio of signal to noise was set at ≥1, and further, they were aligned, and ion-wise extraction of the mass signal was performed. The mass signals of <3 present in samples were eliminated. The extracted mass spectra from the above process were analyzed by using NIST MS Search v 2.0 software. The compound identification was done by comparing their mass spectra with those of the NIST (National Institute of Standard and Technology) mass spectral library. For identifying metabolites, the compound hits with maximum matching factor (MF) value >700 (similarity) and least deviation (± 30) from the retention index (RI) were considered as putative metabolites. The metabolites possessing different trimethylsilyl derivatives were clubbed together as a single entity at the time of identifying metabolites. The metabolite analysis was done by considering only the annotated metabolites present in all the three replicates of samples, and the unknown metabolites were removed.

3.7. *In vitro* propagation studies from nodal and shoot tip explants of *S. glauca*:

In vitro studies were conducted from nodal and shoot tip explants obtained from 6-7-year-old mature trees. The explants were placed in a beaker containing tap water and washed thoroughly with running tap water for 30 min and another 5 min with a 1% aqueous solution of Tween 20. Explants were repeatedly washed with single distilled water and disinfected with 70% ethanol for 3 min and subsequently surface sterilized with 0.1 % (w/v) mercuric chloride for 5 min followed by repeated rinsing with sterile double distilled water under aseptic conditions in the laminar flow chamber. Explants of 1.5-2.0 cm were trimmed (~1 cm) at the base and cultured on MS (Murashige and Skoog 1962) medium with BAP (6-benzylaminopurine; 0.5, 1.0 and 2.0 mg/l) and 3% sucrose for shoot induction. After 4 weeks of culture on initiation medium, the explants

were subcultured on MS medium with 2.0 mg/l BAP for shoot induction and proliferation. The shoot induction frequency and shoot proliferation response from the explants was determined after 4 weeks of subculture on the medium

3.8. *In vitro* seed germination and plant regeneration from seedling-derived explants and genetic stability analysis of regenerated plants of *S. glauca*:

3.8.1. Plant material and *in vitro* seed germination:

Mature seeds of *S. glauca* were collected from 6-7-yr-old trees growing at Professor Jayashankar Telangana State Agricultural University (PJTSAU), Rajendranagar, Hyderabad, and used for *in vitro* seed germination. The seeds used in this study are expected to be heterozygous as they were collected from open-pollinated trees. The seeds were initially treated with 2% Bavistin (BASF India Ltd., Mumbai, India) for 10 min and rinsed 2-3 times with double-distilled water. The sterilization was carried out in the laminar flow chamber with 70% (v/v) ethanol (Hayman, Witham, UK) for 5 min, 0.4% (v/v) sodium hypochlorite (Fisher Scientific, Mumbai, India) for 5 min, and 0.1% (w/v) mercuric chloride (Sisco Research Laboratories Pvt. Ltd., Mumbai, India) with 50 µl (for 100 ml solution) of Tween 20 (Sigma-Aldrich®, St. Louis, MO) for 10 min; seeds were rinsed 2-3 times thoroughly with double-distilled water after each sterilization step. The seeds were inoculated on full-strength or half-strength MS (Murashige and Skoog 1962) or woody plant (WP) (Lloyd and McCown 1981) medium containing 3% (w/v) sucrose (Sisco Research Laboratories Pvt. Ltd.) and 0.8% (w/v) phyto agar. All the growth regulators, including phyto agar used in the study, were procured from Duchefa Biochemie, Haarlem, The Netherlands. The pH of the media was adjusted to 5.6-5.8 with 1 N NaOH or 1 N HCl before adding 0.8% (w/v) phyto agar and autoclaved at 121°C for 15 min. Different concentrations of GA_3 (0.5, 1, and 2 mg/l) were added to the media to study their effects on seed germination. GA_3 (10 mg/ml stock with ethanol as solvent) was filter sterilized using a Millex-GV filter unit (Millipore, Carrigtwohill, Ireland) and added to the autoclaved medium (at about 50°C) in the laminar flow chamber. Two to four seeds were inoculated into each culture bottle (60 × 110 mm) containing about 30 ml of medium. The cultures were incubated in the dark for about 1 week and then moved to culture racks under light provided by white fluorescent tubes (65 µmol photons m^{-2} s^{-1}; OSRAM Lighting India Private Limited, Haryana, India) for a 16-h photoperiod at 25 ± 2°C. After 4 weeks of culture, the

observations on the duration for germination, percentage of germination, and length of the seedlings (cm) were recorded.

3.8.2. *In vitro* shoot regeneration from explants derived from aseptically germinated seedlings:

Various explants like cotyledons, cotyledonary nodes, hypocotyls, epicotyls, root nodes, shoot tips, leaves, and petioles obtained from 25-day-old *in vitro* germinated seedlings of *S. glauca* were used for regeneration studies. In preliminary experiments, the cotyledons were cultured on MS medium with BAP (0.5, 1.0, 2.0, and 3.0 mg/l) or thidiazuron (TDZ; 0.1, 0.5, and 1.0 mg/l), either individually or in combination with NAA (0.1, 0.2, and 0.5 mg/l). MS medium with 3.0% sucrose and 0.8% phyto agar was prepared as described previously for *in vitro* seed germination. The growth regulators were added to the autoclaved media and dispensed into culture bottles in the laminar flow chamber. The stock solution of TDZ (5 mg/ml) was prepared in DMSO (Sigma-Aldrich®), whereas BAP and NAA stocks (5 mg/ml) were prepared using 1 N NaOH as a solvent and sterile double distilled water as diluent. The optimal concentrations of BAP, TDZ, and these cytokinins combined with NAA were selected for further studies based on shoot regeneration frequency, the number of shoot buds, low callusing, and lack of yellowing of shoot buds. The optimized concentrations of growth regulators, *i.e.* 2 mg/l BAP alone or in combination with 0.2 mg/l NAA, or 0.5 mg/l TDZ alone, were used in MS or WP medium to evaluate the shoot regeneration response from different explants. During the initial experiments, the responding explants with shoot buds were repeatedly subcultured on MS medium with BAP (0.5–2.0 mg/l) alone or in combination with 0.2 mg/l NAA for shoot bud proliferation or multiplication. In later studies, clusters of shoot buds/shoots produced from explants on MS medium with 2.0 mg/l BAP were divided into 2-3 pieces and subcultured on medium containing different concentrations of BAP (1.0, 0.5, and 0.2 mg/l) and GA_3 (0.2 and 0.5 mg/l) for promoting shoot elongation. The number of explants placed in each culture bottle (60 × 110 mm) varied from 1 to 4 depending on the explant size (1–2 cm), stage of culture (initiation, shoot multiplication, or shoot elongation), and the number of shoot buds per clump.

3.8.3. *In vitro* root induction from regenerated shoots and establishment of plants in soil:

The regenerated shoots (3-4 cm) derived from different explants were excised and treated by dipping the base of the shoots in 10 mg/ml IBA for 5 min. The IBA-treated and IBA-untreated shoots were cultured on full-strength and half-strength MS or WP media with or without 0.2 mg/l IBA. The half-strength and full-strength media consisted of 3% sucrose and 0.8% phyto agar. The media were prepared as described previously, and 0.2 mg/l of IBA (10 mg/ml stock was prepared with 1 N NaOH as a solvent and sterile double-distilled water as diluent) was added to the autoclaved media. The duration for root induction was recorded for different treatments. The observations on the percentage of root induction, the average number of roots per shoot, and average length of roots (cm) were taken after 6 weeks of culture on different rooting media. For all the experiments on *in vitro* shoot bud induction, shoot elongation, and root induction from the regenerated shoots, the cultures were placed in culture racks and incubated in the light as described previously for *in vitro* seed germination. The rooted plants with well-differentiated leaves were carefully removed from the bottles and washed in water to remove any agar sticking onto the roots. The rooted plants were then transferred to plastic pots containing autoclaved soil, manure, and soil rite (3:1:1 v/v/v; Keltech Energies Ltd., Bangalore, India) and acclimatized for 4 weeks in the culture room before moving to the glasshouse. Regenerated plants were maintained in the greenhouse for 4 weeks before repotting to larger pots (15 cm × 17 cm) containing soil and manure (3:1 v/v). Plant height and survival percentage were recorded 8 weeks after transfer to larger pots in the glasshouse.

3.8.4. Statistical analysis:

The seed germination and shoot regeneration experiments were carried out each time with a minimum of 20 seeds or explants per treatment. The explants obtained from 20 to 25 different seedlings were used in each treatment. The root induction experiments were performed using at least 10 regenerated shoots per treatment. In this study, all the experiments for each treatment were repeated three times. The data scored were presented as means ± SE of three experiments. Treatment means were statistically analyzed by one-way analysis of variance (ANOVA), and mean comparisons were

made by the Newman–Keuls multiple comparison test at the 5% probability level using SigmaPlot for Windows (Systat Software, Inc., Bangalore, India).

3.8.5. Genetic stability analysis of the regenerated plants using RAPDs and ISSRs:

The banding patterns of *in vitro* regenerated plants from cotyledon, hypocotyl, and root node explants originating from a single seedling were studied using RAPD and ISSR markers. The *in vitro* multiplied shoots originating from cotyledons, hypocotyl, and root node explants after ten passages on regeneration medium and the shoot excised from the donor seedling were pulse-treated with IBA (10 mg/ml) for 5 min and placed on half-strength WPM with 0.2 mg/l IBA for root induction. A total of 11 plants, comprising four from cotyledons (R1–R4), three each from hypocotyl (R5–R7), and root node (R8–R10), and the donor seedling, were subjected to RAPD and ISSR analysis. The leaves of the *in vitro* rooted plants were quickly frozen in liquid nitrogen and stored at −80°C until further use. The genomic DNA was extracted from leaves using plant DNA extraction kit (Bioserve Biotechnologies Pvt. Ltd., Hyderabad, India) following the manufacturer's instructions. Quantitative and qualitative assessment of genomic DNA was carried out with a NanoDrop™ ND-1000 spectrophotometer (Thermo Fisher, Wilmington, DE) and 1% (w/v) agarose (Sigma-Aldrich®) gel electrophoresis, respectively. Equal concentrations of DNA were used for PCR for all the samples analyzed. RAPD and ISSR reactions and the PCR conditions used were as indicated in section 3.3.4. Sixteen RAPD primers and 20 ISSR primers were initially screened on a single plant's genomic DNA to determine their ability to produce amplified products. Subsequently, 14 RAPD and 15 ISSR primers that produced polymorphic bands on three different genotypes (female, male and andromonoecious) were selected for analyzing the genetic stability of the regenerated plants.

The amplified products were separated on 1.2% (w/v) agarose gels, stained with ethidium bromide (Sigma-Aldrich®), and photographed in a gel documentation system (Syngene, Frederick, MD). The sizes of the amplified PCR products were determined with a 100-bp DNA ladder (MBI Fermentas Vilnius, Lithuania). All PCR reactions for RAPD and ISSRs were performed at least twice to check for reproducibility, and only precise and reproducible bands were scored. The data were scored as "1" for the presence, and "0" for the absence of a band for each plant analyzed. Bands present in all plants were considered monomorphic, whereas those absent in one or more regenerated

plants or donor seedling were deemed polymorphic. The percentage of polymorphic bands was calculated based on the total number of polymorphic bands and the number of amplified bands produced by all primers across the plants was analyzed. A similarity matrix was constructed based on the combined data from RAPD and ISSR analysis using the NTSYSpc software package (Rohlf 2000). The genetic similarities between samples were calculated by the Jaccard similarity coefficient (Jaccard 1908) with SIMQUAL format. Similarity coefficients were used to construct dendrogram using the UPGMA (Un-weighted pair group method using arithmetic averages) and SAHN (sequential agglomerative hierarchical nested clustering) in NTSYS program.

Results

4. RESULTS:

Simarouba glauca is a commercially important tree species valued for seed oil, where female trees have greater economic use for mass cultivation. A productive plantation requires females and males in a ratio of 25:1. Molecular markers play an important role in assessing the genetic variation and identifying the sex at the seedling stage as it is not possible to determine the sex of the trees before flowering based on morphological or biochemical characteristics. The present study was aimed at analyzing the genetic variations among different sexes (female, male and andromonoecious genotypes) of *S. glauca* collected from PJTSAU (Hyderabad, Telangana) by using RAPD and ISSR markers. The study also focused on the identification of RAPD and ISSR molecular markers linked to sex and their conversion into SCAR markers, which are more specific and reliable. Further, the metabolite differences and variations in antioxidant activities in the leaf and shoot apex samples of female, male and andromonoecious genotypes were evaluated. Studies were also targeted for optimizing the *in vitro* regeneration system for rapid multiplication using explants obtained from mature trees and *in vitro* germinated seedlings. RAPD and ISSR markers were employed to ascertain the genetic uniformity of the regenerated plants in comparison to the donor plant. The results of the current research are presented below:

4.1. DNA extraction and optimization of PCR conditions for RAPD and ISSR analysis:

The genomic DNA was isolated from the mature leaves collected from trees of *Simarouba* using modified CTAB method. The kit method used for DNA extraction was suitable for young leaves obtained from seedlings whereas mature leaf samples of aged plants were too mucilaginous and did not yield good quality DNA. The modified CTAB method used in the study resulted in isolation of good quality DNA from mature and young leaves with higher yield as compared to kit method. Different annealing temperatures and concentrations of Taq DNA polymerase, $MgCl_2$, and template DNA were studied to determine the suitable conditions for good amplification by RAPD and ISSR markers. For RAPD markers, the optimal annealing temperature was 37°C as it resulted in better resolution of bands compared to 35°C and 40°C. For ISSR markers, the optimal annealing temperature was found to be 50°C for good resolution of bands

compared to other temperatures tested. Of the different concentrations of Taq DNA polymerase tested for RAPD and ISSRs, 1U enzyme per reaction volume of 25 µl was optimal for good PCR amplification with other concentrations resulting in poor amplification or low specificity. The optimal $MgCl_2$ concentration for RAPD and ISSR was 2 mM, and other concentrations resulted in amplification with smear. The optimal concentration for the DNA template was 15 ng for RAPD markers and 30 ng for ISSR markers as they resulted in precise amplification without smear whereas lower and higher concentrations did not result in any amplification or smearing was observed.

4.2. Analysis of genetic variation among female, male and andromonoecious genotypes of *S. glauca* using RAPD and ISSR markers:

In *S. glauca*, there are very few studies conducted till today to analyze the genetic variation among different sexes using molecular markers. In the present study, 64 RAPDs and 33 ISSRs were employed to analyze the variations among female, male and andromonoecious genotypes of *S. glauca*.

4.2.1.1. RAPD marker analysis among different sexes:

Initially**,** 64 RAPD primers as listed in Table 1 were used to screen the bulk DNA samples of different sexes of *S. glauca*, out of which 61 random primers resulted in precise and reproducible bands. Three primers (OPA-17, OPS-06 and OPC-07) did not show amplification or resulted in poor amplification with smear. Sixty-one RAPD primers that resulted in good amplification (Fig. 6 & 7) were further tested on 36 individual DNA samples of female, male and andromonoecious plants to assess the polymorphism among different sexes of *S. glauca.* The size of the RAPD bands ranged from 130->3000 bp for different random primers tested. The primer OPD-20 generated the highest number of bands (15), and OPD-14 produced the lowest number of bands (2). The average number of loci and polymorphic loci per primer was 7.62 and 3.43, respectively (Table 7). The percentage of polymorphism detected by RAPD primers ranged from 0 to 100%. Out of 61 random primers used, 55 primers produced polymorphic bands (Fig. 8-13) whereas 6 primers *viz*., OPA-04, OPAW-07, OPAL-08, OPT-07, OPK-07, and OPK-16 resulted in monomorphic bands (Fig. 14-16). Among 55 polymorphic random primers, 2 primers *viz*., OPA-11 and OPC-05, produced 100% polymorphic bands in the genotypes tested. The primers OPK-10 and OPA-05 resulted

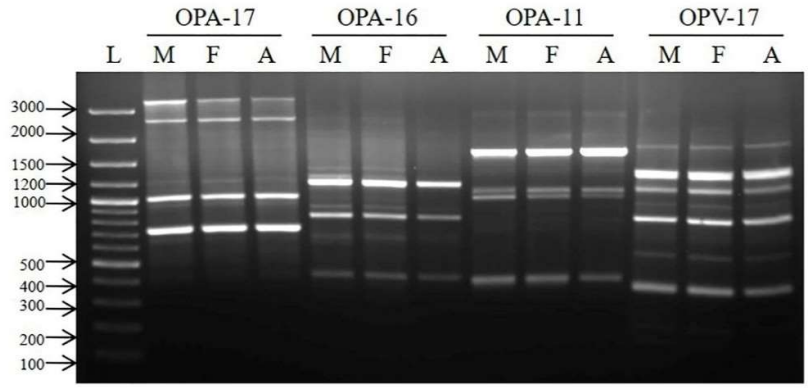

Fig. 6. Screening of bulk DNA of male, female and andromonoecious genotypes of *S. glauca* with RAPD primers, OPA-17, OPA-16, OPA-11 and OPV-17. *Lane L* – 100 bp DNA ladder, M – Bulk male genotypes, F – Bulk female genotypes, A – Bulk andromonoecious genotypes.

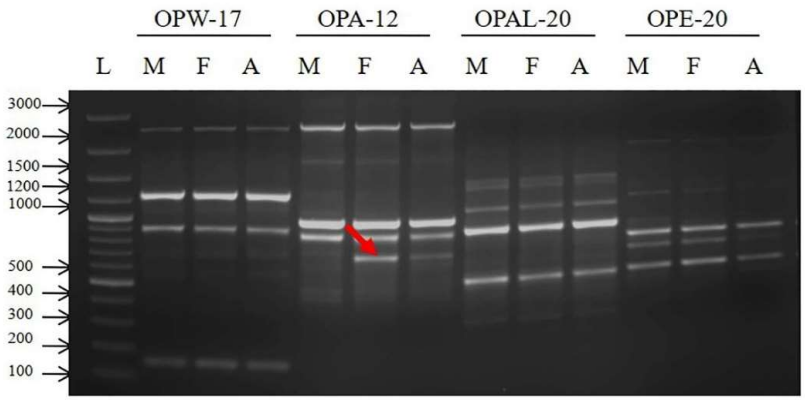

Fig. 7. Screening of bulk DNA of male, female and andromonoecious genotypes of *S. glauca* with RAPD primers, OPW-17, OPA-12, OPAL-20 and OPE-20. *Lane L* – 100 bp DNA ladder, M – Bulk male genotypes, F – Bulk female genotypes, A – Bulk andromonoecious genotypes. *Red arrow* indicates polymorphic band.

Table 7. RAPD amplification profiles of different sexes of *S. glauca* generated with 61 random primers.

S. No.	Primer	Number of loci	Number of monomorphic loci	Number of polymorphic loci	Polymorphism percentage (%)	Size range (bp)
1	OPF-03	5	4	1	20	300-900
2	OPF-11	6	4	2	33.3	440-1450
3	OPA-04	6	6	0	0	200-1400
4	OPD-14	2	1	1	50	900-2400
5	OPS-05	5	2	3	60	180-1300
6	OPAW-07	6	6	0	0	150-1600
7	OPT-18	7	3	4	57.1	700-1400
8	OPAB-06	7	5	2	28.6	480-2000
9	OPAL-08	6	6	0	0	520-1480
10	OPAK-14	7	6	1	14.3	430-1500
11	OPT-17	7	1	6	85.7	350-1750
12	OPZ-01	4	3	1	25	400-1300
13	OPZ-10	9	6	3	33.3	250-2000
14	OPZ-06	9	6	3	33.3	280-1600
15	OPB-12	6	2	4	66.7	400-1500
16	OPA-18	3	1	2	66.7	800-1300
17	OPA-16	9	5	4	44.4	300-1200
18	OPA-11	3	0	3	100	450-1200
19	OPV-17	8	4	4	50	290-1900
20	OPC-05	7	0	7	100	380-1250
21	OPC-18	10	2	8	80	200-1400
22	OPP-03	4	1	3	75	380-1500
23	OPV-08	9	2	7	77.8	130-1350
24	OPW-17	3	1	2	66.7	150-1300
25	OPA-12	9	5	4	44.4	420-2600
26	OPAL-20	10	5	5	50	240-1350
27	OPE-20	6	1	5	83.3	520-1900
28	OPB-07	7	5	2	28.6	250-1000
29	OPF-08	4	2	2	50	340-1500
30	OPG-14	10	5	5	50	250-1500
31	OPK-03	8	3	5	62.5	250-1700
32	OPA-06	9	4	5	55.6	420-2700
33	OPB-05	7	5	2	28.6	380-2600
34	OPB-20	6	3	3	50	520-1180
35	OPO-08	9	5	4	44.4	300-1750
36	OPT-07	8	8	0	0	200-1100
37	OPW-03	10	1	9	90	220-1650
38	OPG-16	11	7	4	36.4	300->3000
39	OPG-08	11	4	7	63.6	350-2200
40	OPG-06	8	3	5	62.5	300-1000

41	OPG-17	7	3	4	57.1	400-1750
42	OPK-01	6	3	3	50	450-2300
43	OPK-09	6	2	4	66.7	400-1050
44	OPK-07	10	10	0	0	220-1400
45	OPC-09	6	3	3	50	220-1600
46	OPC-06	10	3	7	70	600->3000
47	OPC-10	10	8	2	20	320-1700
48	OPK-10	10	9	1	10	220-1750
49	OPK-16	8	8	0	0	350-1450
50	OPC-08	9	6	3	33.3	220-1950
51	OPH-03	10	6	4	40	360-2000
52	OPA-08	7	1	6	85.7	130-1500
53	OPU-10	10	7	3	30	200-2000
54	OPD-19	7	2	5	71.4	400-1700
55	OPU-19	11	7	4	36.4	350-2300
56	OPD-20	15	11	4	26.7	180-1500
57	OPA-05	10	9	1	10	200-1750
58	OPC-16	10	5	5	50	200-1500
59	OPD-15	11	7	4	36.4	340-1400
60	OPE-05	6	1	5	83.3	200-1400
61	OPD-12	5	2	3	60	230-1400
	Total	465	256	209	-	
	Mean	7.62	-	3.43	46.8	

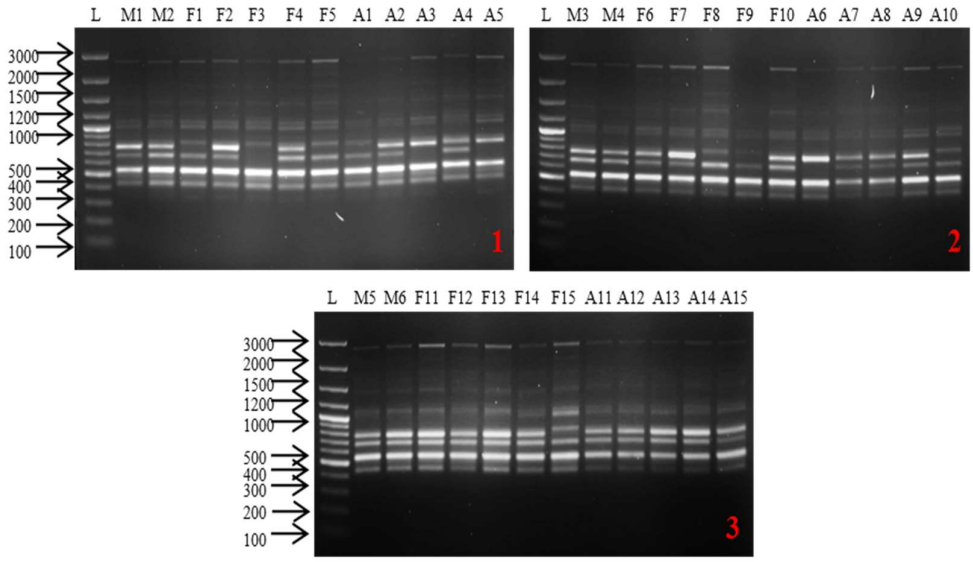

Fig. 8. Screening of individual DNA of male, female and andromonoecious genotypes of *S. glauca* with RAPD primer, OPA-06. *Lane L* – 100 bp DNA ladder, M1-M6 – male genotypes, F1-F15 – female genotypes, A1-A15 – andromonoecious genotypes.

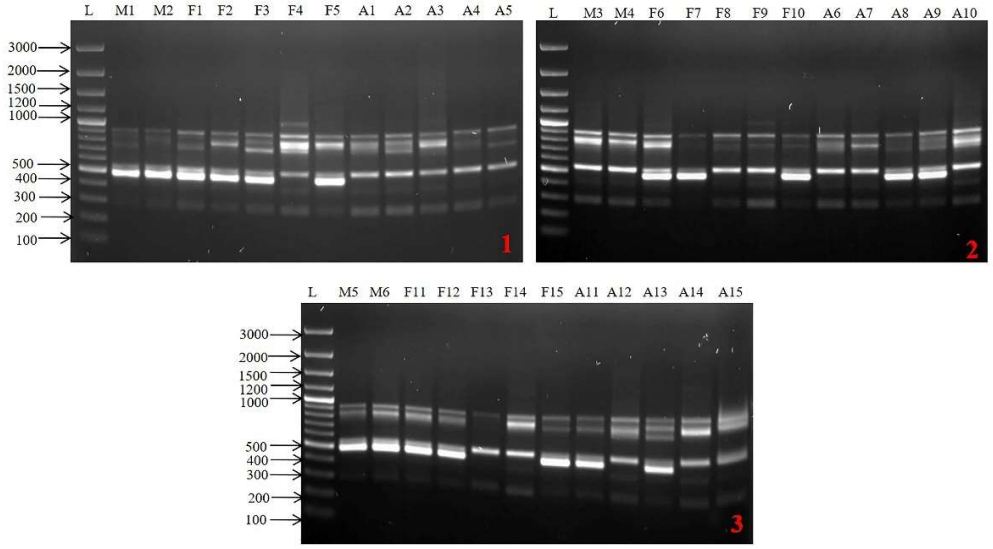

Fig. 9. Screening of individual DNA of male, female and andromonoecious genotypes of *S. glauca* with RAPD primer, OPG-06. *Lane L* – 100 bp DNA ladder, M1-M6 – male genotypes, F1-F15 – female genotypes, A1-A15 – andromonoecious genotypes.

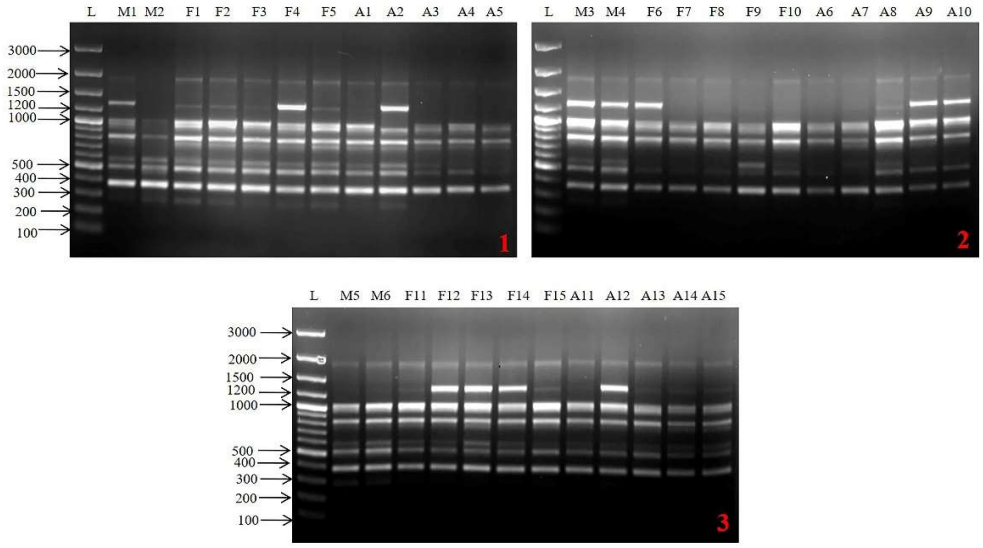

Fig. 10. Screening of individual DNA of male, female and andromonoecious genotypes of *S. glauca* with RAPD primer, OPV-17. *Lane L* – 100 bp DNA ladder, M1-M6 – male genotypes, F1-F15 – female genotypes, A1-A15 – andromonoecious genotypes.

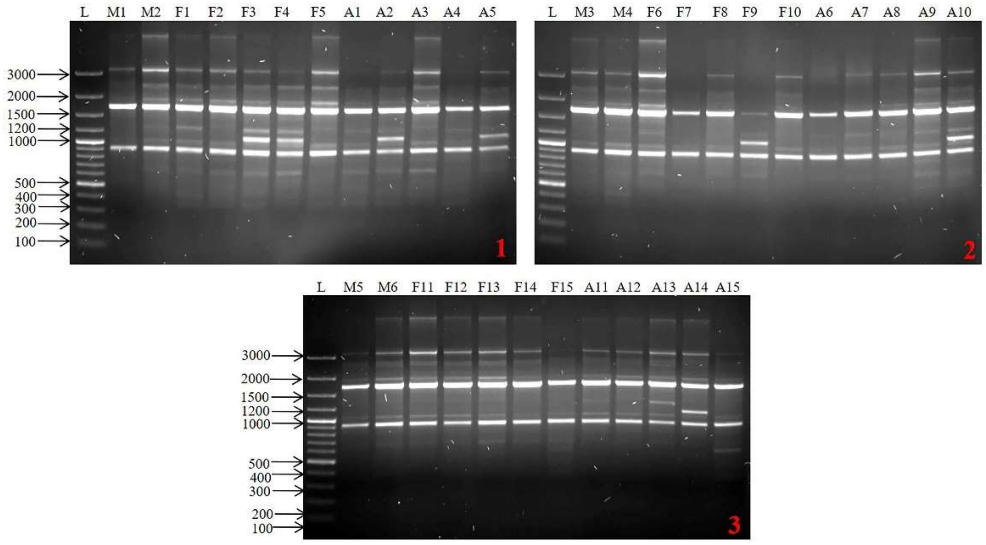

Fig. 11. Screening of individual DNA of male, female and andromonoecious genotypes of *S. glauca* with RAPD primer, OPC-06. *Lane L* – 100 bp DNA ladder, M1-M6 – male genotypes, F1-F15 – female genotypes, A1-A15 – andromonoecious genotypes.

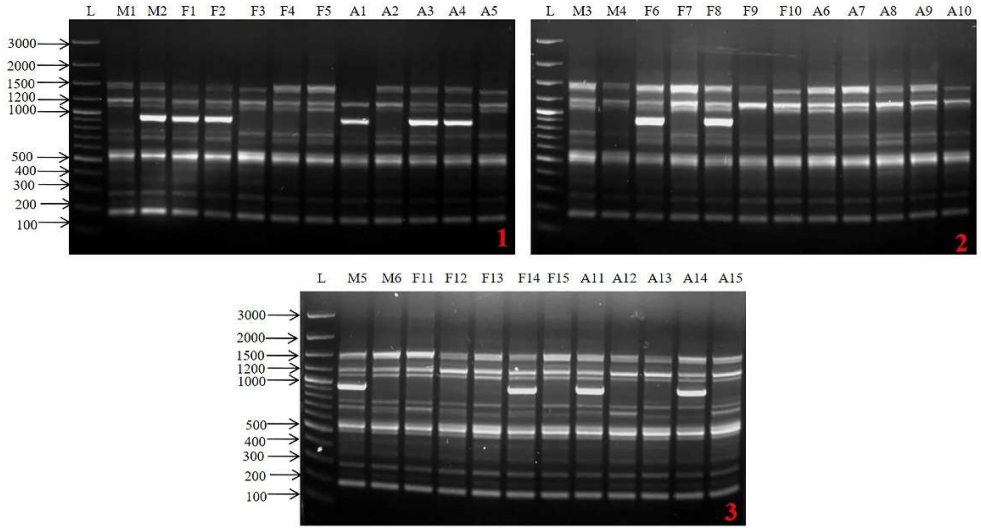

Fig. 12. Screening of individual DNA of male, female and andromonoecious genotypes of *S. glauca* with RAPD primer, OPD-20. *Lane L* – 100 bp DNA ladder, M1-M6 – male genotypes, F1-F15 – female genotypes, A1-A15 – andromonoecious genotypes.

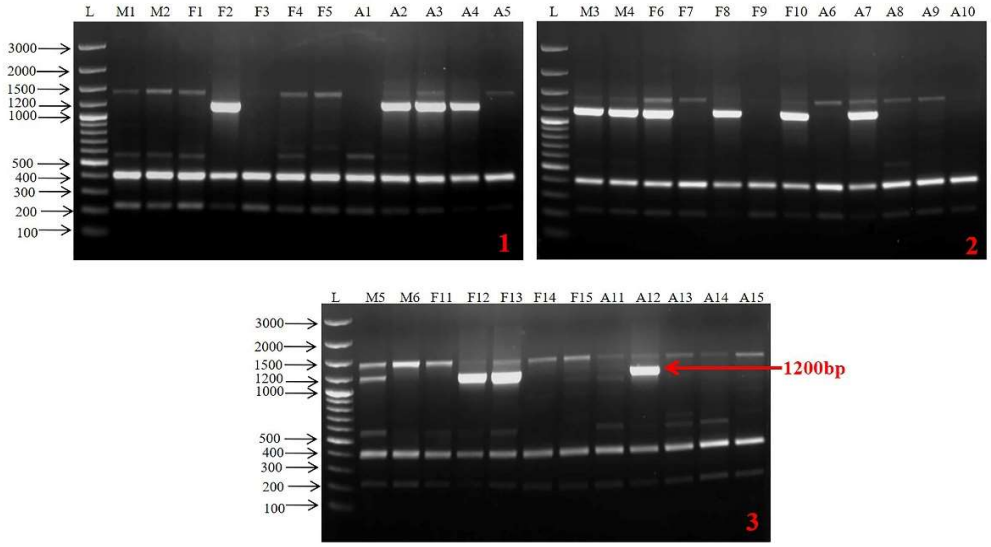

Fig. 13. Screening of individual DNA of male, female and andromonoecious genotypes of *S. glauca* with RAPD primer, OPD-12. *Lane L* – 100 bp DNA ladder, M1-M6 – male genotypes, F1-F15 – female genotypes, A1-A15 – andromonoecious genotypes.

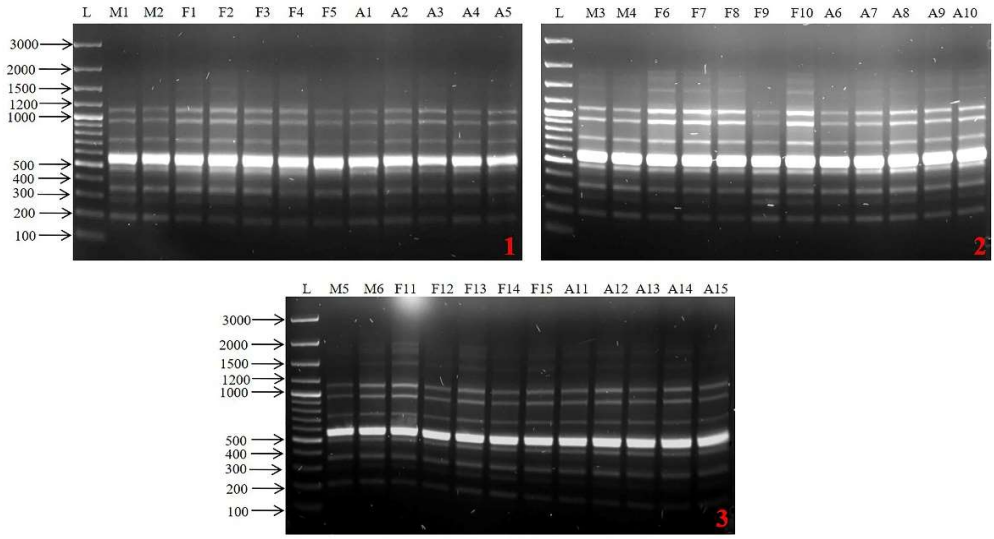

Fig. 14. Screening of individual DNA of male, female and andromonoecious genotypes of *S. glauca* with RAPD primer, OPT-07. *Lane L* – 100 bp DNA ladder, M1- M6 – male genotypes, F1-F15 – female genotypes, A1-A15 – andromonoecious genotypes.

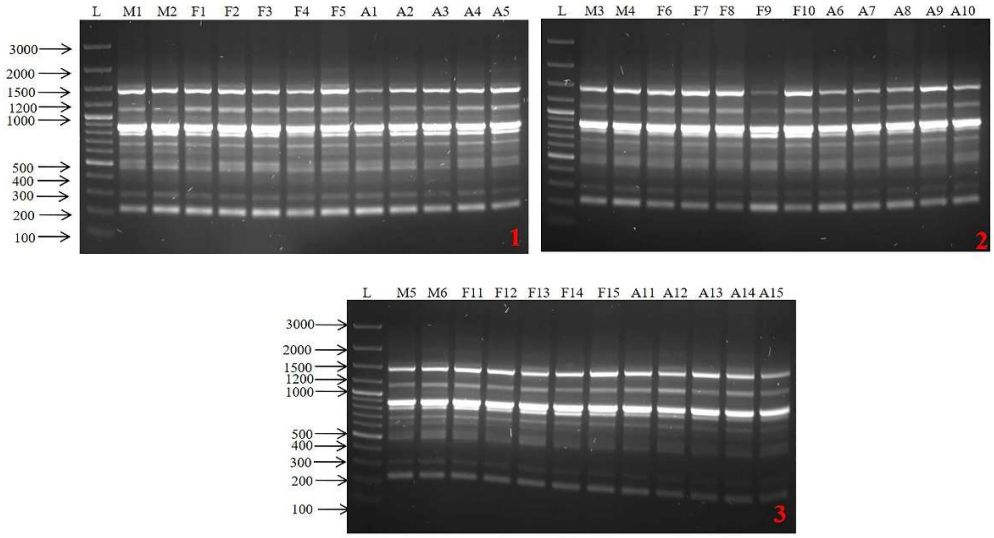

Fig. 15. Screening of individual DNA of male, female and andromonoecious genotypes of *S. glauca* with RAPD primer, OPK-07. *Lane L* – 100 bp DNA ladder, M1- M6 – male genotypes, F1-F15 – female genotypes, A1-A15 – andromonoecious genotypes.

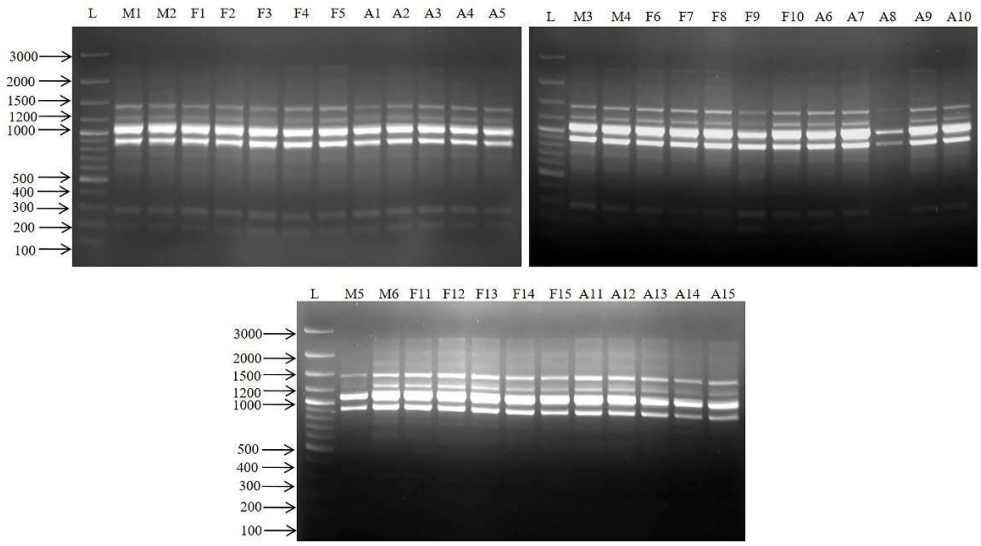

Fig. 16. Screening of individual DNA of male, female and andromonoecious genotypes of *S. glauca* with RAPD primer, OPA-04. *Lane L* – 100 bp DNA ladder, M1-M6 – male genotypes, F1-F15 – female genotypes, A1-A15 – andromonoecious genotypes.

in the lowest polymorphism of 10% in the genotypes analyzed. A total of 465 bands were produced by 61 random primers tested, out of which 209 bands were polymorphic accounting to 46.8% polymorphism.

RAPD dissimilarity matrix of 36 genotypes of *S. glauca* was generated based on Jaccard's dissimilarity index to assess the genetic variations among them. The dissimilarity indices among all genotypes ranged from 0.041 and 0.253. The maximum dissimilarity value of 0.253 was observed between the genotypes M2 and F9, and the minimum dissimilarity value of 0.041 was observed between F1 and F2 genotypes (Table 8). The females showed the dissimilarity range from 0.041 (F1 and F2) to 0.218 (F9 and F11) with an average of 0.098. For males, the dissimilarity values ranged from 0.04 (M5 and M6) to 0.13 (M2 and M5) with an average of 0.091. The dissimilarity values for the andromonoecious genotypes ranged from 0.065 (A9 and A11) to 0.193 (A1 and A12) with an average of 0.111.

4.2.1.2. Genetic variability assessment among different sexes using RAPD markers:

The discriminating power of RAPD was analyzed by calculating the polymorphic information contents (PIC) based on the number and frequency distribution of alleles. In the present study, PIC values for different sexes ranged from 0.00 to 0.21 for different random primers analyzed (Table 9). The maximum PIC value of 0.21 was obtained for primers OPC-06 and OPD-12, whereas PIC value was found to be 0.00 for 7 primers (OPA-04, OPAW-07, OPAL-08, OPT-07, OPK-07, OPK-10 and OPK-16). The mean PIC value for different RAPD primers tested in all genotypes was 0.09. Among male plants, the PIC value ranged from 0.00 to 0.26 with a mean PIC value of 0.07. The OPC-18 primer resulted in a maximum PIC value of 0.26. Among female plants, the PIC value ranged from 0.00 to 0.34 with a mean PIC value of 0.09. The maximum PIC value among females was obtained for primer OPE-05. The PIC value among the andromonoecious plants ranged from 0.00 to 0.28 with a mean PIC value of 0.10. The primers OPD-12 and OPC-06 resulted in the highest PIC value among andromonoecious plants with RAPD markers.

The POPGENE software version 1.32 was used to assess the genetic variation among different sexes of *S. glauca* using the RAPD data matrix generated by scoring of bands. The genetic diversity parameters such as the observed number of alleles (na),

Table 8. Dissimilarity matrix of different sexes of *S. glauca* revealed by RAPD markers

Units	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
2	0.08																																		
3	0.09	0.13																																	
4	0.09	0.12	0.07																																
5	0.11	0.13	0.08	0.06																															
6	0.09	0.12	0.08	0.07	0.04																														
7	0.12	0.14	0.1	0.12	0.09	0.09																													
8	0.12	0.15	0.1	0.11	0.1	0.08	0.04																												
9	0.15	0.18	0.12	0.13	0.12	0.11	0.05	0.05																											
10	0.14	0.17	0.11	0.11	0.11	0.1	0.07	0.06	0.06																										
11	0.13	0.17	0.11	0.12	0.1	0.09	0.06	0.06	0.06	0.07																									
12	0.13	0.17	0.1	0.11	0.12	0.11	0.08	0.08	0.1	0.09	0.09																								
13	0.14	0.18	0.11	0.13	0.12	0.11	0.1	0.09	0.11	0.11	0.09	0.07																							
14	0.18	0.21	0.13	0.15	0.15	0.15	0.11	0.1	0.12	0.12	0.12	0.09	0.1																						
15	0.24	0.25	0.21	0.22	0.21	0.21	0.18	0.19	0.19	0.18	0.2	0.18	0.17	0.16																					
16	0.14	0.17	0.11	0.13	0.12	0.11	0.09	0.06	0.09	0.1	0.09	0.1	0.09	0.11	0.19																				
17	0.13	0.16	0.09	0.1	0.08	0.07	0.07	0.06	0.09	0.08	0.08	0.08	0.11	0.12	0.22	0.09																			
18	0.13	0.15	0.07	0.09	0.09	0.08	0.07	0.07	0.1	0.09	0.08	0.07	0.1	0.12	0.21	0.09	0.05																		
19	0.13	0.16	0.09	0.09	0.09	0.09	0.09	0.07	0.09	0.09	0.08	0.1	0.11	0.13	0.21	0.08	0.06	0.05																	
20	0.13	0.16	0.09	0.09	0.09	0.09	0.08	0.07	0.1	0.09	0.09	0.09	0.11	0.13	0.2	0.09	0.06	0.07	0.05																
21	0.14	0.17	0.11	0.13	0.11	0.11	0.06	0.06	0.07	0.06	0.05	0.09	0.1	0.11	0.18	0.08	0.06	0.06	0.08	0.08															
22	0.21	0.21	0.18	0.19	0.17	0.18	0.16	0.17	0.16	0.17	0.17	0.18	0.18	0.19	0.16	0.18	0.19	0.18	0.17	0.16	0.17														
23	0.13	0.17	0.1	0.11	0.1	0.1	0.07	0.07	0.07	0.06	0.08	0.1	0.11	0.12	0.16	0.1	0.1	0.08	0.08	0.08	0.07	0.13													
24	0.15	0.17	0.13	0.13	0.13	0.13	0.11	0.09	0.1	0.12	0.1	0.09	0.11	0.11	0.19	0.11	0.12	0.11	0.11	0.11	0.1	0.14	0.08												
25	0.17	0.18	0.14	0.15	0.14	0.15	0.14	0.12	0.14	0.13	0.13	0.12	0.11	0.11	0.17	0.14	0.15	0.15	0.15	0.14	0.14	0.17	0.1	0.08											
26	0.17	0.2	0.15	0.16	0.15	0.15	0.12	0.12	0.1	0.13	0.14	0.13	0.13	0.12	0.2	0.13	0.14	0.15	0.14	0.13	0.13	0.18	0.11	0.09	0.09										
27	0.17	0.19	0.14	0.13	0.13	0.13	0.13	0.11	0.14	0.12	0.13	0.12	0.1	0.1	0.16	0.12	0.13	0.13	0.14	0.12	0.13	0.18	0.12	0.12	0.11	0.11									
28	0.15	0.17	0.12	0.12	0.12	0.12	0.1	0.08	0.1	0.09	0.1	0.09	0.09	0.11	0.15	0.1	0.11	0.11	0.12	0.11	0.09	0.17	0.08	0.1	0.1	0.12	0.07								
29	0.13	0.17	0.11	0.11	0.1	0.1	0.08	0.09	0.09	0.09	0.1	0.08	0.08	0.14	0.18	0.1	0.1	0.09	0.1	0.11	0.1	0.17	0.09	0.12	0.14	0.13	0.11	0.08							
30	0.13	0.17	0.12	0.11	0.11	0.11	0.07	0.06	0.08	0.08	0.07	0.07	0.09	0.11	0.19	0.08	0.08	0.09	0.08	0.09	0.07	0.18	0.09	0.11	0.14	0.13	0.11	0.07	0.07						
31	0.17	0.2	0.14	0.15	0.16	0.15	0.11	0.1	0.09	0.1	0.11	0.1	0.12	0.13	0.21	0.12	0.12	0.13	0.13	0.14	0.12	0.18	0.1	0.11	0.14	0.12	0.13	0.1	0.1	0.1					
32	0.14	0.16	0.11	0.13	0.11	0.1	0.08	0.07	0.1	0.09	0.08	0.09	0.09	0.13	0.19	0.07	0.08	0.08	0.09	0.1	0.08	0.17	0.09	0.1	0.12	0.14	0.12	0.08	0.09	0.06	0.12				
33	0.15	0.19	0.13	0.13	0.12	0.12	0.1	0.08	0.1	0.09	0.09	0.11	0.11	0.14	0.21	0.11	0.09	0.1	0.1	0.11	0.1	0.19	0.1	0.12	0.12	0.14	0.13	0.1	0.11	0.08	0.13	0.09			
34	0.16	0.2	0.12	0.14	0.13	0.12	0.08	0.08	0.09	0.08	0.09	0.1	0.11	0.12	0.2	0.09	0.1	0.09	0.1	0.1	0.08	0.18	0.09	0.13	0.14	0.13	0.13	0.09	0.09	0.08	0.13	0.07	0.09		
35	0.15	0.17	0.11	0.12	0.1	0.1	0.08	0.07	0.09	0.07	0.08	0.09	0.11	0.13	0.19	0.09	0.07	0.08	0.08	0.07	0.06	0.16	0.08	0.1	0.13	0.13	0.11	0.08	0.09	0.08	0.11	0.07	0.07	0.08	
36	0.13	0.17	0.12	0.13	0.11	0.11	0.1	0.07	0.11	0.09	0.09	0.1	0.09	0.12	0.19	0.1	0.09	0.11	0.11	0.11	0.08	0.17	0.1	0.1	0.11	0.11	0.1	0.07	0.1	0.08	0.11	0.07	0.09	0.09	0.07

1-6 = Male plants, 7-21 = Female plants, 22-36 = Andromonoecious plants

Table 9. Polymorphic information content (PIC) among different sexes of *S. glauca* using RAPD markers.

S. No.	Marker	PIC values for males	PIC values for females	PIC values for andromonoecious	Total PIC values
1	OPF-03	0.09	0.08	0.10	0.09
2	OPF-11	0.00	0.04	0.04	0.03
3	OPA-04	0.00	0.00	0.00	0.00
4	OPD-14	0.00	0.00	0.06	0.02
5	OPS-05	0.00	0.07	0.00	0.02
6	OPAW-07	0.00	0.00	0.00	0.00
7	OPT-18	0.07	0.07	0.16	0.10
8	OPAB-06	0.07	0.13	0.13	0.11
9	OPAL-08	0.00	0.00	0.00	0.00
10	OPAK-14	0.04	0.00	0.00	0.01
11	OPT-17	0.15	0.06	0.16	0.12
12	OPZ-01	0.00	0.03	0.06	0.03
13	OPZ-10	0.05	0.05	0.07	0.06
14	OPZ-06	0.03	0.01	0.07	0.04
15	OPB-12	0.15	0.19	0.23	0.19
16	OPA-18	0.09	0.18	0.20	0.16
17	OPA-16	0.08	0.09	0.17	0.11
18	OPA-11	0.00	0.08	0.24	0.11
19	OPV-17	0.10	0.15	0.14	0.13
20	OPC-05	0.08	0.00	0.12	0.07
21	OPC-18	0.26	0.06	0.07	0.13
22	OPP-03	0.21	0.10	0.16	0.15
23	OPV-08	0.18	0.12	0.15	0.15
24	OPW-17	0.19	0.00	0.00	0.06
25	OPA-12	0.00	0.08	0.06	0.05
26	OPAL-20	0.17	0.02	0.00	0.06
27	OPE-20	0.22	0.08	0.02	0.11
28	OPB-07	0.06	0.12	0.09	0.09
29	OPF-08	0.07	0.13	0.20	0.13
30	OPG-14	0.18	0.17	0.14	0.16
31	OPK-03	0.17	0.21	0.18	0.19
32	OPA-06	0.00	0.15	0.16	0.10
33	OPB-05	0.00	0.06	0.09	0.05
34	OPB-20	0.07	0.15	0.08	0.10
35	OPO-08	0.14	0.07	0.11	0.10
36	OPT-07	0.00	0.00	0.00	0.00
37	OPW-03	0.18	0.09	0.18	0.15
38	OPG-16	0.00	0.05	0.10	0.05
39	OPG-08	0.11	0.15	0.09	0.11
40	OPG-06	0.15	0.22	0.19	0.18
41	OPG-17	0.00	0.05	0.14	0.07

42	OPK-01	0.08	0.08	0.20	0.12
43	OPK-09	0.07	0.13	0.15	0.12
44	OPK-07	0.00	0.00	0.00	0.00
45	OPC-09	0.00	0.20	0.17	0.13
46	OPC-06	0.11	0.23	0.28	0.21
47	OPC-10	0.00	0.06	0.06	0.04
48	OPK-10	0.00	0.00	0.00	0.00
49	OPK-16	0.00	0.00	0.00	0.00
50	OPC-08	0.08	0.04	0.08	0.07
51	OPH-03	0.04	0.10	0.04	0.06
52	OPA-08	0.08	0.14	0.13	0.12
53	OPU-10	0.00	0.02	0.04	0.02
54	OPD-19	0.11	0.23	0.22	0.18
55	OPU-19	0.00	0.00	0.05	0.02
56	OPD-20	0.03	0.06	0.07	0.05
57	OPA-05	0.00	0.01	0.01	0.01
58	OPC-16	0.07	0.06	0.08	0.07
59	OPD-15	0.04	0.09	0.05	0.06
60	OPE-05	0.08	0.34	0.10	0.18
61	OPD-12	0.10	0.25	0.28	0.21
	Mean for RAPD	0.07	0.09	0.10	0.09

effective number of alleles (ne), Shannon's information index (I), and Nei's gene diversity (h) were estimated (Table 10) to assess the genetic variability within a population. The mean estimated values for the observed number of alleles and the effective number of alleles were 1.45 and 1.27, respectively, for all RAPD primers used in the study. The observed number of alleles, *i.e.* 2 for primers OPA-11 and OPC-05, was higher than other RAPD primers. The effective number of alleles, *i.e.* 1.62, produced by primer OPA-18, was higher than other RAPD primers tested. The detected mean values for Nei's gene diversity and Shannon's information index for different random primers used in the study were 0.16 and 0.24, respectively. The primer OPA-11 resulted in the highest Nei's gene diversity and Shannon's information index values of 0.35 and 0.53, respectively, among all the RAPD primers used.

4.2.1.3. Cluster and Principal coordinate analysis for RAPD markers:

The genetic variation among 36 genotypes of *S. glauca* was assessed by Hierarchical clustering based on UPGMA and Neighbor-Joining analysis based on the UnWeighted Neighbor-Joining method from the dissimilarity matrix generated for Jaccard dissimilarity index. The genetic relationships among the 36 genotypes for RAPD data were revealed based on cluster analysis. The UPGMA tree (hierarchical clustering) resulted in two main clusters where cluster I consisted of F9 and A1 genotypes, and cluster II was divided into sub-clusters consisting of females, males, and andromonoecious genotypes (Fig. 17). In cluster II, the male genotypes were grouped under two sub-clusters, and females and andromonoecious were grouped under sub-clusters and were interspersed.

The Neighbor-Joining tree produced three main clusters (Fig. 18). Cluster I showed only one genotype *i.e.,* F10, which is outgrouped from other female genotypes. The cluster II showed two sub-clusters, where one sub-cluster was further divided into sub-clusters. One sub-cluster comprised of all six male genotypes and other sub-clusters comprised of female genotypes and also one andromonoecious genotype *i.e.* A2. The main cluster III was divided into two sub-clusters comprising of all andromonoecious genotypes except A2 and few females *i.e.* F6, F7, F8 and F9 genotypes. Thus, NJ tree based on UPGMA resulted in grouping of all males together, whereas few females and andromonoecious genotypes exhibited interspersed distribution in II and III clusters.

Table 10. Assessment of genetic polymorphism among different sexes of *S. glauca* using genetic diversity parameters based on RAPD data.

S. No.	Primer	na*	ne*	h*	I*
1	OPF-03	1.20	1.16	0.09	0.13
2	OPF-11	1.33	1.19	0.12	0.18
3	OPA-04	1.00	1.00	0.00	0.00
4	OPD-14	1.50	1.19	0.14	0.23
5	OPS-05	1.60	1.23	0.17	0.27
6	OPAW-07	1.00	1.00	0.00	0.00
7	OPT-18	1.57	1.37	0.22	0.33
8	OPAB-06	1.29	1.24	0.13	0.19
9	OPAL-08	1.00	1.00	0.00	0.00
10	OPAK-14	1.14	1.05	0.04	0.06
11	OPT-17	1.86	1.43	0.27	0.42
12	OPZ-01	1.25	1.17	0.10	0.15
13	OPZ-10	1.33	1.14	0.10	0.15
14	OPZ-06	1.33	1.13	0.09	0.14
15	OPB-12	1.67	1.44	0.26	0.38
16	OPA-18	1.67	1.62	0.32	0.45
17	OPA-16	1.44	1.38	0.20	0.29
18	OPA-11	2.00	1.59	0.35	0.53
19	OPV-17	1.50	1.35	0.20	0.29
20	OPC-05	2.00	1.44	0.30	0.48
21	OPC-18	1.80	1.51	0.30	0.45
22	OPP-03	1.75	1.56	0.32	0.46
23	OPV-08	1.78	1.59	0.33	0.47
24	OPW-17	1.67	1.26	0.19	0.30
25	OPA-12	1.44	1.31	0.17	0.26
26	OPAL-20	1.50	1.19	0.12	0.19
27	OPE-20	1.83	1.31	0.21	0.33
28	OPB-07	1.29	1.09	0.07	0.12
29	OPF-08	1.50	1.46	0.24	0.34
30	OPG-14	1.50	1.48	0.25	0.34
31	OPK-03	1.63	1.39	0.23	0.35
32	OPA-06	1.56	1.39	0.22	0.33
33	OPB-05	1.29	1.19	0.11	0.17
34	OPB-20	1.50	1.42	0.23	0.32
35	OPO-08	1.44	1.36	0.20	0.28
36	OPT-07	1.00	1.00	0.00	0.00
37	OPW-03	1.90	1.47	0.28	0.43
38	OPG-16	1.36	1.19	0.12	0.19
39	OPG-08	1.64	1.26	0.18	0.29
40	OPG-06	1.63	1.34	0.20	0.31
41	OPG-17	1.50	1.39	0.22	0.31
42	OPK-01	1.40	1.08	0.05	0.10

43	OPK-09	1.67	1.55	0.30	0.43
44	OPK-07	1.00	1.00	0.00	0.00
45	OPC-09	1.50	1.24	0.14	0.23
46	OPC-06	1.70	1.54	0.30	0.43
47	OPC-10	1.20	1.18	0.09	0.13
48	OPK-10	1.10	1.09	0.05	0.07
49	OPK-16	1.00	1.00	0.00	0.00
50	OPC-08	1.33	1.20	0.12	0.18
51	OPH-03	1.40	1.16	0.10	0.16
52	OPA-08	1.86	1.41	0.25	0.39
53	OPU-10	1.30	1.15	0.10	0.15
54	OPD-19	1.71	1.52	0.30	0.43
55	OPU-19	1.36	1.14	0.10	0.16
56	OPD-20	1.27	1.15	0.10	0.14
57	OPA-05	1.10	1.06	0.04	0.05
58	OPC-16	1.50	1.24	0.16	0.25
59	OPD-15	1.50	1.30	0.18	0.28
60	OPE-05	1.00	1.00	0.00	0.00
61	OPD-12	1.60	1.45	0.25	0.36
	Mean	1.45	1.27	0.16	0.24

The na, ne, h and I values were generated using POPGENE (version 1.32). (*a*) na: Observed number of alleles, (*b*) ne: Effective number of alleles, (*c*) h: Nei's gene diversity, (*d*) I: Shannon's information index.

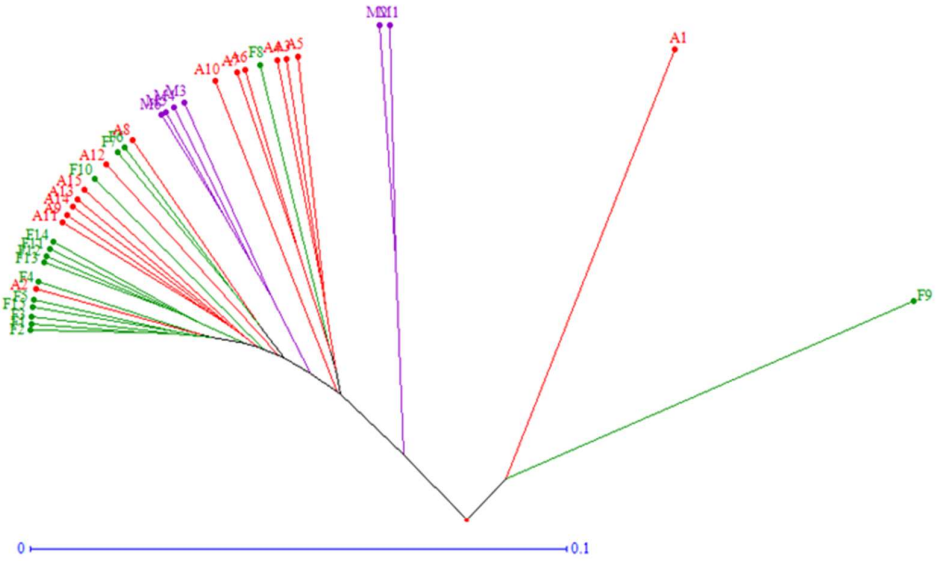

Fig. 17. Hierarchical clustering using UPGMA method obtained from RAPD data based on genetic dissimilarity matrix (Jaccard dissimilarity index) for different sexes of *S. glauca.* F1-F15 – female genotypes, M1-M6 – male genotypes, A1-A15 – andromonoecious genotypes.

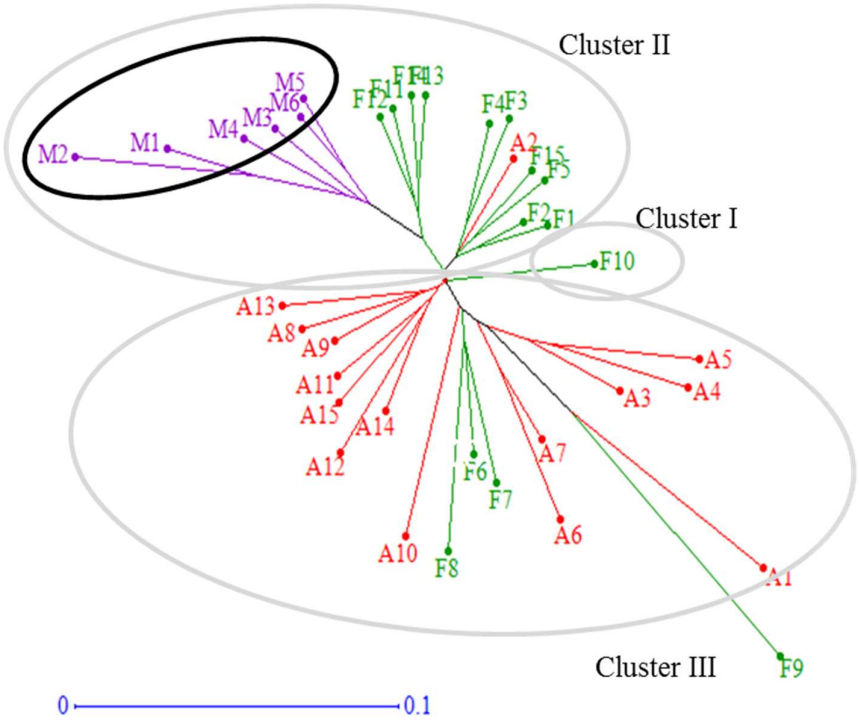

Fig. 18. Neighbor-Joining tree constructed using UnWeighted Neighbor-joining method from RAPD data based on genetic dissimilarity matrix (Jaccard dissimilarity index) for different sexes of *S. glauca.* F1-F15 – female genotypes, M1-M6 – male genotypes, A1-A15 – andromonoecious genotypes.

The principal coordinate analysis (PCoA) plot was produced by factorial analysis of the data produced for all genotypes with different RAPD primers used to analyze genetic variation (Fig. 19). All the genotypes with less genetic dissimilarity were under one quadrant, and genotypes in other quadrants exhibited high genetic dissimilarity. Based on this dissimilarity matrix, the genetic relationships among the genotypes were studied. The PCoA resulted in grouping of a few females in quadrant I, whereas quadrants II and III showed grouping of female and andromonoecious genotypes. The quadrant IV resulted in grouping of all-male genotypes only. The genotypes F9 and A1 exhibited high dissimilarity compared to other genotypes used in the study.

4.2.2.1. ISSR marker analysis among different sexes:

The screening of bulk DNA samples of different sexes was carried out with 33 ISSR primers, out of which 24 primers resulted in precise and reproducible amplification, and 9 primers (UBC 824, UBC 835, UBC 819, UBC 842, UBC 862, UBC 867, UBC 875, UBC 874 and UBC 879) did not show any amplification. Twenty-four ISSR primers that exhibited precise amplification (Fig. 20 & 21) were used to analyze genetic variation in female, male and andromonoecious plants. The size of the amplified bands for different ISSR primers ranged from 150-2900 bp. The number of bands generated for different ISSR primers ranged from 2 (UBC 865) to 14 (UBC 873) with the average number of bands per primer being 7.63 of which 4.92 were polymorphic (Table 11). The percentage polymorphism for different ISSR primers varied from 0 to 100%. Out of 24 ISSR primers screened on individuals of different sexes, 2 primers ISSR-12 and UBC 865, resulted in only monomorphic loci, whereas the other 22 primers resulted in polymorphic loci (Fig. 22-26). Among 22 ISSR primers, one primer *i.e.,* ISSR-14 resulted in 100% polymorphic bands, and the remaining primers showed polymorphic bands ranging from 40-87.5%. A total number of 183 bands were generated by 24 ISSR primers in all the genotypes tested, of which 118 bands were polymorphic revealing 61.48% polymorphism, and 65 bands were monomorphic.

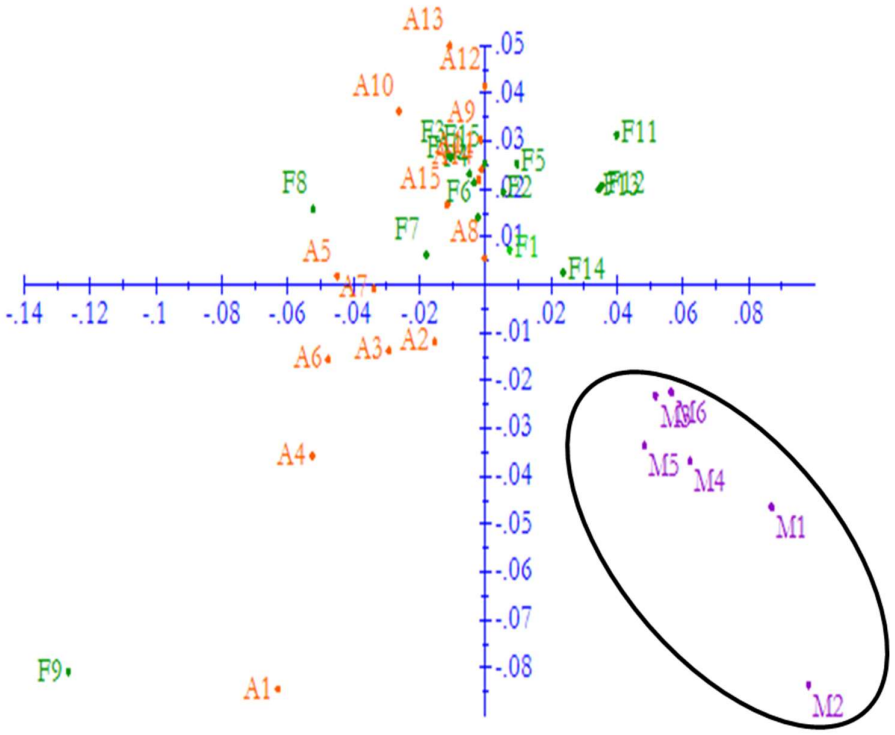

Fig. 19. Principal coordinates analysis (PCoA) of different sexes of *S. glauca* based on RAPD data. F1-F15– female genotypes, M1-M6 – male genotypes, A1-A15 – andromonoecious genotypes.

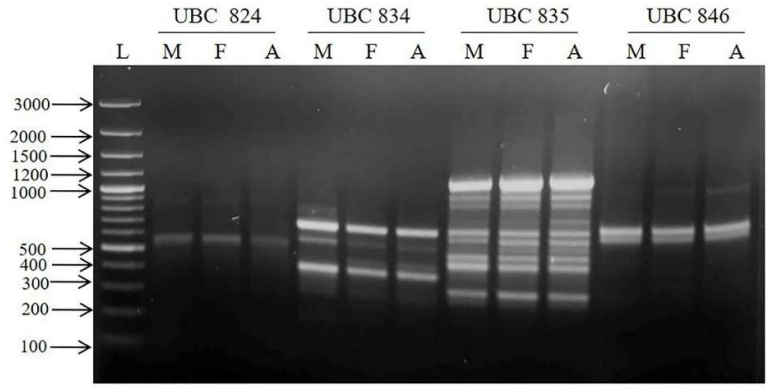

Fig. 20. Screening of bulk DNA of male, female and andromonoecious genotypes of *S. glauca* with ISSR primers, UBC 824, UBC 834, UBC 835 and UBC 846. *Lane L* – 100 bp DNA ladder, M – Bulk male genotypes, F – Bulk female genotypes, A – Bulk andromonoecious genotypes.

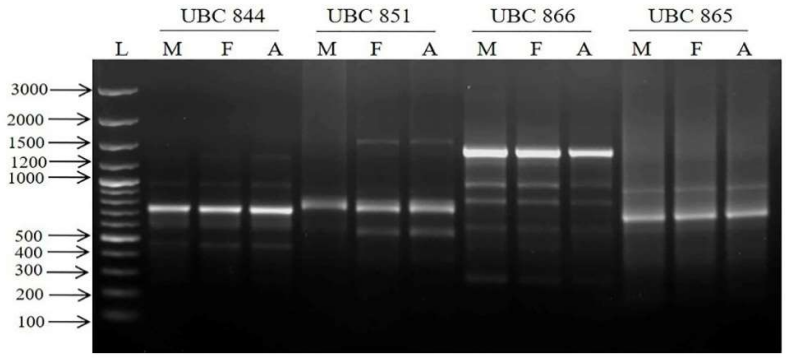

Fig. 21. Screening of bulk DNA of male, female and andromonoecious genotypes of *S. glauca* with ISSR primers, UBC 844, UBC 851, UBC 866 and UBC 865. *Lane L* – 100 bp DNA ladder, M – Bulk male genotypes, F – Bulk female genotypes, A – Bulk andromonoecious genotypes.

Table 11. ISSR amplification profiles of different sexes of *S. glauca* generated with 24 ISSR primers.

S. No.	Primer name	Number of loci	Number of monomorphic loci	Number of polymorphic loci	Polymorphism percentage (%)	Size range (bp)
1	ISSR-12	6	6	0	0	250-620
2	ISSR-13	8	3	5	62.5	200-700
3	ISSR-14	5	0	5	100	540-1650
4	ISSR-17	9	3	6	66.7	250-1850
5	ISSR-18	8	1	7	87.5	300-1050
6	ISSR-19	11	4	7	63.6	200-1450
7	UBC-813	4	2	2	50	500-1100
8	UBC-820	8	2	6	75	150-1500
9	UBC-823	5	1	4	80	430-980
10	UBC-834	9	3	6	66.7	200-1100
11	UBC-846	8	2	6	75	380-1400
12	UBC-880	13	4	9	69.2	220-1400
13	UBC-899	9	2	7	77.8	220-1750
14	UBC-818	5	2	3	60	200-1000
15	UBC-844	5	3	2	40	450-1350
16	UBC-851	6	1	5	83.3	400-1600
17	UBC-866	8	4	4	50	300-2900
18	UBC-865	2	2	0	0	700-950
19	UBC-873	14	3	11	78.6	150-1750
20	UBC-876	5	2	3	60	300-650
21	UBC-881	6	3	3	50	520-1500
22	UBC-814	6	2	4	66.7	150-900
23	ISSR-864	12	5	7	58.3	220-1300
24	ISSR-06	11	5	6	54.6	250-1200
	Total	183	65	118	-	
	Mean	7.63	-	4.92	61.48	150-2900

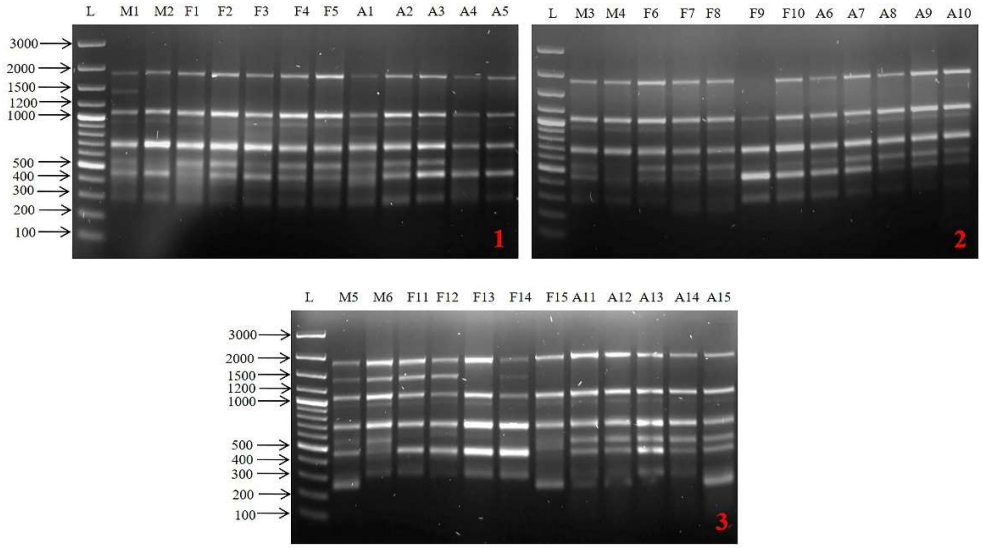

Fig. 22. Screening of individual DNA of male, female and andromonoecious genotypes of *S. glauca* with ISSR primer, ISSR-17. *Lane L* – 100 bp DNA ladder, M1-M6 – male genotypes, F1-F15 – female genotypes, A1-A15 – andromonoecious genotypes.

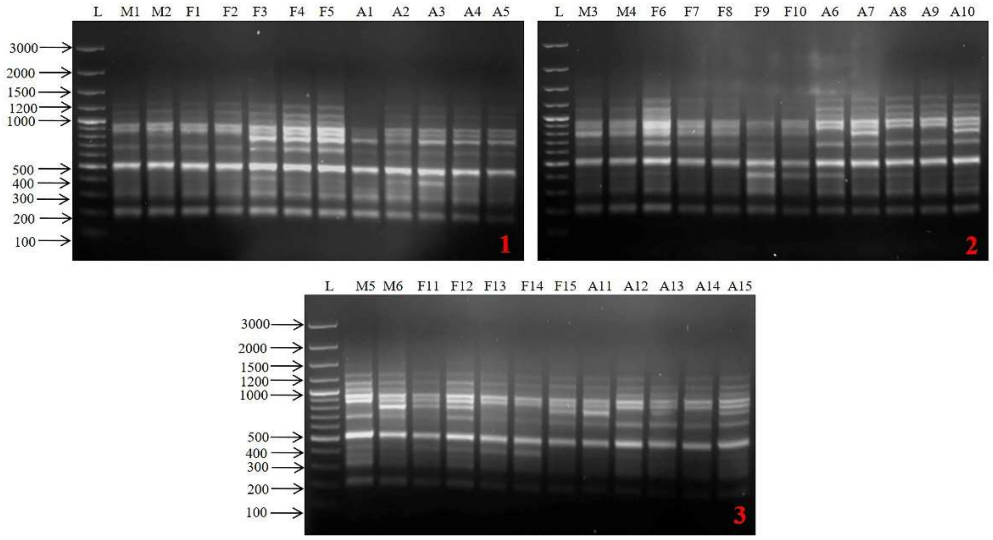

Fig. 23. Screening of individual DNA of male, female and andromonoecious genotypes of *S. glauca* with ISSR primer, ISSR-864. *Lane L* – 100 bp DNA ladder, M1- M6 – male genotypes, F1-F15 – female genotypes, A1-A15 – andromonoecious genotypes.

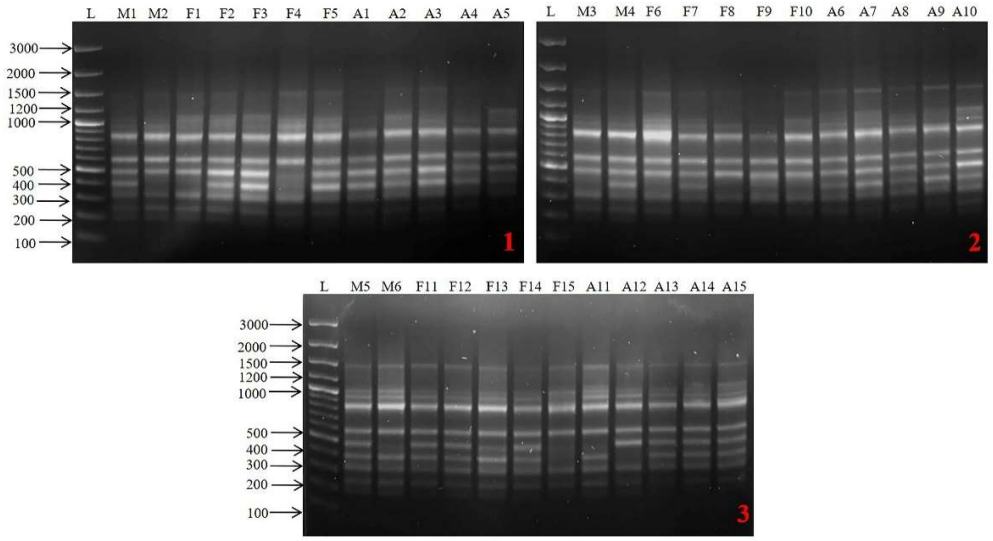

Fig. 24. Screening of individual DNA of male, female and andromonoecious genotypes of *S. glauca* with ISSR primer, ISSR-19. *Lane L* – 100 bp DNA ladder, M1-M6 – male genotypes, F1-F15 – female genotypes, A1-A15 – andromonoecious genotypes.

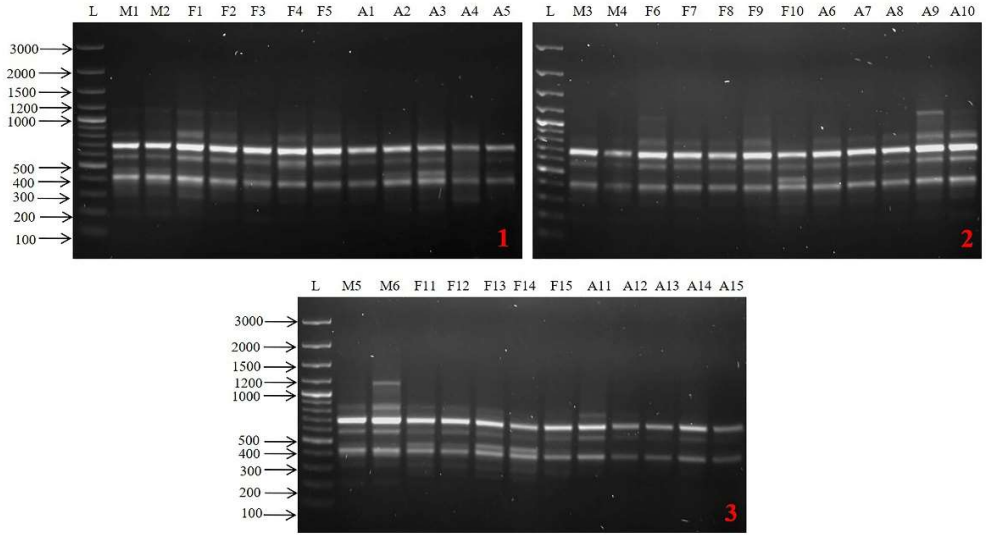

Fig. 25. Screening of individual DNA of male, female and andromonoecious genotypes of *S. glauca* with ISSR primer, UBC-834. *Lane L* – 100 bp DNA ladder, M1-M6 – male genotypes, F1-F15 – female genotypes, A1-A15 – andromonoecious genotypes.

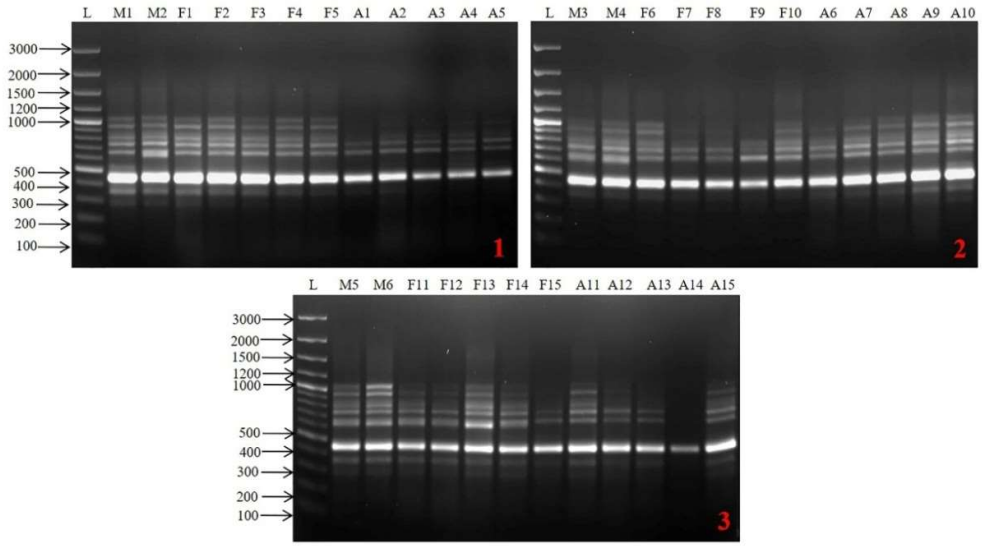

Fig. 26. Screening of individual DNA of male, female and andromonoecious genotypes of *S. glauca* with ISSR primer, ISSR-18. *Lane L* – 100 bp DNA ladder, M1-M6 – male genotypes, F1-F15 – female genotypes, A1-A15 – andromonoecious genotypes.

ISSR dissimilarity matrix of 36 genotypes of *S. glauca* was generated based on Jaccard's dissimilarity index to assess the genetic variations among them. The dissimilarity indices among all genotypes ranged from 0.047 and 0.399. The maximum dissimilarity value of 0.399 was observed between the genotypes F9 and F15, and the minimum dissimilarity value of 0.047 was observed between A7 and A8 genotypes (Table 12). The dissimilarity value ranged from 0.064 (M3 and M4) to 0.172 (M1 and M3) with an average of 0.129 for males. The females showed the dissimilarity values ranging from 0.085 (F4 and F5) to 0.399 (F9 and F15) with an average of 0.224. The andromonoecious genotypes for RAPD data showed dissimilarity values ranging from 0.047 (A7 and A8) to 0.319 (A1 and A6) with an average of 0.184.

4.2.2.2. Genetic variability assessment among different sexes using ISSR markers:

The polymorphism ability of a marker was analyzed by polymorphic information content (PIC). The PIC values among different sexes ranged from 0.00 to 0.22 for different ISSR primers analyzed in the study (Table 13). The maximum PIC value of 0.22 was obtained for primer UBC-899, and the minimum PIC value of 0.00 was obtained for primers ISSR-12 and UBC-865. The mean PIC value for ISSR primers in all genotypes was 0.13. The PIC values for female genotypes ranged from 0.00 to 0.28, with an average PIC value of 0.16. In female genotypes, the highest PIC value of 0.28 was obtained for ISSR primer UBC-899. For male plants, the PIC values were in the range of 0.00 to 0.22, with an average PIC value of 0.08. The ISSR primer UBC-814 showed the highest PIC value of 0.22 in all male genotypes. The PIC values for andromonoecious plants ranged from 0.00 to 0.25, with an average PIC value of 0.13. In andromonoecious genotypes tested for ISSR markers, the highest PIC value of 0.25 was observed for primer UBC-899. All the primers that resulted in zero PIC value were unable to detect polymorphism among the tested genotypes.

The POPGENE software version 1.32 was used to assess the genetic variation among different sexes of *S. glauca* using the ISSR data matrix generated by the scoring of bands. The genetic diversity parameters such as the observed number of alleles (na), effective number of alleles (ne), Shannon's information index (I), and Nei's gene diversity (h) were estimated in the present study to assess the genetic variability in different sexes (Table 14). The mean estimated values for the observed number of

Table 12. Dissimilarity matrix of different sexes of *S. glauca* revealed by ISSR markers.

Units	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
2	0.12																																		
3	0.17	0.14																																	
4	0.15	0.13	0.06																																
5	0.12	0.13	0.14	0.11																															
6	0.15	0.14	0.15	0.13	0.1																														
7	0.14	0.11	0.16	0.14	0.13	0.15																													
8	0.14	0.13	0.14	0.15	0.13	0.18	0.09																												
9	0.16	0.18	0.17	0.16	0.15	0.2	0.13	0.12																											
10	0.16	0.13	0.16	0.14	0.15	0.15	0.1	0.13	0.14																										
11	0.16	0.16	0.18	0.14	0.15	0.15	0.1	0.14	0.14	0.08																									
12	0.16	0.15	0.17	0.15	0.15	0.16	0.1	0.13	0.13	0.12	0.1																								
13	0.17	0.18	0.16	0.15	0.17	0.19	0.17	0.17	0.2	0.18	0.19	0.17																							
14	0.24	0.21	0.19	0.22	0.25	0.28	0.25	0.24	0.25	0.24	0.26	0.24	0.15																						
15	0.33	0.32	0.33	0.34	0.35	0.36	0.35	0.36	0.34	0.34	0.35	0.33	0.35	0.32																					
16	0.25	0.24	0.24	0.24	0.24	0.28	0.24	0.24	0.26	0.24	0.27	0.25	0.23	0.26	0.21																				
17	0.23	0.22	0.25	0.22	0.21	0.24	0.25	0.26	0.25	0.25	0.26	0.25	0.22	0.24	0.32	0.23																			
18	0.18	0.17	0.22	0.19	0.15	0.2	0.2	0.21	0.2	0.19	0.22	0.19	0.21	0.26	0.31	0.21	0.12																		
19	0.21	0.19	0.21	0.17	0.14	0.18	0.19	0.22	0.23	0.21	0.22	0.21	0.23	0.3	0.32	0.2	0.17	0.14																	
20	0.25	0.21	0.26	0.22	0.19	0.22	0.25	0.26	0.27	0.27	0.27	0.27	0.27	0.31	0.29	0.19	0.16	0.16	0.1																
21	0.22	0.25	0.25	0.22	0.2	0.21	0.25	0.25	0.26	0.21	0.23	0.25	0.19	0.27	0.4	0.28	0.25	0.25	0.22	0.26															
22	0.29	0.28	0.25	0.26	0.27	0.28	0.27	0.25	0.24	0.28	0.23	0.23	0.28	0.3	0.3	0.3	0.35	0.32	0.32	0.33	0.32														
23	0.2	0.19	0.22	0.19	0.18	0.22	0.18	0.18	0.18	0.17	0.16	0.15	0.21	0.29	0.31	0.15	0.22	0.19	0.16	0.18	0.22	0.23													
24	0.22	0.19	0.23	0.21	0.19	0.22	0.19	0.19	0.19	0.19	0.2	0.18	0.21	0.3	0.27	0.15	0.24	0.19	0.16	0.18	0.22	0.25	0.07												
25	0.28	0.26	0.28	0.28	0.26	0.28	0.28	0.26	0.27	0.26	0.28	0.26	0.29	0.3	0.26	0.24	0.27	0.29	0.28	0.27	0.33	0.23	0.21	0.21											
26	0.22	0.16	0.2	0.19	0.18	0.22	0.18	0.19	0.22	0.2	0.23	0.19	0.19	0.25	0.29	0.2	0.22	0.21	0.19	0.2	0.24	0.25	0.15	0.14	0.17										
27	0.2	0.18	0.25	0.22	0.18	0.22	0.18	0.16	0.19	0.2	0.21	0.19	0.2	0.26	0.3	0.17	0.22	0.18	0.16	0.2	0.23	0.32	0.16	0.14	0.26	0.15									
28	0.14	0.11	0.15	0.13	0.12	0.18	0.09	0.11	0.12	0.11	0.13	0.09	0.12	0.21	0.32	0.2	0.19	0.14	0.17	0.22	0.2	0.25	0.13	0.13	0.24	0.12	0.12								
29	0.12	0.12	0.16	0.15	0.12	0.18	0.12	0.11	0.14	0.14	0.14	0.11	0.12	0.22	0.32	0.19	0.19	0.14	0.17	0.21	0.22	0.24	0.14	0.14	0.25	0.15	0.11	0.05							
30	0.17	0.16	0.2	0.16	0.14	0.17	0.11	0.12	0.16	0.15	0.11	0.09	0.19	0.25	0.33	0.24	0.24	0.19	0.21	0.25	0.25	0.24	0.16	0.18	0.27	0.19	0.16	0.09	0.11						
31	0.16	0.15	0.15	0.15	0.14	0.16	0.1	0.13	0.12	0.13	0.11	0.09	0.17	0.25	0.33	0.26	0.24	0.18	0.21	0.27	0.25	0.24	0.17	0.19	0.27	0.17	0.19	0.08	0.1	0.09					
32	0.19	0.2	0.19	0.16	0.15	0.17	0.14	0.16	0.18	0.14	0.18	0.14	0.14	0.24	0.29	0.17	0.23	0.2	0.19	0.25	0.16	0.29	0.2	0.22	0.31	0.21	0.19	0.12	0.15	0.18	0.16				
33	0.22	0.19	0.22	0.18	0.14	0.21	0.14	0.17	0.2	0.18	0.16	0.15	0.18	0.23	0.29	0.26	0.27	0.2	0.21	0.25	0.22	0.25	0.19	0.21	0.3	0.21	0.19	0.13	0.15	0.14	0.18	0.17			
34	0.25	0.2	0.2	0.21	0.2	0.22	0.19	0.22	0.24	0.21	0.21	0.19	0.17	0.22	0.32	0.27	0.26	0.21	0.25	0.27	0.23	0.23	0.24	0.22	0.3	0.19	0.23	0.17	0.17	0.22	0.2	0.21	0.14		
35	0.22	0.2	0.23	0.21	0.17	0.18	0.18	0.18	0.23	0.22	0.18	0.15	0.21	0.29	0.39	0.28	0.3	0.24	0.23	0.27	0.23	0.23	0.2	0.2	0.3	0.21	0.21	0.18	0.16	0.16	0.19	0.21	0.15	0.14	
36	0.2	0.19	0.2	0.18	0.15	0.17	0.15	0.15	0.18	0.18	0.15	0.1	0.18	0.25	0.35	0.24	0.28	0.22	0.22	0.25	0.25	0.2	0.16	0.18	0.26	0.17	0.19	0.15	0.15	0.15	0.14	0.18	0.12	0.15	0.11

1-6 = male plants, 7-21 = female plants, 22-36 = andromonoecious plants

Table 13. Polymorphic information content (PIC) among different sexes of *S. glauca* using ISSR markers.

S. No.	Marker	PIC values for males	PIC values for females	PIC values for andromonoecious	Total PIC values
1	ISSR-12	0.00	0.00	0.00	0.00
2	ISSR-13	0.11	0.19	0.11	0.14
3	ISSR-14	0.06	0.10	0.22	0.13
4	ISSR-17	0.12	0.18	0.06	0.12
5	ISSR-18	0.00	0.11	0.22	0.11
6	ISSR-19	0.11	0.19	0.12	0.14
7	UBC-813	0.00	0.11	0.06	0.06
8	UBC-820	0.03	0.19	0.16	0.13
9	UBC-823	0.16	0.20	0.15	0.17
10	UBC-834	0.11	0.24	0.24	0.20
11	UBC-846	0.15	0.27	0.22	0.21
12	UBC-880	0.08	0.21	0.08	0.13
13	UBC-899	0.14	0.28	0.25	0.22
14	UBC-818	0.17	0.22	0.12	0.17
15	UBC-844	0.06	0.09	0.07	0.07
16	UBC-851	0.00	0.19	0.22	0.14
17	UBC-866	0.06	0.18	0.10	0.12
18	UBC-865	0.00	0.00	0.00	0.00
19	UBC-873	0.11	0.22	0.22	0.18
20	UBC-876	0.10	0.12	0.14	0.12
21	UBC-881	0.00	0.06	0.00	0.02
22	UBC-814	0.22	0.21	0.20	0.21
23	ISSR-864	0.14	0.20	0.14	0.16
24	ISSR-06	0.07	0.11	0.10	0.09
	Mean	0.08	0.16	0.13	0.13

Table 14. Assessment of genetic polymorphism among different sexes of *S. glauca* using genetic diversity parameters based on ISSR data.

S. No.	Primer	na*	ne*	h*	I*
1	ISSR-12	1.00	1.00	0.00	0.00
2	ISSR-13	1.63	1.38	0.22	0.33
3	ISSR-14	2.00	1.61	0.36	0.54
4	ISSR-17	1.67	1.33	0.20	0.32
5	ISSR-18	1.88	1.56	0.32	0.48
6	ISSR-19	1.64	1.49	0.27	0.39
7	UBC-813	1.50	1.32	0.19	0.28
8	UBC-820	1.75	1.37	0.23	0.35
9	UBC-823	1.80	1.47	0.26	0.40
10	UBC-834	1.67	1.41	0.24	0.36
11	UBC-846	1.75	1.33	0.20	0.32
12	UBC-880	1.69	1.55	0.30	0.43
13	UBC-899	1.78	1.61	0.33	0.47
14	UBC-818	1.60	1.56	0.29	0.41
15	UBC-844	1.40	1.32	0.18	0.25
16	UBC-851	1.83	1.53	0.29	0.44
17	UBC-866	1.50	1.33	0.18	0.27
18	UBC-865	1.00	1.00	0.00	0.00
19	UBC-873	1.79	1.28	0.19	0.30
20	UBC-876	1.60	1.31	0.18	0.27
21	UBC-881	1.50	1.01	0.01	0.04
22	UBC-814	1.67	1.28	0.19	0.31
23	UBC-864	1.58	1.44	0.25	0.36
24	ISSR-06	1.55	1.23	0.14	0.22
	Mean	1.62	1.36	0.21	0.31

The na, ne, h and I values were generated using POPGENE (version 1.32). (*a*) na: Observed number of alleles, (*b*) ne: Effective number of alleles, (*c*) h: Nei's gene diversity, (*d*) I: Shannon's information index.

alleles and the effective number of alleles were 1.62 and 1.36, respectively, for different ISSR primers used in the study. The highest values of the observed number of alleles and the effective number of alleles i.e. 2.00 and 1.61 were produced by the primer ISSR-14 compared to other ISSR primers tested. The detected mean values for Nei's gene diversity and Shannon's information index for different ISSR primers used in the study were 0.21 and 0.31, respectively. The primer ISSR-14 resulted in the highest Nei's gene diversity and Shannon's information index values of 0.36 and 0.54, respectively among all the ISSR primers analyzed.

4.2.2.3. Cluster and Principal coordinate analysis for ISSR markers:

The genetic variation among 36 genotypes of *S. glauca* was assessed by Hierarchical clustering based on the UPGMA method and Neighbor-Joining analysis based on the Unweighted Neighbor-Joining method from the dissimilarity matrix generated based on the Jaccard dissimilarity index. Based on the clustering patterns obtained for Hierarchical clustering and Neighbor-Joining, the genetic relationships among the 36 genotypes for ISSR data were determined. The UPGMA tree (Hierarchical clustering) resulted in grouping of genotypes into two main clusters (Fig. 27). The cluster I was divided into two sub-clusters consisting of F9, A1, and A4 genotypes. Cluster II was divided into two sub-clusters, which were further divided into sub-clusters. All male genotypes with more similarity were grouped in one sub-cluster of cluster II. In cluster II, female and andromonoecious were also grouped under sub-clusters. The Neighbor-Joining tree resulted in three clusters (Fig. 28), of which clusters I and II were comprised of female and andromonoecious genotypes. Cluster III formed 3 sub-clusters with grouping of genotypes of all three sexes. All male genotypes that showed distinct grouping as one sub-cluster of Cluster III exhibited the least genetic dissimilarity.

The principal coordinate analysis (PCoA) plot was produced by factorial analysis of all genotypes with different ISSR primers to analyze the genetic variation. All the genotypes with less genetic dissimilarity were under one quadrant and genotypes in other quadrants exhibit high genetic dissimilarity (Fig. 29). Based on this dissimilarity matrix obtained for ISSR binary data, the genetic relationships of the

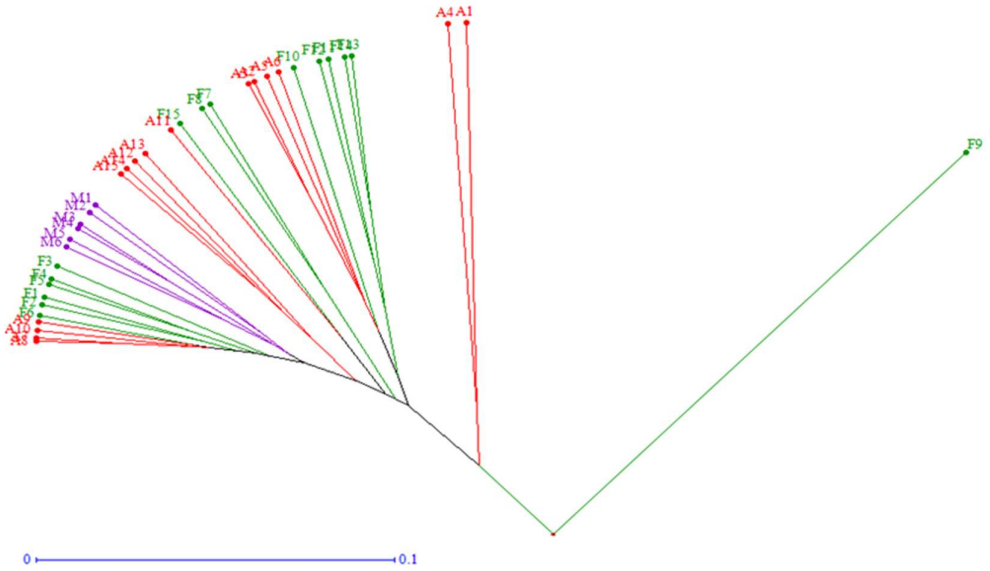

Fig. 27. Hierarchical clustering using UPGMA method from ISSR data based on genetic dissimilarity matrix (Jaccard dissimilarity index) for different sexes of *S. glauca.* F1-F15 – female genotypes, M1-M6 – male genotypes, A1-A15 – andromonoecious genotypes.

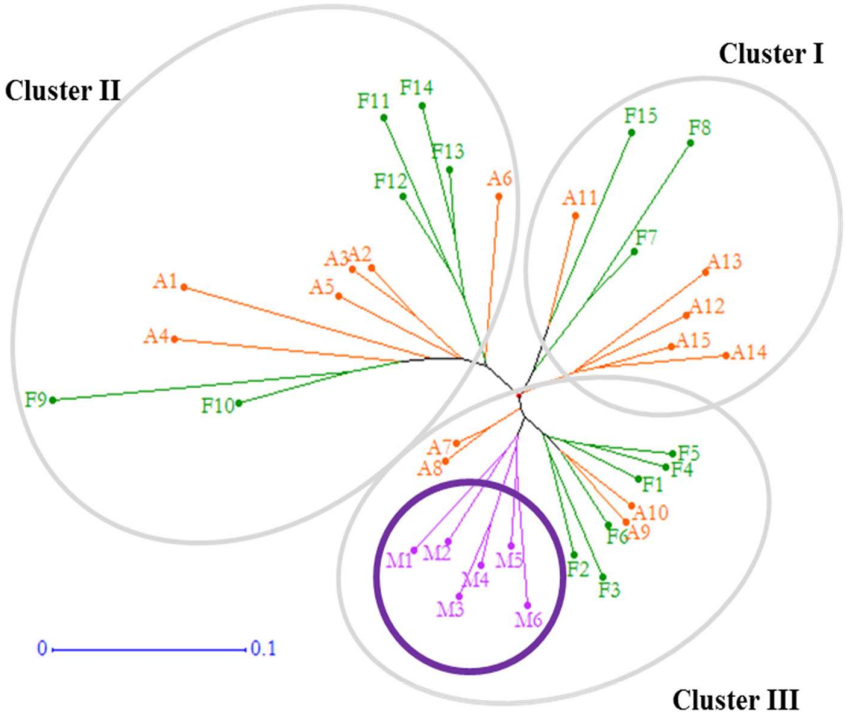

Fig. 28. Neighbor-Joining tree constructed using UnWeighted Neighbor-Joining method from ISSR data based on genetic dissimilarity matrix (Jaccard dissimilarity index) for different sexes of *S. glauca.* F1-F15 – female genotypes, M1-M6 – male genotypes, A1-A15 – andromonoecious genotypes.

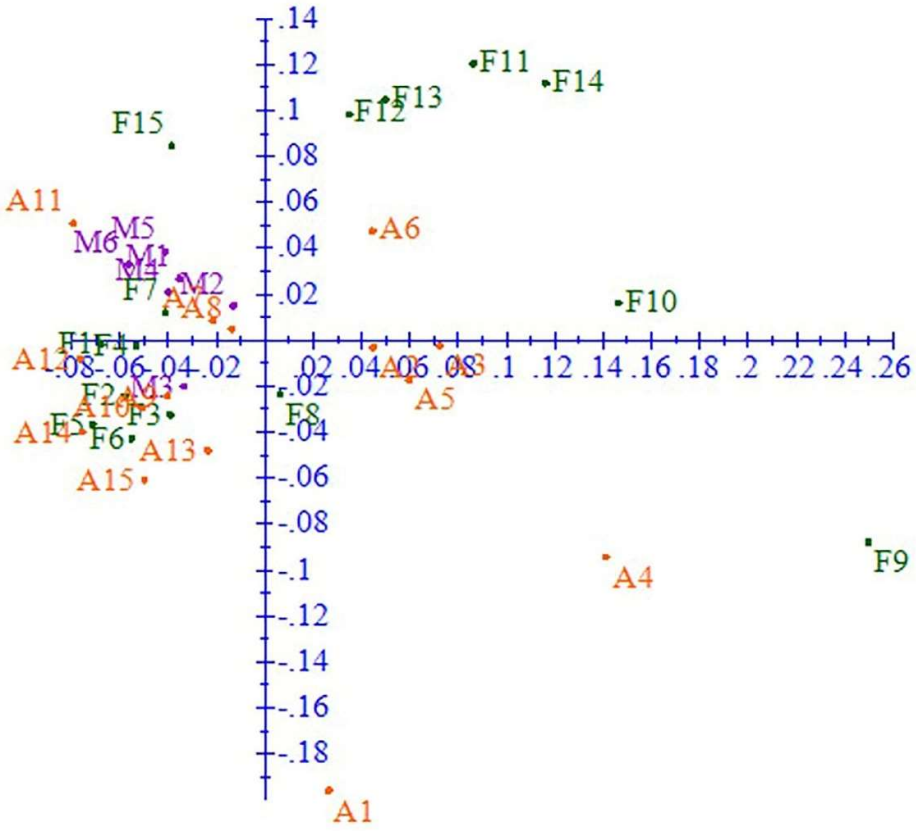

Fig. 29. Principal coordinates analysis (PCoA) based on ISSR data for different sexes of *S. glauca.* F1-F15 – female genotypes, M1-M6 – male genotypes, A1-A15 – andromonoecious genotypes.

genotypes were studied. The PCoA resulted in the grouping of females and andromonoecious genotypes present in all the quadrants. The quadrant II resulted in the grouping of 5 male genotypes (M1, M2, M4, M5, and M6) except M3, which grouped under the quadrant IV. The genotypes F9, A4, and A1, exhibited high dissimilarity compared to other genotypes used in the study.

4.2.3.1. Genetic variability analysis for combined RAPD and ISSR markers in different sexes of *S. glauca*:

The combined RAPD and ISSR dissimilarity matrix of 36 genotypes of *S. glauca* was generated based on Jaccard's dissimilarity index to assess the genetic variations among them. The dissimilarity indices among all genotypes ranged from 0.054 to 0.270. The maximum dissimilarity value of 0.270 was observed between the genotypes M2 and F9, and the minimum dissimilarity value of 0.054 was observed between F1 and F2 genotypes (Table 15). The females showed the dissimilarity ranging from 0.054 (F1 and F2) to 0.245 (F9 and F11) with an average of 0.131. The dissimilarity values ranged from 0.059 (M5 and M) to 0.131 (M2 and M3) with an average of 0.101 for males. The andromonoecious genotypes showed dissimilarity values ranging from 0.071 (A7 and A8) to 0.218 (A1 and A6) with an average of 0.131 with combined RAPD and ISSR data.

The overall mean PIC value for females, males and andromonoecious genotypes for all RAPD and ISSR primers tested were 0.11, 0.07, and 0.11, respectively. The mean PIC value for all 36 genotypes for combined RAPD and ISSR primers was 0.10. The mean values obtained by combined RAPD and ISSR markers for genetic diversity parameters such as observed number of alleles, the effective number of alleles, Nei's gene diversity, and Shannon's information index were 1.49, 1.29, 0.18 and 0.26, respectively.

4.2.3.2. Cluster and principal coordinate analysis for combined RAPD and ISSR markers in different sexes of *S. glauca*:

The combined RAPD and ISSR binary data for all the genotypes were used for UPGMA tree, Neighbor-Joining tree, and Principal coordinate analysis. The UPGMA tree (hierarchical clustering) with combined RAPD and ISSR data resulted in two

Table 15. Dissimilarity matrix of different sexes of *S. glauca* revealed by RAPD and ISSR markers.

Units	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
2	0.09																																		
3	0.11	0.13																																	
4	0.11	0.12	0.07																																
5	0.11	0.13	0.1	0.07																															
6	0.11	0.13	0.1	0.08	0.06																														
7	0.12	0.14	0.12	0.13	0.1	0.1																													
8	0.13	0.15	0.11	0.12	0.1	0.11	0.05																												
9	0.15	0.18	0.13	0.13	0.13	0.13	0.07	0.07																											
10	0.14	0.16	0.12	0.12	0.12	0.12	0.08	0.08	0.08																										
11	0.14	0.16	0.13	0.12	0.11	0.11	0.07	0.08	0.08	0.07																									
12	0.14	0.16	0.12	0.12	0.12	0.12	0.09	0.09	0.11	0.1	0.09																								
13	0.15	0.18	0.12	0.13	0.13	0.14	0.12	0.12	0.13	0.13	0.12	0.09																							
14	0.19	0.21	0.15	0.17	0.18	0.18	0.15	0.13	0.15	0.15	0.16	0.13	0.1																						
15	0.26	0.27	0.24	0.25	0.25	0.25	0.23	0.23	0.23	0.22	0.24	0.22	0.22	0.2																					
16	0.17	0.19	0.14	0.16	0.15	0.16	0.13	0.11	0.13	0.14	0.14	0.14	0.13	0.15	0.19																				
17	0.15	0.17	0.13	0.13	0.11	0.12	0.12	0.11	0.13	0.12	0.13	0.13	0.14	0.15	0.25	0.13																			
18	0.14	0.16	0.11	0.12	0.11	0.11	0.11	0.1	0.12	0.12	0.12	0.1	0.13	0.15	0.24	0.12	0.07																		
19	0.15	0.17	0.12	0.11	0.1	0.11	0.11	0.11	0.12	0.12	0.12	0.13	0.14	0.18	0.24	0.11	0.09	0.07																	
20	0.16	0.17	0.14	0.12	0.11	0.12	0.12	0.12	0.14	0.14	0.14	0.14	0.15	0.18	0.22	0.11	0.09	0.09	0.06																
21	0.16	0.19	0.14	0.15	0.13	0.13	0.11	0.11	0.12	0.1	0.1	0.13	0.12	0.15	0.24	0.13	0.11	0.1	0.11	0.12															
22	0.23	0.23	0.2	0.21	0.2	0.21	0.19	0.19	0.18	0.2	0.19	0.19	0.21	0.22	0.2	0.21	0.23	0.22	0.21	0.21	0.21														
23	0.15	0.17	0.13	0.13	0.12	0.13	0.1	0.1	0.1	0.09	0.1	0.11	0.14	0.17	0.2	0.11	0.13	0.11	0.1	0.11	0.11	0.16													
24	0.17	0.18	0.15	0.15	0.14	0.15	0.13	0.11	0.13	0.14	0.13	0.12	0.14	0.16	0.21	0.12	0.15	0.13	0.12	0.12	0.13	0.17	0.08												
25	0.2	0.2	0.18	0.18	0.17	0.19	0.18	0.16	0.17	0.17	0.17	0.16	0.16	0.16	0.19	0.16	0.18	0.19	0.18	0.18	0.19	0.18	0.13	0.12											
26	0.18	0.19	0.16	0.17	0.16	0.17	0.14	0.14	0.15	0.15	0.17	0.15	0.14	0.15	0.23	0.15	0.16	0.16	0.15	0.15	0.16	0.2	0.12	0.1	0.11										
27	0.18	0.18	0.17	0.16	0.14	0.16	0.15	0.12	0.15	0.14	0.15	0.14	0.15	0.14	0.2	0.13	0.15	0.14	0.14	0.14	0.16	0.22	0.13	0.12	0.15	0.12									
28	0.15	0.15	0.13	0.12	0.12	0.14	0.1	0.09	0.11	0.1	0.1	0.09	0.1	0.13	0.2	0.13	0.13	0.12	0.13	0.14	0.12	0.19	0.09	0.11	0.14	0.12	0.08								
29	0.13	0.15	0.12	0.12	0.11	0.12	0.09	0.1	0.1	0.1	0.11	0.09	0.09	0.16	0.22	0.13	0.12	0.1	0.12	0.14	0.13	0.19	0.1	0.13	0.17	0.14	0.11	0.07							
30	0.14	0.17	0.14	0.13	0.12	0.12	0.08	0.08	0.1	0.1	0.08	0.08	0.12	0.15	0.23	0.13	0.12	0.12	0.12	0.13	0.12	0.19	0.11	0.13	0.17	0.14	0.12	0.07	0.08						
31	0.17	0.18	0.14	0.15	0.15	0.15	0.11	0.11	0.1	0.11	0.11	0.1	0.13	0.16	0.24	0.16	0.15	0.14	0.15	0.17	0.15	0.2	0.12	0.13	0.18	0.14	0.15	0.1	0.1	0.09					
32	0.16	0.17	0.13	0.14	0.12	0.12	0.1	0.09	0.12	0.1	0.11	0.1	0.1	0.15	0.25	0.13	0.12	0.11	0.12	0.14	0.1	0.2	0.12	0.13	0.17	0.16	0.14	0.09	0.11	0.09	0.13				
33	0.17	0.19	0.15	0.15	0.12	0.15	0.11	0.11	0.13	0.11	0.11	0.12	0.13	0.16	0.26	0.15	0.14	0.13	0.12	0.15	0.13	0.21	0.12	0.14	0.17	0.16	0.15	0.11	0.12	0.1	0.14	0.11			
34	0.18	0.2	0.14	0.16	0.15	0.15	0.11	0.12	0.13	0.11	0.12	0.13	0.13	0.14	0.23	0.14	0.14	0.12	0.14	0.14	0.12	0.2	0.13	0.13	0.18	0.15	0.13	0.11	0.11	0.11	0.13	0.1	0.11		
35	0.17	0.18	0.14	0.14	0.12	0.12	0.1	0.1	0.13	0.11	0.11	0.11	0.14	0.17	0.24	0.14	0.13	0.12	0.12	0.12	0.11	0.18	0.11	0.13	0.18	0.13	0.13	0.11	0.11	0.1	0.13	0.1	0.09	0.09	
36	0.15	0.17	0.14	0.14	0.12	0.13	0.11	0.1	0.12	0.12	0.1	0.1	0.12	0.15	0.24	0.14	0.14	0.14	0.14	0.13	0.13	0.18	0.11	0.12	0.15	0.13	0.12	0.09	0.11	0.09	0.12	0.1	0.1	0.11	0.08

1-6 = male plants, 7-21 = female plants, 22-36 = andromonoecious plants

clusters I and II (Fig. 30). The cluster I comprised of only one genotype, i.e. F9, which was out-grouped indicating that it is more genetically divergent from other genotypes used in the study. The main cluster II was divided into several sub-clusters. The resulted sub-clusters showed grouping of females, males, and andromonoecious genotypes. Males were grouped into two different sub-clusters, which showed that M1 and M2 have less dissimilarity compared to M3, M4, M5, and M6 genotypes. All female genotypes except F9 were grouped under 6 sub-clusters where F10 and F15 exhibited more genetic dissimilarity with other female genotypes. All andromonoecious genotypes were also grouped into 6 sub-clusters.

The Neighbor-Joining tree analysis resulted in 3 main clusters with distribution of females and andromonoecious genotypes as shown in Fig. 31. The cluster I formed two sub-clusters, one sub-cluster comprised of all male genotypes and the other sub-cluster consisting of females. The clusters II and III had sub-clusters with grouping of females and andromonoecious genotypes.

The PCoA analysis for combined RAPD and ISSR data resulted in an interspersed distribution of all females and andromonoecious genotypes in quadrants I, II, and III except F12, 13, and 14 in quadrant IV. All male genotypes resulted in clear and separate grouping in quadrant IV, showing less dissimilarity than others (Fig. 32).

4.3. Sex-identification and validation of sex-specific bands using RAPD, ISSR and SCAR markers in *S. glauca*:

4.3.1. Testing of previously reported RAPD and ISSR primers:

Initially, RAPD primers (OPU-10, OPD-19, OPU-19, OPS-05, OPW-03, OPE-05, OPA-18, OPD-20, OPS-06, and OPA-08/RP-07) that were reported for sex identification previously (Vaidya and Naik 2014; Ghumatkar *et al*. 2015; Baratakke and Patil 2014; Simon *et al*. 2009; Savitha *et al*. 2008; Choudhary 2014) were first tested for their ability to distinguish *Simarouba* sexes as shown in Table 3. The primer named RP-49 (ACCCGGTCAC) has the same sequence as OPD-20 (ACCCGGTCAC) universal random primer; hence OPD-20 was used in the present study. Similarly, the primer named RP-07 (GTGACGTAGG) has similar sequence as the RAPD primer, OPA-08 (GTGACGTAGG) and therefore OPA-08 was used in the study. The primer

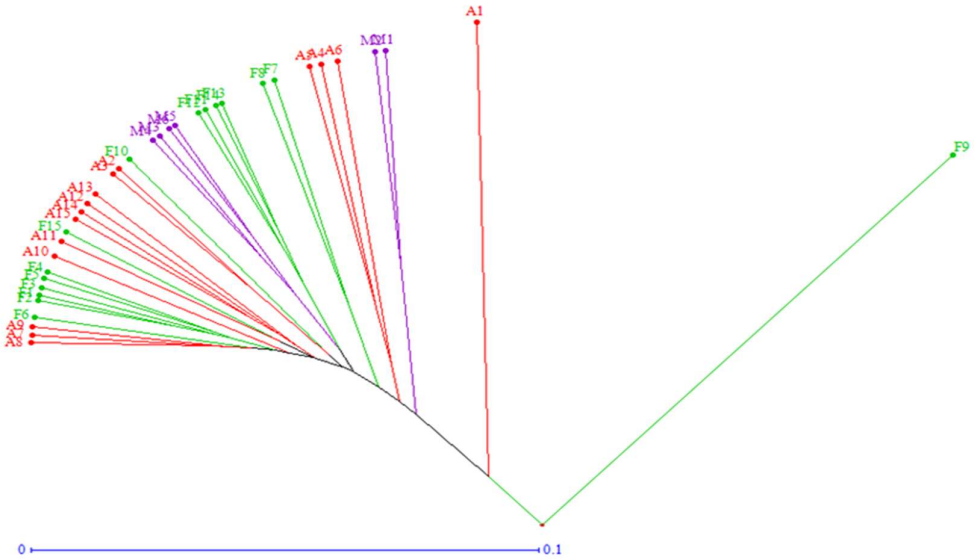

Fig. 30. Hierarchical clustering using UPGMA method from combined data of RAPD and ISSR based on genetic dissimilarity matrix (Jaccard dissimilarity index) for different sexes of *S. glauca.* F1-F15 – female genotypes, M1-M6 – male genotypes, A1-A15 – andromonoecious genotypes.

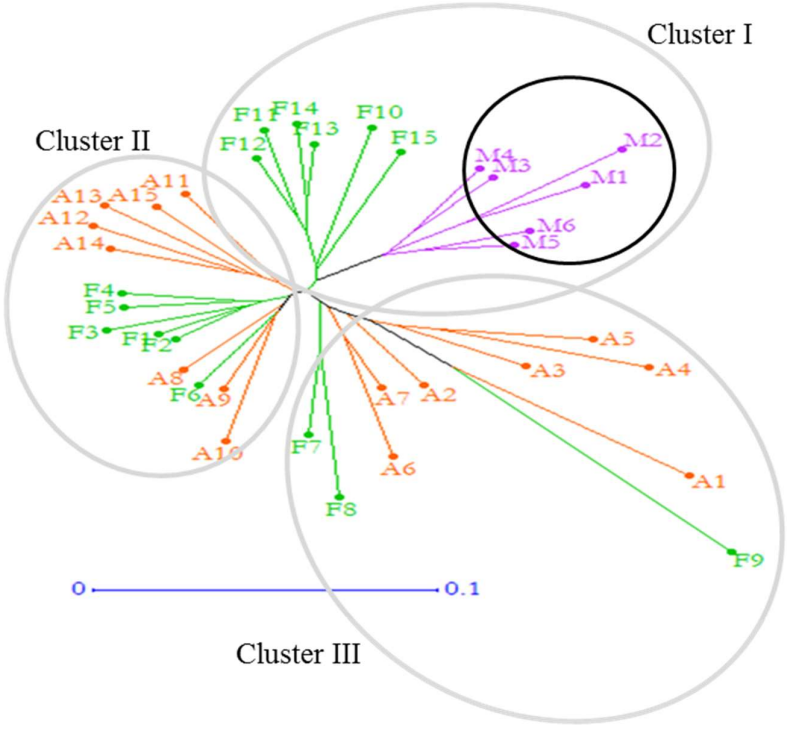

Fig. 31. Neighbor-Joining tree constructed based on UnWeighted Neighbor-Joining method (Jaccard dissimilarity index) from combined data of RAPD and ISSR for different sexes of *S. glauca.* F1-F15 – female genotypes, M1-M6 – male genotypes, A1-A15 – andromonoecious genotypes.

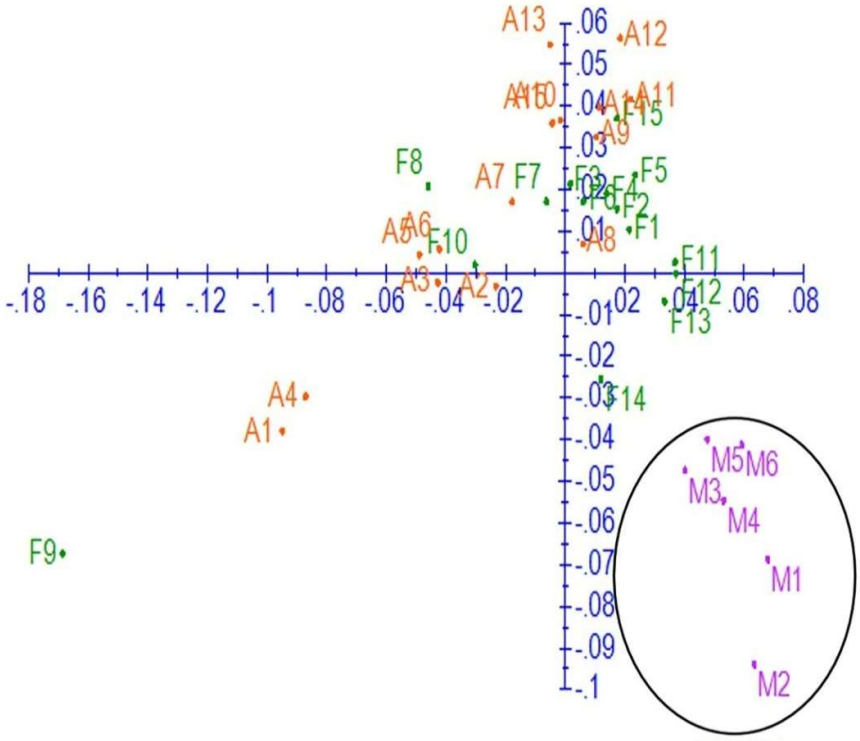

Fig. 32. Principal coordinates analysis (PCoA) based on combined data of RAPD and ISSR for different sexes of *S. glauca.* F1-F15 – female genotypes, M1-M6 – male genotypes, A1-A15 – andromonoecious genotypes.

OPU-10 resulted in ~1150 bp (>1 kbp as reported previously) in both bulk and individual DNA samples of all 3 sexes. The primer OPD-19 and OPU-19 did not result in 350 bp and 500 bp bands, respectively, as reported previously in bulk and individual samples. The primer OPS-05 amplified ~1300 bp band (>1 kbp as reported previously) in both bulk and individual DNA samples of three sexes. As previously reported, the primer OPW-03 did not result in a 600 bp band in bulk and individual samples. The primer OPE-05 showed ~521 bp band as reported previously but did not result in male sex-specific amplification and was present in 3 bulk samples of female, male and andromonoecious genotypes. The band ~521 bp was present in 11 females and all males and andromonoecious genotypes tested in the study. The primer OPA-18 resulted in ~1100 bp, as reported previously. However, the band was not sex-specific and was present in all 3 bulk DNA samples as well as one male, four females, and five andromonoecious individual genotypes, thus exhibiting only polymorphism. The primer OPD-20 resulted in amplification of ~900 bp band as reported earlier. In the present study, the band was amplified in all 3 bulk samples, and 2 males, 5 females, and 5 andromonoecious individual genotypes, thus exhibiting only polymorphism and not sex-specific amplification. For the primer OPS-06, the band size was not mentioned in the previous report by Savitha *et al*. (2008). However, in the present study the primer resulted in ~2800 bp band specific to male bulk sample only and did not show amplification in all individuals tested. Choudhary (2014) reported that the primer RP-07 (OPA-08) generated 900 bp band that was specific to male and bisexual genotypes. In the present study, the primer resulted in ~900 bp band in bulk DNA samples of female and andromonoecious sexes and was absent in males. When screened on individuals, the band was present in one male, four females, and five andromonoecious genotypes; thus, the band exhibited only polymorphism and was not sex-specific.

Thus, RAPD markers reported by other researchers did not show association with sex in the genotypes analyzed in this study. We, therefore, have tested 9 RAPD primers that produced polymorphic bands in bulk DNA samples of different sexes in the genetic variation studies on individual female, male and andromonoecious genotypes for identifying markers that are sex associated in *S. glauca*.

4.3.2. Testing of previously reported SCAR markers:

The previously reported few SCAR markers for sex identification as shown in Table 4, were tested on the genotypes selected in the study to determine their ability to differentiate the sex (Fig. 33). The SCAR marker RP-49 SCAR 353 resulted in a 353 bp band in bulk samples of all three sexes, and in 2 males, 5 females, and 5 andromonoecious individuals (Fig. 33 & Fig. 34a). The band 300 bp was present in the male, female, and andromonoecious bulk samples and almost in all individuals tested except for one male individual. Thus, the SCAR primers in our study did not result in sex-specific amplification as reported but exhibited polymorphism among individuals tested.

The UBC-815 SCAR was reported to produce a 721 bp band specific to males and andromonoecious genotypes. In the present study, the primer was tested on bulk samples and individually in all sexes to differentiate the sexes. The primer resulted in amplification of the 721 bp band in all 3 bulks, and all individuals tested and thus failed to distinguish the sexes (Fig. 33 & Fig. 34b). The SCAR marker for OPA-18 (MSSMS-01F and R) has previously been reported to produce 1100 bp band that is male specific. In the present study, the OPA-18 (MSSMS-01F and R) amplified ~150 bp band present in all 3 bulks (Fig. 33), and 1100 bp band specific to males was not observed in the genotypes analyzed in this study. The SCAR markers OPA-08 SCAR1-F/R and SCAR2-F/R were previously reported to amplify 91 bp and 915 bp band in male and andromonoecious genotypes. In the present study, by using the same conditions mentioned in the publication as well as by using other PCR conditions, the OPA-08 SCAR1-F and R showed no amplification. Another marker OPA-08 SCAR2-F/R resulted in amplification of ~950 bp band in all bulk samples whereas in individuals it was randomly present in few genotypes of all 3 sexes (Fig. 33) and could not differentiate the sexes in this study.

4.3.3. Screening of male, female, and andromonoecious trees for sex identification by RAPD primers:

Here, 9 RAPD primers out of 61 primers, namely OPZ-10, OPC-18, OPA-12, OPS-06, OPG-08, OPK-10, OPC-08, OPA-08, and OPE-05 produced polymorphic bands among the bulk DNA samples of different sexes (Table 16; Fig 35a, b & c). The

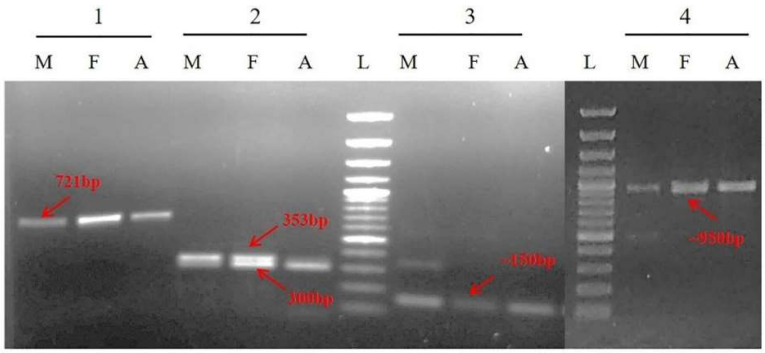

Fig. 33. Previously reported SCAR markers tested for sex-specific amplification with bulk DNA and individual genotypes of male, female and andromonoecious genotypes of *S. glauca.* 1 = UBC-815 SCAR 721, 2 = RP-49 SCAR 353, 3 = OPA-18 (MSSMS-01), 4 = OPA-08 SCAR2. *Lane L* – 100 bp DNA ladder, M – male, F – female, A – andromonoecious genotypes.

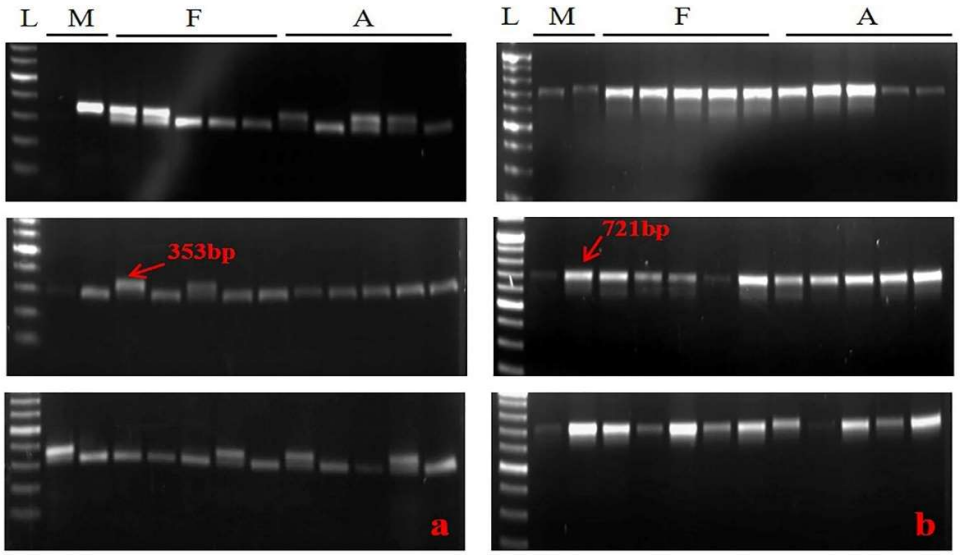

Fig. 34. Previously reported SCAR markers tested for sex-specific amplification with individual genotypes of male, female and andromonoecious genotypes of *S. glauca*. (*a*) RP-49 SCAR 353, (*b*) UBC-815 SCAR 721. *Lane L* – 100 bp DNA ladder, M – male genotypes (M1-M6), F – female genotypes (F1-F15), A – andromonoecious genotypes (A1-A15).

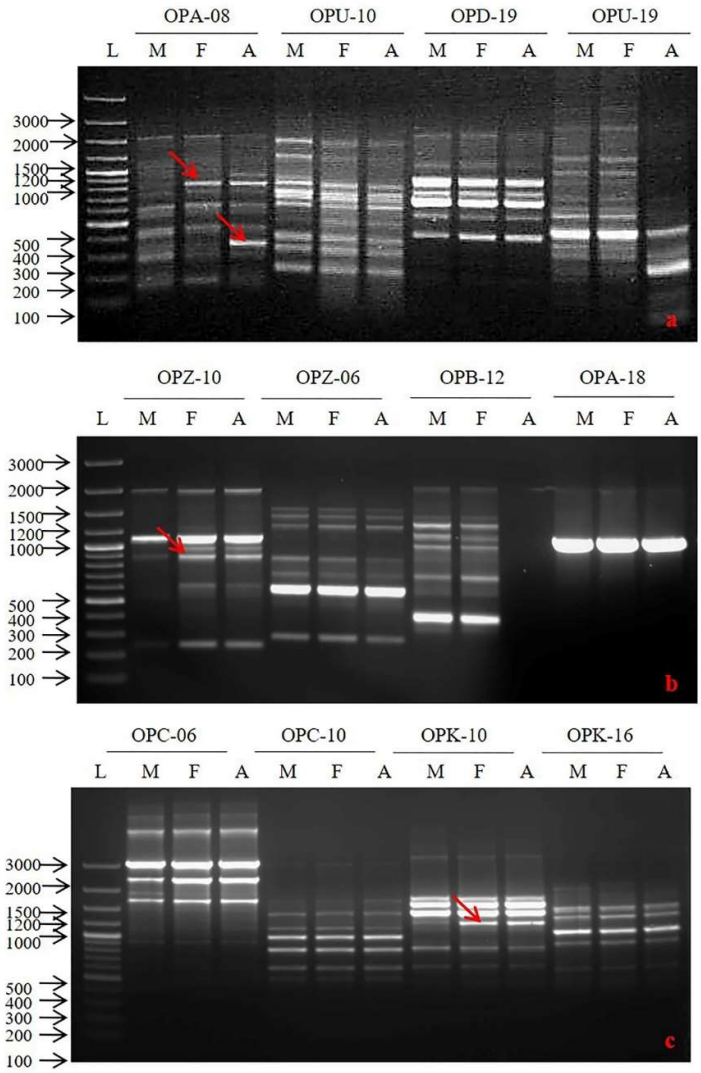

Fig. 35. Screening of bulk DNA samples of male, female and andromonoecious genotypes of *S. glauca* with RAPD primers. (*a*) OPA-08, OPU-10, OPD-19 and OPU-19, (*b*) OPZ-10, OPZ-06, OPB-12 and OPA-18, (*c*) OPC-06, OPC-10, OPK-10 and OPK-16 for sex identification. *Lane L* – 100 bp DNA ladder, M – Bulk male genotypes, F – Bulk female genotypes, A – Bulk andromonoecious genotypes. *Red arrows* indicate polymorphic bands among sexes.

Table 16. Sex-specific bands produced by RAPD and ISSR primers in bulk and individual DNA samples of different sexes of *S. glauca*.

S. No.	Primer	Unique band size (bp)	Presence of band in bulked genotypes			Presence of band in individual genotypes		
			Males	Females	Andromonoecious	Males (6 No.)	Females (15 No.)	Andromonoecious (15 No.)
1	OPZ-10	~1000	Absent	Present	Present	6	15	15
2	OPC-18	>3000	Present	Present	Absent	0	0	0
3	OPA-12	~700	Absent	Present	Present	0	7	5
		~2900	Absent	Present	Present	0	14	15
		~1900	Absent	Present	Present	0	14	15
4	OPS-06	~3000	Present	Absent	Absent	No amplification		
5	OPG-08	~2800	Present	Present	Absent	0	0	0
6	OPK-10	~1200	Absent	Present	Present	0	15	15
7	OPC-08	~2700	Absent	Present	Present	0	0	0
		~1100	Absent	Present	Present	0	0	0
8	OPA-08	~900	Absent	Present	Present	1	4	5
		~350	Absent	Absent	Present	0	0	15
9	OPE-05	~1300	Present	Present	Absent	6	8	2
10	UBC-899	~800	Absent	Present	Present	3	14	14
11	UBC-851	~1600	Absent	Present	Present	6	13	12
		~580	Absent	Present	Present	6	14	10
12	UBC-873	~1300	Present	Absent	Present	6	9	13
		~900	Present	Absent	Present	5	4	7

primers OPZ-10, OPA-12, and OPK-10 resulted in amplification of ~1000 bp, ~700 bp, and ~1250 bp bands specific to female and andromonoecious bulk samples and absent in the male bulk. The primers OPC-18, OPG-08, and OPE-05 amplified >3000 bp, ~2800 bp, and ~1300 bp bands specific to male and female bulk samples and absent in andromonoecious samples. The primer OPS-06 showed amplification of ~3000 bp band specific to male bulk DNA, whereas absent in females and andromonoecious bulk samples. The primer OPC-08 amplified ~2700 bp and ~1100 bp bands in female and andromonoecious bulk samples and no amplification was observed in the bulk sample of males. The random primer OPA-08 showed specific amplification of ~900 bp band in female and andromonoecious bulk samples and ~350 bp band specific to andromonoecious bulk sample. Among the 9 primers, the primers OPK-10, OPA-08 and OPA-12 produced amplification specific to sex type when tested on individual genotypes of all 3 sexes (Fig. 36, 37, 38). These primers resulted in sex-specific amplification of ~2900 bp (OPA-12) and ~1250 bp (OPK-10) bands in females and andromonoecious genotypes, and ~350 bp (OPA-08) band in andromonoecious genotypes. The other primers resulted in polymorphic bands but not specific to sex type (Table 14).

4.3.4. Screening of female, male and andromonoecious genotypes for sex identification by ISSR primers:

In the genetic variation studies performed with 24 ISSR primers, 3 primers *viz*., UBC-899, UBC-851 and UBC-873 resulted in sex-specific amplification in bulk DNA samples of 3 sexes (Table 16; Fig. 39). The primer UBC-899 amplified ~800 bp band in bulk DNA samples of female and andromonoecious genotypes and absent in males. The primer UBC-851 resulted in two sex-specific bands, *i.e.* ~1600 bp and ~580 bp specific to female and andromonoecious genotypes and absent in the male bulk sample. The ISSR primer, UBC-873 amplified ~1300 bp and ~ 900 bp bands in bulk samples of male and andromonoecious genotypes, but not in females. These 3 primers were further used to screen individual DNA samples of 36 genotypes where none of these 3 primers resulted in sex-specific amplification among individual genotypes. The primer UBC-899 resulted in a ~800 bp band that exhibited polymorphism among the individuals without any association with sex as the band was amplified in 3 males, 14 females, and 14 andromonoecious genotypes. The primer UBC-851 resulted in

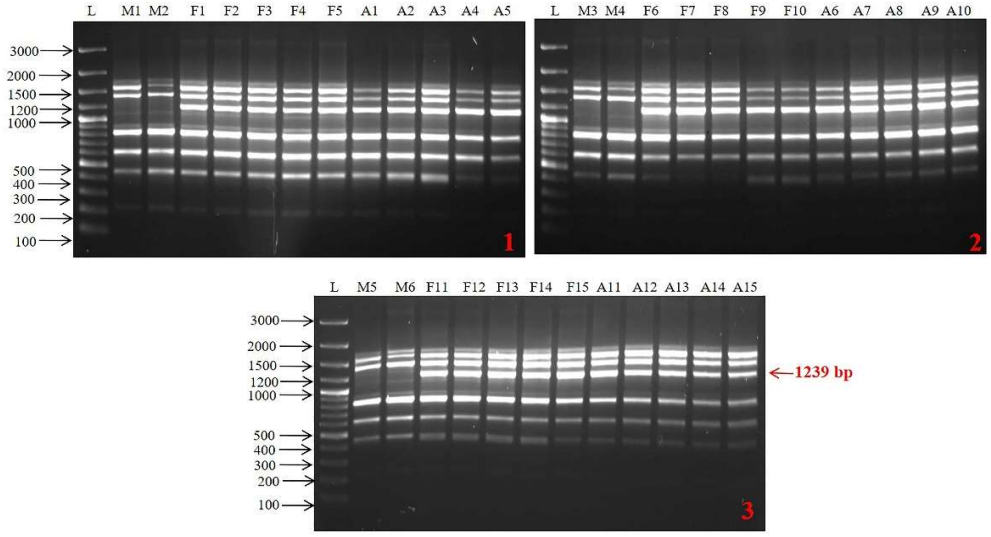

Fig. 36. Screening of individual DNA of male, female and andromonoecious genotypes of *S. glauca* with RAPD primer, OPK-10. *Lane L* – 100 bp DNA ladder, M1-M6 – male genotypes, F1-F15 – female genotypes, A1-A15 – andromonoecious genotypes.

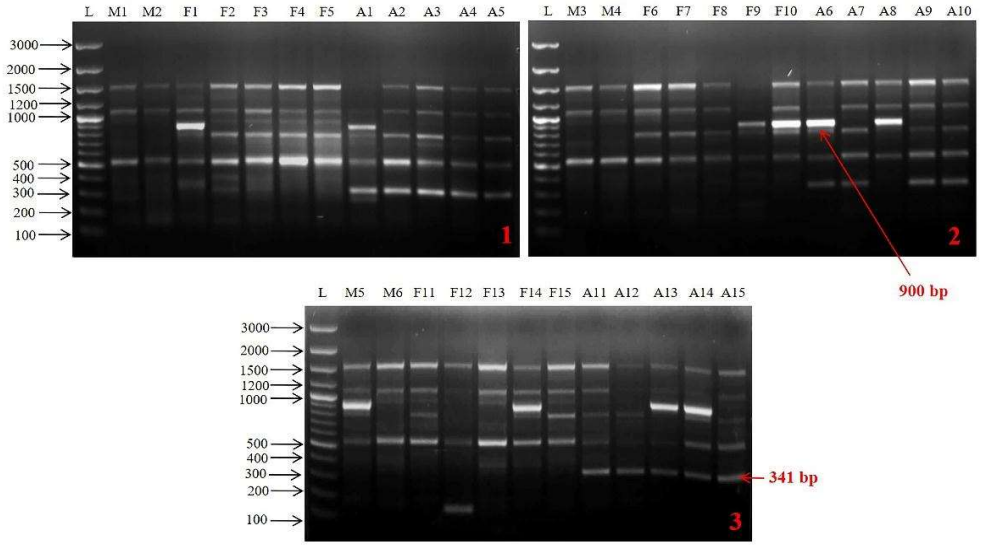

Fig. 37. Screening of individual DNA of male, female and andromonoecious genotypes of *S. glauca* with RAPD primer, OPA-08. *Lane L* – 100 bp DNA ladder, M1- M6 – male genotypes, F1-F15 – female genotypes, A1-A15 – andromonoecious genotypes.

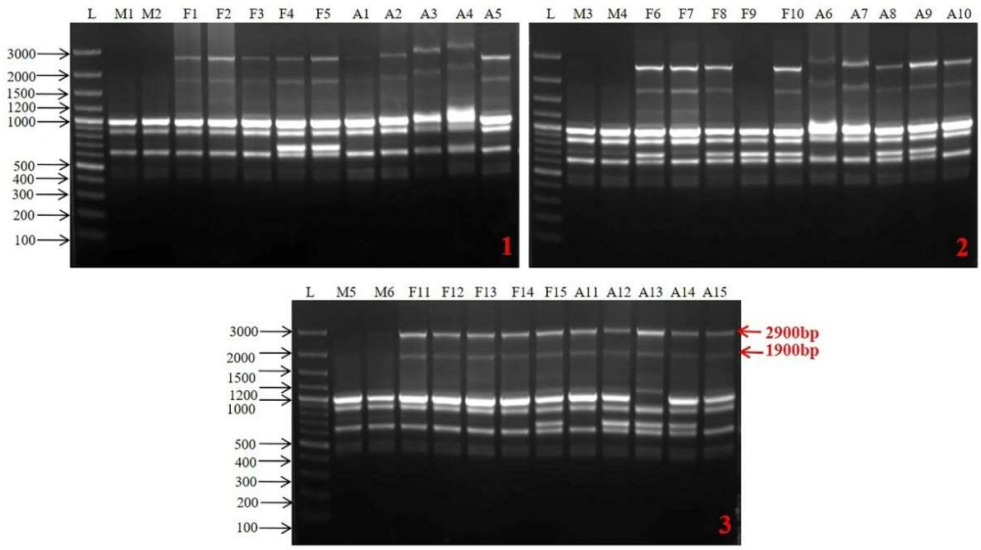

Fig. 38. Screening of individual DNA of male, female and andromonoecious genotypes of *S. glauca* with RAPD primer, OPA-12. *Lane L* – 100 bp DNA ladder, M1-M6 – male genotypes, F1-F15 – female genotypes, A1-A15 – andromonoecious genotypes.

~1600 bp and ~580 bp bands, which were polymorphic with ~1600 bp band present in all males, 13 females and 12 andromonoecious genotypes and the band ~580 bp was present in all males, 14 females and 10 andromonoecious genotypes. The primer UBC-873 also resulted in ~1300 bp and ~900 bp bands in individual genotypes which were not sex-specific, and the band ~1300 bp was present in all males, 13 andromonoecious, and 9 females genotypes; the band ~900 bp was present in 5 males, 4 females and 7 andromonoecious genotypes (Fig. 40). Thus, none of the ISSR primers used in the study resulted in sex-specific bands.

Among the previously reported sex-specific ISSR primers, UBC-814 and UBC-815; the primer UBC-814 was used in the present study. In previous study by Choudhary (2014), it was reported that UBC-814 resulted in sex-specific amplification of 1000 bp in males and absent in females and bisexual genotypes (Table 3). In contrast, the primer UBC-814 in our work resulted in ~1000 bp band amplified in few females (3 No.) and few andromonoecious (5 No.) genotypes and absent in rest of the genotypes including all males. The ISSR primer, UBC-815 was not used as the reported SCAR marker (UBC-815 SCAR) developed for that primer was used for sex identification as shown in section 4.3.2 of results.

4.3.5. Development of SCAR markers from sex-specific bands in *S. glauca*:

The SCAR markers were developed for the sex-specific bands (~1250 bp, ~2900 bp, and ~350 bp) produced by RAPD primers, OPK-10, OPA-12 and OPA-08.

4.3.5.1. Cloning, confirmation, and sequencing of sex-specific bands:

The RAPD primers OPK-10 (~1250 bp) and OPA-12 (~2900 bp) resulted in bands specific to female and andromonoecious genotypes whereas the primer OPA-08 (~350 bp) resulted in band-specific to andromonoecious genotypes. Therefore, these 3 bands, *i.e.* ~1250 bp, ~2900 bp, and ~350 bp, along with positive control 953 bp (provided with the kit), were selected for cloning (Table 5). The bands generated by these primers were eluted from agarose gel using QIAquick Gel Extraction Kit and were cloned into pTZ57R/T cloning vector. The recombinant vector with desired DNA inserts was transformed into DH5α competent cells. The positive white colonies were picked through blue-white selection method and the recombinant clones were confirmed

by performing colony PCR (Fig. 41a-f). The PCR confirmed positive colonies that resulted in desired amplification were picked for plasmid isolation. The isolated plasmids (Fig. 41b & e) were further confirmed for desired DNA inserts by restriction digestion with *EcoR*I and *Hind*III restriction endonucleases.

The recombinant plasmids with ~1250 bp (OPK-10) insert released two bands of sizes ~850 bp and ~400 bp as shown in Fig. 41c, which was due to the presence of a restriction site for one of the restriction enzymes used. The recombinant plasmid with ~2900 bp (OPA-12) resulted in 6-7 bands of different sizes which could be due to the presence of restriction sites. The plasmid with the desired insert ~350 bp (OPA-08) released a ~350 bp band (Fig. 41f). The positive control recombinant plasmid released a band of ~953 bp (Fig. 41c). Thus, these 3 recombinant plasmids with desired DNA inserts that were confirmed by double digestion with endonucleases were sequenced by M13 forward and reverse sequencing primers. The forward and reverse sequences for ~1250 bp, ~2900 bp, and ~350 bp obtained in FASTA format for each plasmid were analyzed and transformed into contigs of sex-specific bands. The contamination of sequences for vector regions was analyzed by VecScreen, and the vector sequences were removed. Finally, 1239 bp (OPK-10), ~2900 bp (OPA-12), and 341 bp (OPA-08) sequences were obtained as shown in Fig. 42, 43 & 44. The sequencing of ~2900 bp sex-specific band resulted in only 1856 bp from forward (928 bp) and reverse (928 bp) sequences, as the size of the band was larger. The sequences of 3 sex-specific bands obtained for primers OPK-10, OPA-12, and OPA-08 were as follows-

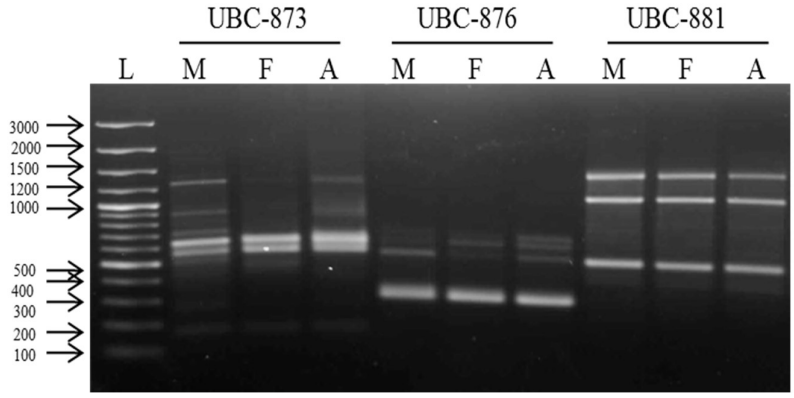

Fig. 39. Screening of bulk DNA of male, female and andromonoecious genotypes with ISSR primers, UBC-873, UBC-876 and UBC-881. *Lane L* – 100 bp DNA ladder, M – male, F – female, A – andromonoecious genotypes.

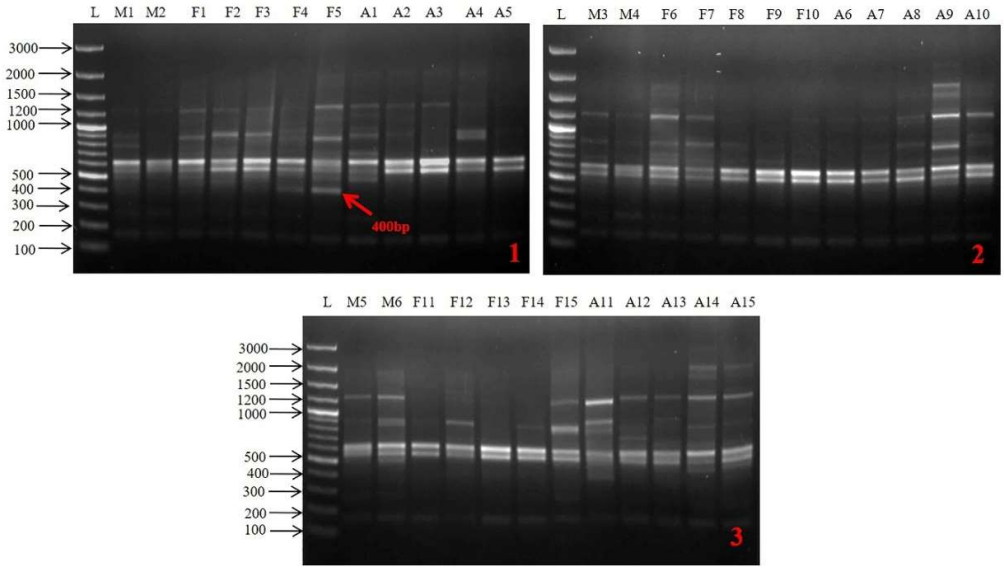

Fig. 40. Screening of individual DNA of male, female and andromonoecious genotypes with ISSR primer, UBC-873. *Lane L* – 100 bp DNA ladder, M – male, F – female, A – andromonoecious genotypes.

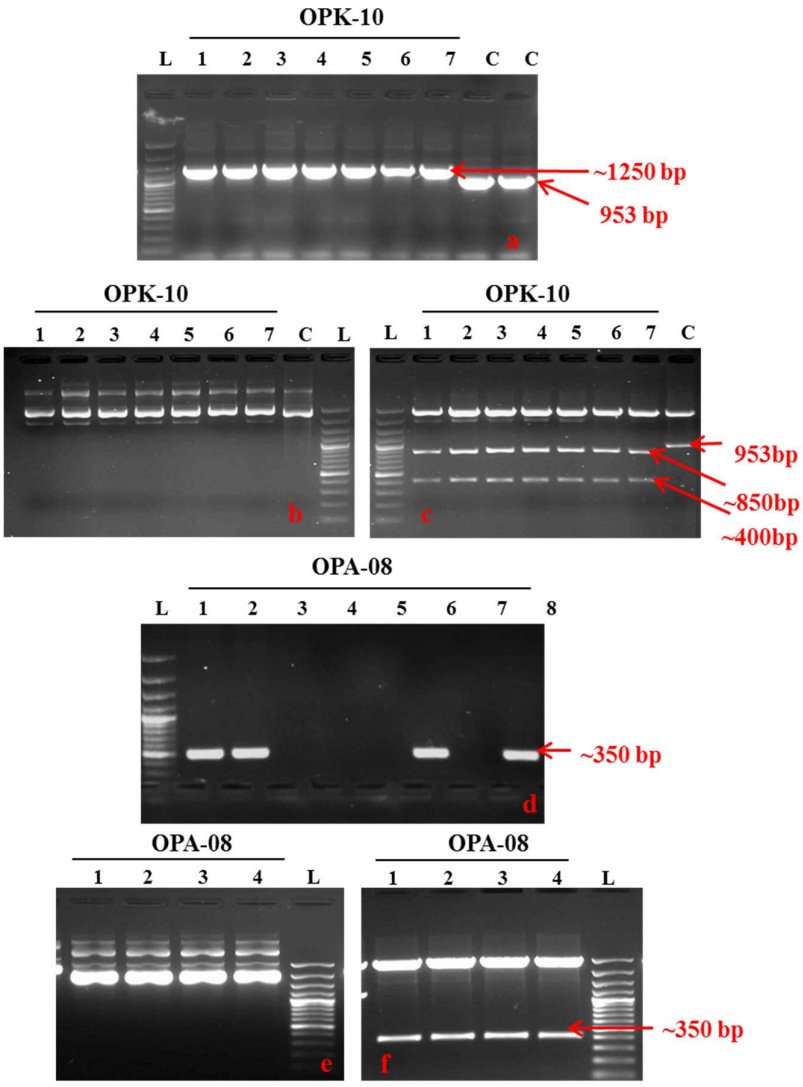

Fig. 41. Cloning and confirmation of recombinant plasmids by colony PCR amplification and double digestion. (*a*) Colony PCR amplification of desired ~1250 bp RAPD band for OPK-10 primer (*Lanes 1-7*) and control of 953 bp band (*Lanes C*) of recombinant plasmids by using M13 primers, (*b*) Plasmid isolation of pTZ57R/T recombinant plasmids having ~1250 bp RAPD band (*Lanes 1-7*) and 953 bp control PCR band (*Lanes C*), (*c*) Double digestion of plasmids resulted in release of 2 bands (~850 bp and ~400 bp) for ~1250 bp (*Lanes 1-7*) and a single band of 953 bp for control PCR product (*Lane C*), (*d*) Colony PCR amplification of desired RAPD band of size ~350 bp (*Lanes 1-8*) of recombinant plasmids by using M13 primers, (*e*) Plasmid isolation of recombinant plasmids with desired RAPD band of size ~350 bp (*Lanes 1-4*), (*f*) Double digestion of pTZ57R/T recombinant plasmids having inserts of size ~350 bp (*Lanes 1-4*). *Lane L* – 100 bp DNA ladder.

GTGCAACGTGTCGAATCTTCCATATCTGACATATTGGCCTATTGCTGAAGTCTCTTCTAAATTGACTAT
TCTGAGAGCCATATTGATTTTATCAACATAACCACCTCTCCAAAATTTACTGTTCCTATTAAATCCTCTA
CGCTTGCCTCCATCCCTAAAGAAACCTCTGCTATTTGGTCCAGTGTTTTTGGGTCCAAGTCTATTGCTT
TGATTCACATTATAACTGTTATTTTTTCTGAGTTGGCTTATCTCTTGCATAGTTTGATTCTTCTTTCTGATT
CGAGTTTCTTAGACTGATTCTACTCTCATGTACTAATAACAAGGACATCACCTCATTCAGATTAATAATT
CTCAATCTACTTTGAATATTCTTTACTGCCGAATCAAAGCTTTTATCTAAGCCATATAAAGAATACATAA
TCAGGTCATGTTCATTTAAAGGACAGCCAGCAATTGTCAAATTATCAGCATGACTCTTAATCTTCCTAA
AATAATCTGTTATGGATAAATAACCTTGCTTCATATGTGAGAGCTGAGCCTTAATCTGGATTTCCAAAA
CTTTTGATTGAGATCCAAACTGCTTATCAATTGCAAGCCACAGTTTAGATGTTGTTCCGCATCCAATTAT
AAATCGTAGCACTCCTTCACTTATTAAAAACATGAGCCAACCTAGAATGATTTGATCCTGTTTTTCAACT
TGTCCAACTCTTCTTGCATTTGGATTTAAGGACTCACTGATCGTTGAACCAGCAAACCTGTTGATATCCA
TCTATAAAGCTCTCCAAATCATGACCTCGGATGATTGCAACAATTTGTGCCTTCCAAACCAAATAGTTT
GCTCTTTCAATTTTGATCTGAGTTGGAAACGTAAATGTTATCAATTGAGAAAACGAAATTTGAGTTGTC
ATGTTTGTTTGGCTCTGATACCAAGTGAAAGTTTAAAGAGAAAGCTGTTTGGTTATAGAGAGTGAAGGA
AAATATTCAAGAAGAGAATCCTGCTATTTCTGTTTTTATTGATTTTTAGTCTTAATGATTTATACAAAGA
TCATAGCTATTTATACTTAATGAAAACTAGGGCTAAAATAGCAGGAATTAGTTGGAGTGAAATATTTGA
GCTGGACTTCAATTCTGAGCTGCTGGAGCTGACCTCTAATAGTCTATTCTCAGTGACACCACAACTGCT
GGAGCTTCAGCTTTGATTATTATCTCTCAGGTCGGCTCTCTTTTAGCACGTTGCAC

Fig. 42. Nucleotide sequence of cloned female and andromonoecious specific 1239 bp band. The sequence in red-colored bold represents the RAPD primer (OPK-10) sequence used for screening. The green color highlighted bold letters represent the SCAR forward and reverse primer sequences, which amplified 1063 bp band. The highlighted yellow color sequence is the ORF found in the sequence.

GACGCGAACCAGAACAACCTTTTTCAGAACCATCACTTGCCCGGATTGAGATGATCTTACGAGGCTGC
TACATTGGGAGTGGAATCAGCCAGTTGTTTACTAATGCTATTGGTCTAAAGTTCTTTTGCACACTCAGC
TTGAGATAATAAGAATAATTTAGGTAGATAGATCTATCCCACTAGATCGAGAAGATTGGACGAGCGCT
ACTTCAACGTCAGGCCTATACCATAAGGATCGGGAAGAAATATATATTAGGCTGGTCATGCACCCGTGT
GTCTCACAACAGCAGAACAGAACGAAACCGAAGACTTTTATCTAGGGTAGTTCAATGGCAGAGCACCA
GGACGAAGGTCCCTAAGTGATGGGCTTGAATCCATTTCCGAATGGCACAGATCAAACACAAAAAATTA
CTTCTATTGAATAGAGGTTCACTTATATAAGAATAAGAAATTATAGCATTGCTATTGTAATATTTAATTG
AGCTCGATCGAGCCTTTGAATTAGAACTAGAAGGTAAGGCCAAAGCCTTCTCAAGGTCCTTCCTACACT
ATAAATAAAAGCTGTCGCTAGAATTAATAGACTAGCGCACTCTATTCAGCTCGTACCTTCCCACTCTGA
CATATGTTGAATTAGAGATCTCTTACCGACAACAAACCAAGTGAAGCAGATTATCGTCCTCCAACTAAA
GCTTCAAGATCGGGCAGCTCTCACTTTAAGCCTTGCCCGCTGCTTTTATATGTATGGAATATAGGTAGGC
AGTAAATAAGATCTTTGATTGTTTCTACATCCTCTGGGTAGTCACCTCAAGATCCCAACTCCTCAGCGC
GGTCAGTATCATCTTCTTAGATCGGATCAGCTACGTCTTGGTGAACAAGAATGAAAGCAGCGCCGAGA
GAACCCTCATGGACTAAATAGAAGGACCTGCGGGCCCGCAGGTCCTTCTATTTAGTCCATGAGGGTTCT
CTCGGCGCTGCTTTCATTCTTGTTCACCAAGACGTAGCTGATCCGATCTAAGAAGATGATACTGACCGC
GCTGAGGAGTTGGGATCTTGAGGTGACTACCCAGAGGATGTAGAAACAATCAAAGATCTTATTTACTGC
CTACCTATATTCCATACATATAAAAGCAGCGGGCAAGGCTTAAAGTGAGAGCTGCCCGATCTTGAAGCT
TTAGTTGGAGGACGATAATCTGCTTCACTTGGTTTGTTGTCGGTAAGAGATCTCTAATTCAACATATGTC
AGAGTGGGAAGGTACGAGCTGAATAGAGTGCGCTAGTCTATTAATTCTAGCGACAGCTTTTATTTATAG

TGTAGGAAGGACCTTGAGAAGGCTTTGGCCTTACCTTCTAGTTCTAATTCAAAGGCTCGATCGAGCTCA
ATTAAATATTACAATAGCAATGCTATAATTTCTTATTCTTATATAAGTGAACCTCTATTCAATAGAAGTA
ATTTTTTGTGTTTGATCTGTGCCATTCGGAAATGGATTCAAGCCCATCACTTAGGGACCTTCGTCCTGGT
GCTCTGCCATTGAACTACCCTAGATAAAAGTCTTCGGTTTCGTTCTGTTCTGCTGTTGTGAGACACACGG
GTGCATGACCAGCCTAATATATATTTCTTCCCGATCCTTATGGTATAGGCCTGACGTTGAAGTAGCGCTC
GTCCAATCTTCTCGATCTAGTGGGATAGATCTATCTACCTAAATTATTCTTATTATCTCAAGCTGAGTGT
GCAAAAGAACTTTAGACCAATAGCATTAGTAAACAACTGGCTGATTCCACTCCCAATGTAGCAGCCTCG
TAAGATCATCTCAATCCGGGCAAGTGATGGTTCTGAAAAAGGTTGTTCTGGTTCGCGTC

Fig. 43. Nucleotide sequence of cloned female and andromonoecious specific 1856 bp (~2900 bp) band. The sequence depicted in red color bold represents the RAPD primer (OPA-12) sequence used for screening. The sequence highlighted in blue colour corresponds to reverse complementary sequence with random primer sequence. The highlighted green color bold letters represent the SCAR forward and reverse primer sequences, which amplified 762 bp band.

GTGACGTAGGTGTTCGTGGAAGAGACCGAACCACTATAAAAGAGTTTAAGCACTTCTCTCCCTATAC
TCTTTTATTTTCTTGTCAATTTGCCTTAATTAATTATTTGATTACATTTGAGCATCTTTTATATATTATCTT
AACATAATCAAATATTTAGGGAGAATTGTATGTTTACATAATTTTGGTTTATTAAACTCCCGCATTGCAC
TTTTGAACACACACTTTGAGTAATTACATACTAGGAGTTTATTTTACTTATTAAGTGCTTTCAAATTAAA
AAAAAAAAGATCTAATTCACCCCCCTTTTGGGTTTCCTAAGGGATCAACAAGCCTACGTCAC

Fig. 44. Nucleotide sequence of cloned male and andromonoecious specific 341 bp band. The sequence in red color highlighted bold represents the RAPD primer (OPA-08) sequence used for screening. The green color highlighted bold letters represent the SCAR forward and reverse primer sequences, which amplified 341 bp band.

4.3.5.2. Homology analysis of sex-specific cloned RAPD bands:

The FASTA sequences were analyzed for the homology of the cloned bands with the known sequences in the non-redundant database of NCBI by nucleotide BLAST search. The nucleotide BLAST search of the band 1239 bp (OPK-10) that was female and andromonoecious specific did not show any similarity with the nucleotide sequences available in the database. The ORF finder resulted in 6 ORF's (180, 85, 60, 58, 44, and 28 aa), and the addgene program resulted in ORF-3 consisting of 180 aa. The BLASTP analysis for 180 aa resulted in a putative conserved domain for retrotran_gag_2 representing gag-polypeptide of LTR Copia-type which are present in plants and fungi (Fig. 45a & b). The 180 amino acid sequence blast results showed less homology, *i.e.* 37.09 % identity with retrovirus-related pol polyprotein from transposon tnt 1-94 (*Thalictrum thalictroides*) and 38.06% with retrovirus-related pol polyprotein from transposon RE1 (*Vitis vinifera*). The sequence of 1239 bp possessed sites for restriction enzymes *Ase*I (342), *Hind*III (387), *Nde*I (522), *ECoR*V (763), and *Pml*I (1239) as shown in Fig. 45c.

The female and andromonoecious specific ~2900 bp band generated by primer OPA-12 was analyzed for sequence homology using nucleotide BLAST. As the complete sequence of ~2900 bp band could not be obtained due to larger size of the band, the forward (928 bp) and reverse (928 bp) sequences obtained from sequencing results were analyzed separately. The forward sequence of ~2900 bp resulted in a high similarity of 98.91 % with *Phoenix dactylifera* mitochondrion, complete genome. The ORF finder resulted in two short ORF's (35 and 31 aa) with no similarity to the sequences in the database. The forward sequence of ~2900 bp has restriction sites for enzymes *Stu*I (219), *Sac*I (484), *Ase*I (573), *Nde*I (620), and *Hind*III (686). The reverse sequencing result analyzed was searched for homology analysis which resulted in a high similarity of 98.91 % with *Phoenix dactylifera* mitochondrion, complete genome. The ORF finder resulted in no ORF's and the reverse sequence of ~2900 bp has restriction sites for enzymes *Hind*III (238), *Nde*I (306), *Ase*I (353), *Sac*I (448), and *Stu*I (709).

The male and andromonoecious specific 341 bp generated by primer OPA-08 was analyzed for sequence similarity with sequences in the database using nucleotide BLAST. A nucleotide BLAST search of the band 341 bp (OPA-08) did not show any

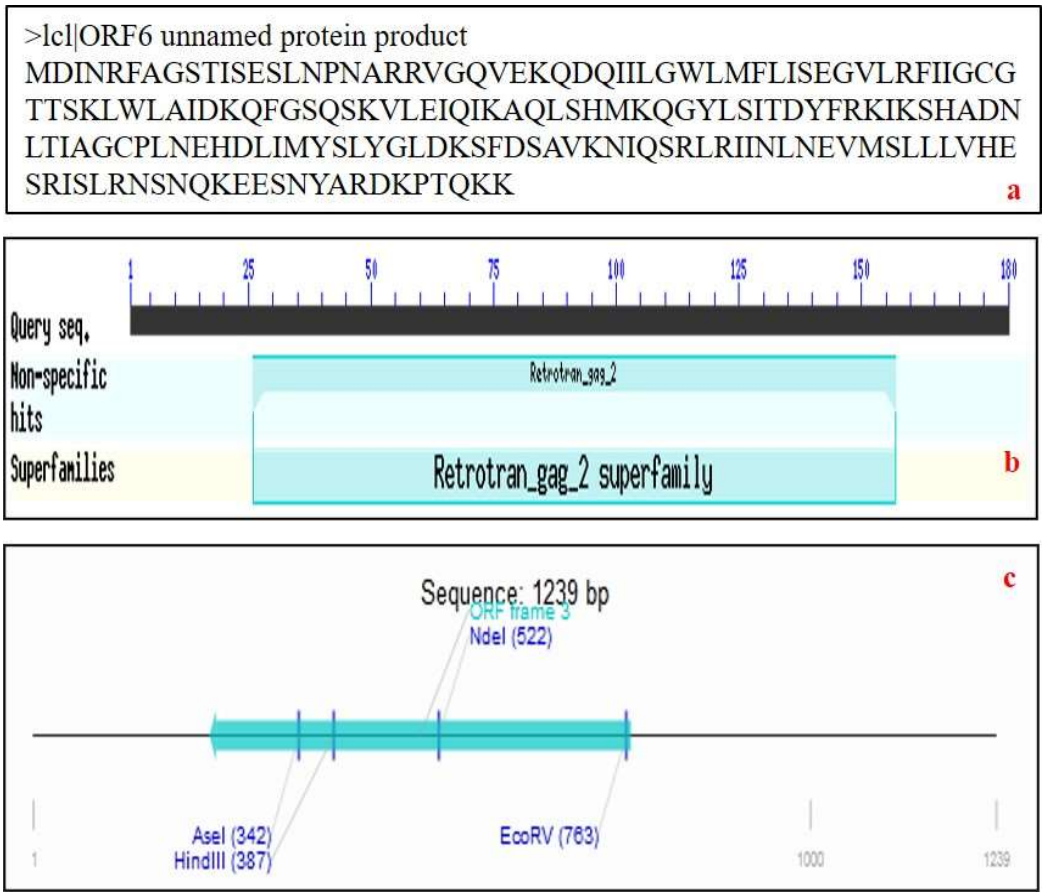

Fig. 45. Sequence analysis of desired sex-specific RAPD band of size 1239 bp obtained for OPK-10 random primer. (*a*) Sequence of translated protein (180 aa) of 1239 bp RAPD fragment, (*b*) BLASTp analysis of 180 aa (ORF) of 1239 bp RAPD band showed the presence of putative conserved domain, *i.e.,* retrotran_gag_2, (*c*) Map for 1239 bp showing ORF and sites for restriction enzymes.

similarity with the nucleotide sequences available in the database. The ORF finder revealed the absence of ORF's in the sequence. The sequence of 341bp showed sites for restriction enzymes *Pac*I (97), *Ase*I (97), *Bgl*II (287) and *Pml*I (341).

4.3.5.3. Designing of SCAR primers and screening on bulk and individual samples of PJTSAU:

The SCAR primer sequences in the present study were designed based on the sequences of cloned RAPD bands specific to sex. The Sg SCAR1 and Sg SCAR2 forward (FP) and reverse (RP) primers were designed for sex-specific bands of sizes 1239 bp and 341 bp to amplify 1063 bp and 341 bp, respectively. The Sg SCAR2 resulted in amplification of 341 bp not only in all andromonoecious genotypes but also in all male genotypes. The Sg SCAR3 forward (FP) and reverse (RP) primers were designed for a sex-specific band of ~2900 bp (OPA-12) to amplify the 762 bp band. The sequences of the forward and reverse primers for 3 SCAR markers were shown in Table 6. The SCAR primers were tested on DNA samples of bulk and 36 individual genotypes of female, male, and andromonoecious plants using SCAR-PCR amplification program. The SCAR markers Sg SCAR1 and Sg SCAR2 resulted in amplification of sex-specific bands of 1063 bp and 341 bp, respectively (Fig. 46a1, a2, b1 & b2). These sex-specific bands (1063 bp and 341 bp) amplified with SCAR primers were again sequenced and the sequence obtained matched 100% with RAPD band sequences (1239 bp and 341 bp). The Sg SCAR3 primers did not result in sex-specific amplification where the band 762 bp was amplified in all bulk and individual genotypes tested regardless the sex-type (Fig. 46c1& c2). Thus, only two RAPD primers OPK-10 (female & andromonoecious specific) and OPA-08 (male & andromonoecious specific) resulted in sex-specific amplification.

4.3.5.4. Validation of SCAR primers on different genotypes of known and unknown sexes:

The ability of the SCAR markers developed in the present study was tested by validating on 15 genotypes, 5 each of female, male and andromonoecious plants of IIOR, Narkhoda location. The Sg SCAR1 and Sg SCAR2 were tested on bulk and individual genotypes of 3 sexes. The SCAR primer pairs resulted in

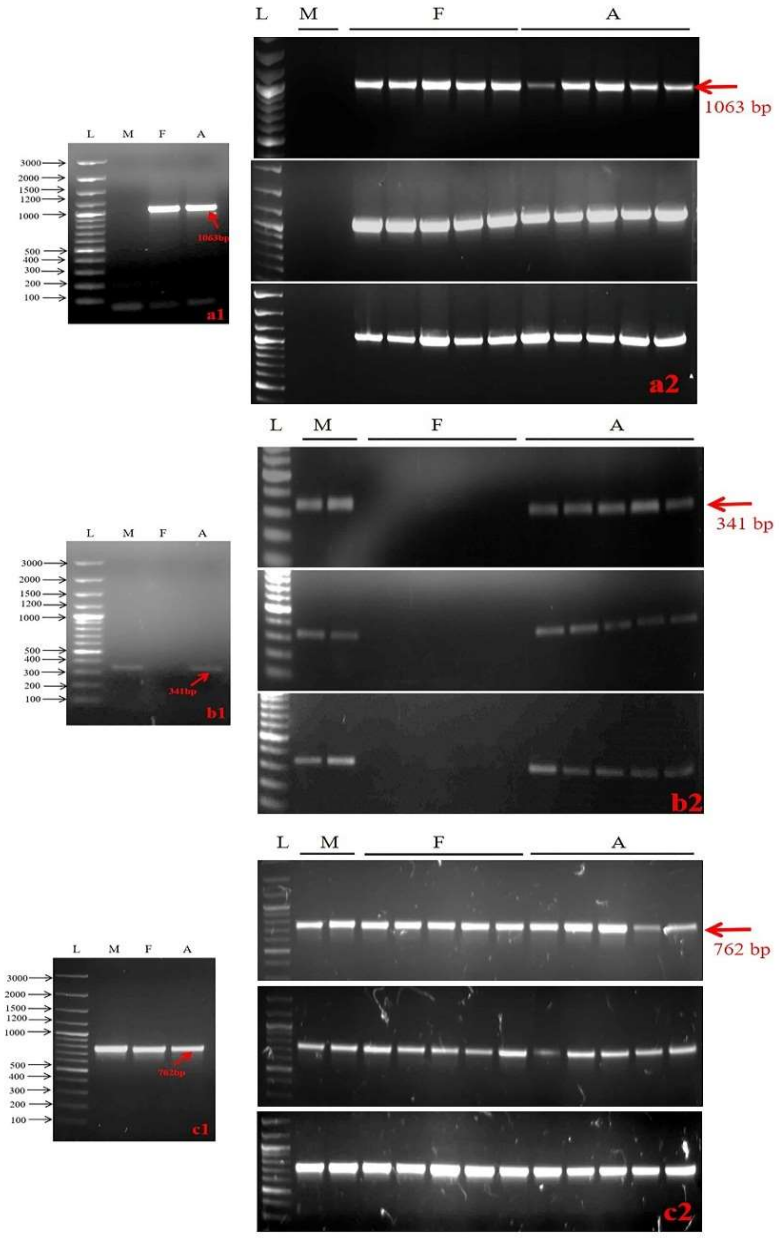

Fig. 46. Validation of designed SCAR markers for sex-specific amplification on bulk and individual DNA samples of *S. glauca* obtained from PJTSAU. (*a1 & a2*) Amplification of 1063 bp band by Sg SCAR1 marker (OPK-10) in bulk and individual genotypes, (*b1 & b2*) Amplification of 341 bp band by Sg SCAR2 marker (OPA-08) in bulk and individual genotypes, (*c1-c2*) Amplification of 762 bp band by Sg SCAR3 marker (OPA-12) in bulk and individual genotypes. *L* – 100 bp DNA ladder, M – male, F – female, and A – andromonoecious genotypes.

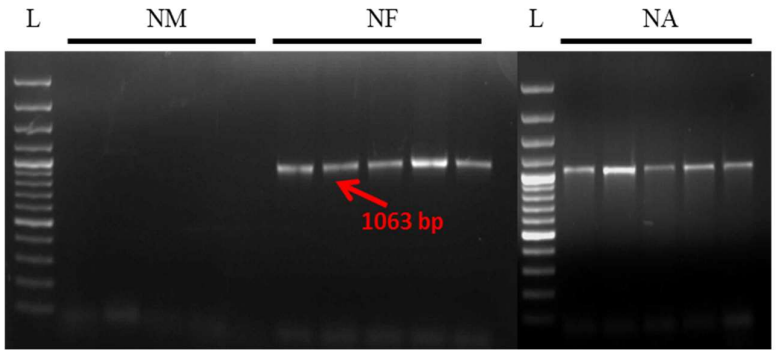

Fig. 47. Validation of Sg SCAR1 marker (OPK-10) on different sexes obtained from IIOR, Narkhoda. *L* – 100 bp DNA ladder, NF – Females of Narkhoda, NM – Males of Narkhoda, NA – Andromonoecious of Narkhoda.

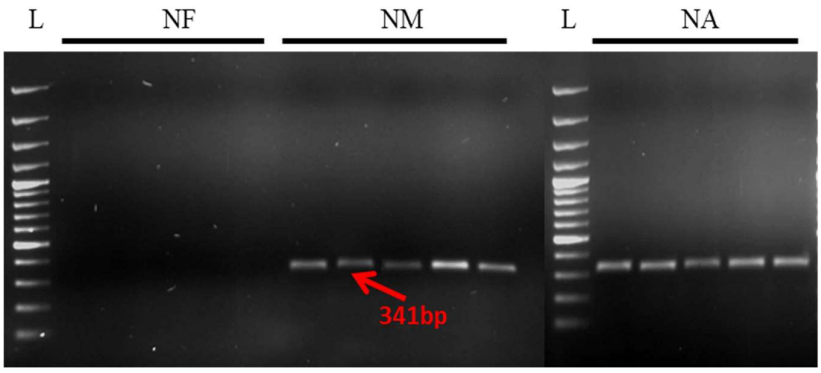

Fig. 48. Validation of Sg SCAR2 marker (OPA-08) on different sexes obtained from IIOR, Narkhoda. *L* – 100 bp DNA ladder, NF – Females of Narkhoda, NM – Males of Narkhoda, NA – Andromonoecious of Narkhoda.

sex-specific amplification where Sg SCAR1 amplified 1063 bp band in female and andromonoecious genotypes of bulk, and all individuals as in Fig. 47 and the Sg SCAR2 generated 341 bp band in male and andromonoecious genotypes of bulk and all individuals tested (Fig. 48).

The SCAR primers Sg SCAR1 and Sg SCAR2 were also tested on DNA samples of unknown sexes of 10 *in vitro* raised seedlings maintained at the greenhouse of plant culture facility and 12 plants (2-3-year-old) growing in the campus of University of Hyderabad (UoH). The Sg SCAR1 primer pairs when tested on these 22 samples amplified 1063 bp band in 8 seedlings and 12 UoH plants and therefore these plants might be either female or andromonoecious sexes (Fig. 49a & Fig. 50a). The other 2 seedlings where there was no amplification might be males. The Sg SCAR2, when tested on 22 samples, resulted in amplification of 341 bp in 6 seedlings and 4 UoH plants; therefore, might be either male or andromonoecious plants, and rest of the seedlings and plants with no amplification were females (Fig. 49b & Fig 50b). Based on the bands amplified with both primers, the sex of the unknown plants was determined. The amplification pattern of 1063 bp and 341 bp with two SCAR primers for a particular sample was compared to identify the sex. When both 1063 bp and 341 bp were amplified for a sample, the sex of the plant was determined as andromonoecious. If the 1063 bp band was present and 341 bp was absent, then the sex for the plant was ascribed as females. If the band 1063 bp was absent and 341 bp was amplified for a particular sample, then the sex of those plants was considered as males. Thus, by adapting this two-level screening approach as shown in Fig. 51, the sexes of seedlings were identified as four females, two males, and four andromonoecious plants. Among UoH maintained plants, 8 were females, and 4 were andromonoecious and no males were detected. Thus, the two-step screening approach developed in the study was successfully used to identify the sex of unknown seedlings or plants with precision.

4.3.6.1. Cloning, confirmation, and sequencing of RAPD and ISSR polymorphic bands:

RAPD and ISSR primers OPD-20 (~950 bp), OPD-12 (~1200 bp), OPC-06 (~1100 bp), ISSR-19 (~400 bp), and UBC 873 (~400 bp) resulted in bands which exhibited polymorphism but not specific to sexes of *S. glauca* (Table 5). The band

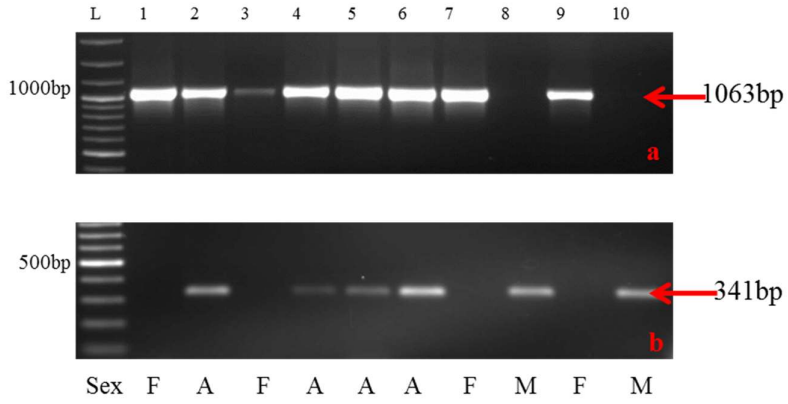

Fig. 49. Validation of SCAR markers for identification of sex of seedlings of *S. glauca*. (*a*) Sg SCAR1 marker (OPK-10) amplification, (*b*) Sg SCAR2 marker (OPA-08) amplification. *L* – 100 bp DNA ladder, 1-10 – seedlings.

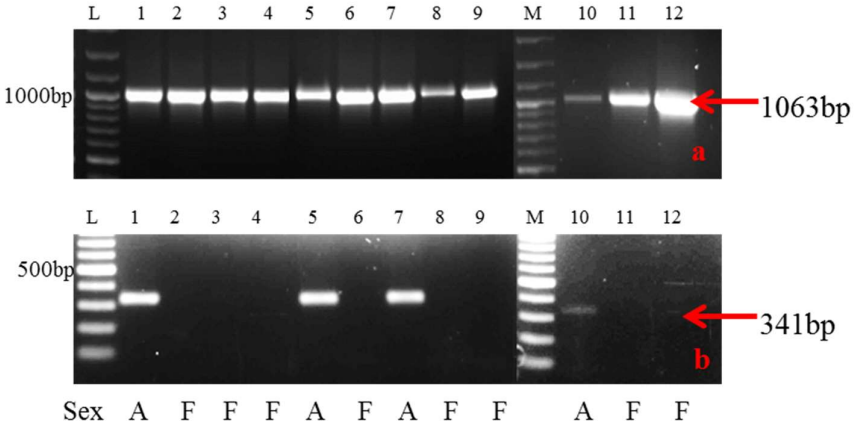

Fig. 50. Validation of SCAR markers for identification of sex of *S. glauca* plants at University of Hyderabad. (*a*) Sg SCAR1 marker (OPK-10) amplification, (*b*) Sg SCAR2 marker (OPA-08) amplification. L – 100 bp DNA ladder, 1-12 – Plants of unknown sex at University of Hyderabad.

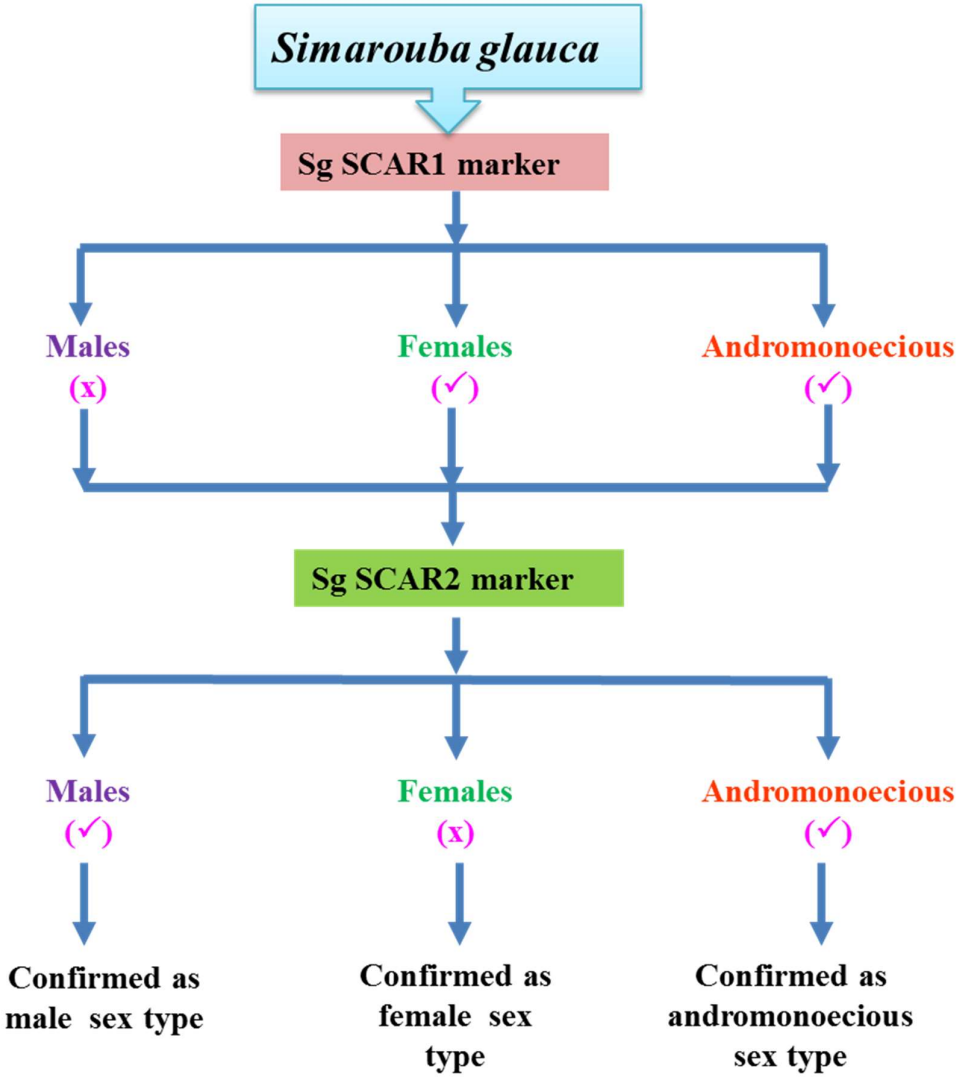

Fig. 51. Schematic representation of two-step screening approach for Sg SCAR markers for sex identification (females, males and andromonoecious plants) in *S. glauca.*

~950 bp (OPD-20) was amplified in 2 males, 5 females, and 5 andromonoecious genotypes (Fig. 12). The band ~1200 bp (OPD-12) was amplified in 3 males, 6 females, and 5 andromonoecious genotypes (Fig. 13). The band ~1100 bp (OPC-06) was amplified in 3 females and 4 andromonoecious genotypes and absent in males. The ~400 bp band (ISSR-19) was amplified in 4 males, 8 females, and 14 andromonoecious genotypes. The ~400 bp band (UBC-873) was amplified in two females and one andromonoecious genotype and absent in males. To know the sequence information of polymorphic bands, few sequences were selected for cloning and sequencing. Therefore, these 5 bands, *i.e.* ~950 bp, ~1200 bp, ~1100 bp, ~400 bp, and ~400 bp along with positive control 953 bp, were selected for cloning.

The bands generated by these primers were eluted from agarose gel using QIAquick Gel Extraction Kit and were cloned into pTZ57R/T cloning vector. The recombinant vector with desired DNA inserts was transformed into DH5α competent cells. The positive white colonies were picked through the blue-white selection method and the recombinant clones were confirmed by performing colony PCR (Fig. 52a & b). The PCR confirmed positive colonies that resulted in desired amplification were picked for plasmid isolation. The isolated plasmids were further confirmed for desired DNA inserts by restriction digestion with *EcoRI* and *HindIII* restriction endonucleases. The recombinant plasmids with ~950 bp (OPD-20) insert released two bands of sizes ~350 bp and ~620 bp as shown in Fig. 52c, due to the presence of a restriction site for one of the restriction enzymes (*ECoR*I) used. The recombinant plasmid with ~1200 bp (OPD-12) resulted in 2 bands of different sizes, *i.e.* ~900 bp and ~300 bp due to the presence of restriction site for *Hind*III (Fig. 52c). The plasmid with desired insert of 1100 bp (OPC-06) released ~1100 bp band. The recombinant plasmid with desired insert size of ~400 bp (ISSR-19) band released the band of same size when digested with restriction enzymes (Fig. 52d). The plasmid for 400 bp (UBC 873) released two bands, *i.e.* ~200 bp and ~220 bp as visualized on the agarose gels due to presence of site for restriction enzyme *ECoR*I (Fig.52d). Thus, these 5 recombinant plasmids with desired DNA inserts confirmed by double digestion with endonucleases were sequenced by M13 forward and reverse sequencing primers. The forward and reverse sequences for ~950 bp (OPD-20), ~1200 bp (OPD-12), ~1100 bp (OPC-06), ~400 bp (ISSR-19), and ~400 bp (UBC 873) obtained in FASTA format for each plasmid were analyzed and transformed into contigs

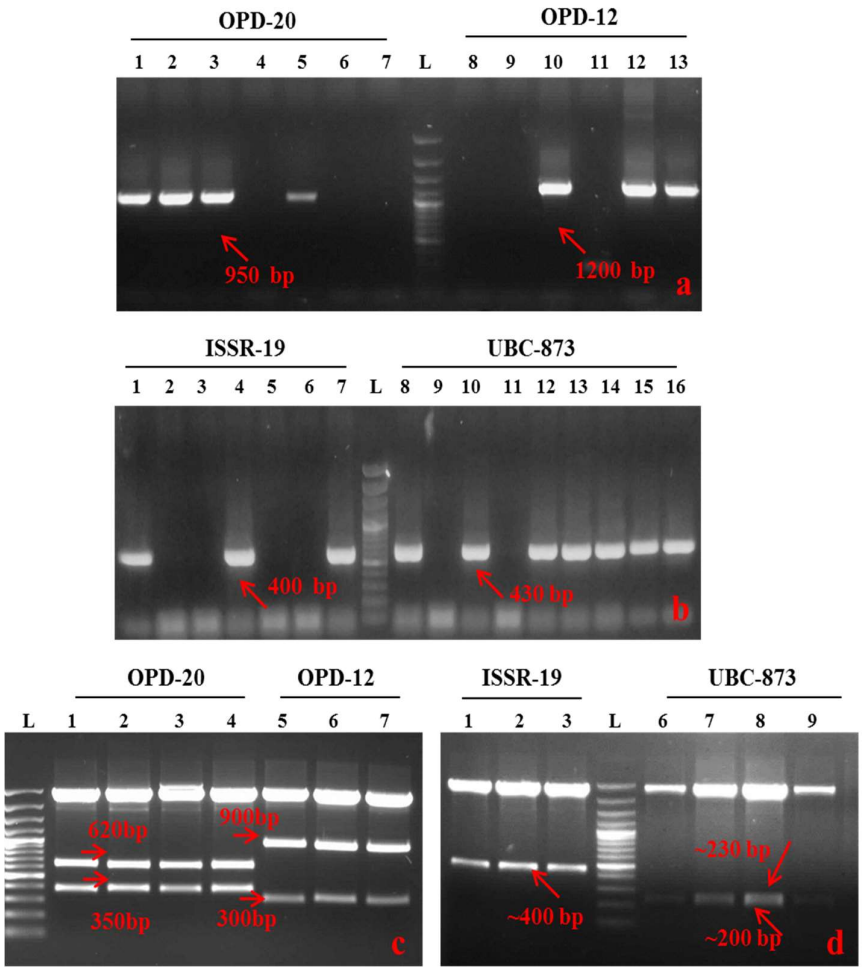

Fig. 52. Cloning and confirmation of recombinant plasmids by colony PCR amplification and double digestion resulting in desired polymorphic RAPD and ISSR bands (*a*) Colony PCR amplification of desired RAPD for primers OPD-20 (*Lanes 1-7*) and OPD-12 (*Lanes 8-13*) by using M13 primers, (*b*) Colony PCR amplification of desired ISSR bands for primers ISSR-19 (*Lanes 1-7*) and UBC-873 (*Lanes 8-16*) by using M13 primers, (*c*) double digestion of pTZ57R/T recombinant plasmids having inserts of ~950 bp (*Lanes 1-4*) and ~1200 bp (Lanes 5-7), (*d*) double digestion of pTZ57R/T recombinant plasmids having inserts of size ~400 bp for ISSR-19 (*Lanes 1-3*) and ~420 bp for UBC-873 (*Lanes6-9*). *L* – 100 bp DNA ladder.

of sequenced bands. The contamination of sequences for vector regions was analyzed by VecScreen, and the vector sequences were removed. Finally, 972 bp (OPD-20), 1201 bp (OPD-12), 1130 bp (OPC-06), 409 (ISSR-19) and 414 bp (UBC-873) sequences were obtained as indicated in Figs. 53-57. The sequences of 5 polymorphic bands obtained were as follows:

ACCCGGTCACGCGCTGACAATAATAGTAATCCTAGATATAAACATCAAAAGTTGTACATTAGTAAGTT
ATGTCAAGTAGACAATCTTTTAATCTTTGCAAGACTTGTGCTTTGGTCTTTGCCTCGATTGGATTTCCTTG
CATGACTAAAGTCGACGAATGAGAGCTGTGAAGTCAGACAAGTAATTGATCGATCGTCAATGTTCATTT
GTACTATTTGTTAGTAGAAAGTTATCTTCTACATTATTGATAGCTGTTTTTTATCCCTGAGTCTGATTAAT
TTCTAGTTGAATTGTTAGTTGAAGTTTTCTAAATCTCAACTTCACCATAAATTACTCTGTTGTTTATAAAT
ACACATTGAATTCTTTAAGGGATACTTCATTCTTCTTCCAAAAAAAAAAACAAATATTTCAAAGCTCAGA
TCAATGAGATTTTAAAACAAAAGTGCAACAATGTGGAAATATCTTAGCCATACAAGTAGTGGTGCCGG
TGCTAATTTTGCTACGCAGGCTACGTCATCTATGAAGGTTATATATAGGCAAATATTATGCATATTTGAA
TCATATTATGATAATTATTAAAATTAAGGCCTATGGCATTAATCTAAACCATTTTCGAGTAATGAATTTT
TAACTAAGATTTGAAGACGACTATTCCATAATCGCCTAGTGGATTCTGCTTGCCAAACTATCATCAACT
CCGCTGAAAGTTAATAAGCAATACACAAACATTAGGGGACGTGAAATCAGCTTATGGGATTTAATTAA
ATAGCCATATAAGTTAGGCCATTGCATTAAACACTAGCTTCTACAAGTGGGAAGCAATCTTTTTTAACA
AAAATGATAATAAAACCTAGAAATATAGCTCTTGCTATGTAACCCATGATTACTTTCAGGTTGCGTTCA
AGACTGTAATGTACCCTAATCTCATTTAACTCTAAATTAAGACATATAATGATGATGATG**GTGACCGG
GT**

Fig. 53. Nucleotide sequence of cloned 972 bp band. The sequence in red-colored bold represents the RAPD primer (OPD-20) sequence used in the analysis.

CACCGTATCCATTCGCTAAGGCAGTTATTGGCAACTTGCAAGCATCTCGTGCGACTGCATTAATGCTCT
CTGCAATATTTGTGGTCATTATGTCGTATCTATTGTAAGCGCCCTGTGACCTTGCCTATTTCATCAAACC
AACCTCATTTTCCAAATAATCTGTCATATCAAGCCTCATTTGTCTCATGTTCTCGAACATTTCTCGAAAT
TCACCATTACGATATACCTTTGCAGCTATAAAGTACGCAGCTAAATATCTTCCTTCTTTTCTCTTTTCAGT
TGAAAGCTTAAATCTGGTAACCAAATTCCCTTTTAGATGATATATACAGAACCCATGATGCGTAATTGG
GAACACATTATGAATTGTTTTCTCAATACTTGCATGTCGATTAGATATGAATACTAAGTTTTTCATGTCT
CCAACAACTTCGTGTAATTTTTCCATGAACCAATGCCAAGATGCATCATTCCTTGAGTCTACAATTGCCC
ATGCCAATAGAAATATTTGATTGTTTCCATCTTTGTAAACCACAACAAACATCACACCAAGGTAAGGGC
TTTTCAAGAAAGTACCGTTAACTACAATAACCAGTCGGATGGATGAATGAAAACAAATGATGCACGGT
GCCAACGACAGAAAGTCATATTTGAAGCATCGTGTCATTGTACCAAGGATTTTTCATGCATCAACGCAT
ATGAATATGCAGGGAGTAATCGATAAGAATGCTCTGGCGTGCCAAACAATGAATCTTTTTCGCAAGTTA
TCACGTATGTTATCTAGACACCAAATTCCTCAAGAATGTCTGCTTGAACTTCTTTTGGTCTGTAGTTCCT
AATAACAGAAGCAAATTTGTTTTTTATTATTTTGCCAACAATTCGACTTTTCGCTTGCCGATGGCCATGG
CGTAATTGTTCTGTTATACATTTGTGATTAGGATAGATCCTTCGAATAATCCACTAACTTCGCCCTTCAG
TCGTTACTGCTCATAATCGCCAGTTACAATTATTTGCATCGCATTGTGCTTCAAATCGATCTTTGGTTGA
CCTAATGACTTTGTAGCTCCAATTACCCCTCAACGCTAGTATGTTAATAGCCTCTTGAATTTCAGTTTTT
GTGTTATACAACTTGTTCACAAAAACATTTCCTATTCCACTACAACCTTCATTCTCGACCACAGTTGGAT
TTGATGG**GGATACGGTG**

Fig. 54. Nucleotide sequence of cloned 1201 bp band. The sequence in red-colored bold represents the RAPD primer (OPD-12) sequence used in the analysis.

GAACGGACTCTCTGCCAAAGATTGAATTTCAGAAAAAGAGCACTGACAAATACAGGATGGTAACTAA
TTTTAAGGGAAAAAACAACATTGCTAAGGAGGATAAATCATAAGTTGAAAGGCTTATTAGAAAAAGGA
TACCAACAGTATCCTCGTTGACAGGCACTTACGCAATGGCTTGTCCATCTGAAGTTGATGTCCCACGTC
CAAGTTCATCTAGCACCACCAATGAGTTACAAGTTGCTGCTGACTGCCATAAGTATGGTAAGCAGTTAA
GGCTAAAGGGCTTAAAAATCAAAATGTTACACATTTATAGCAAAATGTACTTGCATGTTTGAGACCTCA
CAGAGTTCCAAATTCAACTGAAAAATATAGGACTCAATAAAGGGGAGGAAGCCTTATATCAATGTCTA
GCATGTAAACTGATTCAAATATAATGAAGGCCATAGTTGCATCCAGAATAAACCAAACAATCTATGAG
TCTTAATGGATAATGCAAGGAAAATCAATGATAGTTTCCCAATGTTTGTTTGTCGATAAGTTGCAAACA
AGTCAAAAAGATCCAAAGAGATTTATTAACTTCCAACATTTTACTCAATAATAACTTTTCATGTCCTTGT
TTGCCCACATTTCCATACTTCGTACAAGCCATAATGAGATATGCCTAAAATATAGGTTTACAGTATGAA
AAAAATCTAACCAGCATTTGTGCAGTTTCTGTAAGCTCTGTTAAAAAGGTACTCTGGCCTGCCATAATA
TGATCCTTAGCACCCATCCAGACAAAGATTCGGTCAACAGGTGAGAGCTCAAAACTTTCAGCTGGGAC
ATCTGCTCCTACCTGGGTAATAACCAAAGATAGAGATTACTGAATAGCAGAACAACAAACTAAAATTT
GATGACAACAAAATACTTTACCTGAGCCAAGATCACAGCTGAGCACACCTGGCGAAGAAGGGTTGACT
TTCCTCCCATGTTAGGACCAGTGAGGAGTATAAAACTGGCATTGCCAGAACCACCAAGAGTAATATCAT
TGGCAACAAATGCACCCTTGCCTAAGGAATCACTACTAAGAACAGGATGTCCTAAGCGTTTAGCAGAT
ACACATGGCACTTCAATTGAACAT**GAGTCCGTTC**

Fig. 55. Nucleotide sequence of cloned 1130 bp band. The sequence in red-colored bold represents the RAPD primer (OPC-06) sequence used in the analysis.

ACACACACACACACACCCAAAAGAGAACATGCTGTAAATTTTGTCATTGTGTTTTCAAAATGTAGAAG
AAAGTGTAATAGAAGAAATTTATATTGTAAATATGGATTTAAATGAGCATTAAGATAGTGATTCAGAA
GTTATTCATTCCATCTTCTCAAATAAGAAATGGATTTGAATCCCTTCACCTTTTACCTATTAAAAGAAAA
TCTGGATTTGAATTGTCTAAATAAATTCAATAGGTTAAAGATAATTTGGTTTCTCAACTTTAATAAAAAT
GACTATTTGATTCTCTTAATTTAAAATGTGGATAATTTGATTTTTCGTTGTTTATTTCTTTTGTGAAAGTT
AACGAATGAAGGGGATTGTGGGATGCAGGGTGTCGGAGCGATTAT**GGTGTGTGTGTGTGTGT**

Fig. 56. Nucleotide sequence of cloned 409 bp band. The sequence in red-colored bold represents the ISSR primer (ISSR-19) forward and reverse sequence used in the analysis.

GACAGACAGACAGACAGAGACAGAGGGAGCGAAAGAAAAAGACAAAAGAGAGAGAGGGAGAGGGA
GAAGACATCGTGAAGCTGTGAAGGAGAAGGAAACATAAAAAATTGTGCATATGAGTAGTTGTGACTTG
TATCTTGCACGCTTGTAGAGTTCCTCTTGTTTATTTGTTTTTGTTTTATCGGCGTGAGAATTCGTCAAGAG
GAAAAACATACAAGATGAAAGGTGTAGTAAAACGTATTCTTTGTTTTATATGGTCGTATTTTAACTTATT
TTCTCTGCTTCTCAGTGTTATTTTCCTTGTCATTTTATATTTTTGTCGCCAGTTGTTTCTCCTTATTTTGCTT
CTACGCGTTAAATGATATTTTTTCTTCTTCTTCTCTATTCTGTGTGTGTGT**TGTCTGTCTGTCTGTC**

Fig. 57. Nucleotide sequence of cloned 414 bp band**.** The sequence in red-colored bold represents the ISSR primer (UBC 873) forward and reverse sequence used in the analysis.

4.3.6.2. Homology analysis of polymorphic RAPD and ISSR cloned bands:

The FASTA sequences were analyzed for the homology of the cloned bands with the known sequences in the non-redundant database of NCBI by nucleotide BLAST. A nucleotide BLAST search of the band 972 bp (OPD-20) showed the highest similarity of 98.76% with *S. glauca* SCAR marker SGS1 genomic sequence (accession no. KJ572402.1) in the database, which was titled as female-specific SCAR for *S. glauca* as shown in Fig. 58a. However, the band exhibited polymorphism regardless the sex type of the genotype. The ORF finder resulted in 8 short ORF's (44, 42, 42, 39, 31, 30, 29, and 27 aa). The map generated by the addgene program for the sequence of 972 bp has the sites for restriction enzymes like *Sal*I (150), *Cla*I (189), *EcoR*I (358), *Dra*I (432), *Stu*I (586), *Pac*I (761), and *Kpn*I (2) (Fig. 58b).

A nucleotide BLAST search of the band 1201 bp (OPD-12) did not show similarity with the known sequences present in the database. The ORF finder resulted in 9 ORF's (179, 73, 44, 39, 38, 37, 30, 29, and 28). The blast analysis of 179 aa sequence revealed the presence of a putative conserved domain, *i.e.* mule transposase domain of DDE_Tnp_ISL3 superfamily (Fig. 58c). The blast results of the amino acid sequence showed 46.59% homology with PKS-NRPS hybrid synthetase CHGG_01239-like (*Malus domestica*) and 44.91% homology with Squalene synthase 1 (*Vitis vinifera*). The map and restriction sites present in 1201 bp were obtained by addgene analysis. The sites for restriction enzymes in the sequence 1201 bp were *Ase*I (60), *Hind*III (284), *Hpa*I (577), *Nde*I (694), *Xba*I (777), *Msc*I (898), *Nco*I (899), *BstB*I (946), and *ApaL*I (1199) (Fig. 58d).

A nucleotide BLAST search of the band 1130 bp (OPC-06) showed 79.96% similarity with *Gossypioides kirkii* chromosome K1_07 and 88.73% with predicted *Pistacia vera* DNA mismatch repair protein MSH6 (LOC116146751), mRNA present in the database. The ORF finder resulted in 12 ORF's (54, 53, 48, 47, 36, 35, 34, 32, 29, 28, 26, and 25). The blast analysis of 54 aa sequence showed 50% homology with a hypothetical protein Ahy_B06g080669 isoform B [*Arachis hypogaea*]. The map and restriction sites present in 1130 bp were obtained by addgene analysis. The sites for restriction enzymes in the sequence 1130 bp were *Sac*I (804) and *BstB*I (1129).

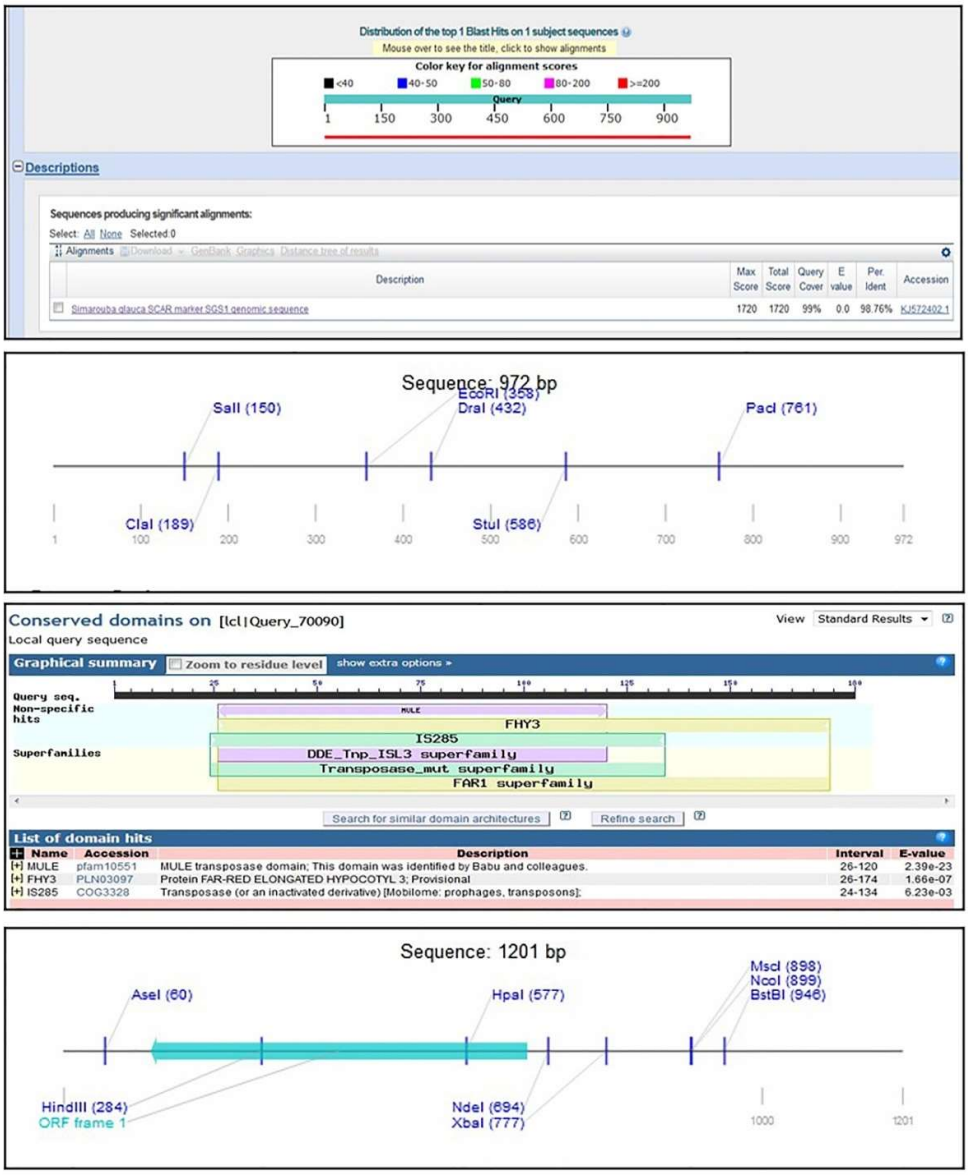

Fig. 58. Sequence analysis and maps of desired polymorphic bands obtained for OPD-20 and OPD-12 RAPD primers. (*a*) Sequencing analysis of 972 bp (OPD-20) RAPD band showing 99% homology with *S. glauca* SCAR marker, SGS1 genomic sequence with accession number '>KJ572402.1', (*b*) Map for 972 bp showing sites for restriction enzymes (including *ECoR*I), (*c*) Sequencing analysis 179 aa (ORF) of 1201 bp (OPD-12) RAPD band showing the presence of putative conserved domain, *i.e.* mule transposase domain of DDE_Tnp_ISL3, (*d*) Map for 1201 bp showing ORF and sites for restriction enzymes (including *Hind*III).

A nucleotide BLAST search of the band 409 bp (ISSR-19) did not show any similarity with the sequences present in the database. The ORF finder revealed 2 short ORF's (44 and 39 aa), and the amino acid sequence did not show any homology. The map and restriction sites present in 409 bp were obtained by addgene analysis. The restriction site present in the sequence was *Hpa*I (347).

A nucleotide BLAST search of the band 414 bp (UBC-873) did not show any similarity with the sequences present in the database. The ORF finder showed the presence of 5 short ORF's (70, 47, 42, 37, 32 aa), and the amino acid sequence did not show any homology. The map and restriction sites present in 414 bp were obtained by addgene analysis. The sites for restriction enzymes present in the sequence were *Nde*I (114), *EcoR*I (191), *Sal*I (412).

4.4. Analysis of phytochemicals, antioxidant activities and identification of sex-related metabolites in leaf and shoot apex extracts of *S. glauca*:

4.4.1. Preliminary phytochemical analysis of leaf and shoot apex extracts:

The preliminary qualitative assays for phytochemicals such as phenols, flavonoids and tannins were carried out using selected solvents based on polarities such as methanol, acetone and hexane for leaf and shoot apex samples of 3 genders of *S. glauca*. The phytochemical differences were observed based on polarity of solvents as shown in Table 17. Phenols, flavonoids and tannins were absent in hexane leaf and shoot apex extracts. The methanolic leaf and shoot apex extracts of *S. glauca* revealed the strong presence of phenols and moderate presence of tannins whereas flavonoids were weakly present. The acetone leaf and shoot apex extracts of *S. glauca* showed the presence of phenols and tannins moderately. Flavonoids were found to be moderately present in shoot apex extracts and weakly present in leaf extracts. Overall, phenolics were found to be strongly present in leaf and shoot apex methanolic extracts as compared to other extracts and gender specific differences were not detected in the preliminary analysis of the phytochemicals.

4.4.2.1. Total phenolic, flavonoid, and condensed tannin contents of leaf extracts:

The total phenolics expressed in gallic acid equivalents were recorded in higher amounts than total flavonoids and tannins in leaf extracts. The total phenolic content

Table 17. Preliminary screening of phytochemicals in leaf and shoot apex extracts of *S. glauca*.

S. No.	Extracts	Plant extracts	Phenols			Flavonoids			Tannins		
			M	F	A	M	F	A	M	F	A
1	Methanol extract	Leaf	+++	+++	+++	+	+	+	++	++	++
		Shoot apex	+++	+++	+++	+	+	+	++	++	++
2	Acetone extract	Leaf	++	++	++	+	+	+	++	++	++
		Shoot apex	++	++	++	++	++	++	++	++	++
3	Hexane extract	Leaf	-	-	-	-	-	-	-	-	-
		Shoot apex	-	-	-	-	-	-	-	-	-

(+++) = strongly present, (++) = moderately present, (+) = weakly present, (-) = absent
M = male, F = female, A = andromonoecious plants.

among leaf methanolic and acetone extracts of females (FLMe and FLAc), males (MLMe and MLAc), and andromonoecious samples (ALMe and ALAc) varied from 141.26 to 267.28 mg GAE/g DW (Fig. 59a). The leaf methanolic extracts of all genders exhibited significantly higher phenolic contents than acetone extracts. The methanolic extract of male leaves (267.28 mg GAE/g DW) showed highest phenolic content than females and andromonoecious extracts (236.65 and 257.52 mg GAE/g DW). The phenolic content in acetone leaf extracts of females and andromonoecious plants (141.26 and 142.28 mg GAE/g DW, respectively) were significantly higher than males (126.02 mg GAE/g DW).

The total flavonoid content of leaf methanolic and acetone extracts was found to be in lesser amounts than total phenolics as expressed in rutin equivalents. The total flavonoid content of leaf methanolic and acetone extracts of all genders was in the range of 61.69 to 101 mg RE/g DW (Fig. 59a). Significant differences ($P<0.05$) in flavonoid contents were observed among methanolic and acetone extracts of all genders. Higher content of total flavonoids was observed in methanolic extracts (82.71 to 101 mg RE/g DW) of all genders than acetone extracts (61.69 to 70.77 mg RE/g DW). The leaf methanolic and acetone extracts of males (101 and 70.77 mg RE/g DW, respectively) showed higher flavonoid content than female and andromonoecious extracts.

The total tannin content of methanolic and acetone extracts remained lower than the total flavonoids and phenolics as expressed in catechin equivalents. The methanolic leaf extracts did not show significant differences ($P<0.05$) among three genders (14.28 mg CE/g DW in females; 14.29 mg CE/g DW in males; 13.62 mg CE/g DW in andromonoecious plants). The acetone extracts showed significant differences among three genders with the highest content of condensed tannins found in females (38.29 mg CE/g DW) compared to males (24.05 mg CE/g DW) and andromonoecious (35.47 mg CE/g DW) plants (Fig. 59a).

4.4.2.2. Total phenolic, flavonoid, and condensed tannin contents of shoot apex samples:

The shoot apex extracts showed higher total phenolic content than flavonoid and condensed tannin contents. Significant differences in total phenolic content were observed among genders ranging from 104.88 to 345.53 mg GAE/g DW, except for

andromonoecious and male acetone extracts. Shoot apex methanolic extracts exhibited high content of phenolics compared to the acetone extracts (Fig 59b). Among shoot apex methanolic extracts of 3 genders, males (345.53 mg GAE/g DW) showed significantly higher content of polyphenols compared to females (340.04 mg GAE/g DW) and andromonoecious (321.95 mg GAE/g DW) plant extracts. Shoot apex acetone extracts of females (144.92 mg GAE/g DW) showed a significantly higher content of total polyphenols compared to males (106.91 mg GAE/g DW) and andromonoecious (104.88 mg GAE/g DW) plant extracts.

Flavonoids in shoot apex methanolic and acetone extracts of different genders ranged from 105.6 to 138.18 mg RE/g DW (Fig 59b). The highest total flavonoid content was observed in acetone shoot apex extracts of andromonoecious plants. Among shoot apex methanolic extracts, females (117.41 mg RE/g DW) exhibited a significantly higher content of flavonoids compared to males (106.34 mg RE/g DW) and andromonoecious (105.60 mg RE/g DW) extracts. The acetone extracts of all genders showed significant differences ($P<0.05$) in flavonoid contents in order of andromonoecious (138.18 mg RE/g DW) > male (125.62 mg RE/g DW) > female (117.66 mg RE/g DW) extracts. The methanolic and acetone extracts of females did not show any significant differences ($P<0.05$) in flavonoid content.

The total condensed tannins were recorded at low levels compared to total phenolics and flavonoids. The tannin contents among the genders of methanolic and acetone extracts ranged from 12.17 to 15.7 mg CE/g DW (Fig 59b). The methanolic extracts of females (15.7 mg CE/g DW) showed higher tannin content compared to males (12.17 mg CE/g DW) and andromonoecious (13.54 mg CE/g DW) extracts. Significant differences in tannin content were not observed in the acetone extracts of shoot apices of females (15.67 mg CE/g DW) and andromonoecious (15.65 mg CE/g DW) plants.

4.4.3. Total antioxidant activity of leaf and shoot apex extracts:

The leaf methanolic extracts of males (182.93 mg AAE/g DW) showed higher total antioxidant activity compared to females (173.39 mg AAE/g DW) and andromonoecious (156.21 mg AAE/g DW) (Fig 59a) plants. Analysis of

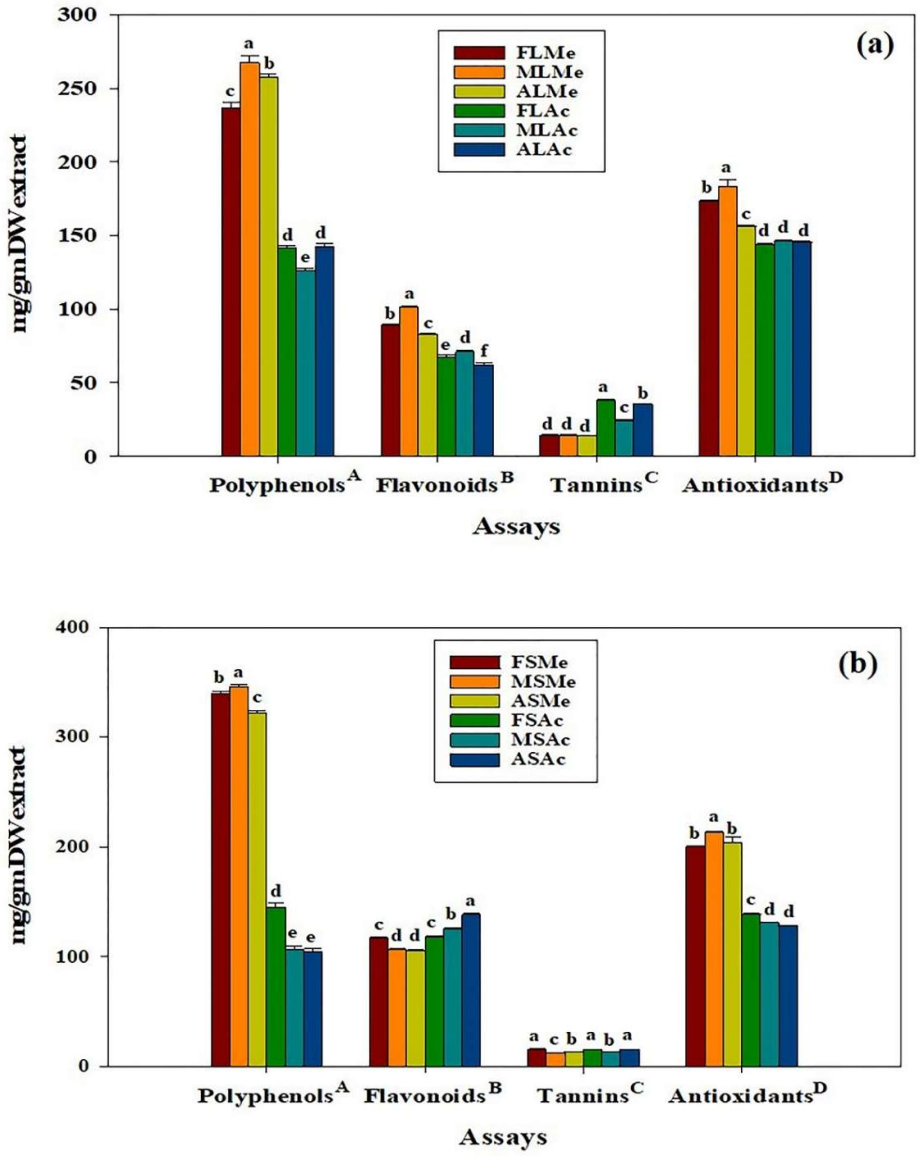

Fig. 59. Quantitative analysis of total phenolics, flavonoids, condensed tannins and antioxidants of different extracts of leaves from female, male and andromonoecious plants of *S. glauca*. (*a*) Leaf methanolic and acetone extracts of different genders, (*b*) Shoot apex methanolic and acetone extracts of different genders. F = female, M = male, A = andromonoecious, L = leaf, S = shoot apex, Me = methanol, Ac = acetone. A: Gallic acid, B: Rutin, C: Catechin, D: Ascorbic acid equivalents mg/g dry weight plant material, respectively. Values are means ± Standard deviation of three replicates (n = 3). Means followed by the same letter in the group are not significantly different at 5% probability level by Newman-Keul's multiple comparison tests.

total antioxidant activity in the leaf acetone extracts did not reveal significant differences ($P<0.05$) among all genders.

The total antioxidant activity of shoot apex methanolic and acetone extracts of 3 genders varied as indicated in Fig 59b. The methanolic extracts exhibited significantly higher antioxidant activity than acetone extracts ranging from 128.38 to 213.60 mg AAE/g DW. The methanolic shoot apex extracts of males (213.60 mg AAE/g DW) exhibited highest total antioxidant activity compared to andromonoecious (203.84 mg AAE/g DW) and female (200.78 mg AAE/g DW) extracts. Among acetone extracts, females (138.77 mg AAE/g DW) displayed significantly higher amount of antioxidant activity than males (131.04 mg AAE/g DW) and andromonoecious (128.38 mg AAE/g DW) extracts. Overall, the highest total antioxidant activity was recorded in shoot apex methanolic extracts as compared to other extracts.

The methanolic extracts of leaf and shoot apex were considered a good source of antioxidants as they exhibited significant total antioxidant activity than acetone extracts. Among all the leaf and shoot apex extracts, the shoot apex methanolic extracts of males was found to be a potent antioxidant than extracts of other genders.

4.4.4. DPPH radical scavenging activity of leaf and shoot apex extracts:

The leaf and shoot apex extracts exhibited good antioxidant power to scavenge the free radicals of DPPH as reflected from IC_{50} values obtained for the extracts. (Table18). All the extracts quenched DPPH free radicals in a concentration-dependent manner where inhibition percentage increased with an increase in concentrations. Among all the extracts tested, the shoot apex extracts exhibited significant DPPH radical scavenging activity than the leaf extracts. The male leaf methanolic (MLMe) extract showed higher radical scavenging activity with the inhibition percentage up to 94.89% with a slightly higher IC_{50} value of 8.97 µg GAE/ml than the ascorbic acid (5.64 µg GAE/ml) showing its antioxidant potential (Fig. 60a). The male leaf acetone extracts (MLAc) showed the lowest IC_{50} value of 6.81 µg GAE/ml, which is closer to ascorbic acid's value, with percentage inhibition of 93.26%, thus considered to have substantial antioxidant potential (Fig. 60b). The other leaf extracts also exhibited low IC_{50} values with antioxidant potential (Table 18).

Table 18. IC_{50} values for DPPH radical scavenging assay for different extracts isolated from leaf and shoot apex samples of female, male and andromonoecious plants of *S. glauca.*

Extract type	IC_{50} value (µg/ml)
AA	5.64 ± 0.05 de
FLMe	10.46 ± 0.83 b
MLMe	8.97 ± 0.71 c
ALMe	10.48 ± 0.69 b
FLAc	12.09 ± 0.87 a
MLAc	6.81 ± 0.41 d
ALAc	9.33 ± 0.56 c
FSMe	7.16 ± 0.38 d
MSMe	5.76 ± 0.19 de
ASMe	6.23 ± 0.26 de
FSAc	6.04 ± 0.22 de
MSAc	6.23 ± 0.31 de
ASAc	7.79 ± 0.53 d

AA= ascorbic acid; FLMe & FLAc = female leaf methanolic and acetone extracts, respectively; MLMe & MLAc = male leaf methanolic and acetone extracts, respectively; ALMe & ALAc = andromonoecious leaf methanolic and acetone extracts, respectively; FSMe & FSAc = female shoot apex methanolic and acetone extracts, respectively; MSMe & MSAc = male shoot apex methanolic and acetone extracts, respectively; ASMe & ASAc = andromonoecious shoot apex methanolic and acetone extracts, respectively.

Values are means ± Standard deviation of three replicates (n = 3). Means followed by the same letter in a column are not significantly different at the 5% probability level by Newman-Keul's multiple comparison tests.

The shoot apex extracts, exhibited significantly higher antioxidant potential, and scavenged the DPPH free radicals with an increased percentage of inhibition at increased concentrations of extracts (Fig 60c & d). The radical scavenging activity of shoot apex methanolic extract of males (MSMe) reached to 94.66% (Fig. 60c). It resulted in the lowest IC_{50} value of 5.76 µg GAE/ml, which showed value closer to ascorbic acid and was considered to possess potent antioxidant potential followed by female shoot apex acetone (FSAc; 6.04 µg GAE/ml) extract (Table 18). The other shoot apex methanolic and acetone extracts also exhibited low IC_{50} values and high antioxidant potential similar to the ascorbic acid standard. Among the different extracts of 3 genders, male extracts showed strong antioxidant activity with low IC_{50} values.

4.4.5.1. GC-MS analysis of leaf and shoot apex samples of three genders of *S. glauca*:

GC-MS analysis of leaf and shoot apex of male, female and andromonoecious genotypes revealed a distinctive metabolite pattern as reflected by Total Ion Chromatogram (TIC) of different sexes (Fig. 61a-c & Fig 62a-c). The TICs were subjected for metabolite identification based on their peak pattern and m/z ratios by comparing with the known metabolites mass spectral details using NIST (National Institutes of Standards and Technology) library. Comparative analysis of leaf metabolites of male and andromonoecious plants displayed higher similarity than female plants. Similarly, the metabolite analysis of shoot apices also varied among the 3 genders used in the study.

The metabolome of leaf extract based on similarity index revealed a total of 163, 147 and 150 known metabolites in females (FL), males (ML) and andromonoecious (AL) genders respectively, of which 49 metabolites were found to be common among three different sexes whereas, 17, 24 and 21 metabolites were common between females and males; females and andromonoecious; and male and andromonoecious genotypes, respectively. The metabolites detected were classified based on their chemical nature and the percentage of individual compounds of particular kind was assigned for different genders. The metabolites from female leaf extracts (FL) were grouped in to 11 classes *viz*., carboxylic acids (22%), carbohydrates (15%), organic compounds (12%),

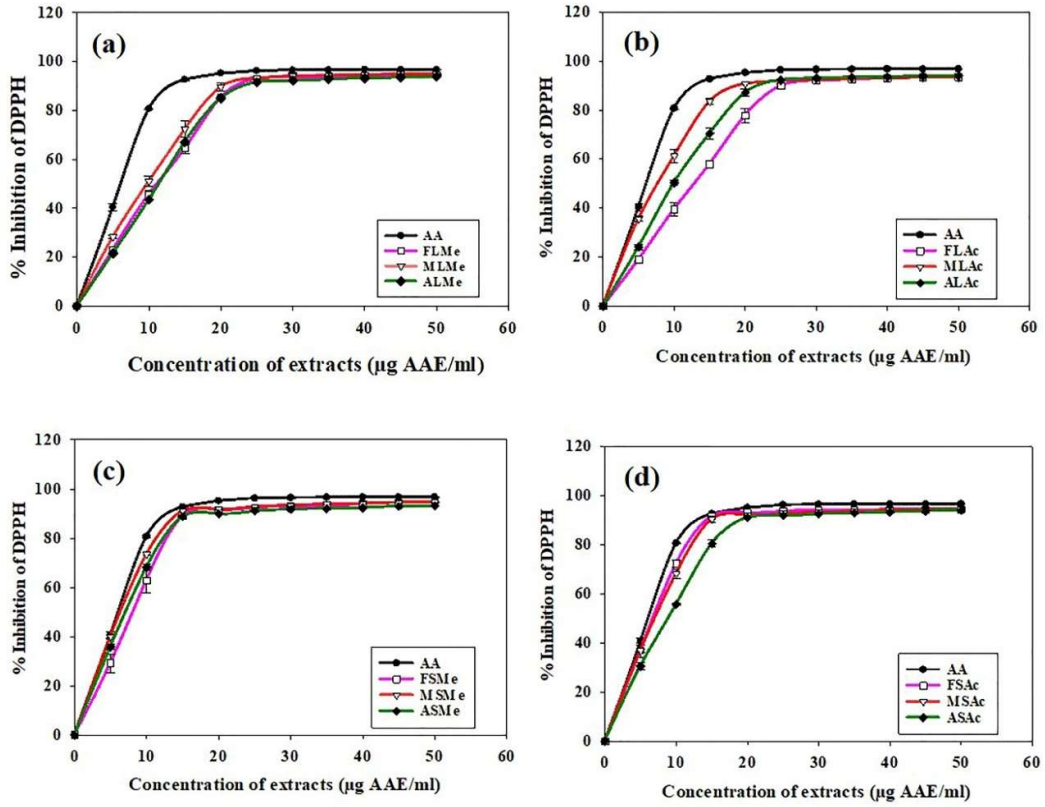

Fig. 60. DPPH radical scavenging activity for various concentrations of leaves and shoot apex extracts of different sexes of *S. glauca*. (*a*) Leaf methanolic extracts, (*b*) Leaf acetone extracts, (*c*) Shoot apex methanolic extracts, (*d*) Shoot apex acetone extracts. AA= ascorbic acid; FLMe & FLAc = female leaf methanolic and acetone extracts, respectively; MLMe & MLAc = male leaf methanolic and acetone extracts, respectively; ALMe & ALAc = andromonoecious leaf methanolic and acetone extracts, respectively; FSMe & FSAc = female shoot apex methanolic and acetone extracts, respectively; MSMe & MSAc = male shoot apex methanolic and acetone extracts, respectively; ASMe & ASAc = andromonoecious shoot apex methanolic and acetone extracts, respectively.

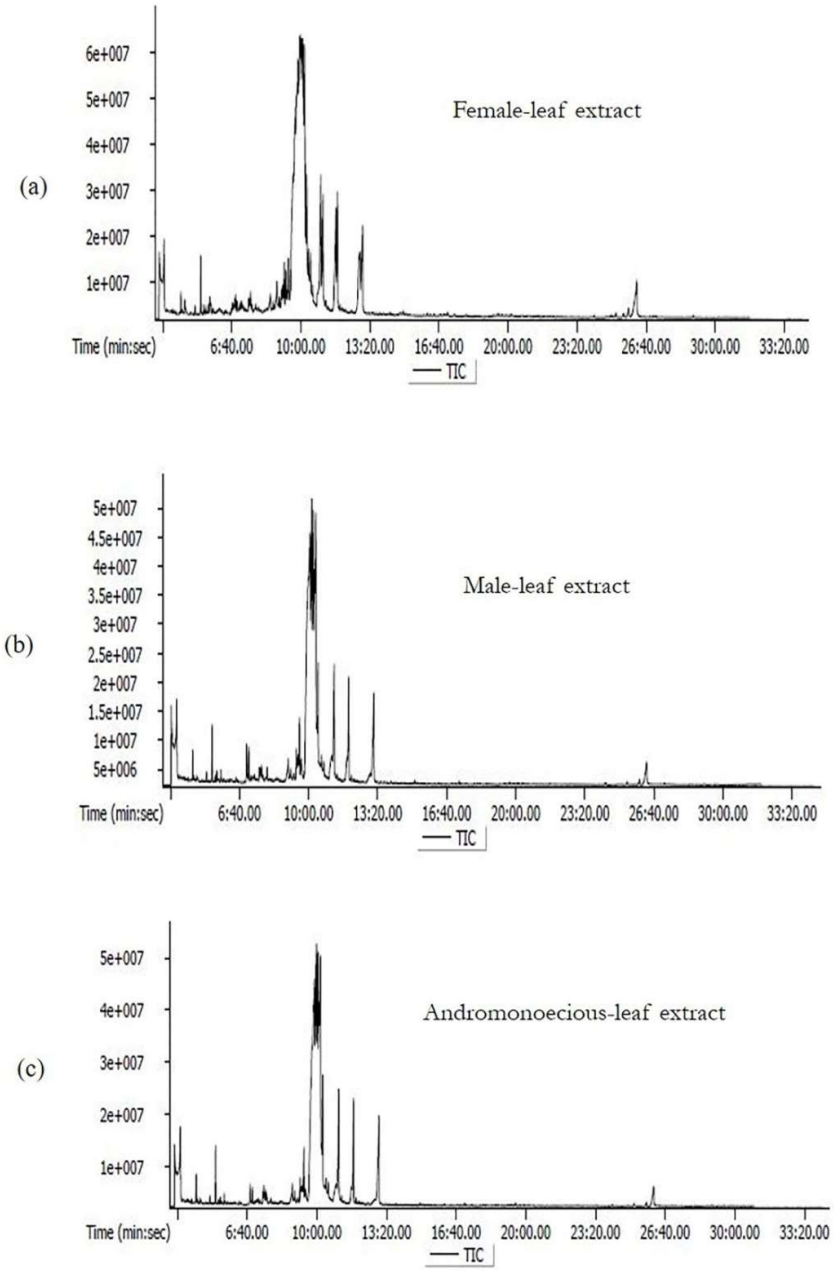

Fig. 61. GC-MS chromatogram of leaf extracts of different sexes of *S. glauca*. (*a*) Female, (*b*) Male, (*c*) Andromonoecious genotypes.

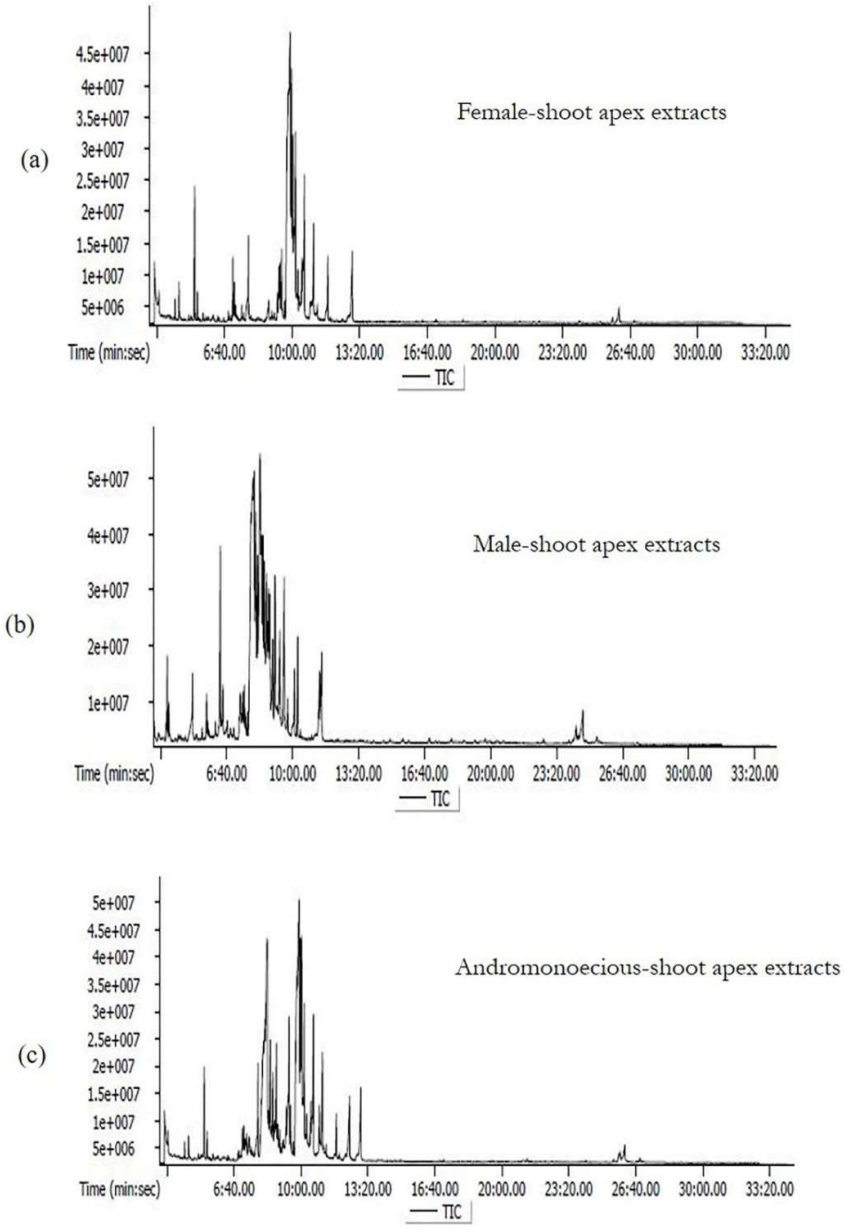

Fig. 62. GC-MS chromatogram of shoot apex extracts of different sexes of *S. glauca*. (*a*) Female, (*b*) Male, (*c*) Andromonoecious genotypes.

lipids (14%), sugars and their derivatives (7%), amino acids (10%), organic acids (6%), esters (4%), amines (1%), amides (1%), and others (8%) as shown in Fig. 63a. The male leaf extract (ML) was grouped in to 10 classes *viz*., carboxylic acid (26%), carbohydrates (16%), organic compounds (12%), lipids (11%), sugars and their derivatives (12%), amino acids (8%), organic acids (2%), amines (4%), amides (5%), and others (4%) as shown in Fig. 63b. The andromonoecious leaf extract (AL) was grouped into 9 classes *viz*., carboxylic acids (13%), carbohydrates (12%), organic compounds (13%), lipids (13%), sugars and their derivatives (14%), amino acids (14%), organic acids (7%), amines (4%) and others (10%) as shown in Fig. 63c. The metabolite analysis between leaf extracts among the genders showed differences in percentages of some classes of metabolites that were grouped together. The percentage of lipids was similar in case of FL (14%) and AL (13%) than ML (11%). ML displayed a higher percentage (26%) of carboxylic acids followed by FL (22%) and AL (13%). The combined percentages of carbohydrates and sugars and their derivatives were higher in ML (28%), followed by AL (26%) and comparably less in FL (22%) extracts. The amino acids were high in AL (14%) as compared to FL (10%) and ML (8%) extracts.

The metabolome of female, male, and andromonoecious shoot apex extracts (FS, MS and AS) revealed 153, 154, and 146 metabolites, respectively. Out of these metabolites, 50 metabolites were found commonly in all 3 genders, 21 metabolites were common to females and males; 18 metabolites were common to female and andromonoecious genders; and 21 metabolites were common to male and andromonoecious genders. The percentages of individual compounds for all the genders were analyzed. The FS comprised of 10 classes *viz*., carboxylic acids (17%), carbohydrates (12%), organic compounds (12%), lipids (9%), sugars and their derivatives (3%), amino acids (16%), organic acids (14%), amines (4%), amides (4%), and others (9%) as shown in Fig. 63d. The MS comprised of 11 classes *viz*., carboxylic acids (21%), carbohydrates (8%), organic compounds (16%), lipids (12%), sugars and their derivatives (4%), amino acids (11%), organic acids (7%), esters (4%), amines (6%), amides (2%), and others (9%) as shown in Fig. 63e. The AS consisted of 11 classes *viz*., carboxylic acids (19%), carbohydrates (11%), organic compounds (10%), lipids (16%), sugars and their derivatives (3%), amino acids (19%), organic acids (3%), esters (3%), amines (5%), amides (3%) and others (8%) as shown in Fig. 63f. The

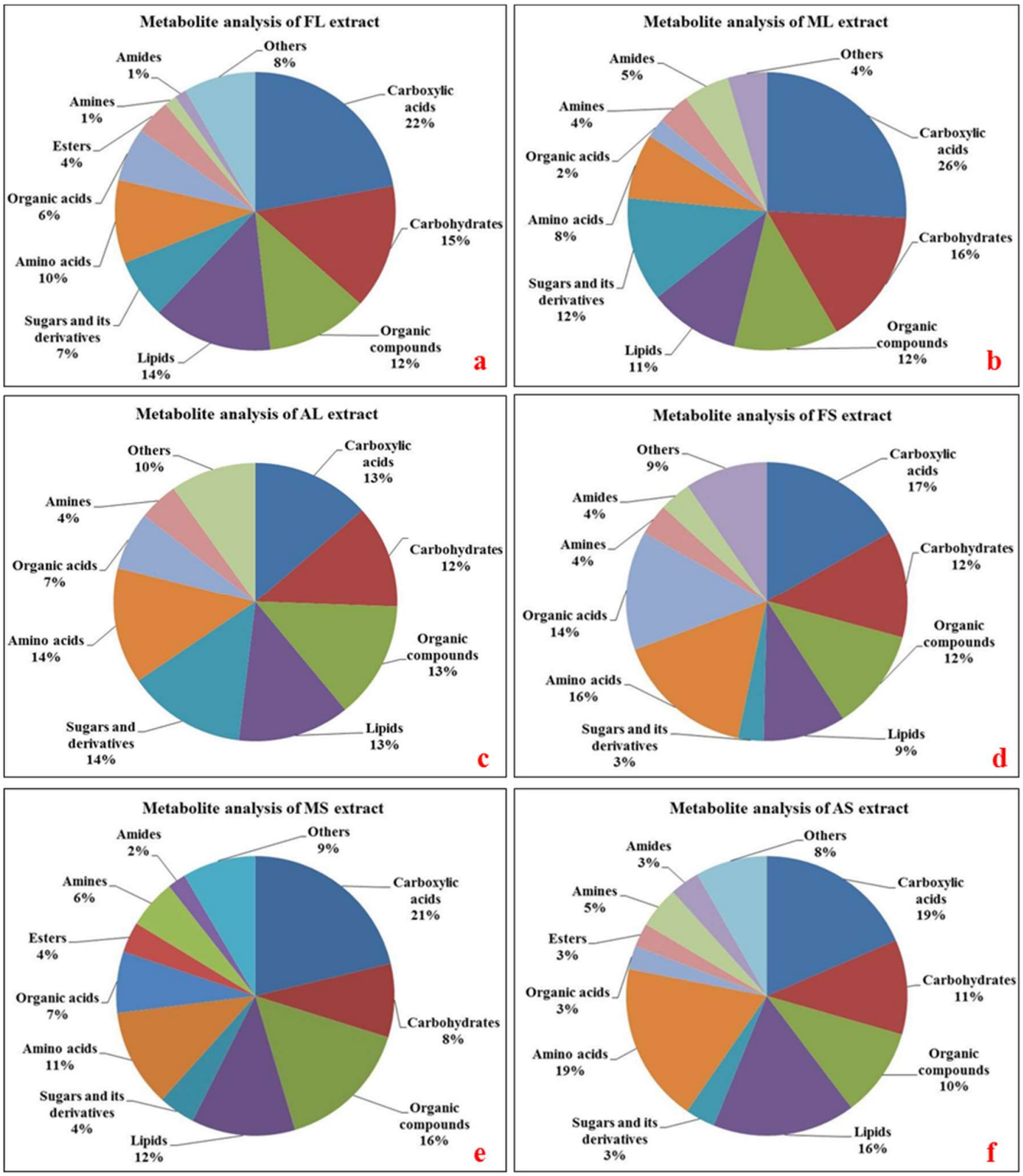

Fig. 63. GC-MS analysis of metabolites in leaf and shoot extracts of different sexes of *S. glauca*. (*a*) Leaf extract of females, (*b*) Leaf extract of males, (*c*) Leaf extract of andromonoecious genotypes, (*d*) Shoot apex extract of females, (*e*) Shoot apex extract of males, (*f*) Shoot apex extract of andromonoecious plants.

metabolite analysis of shoot apex extracts among the genders showed differences in percentage and metabolite types. The percentage of lipids varied among the genders with a high percent observed in AS (16%) and MS (12%) compared to FS (9%) extracts. A higher percent of carboxylic acids was observed in MS (21%) compared to AS (19%) and FS (17%) extracts. The organic compounds were high in MS (16%) compared to FS (12%) and AS (10%) extracts. The amino acids were high in AS (19%) compared to FS (16%) and MS (11%) extracts. A higher percentage of organic acids was observed in FS (14%) compared to MS (7%) and AS (3%) extracts. In shoot apex extracts, the combined percentages of carbohydrates and sugars and their derivatives were higher in FS (15%) and AS (14%) than in MS (12%) extracts.

4.4.5.2. Sex-related metabolites identified by GC-MS analysis of leaf and shoot apex extracts:

The GC-MS analysis of leaf and shoot apex extracts of all genders revealed differences among the genders concerning few metabolites (Table 19). The metabolites such as o-tetramethylen (tert-butyl) silylepiandrosterone; estra-1,3,5(10)-trien-4-amine,3,16,17-tris[(trimethyl silyl) oxy]-,(16à,17á)-; testosterone, 17-O-(t-butyldimethylsilyl)-; prostaglandin D(2), O,O'-bis(trimethylsilyl)-, trimethylsilyl ester exhibited differences among the genders in the present study. The metabolite o-tetramethylen(tert-butyl)silylepiandrosterone, was detected in shoot apex sample of andromonoecious genotype (AS) and absent in females and males in this study. It is a steroid hormone with weak androgenic activity and acts as a precursor for testosterone (Hirotani and Furuya 1974; Šaden-Krehula and Kolbah 1983; Janeczko and Skoczowski 2005). The metabolite estra-1,3,5(10)-trien-4-amine, 3,16,17-tris [(trimethyl silyl)oxy]-, (16à,17á) was present in leaf extracts of female (FL) and andromonoecious samples (AL) and absent in leaf extract of males. It is a steroid hormone and known to have varied functions such as stimulate embryo germination, promotion of the growth of seedlings, and influences reproductive function such as sex determination in plants (Helmkamp and Bonner 1953; Bhattacharya and Gupta 1981; Janeczko and Skoczowski 2005). The metabolite testosterone, 17-O-(t-butyldimethylsilyl)- was present in leaf extract of males (ML) and absent in female and andromonoecious extracts. It is an anabolic steroid hormone that affects the reproductive system, especially sex determination and sometimes also aids vegetative growth in plants (Geuns 1978;

Table 19. Sex-related metabolites found in different extracts of genders of *S. glauca.*

S. No.	Metabolite	Nature of metabolite	Uses	Presence	References
1	o-tetramethylen(tert-butyl)silylepiandrosterone	Steroid	Steroid hormone with weak androgenic activity and acts as a precursor for testosterone.	AS	Šaden-Krehula and Kolbah (1983); Hirotani and Furuya (1974); Janeczko and Skoczowski (2005)
2	estra-1,3,5(10)-trien-4-amine, 3,16,17-tris[(trimethylsilyl)oxy]-, (16à,17á)-	Steroid	The steroidal estrogens have effects such as stimulate embryo germination, promote the growth of seedlings and influence reproductive function such as sex determination in plants.	FL & AL	Janeczko and Skoczowski (2005); Bhattacharya and Gupta (1981); Helmkamp and Bonner (1953)
3	testosterone, 17-O-(t-butyldimethylsilyl)-	Steroid	Testosterone in plants affect the reproductive system especially sex determination and sometimes also aids vegetative growth in plants.	ML	Geuns (1978); Janeczko and Skoczowski (2005); Tarkowská (2019)
4	prostaglandin D(2), O,O'-bis(trimethylsilyl)-, trimethylsilyl ester	Lipid	Prostaglandin and prostaglandin-like compounds possibly act as physiological regulators for flowering, fruit ripening, tuberization, tendril coiling and storage.	FS, MS, AS, ML & AL	Groenewald and van der Westhuizen (1997& 2005); Mueller (1997).

F = Female, M = Male, A = Andromonoecious plants, L = Leaf, S = Shoot apex.

Janeczko and Skoczowski 2005; Tarkowská 2019). The metabolite prostaglandin D (2), O, O'-bis(trimethylsilyl)-, trimethylsilyl ester was present in leaf and shoot apex extracts of all genders except female leaf extract used in this study. It is a lipid molecule, and these prostaglandins or prostaglandin-like compounds possibly act as physiological regulators of flowering, fruit ripening, tuberization, tendril coiling, and storage in plants (Groenewald and van der Westhuizen 1997 & 2005; Mueller 1997).

4.5. *In vitro* regeneration of nodal, shoot tip and seedling-derived explants and genetic stability analysis of regenerated plants of *S. glauca*:

4.5.1. Regeneration response from nodal and shoot tip explants:

A high proportion of nodal explants collected from mature trees underwent browning within 6-7 days after culture on MS medium with BAP (0.5-2.0 mg/l) possibly due to the high content of phenolics which might have got oxidized and leached out into the medium. About 1-2% of the nodal explants that survived were subcultured on the medium with 2 mg/l BAP. Slight proliferation of callus was observed from the nodal explants after subculture without any shoot induction even after 30 days of culture (Fig. 64a). Shoot tip explants exhibited relatively less browning with shoot initiation observed after 20-25 days in 47.36% of the cultures on MS medium with 2 mg/l BAP (Fig. 64b). Shoots grew to a height of 3-4 cm after 20 days of subculture (Fig. 64c) without any formation of multiple shoots.

4.5.2. *In vitro* seed germination:

Seed germination frequency was influenced by the type of medium and the concentration of GA_3 used (Fig. 65a). The seed germination frequency remained low (3.4–9.2%) on full-strength or half-strength MS or WP medium without GA_3. The addition of GA_3 enhanced the germination rate, with a significantly higher response (64.3–88.8%) observed on full-strength MS medium than on half-strength MS or on either strength of WP medium. Maximum germination (88.8%) was achieved on full-strength MS medium with 2 mg /l GA_3, with emergence of radicle in 8–10 days and development of seedlings of 5–6 cm length after 4 weeks of culture (Fig. 65b & 66).

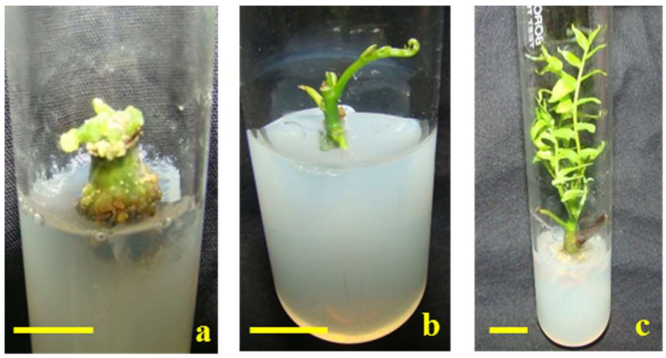

Fig. 64. *In vitro* response from nodal and shoot tip explants cultured on MS medium with 2 mg/l BAP. (*a*) Callus formation after 15 days of subculture from nodal explants, (*b*) Shoot initiation after 25 days of culture on initiation medium containing 2 mg/l BAP, (*c*) *In vitro* developed shoot with leaves from shoot tip explant after 20 days of subculture of MS medium with 2 mg/l BAP. *Bars* = 1 cm (*a–c*)

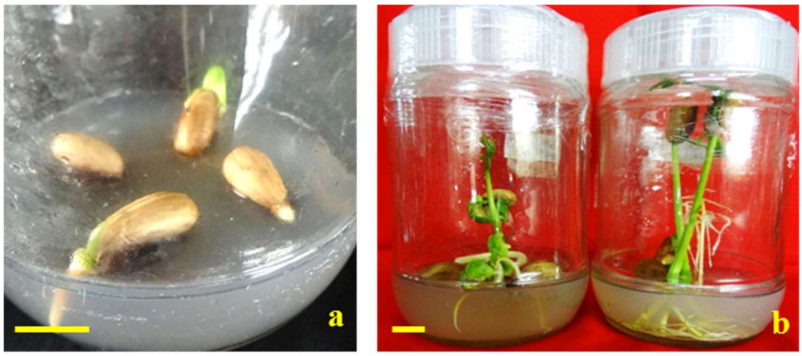

Fig. 65. *In vitro* seed germination and growth of seedlings of *S. glauca*. (*a*) Emergence of radicles after 6 days of culture, (*b*) Seedlings developed after 25 days of culture. *Bars* = 1 cm (*a* & b)

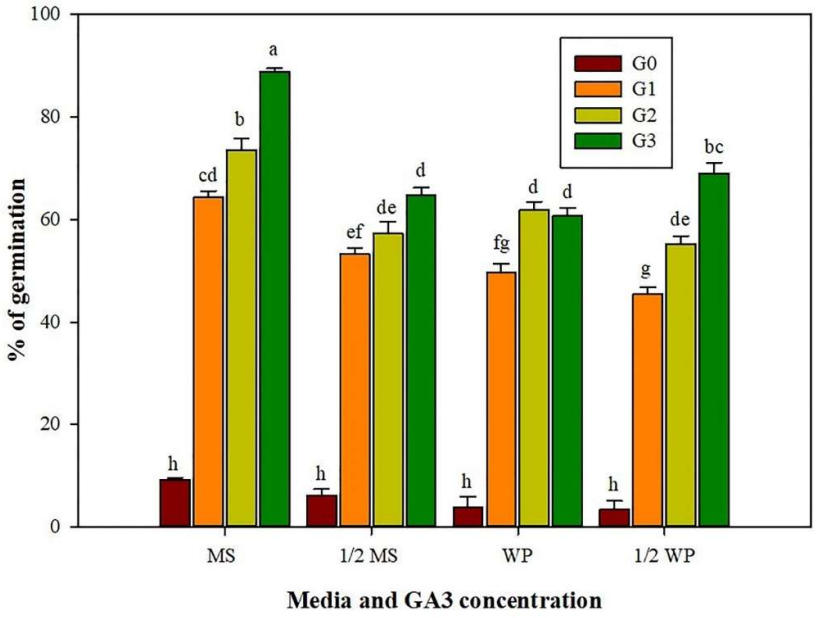

Fig. 66. Germination response from seeds of *S. glauca* on different media. Media used were either full-strength or half-strength MS (Murashige and Skoog) or WP (Woody Plant). G0, basal medium (without GA_3); G1, 0.5 mg/l GA_3; G2, 1.0 mg/l GA_3; G3, 2.0 mg/l GA_3. The values presented are percentages of germination (number of seeds germinated per total number of seeds cultured × 100) for different treatments. Values represent means ± SE (standard error) of three experiments. Bars with the same letter are not significantly different at the 5% probability level by one-way analysis of variance (ANOVA) followed by the Newman–Keuls multiple comparison test.

4.5.3. Shoot bud proliferation and elongation in explants derived from *in vitro*-germinated seedlings:

Shoot buds were induced directly without any callus phase in all explants except leaves and petioles after culture on MS or WP medium with different growth regulators. Shoot bud initiation was observed in the explants after 15–20 days of culture. The frequency of shoot bud induction in different explants varied from 10.8 to 76.2% on the media tested (Fig. 67). MS medium with 2 mg/l BAP induced shoot buds in most explants at a higher frequency (44.8–76.2%) than the WP medium containing a similar concentration of BAP (30.8–63.8%). A decrease in shoot regeneration frequency (down to 10.8 from 52.0%) was observed in all explants when they were cultured on MS or WP medium either with BAP and NAA or with TDZ alone. Among the different explants, cotyledons exhibited a higher frequency of shoot regeneration (76.2%) than other explants on MS medium with 2.0 mg/l BAP (Fig. 67).

To induce shoot multiplication, a series of experiments were conducted. The explants were subcultured on a medium with various concentrations of BAP (0.5–2.0 mg/l) alone or in combination with 0.2 mg/l NAA. However, only one to two shoots continued to elongate after repeated subcultures on medium with BAP (0.5–2.0 mg/l) and 0.2 mg/l NAA, and the shoots that formed exhibited retarded growth associated with leaf yellowing and dropping at the end of subcultures. This difficulty was successfully overcome by gradually reducing BAP concentration (1.0, 0.5, and 0.2 mg/l) along with the addition of GA_3 (0.2 and 0.5 mg/l) in the medium during subcultures. To achieve higher shoot bud induction, the explants were subjected to at least three passages (2 weeks each) on MS medium with 2 mg/l BAP before transferring to medium with 1 mg/l BAP (Fig. 68a-d). Significantly higher numbers of shoot buds per explant were induced in root nodes (28.5) and hypocotyls (27.5), followed by cotyledons (14.5) and cotyledonary nodes (11.4). In contrast, the lowest values were obtained for epicotyl (7.9) and shoot tip (5.2) explants (Table 20). A shoot bud proliferation rate of 3- to 5-fold was achieved per cycle in root nodes, hypocotyls, and cotyledonary explants, without any decrease over the six passages examined in this study. The induced shoot buds started to elongate upon transfer to MS medium with 1 mg/l BAP (no GA_3), regardless of the explant type. Further elongation of the shoots was achieved after subcultures on MS medium with 1 mg/l BAP and 0.2 mg/l GA_3, followed

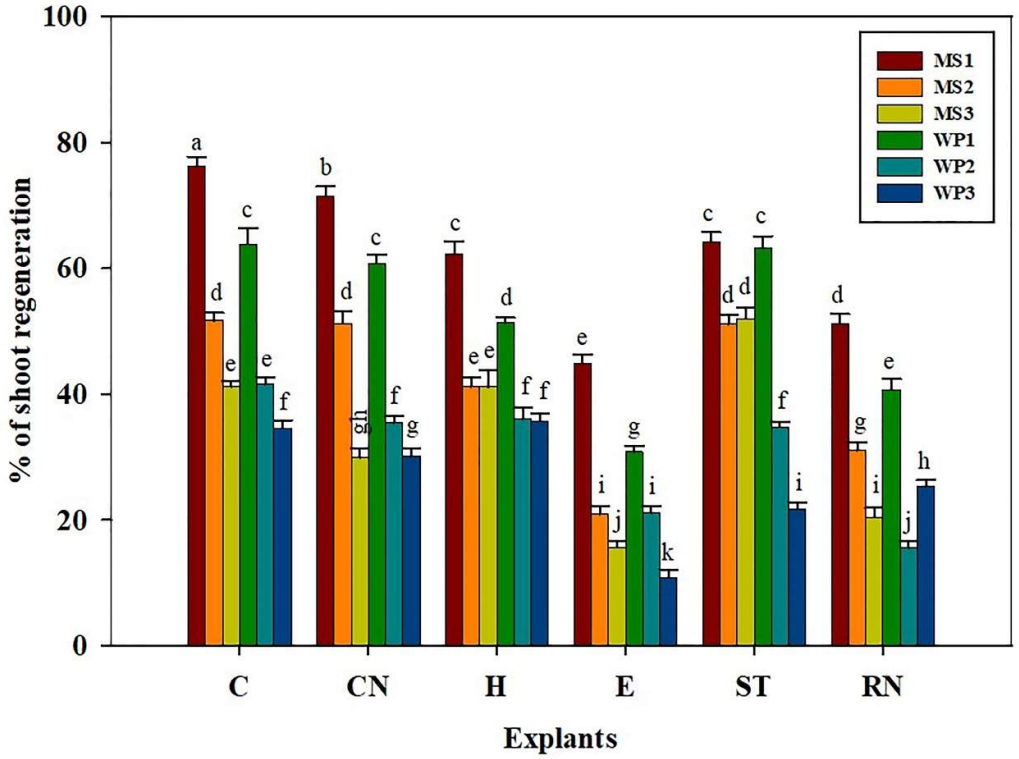

Fig. 67. Shoot regeneration frequency from different explants of *S. glauca* cultured on different media. The explants used for regeneration were cotyledons (*C*), cotyledonary nodes (*CN*), hypocotyls (*H*), epicotyls (*E*), shoot tips (*ST*), and root nodes (*RN*). Media used were MS (Murashige and Skoog) and WP (Woody Plant). MS1, MS with 2 mg/l BAP; *MS2*, MS with 2 mg/l BAP and 0.2 mg/l NAA; MS3, MS with 0.5 mg/l TDZ; *WP1*, WP with 2 mg/l BAP; WP2, WP with 2 mg/l BAP and 0.2 mg/l NAA; WP3, WP with 0.5 mg/l TDZ. Values are percentages of shoot regeneration (number of explants with shoot buds per total number of explants × 100) for different treatments. Values represent means ± SE of three experiments. Bars with the same *letter* are not significantly different at the 5% probability level by one-way ANOVA followed by the Newman–Keuls multiple comparison test.

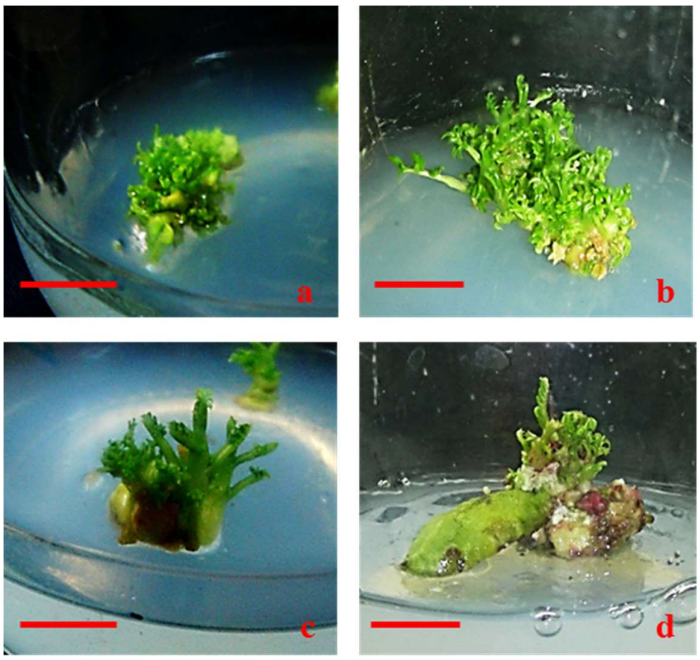

Fig. 68. Direct shoot organogenesis from seedling-derived explants of *S. glauca* after 3 passages (2 weeks each) on MS medium with 2.0 mg/l BAP followed by subculture on MS medium with 1.0 mg/l BAP. (*a*) Induction of multiple shoot buds from hypocotyl explants, (*b*) Induction of multiple shoots from root node explant, (*c*) Induction of multiple shoots from cotyledonary node explants, *(d)* Induction of multiple shoots from cotyledonary explants. *Bars* = 1 cm (*a–d*).

Table 20. Response of shoot proliferation and elongation from shoot buds induced from different explants of *S. glauca.*

Explant type	Average number of shoot buds/explant[z]	Average number of elongated shoots/explant[y]	Average length of shoots (cm)[y]
Cotyledons	14.5 ± 0.5 b	9.1 ± 0.3 a	3.3 ± 0.2 a
Cotyledonary nodes	11.4 ± 0.7 b	5.2 ± 0.3 b	1.9 ± 0.2 b
Hypocotyls	27.5 ± 3.3 a	5.7 ± 0.4 b	3.0 ± 0.1 a
Epicotyls	7.9 ± 0.8 c	0.0 ± 0.0 d	0.0 ± 0.0 d
Shoot tips	5.2 ± 0.5 c	1.8 ± 0.2 c	0.7 ± 0.1 c
Root nodes	28.5 ± 1.2 a	5.1 ± 0.5 b	1.8 ± 0.1 b

Values are means ± SE of three experiments and in each experiment 20 explants were used per treatment. Means followed by the same letter in a column are not significantly different at the 5% probability level by one-way ANOVA followed by the Newman–Keul's multiple comparison test.

[z] Data were scored after 3 passages (2 weeks each) on MS medium with 2.0 mg/l BAP.

[y] Data were scored after 3 passages (2 weeks each) on MS medium with 2.0 mg/l BAP, followed by subcultures on MS medium with 1.0 mg/l BAP, MS medium with 1.0 mg/l BAP and 0.2 mg/l GA_3, MS medium with 0.5 mg/l BAP and 0.2 mg/l GA_3, and MS medium with 0.2 mg/l BAP and 0.5 mg/l GA_3, with each subculture lasting for 3 weeks.

by MS medium with 0.5 mg/l BAP and 0.2 mg/l GA_3 (Fig. 69a), followed by MS medium with 0.2 mg/l BAP and 0.5 mg /lGA_3, with each subculture lasting for 3 weeks (Fig. 69b). This approach yielded consistent results in inducing proliferation and elongation of shoots from all the explants except for those induced in epicotyl explants, which failed to elongate (Table 20).

The shoot buds induced in different explants exhibited differences in their capability for shoot elongation and development. The maximum shoot elongation response was observed from cotyledon explants, with the induction of 9.1 elongated shoots per explant and an average shoot length of 3.3 cm at the end of the last passage (medium with 0.2 mg/l BAP and 0.5 mg/l GA_3) (Table 20). Although a large number of shoot buds proliferated from the root node and hypocotyl explants, the shoot elongation response remained relatively low, with the induction of 5.1 and 5.7 elongated shoots per explant, respectively (Table 20). About 50% of shoots induced from the root node and cotyledonary node explants exhibited stunted growth with premature differentiation of leaves and without subsequent shoot elongation. The shoot proliferation rate from shoot tip explants remained low with the induction of 5.2 shoot buds per explant, from which only 1.8 shoots elongated and formed well-developed shoots.

4.5.4. Root induction and acclimatization:

In initial experiments, the explant source from which the shoots were regenerated did not have a marked effect on root induction. Thus, rooting experiments were performed using the regenerated shoots from different explants. Rooting was achieved more efficiently from IBA pulse-treated shoots than from untreated shoots regardless of medium type (Table 21). Pulse treatment of shoots with IBA resulted in high root induction frequency (66.7%), with the induction of 11.3 roots per shoot of 3.4 cm average length, after culture on half-strength WP medium containing 0.2 mg/l IBA (Table 21 and Fig. 69c & d). In contrast, the untreated shoots were grown on the same medium rooted at a low frequency (33.3%), with the induction of 4.7 roots per shoot of 1.7 cm average length (Table 21). There were no significant differences in root induction from IBA-treated shoots, whether cultured on full-strength MS or full-strength WP basal medium. However, the root induction frequency from IBA-treated

Fig. 69. Regeneration of plants from cotyledon explants of *S. glauca* and establishment in soil. (*a*) Development of multiple shoots after 3 week of subculture on MS medium with 0.5 mg/l BAP and 0.2 mg/l GA_3, (*b*) Elongation of shoots after the last subculture on MS medium with 0.2 mg/l BAP and 0.5 mg/l GA_3, (*c*) Root induction (arrow) from pulse-treated shoots (10 mg/ml IBA for 5 min) after 4 week of culture on half-strength WP medium with 0.2 mg/l IBA, (*d*) Pulse-treated shoot with well-developed roots taken out of culture medium, (*e*) Regenerated plant acclimatized in culture room, (*f*) Regenerated plant established in soil. *Bars* = 1 cm (*a–d*, *f*).

Table 21. Root induction from regenerated shoots of *S. glauca* on different media.

Medium and concentration of IBA (mg/l)	With IBA pulse treatment					Without pulse treatment				
	No. of shoots cultured	No. of shoots rooted	Rooting frequency (%)	Average no. of roots per shoot	Average root length (cm)	No. of shoots cultured	No. of shoots rooted	Rooting frequency (%)	Average no. of roots per shoot	Average root length (cm)
WPM	30	10	33.2±1.8 d	6.3±0.3 cd	1.2±0.1 d	30	0	0.0±0.0 c	0.0±0.0	0.0±0.0 d
WPM + 0.2 mg/l IBA	30	13	43.3±1.7 c	7.3±0.3 bc	2.2±0.2 c	30	0	0.0±0.0 c	0.0±0.0	0.0±0.0 d
½ WPM	33	18	54.3±2.3 b	9.3±0.3 b	2.8±0.1 b	27	3	11.2±0.7 b	3.3±0.3 c	1.1±0.1 c
½ WPM + 0.2 mg/l IBA	30	20	66.7±3.3 a	11.3±0.9 a	3.4±0.1 a	30	10	33.3±3.3 a	4.7±0.3 a	1.7±0.1 a
MS	29	10	34.4±1.0 d	5.0±0.6 c	1.2±0.2 d	30	0	0.0±0.0 c	0.0±0.0	0.0±0.0 d
MS + 0.2 mg/l IBA	32	14	44.0±1.0 c	8.0±0.6 b	2.4±0.1 c	30	0	0.0±0.0 c	0.0±0.0	0.0±0.0 d
½ MS	33	14	42.2±1.7 c	7.0±0.6 b	2.9±0.1 b	29	3	10.4±0.4 b	2.3±0.3 b	0.5±0.0 d
½ MS + 0.2 mg/l IBA	32	17	53.0±1.5 b	9.0±0.6 b	3.0±0.1 ab	28	3	10.7±0.4 b	4.3±0.3 a	1.4±0.1 b

Shoots elongated on MS medium with 0.2 mg/l BAP and 0.5 mg/l GA_3 with pulse treatment (10 mg/ml solution for 5 min) or without treatment were cultured on different rooting media and the data were scored after 45 days of culture.

Values are means ± SE of three experiments. Means followed by the same letter in a column are not significantly different at the 5% probability level by one-way analysis of variance (ANOVA) followed by the Newman–Keul's multiple comparison test.

shoots was higher on half-strength WP medium (54.3%) than on half-strength MS medium (42.2%). Roots were initiated within 12–20 days from IBA-treated shoots on half-strength WP medium with or without IBA. In contrast, it varied from 15 to 30 days from treated or untreated shoots cultured on other media.

The regenerated plants transferred to potting mixture were initially acclimatized in culture room for 4 weeks (Fig. 69e). A total of 30 regenerated plants derived from explants of different seedlings were transferred to soil, out of which 24 plantlets were successfully acclimatized and established in the soil in 7–8 months with a survival rate of 80% (Table 22). The plants exhibited healthy growth and reached a height of 10–11 cm with well-developed leaves after 12 weeks of growth in the glasshouse (Fig. 69f). The regeneration procedure established in this study resulted in the recovery of about 25 plants from different explants of a single seedling (Table 22 and Fig. 70).

4.5.5. Genetic stability analysis of the regenerated plants using RAPD and ISSR primers:

Fourteen RAPD and 15 ISSR primers that resulted in clear amplification were employed to assess the genetic stability of plantlets regenerated from cotyledons, hypocotyl, and root node explants derived from a single seedling. The number of scorable loci for each RAPD primer varied from 3 (OPB-07 and OPV-08) to 13 (OPC-18), with an average of 8.1 loci per primer (Table 23). Eight RAPD primers produced monomorphic bands across the regenerated plants and the donor seedling. The number of amplified fragments generated from the RAPD primers varied from 33 (OPB-07 and OPV-08) to 143 (OPC-18), with size ranging from 200 to 2800 bp. Out of 113 loci generated by RAPD primers, six loci (5.3%) were polymorphic. A total of 1223 bands were amplified in the regenerated plants (across 11 plants analyzed with 14 RAPD primers), of which 20 bands (1.6%) were found to be polymorphic (Fig. 71a-c).

The ISSR primers used in this study produced an average of 6.9 loci per primer, with the lowest number (2 loci) for UBC-865 and the highest number (10 loci) from three primers, *viz*., UBC-815, UBC-826, and UBC-835. A total of 103 loci were generated by 15 ISSR primers, of which 6 loci (5.8%) were found to be polymorphic (Fig. 72a-c). Out of 15 ISSR primers, 10 produced monomorphic bands. The UBC-835 primer generated the highest number of amplified fragments (110), whereas the lowest

Table 22. Regeneration of plants from different explants of *S. glauca* and their establishment in soil.

Explant	Average no. of regenerated plants per seedling[z]	Total no. of regenerated plants transferred to soil[y]	Total no. of regenerated plants established in soil[y]
Cotyledons	12.3±0.2 a	13	11
Cotyledonary nodes	3.7±0.1 b	5	4
Hypocotyls	3.8±0.1 b	6	5
Shoot tips	1.6±0.1 c	3	2
Root nodes	3.8±0.1 b	3	2

[z] Values are means ± SE of three experiments. Means followed by the same *letter* in a column are not significantly different at the 5% probability level by one-way ANOVA followed by the Newman–Keuls multiple comparison test.

[y] Regenerated plants from different seedlings.

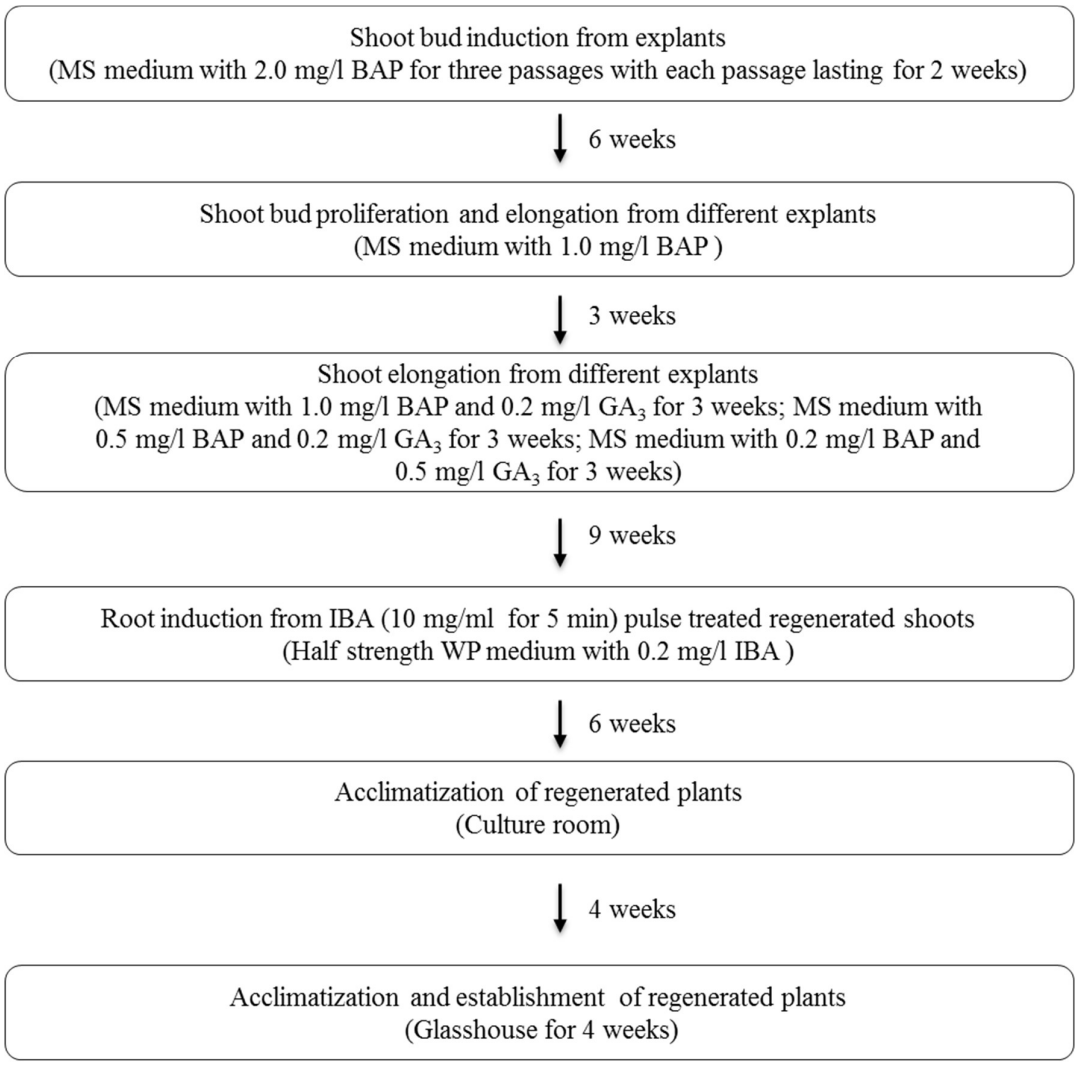

Fig.70. Schematic representation of plant regeneration from seedling-derived explants of *S. glauca*.

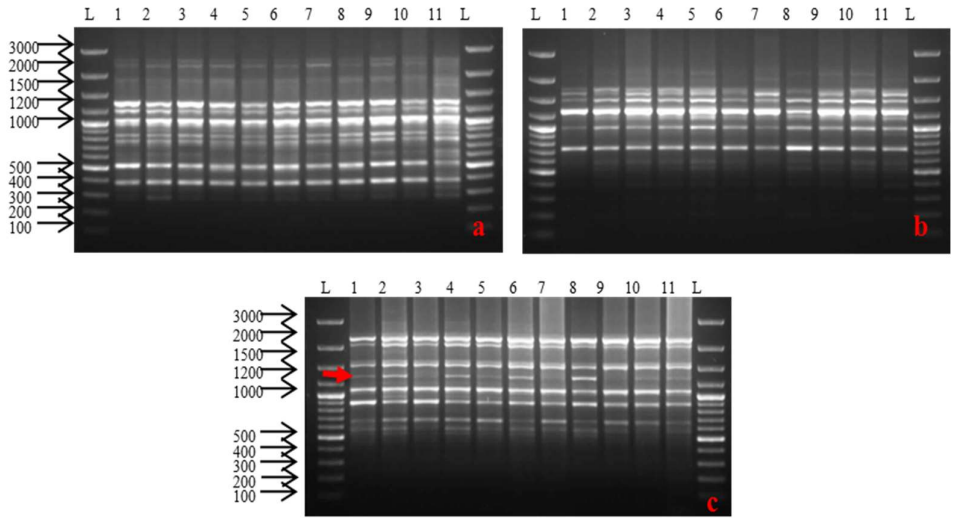

Fig. 71. RAPD profiles of *in vitro* regenerated plants along with donor seedling of *S. glauca* obtained with different primers. (*a*) RAPD primer OPV-17, (*b*) RAPD primer OPAK-14, (*c*) RAPD primer OPD-14. *Lane L*, molecular weight DNA marker. *Lane 1*, donor seedling; *Lanes 2–5*, regenerated plants from cotyledonary explants; *Lanes 6–8*, regenerated plants from hypocotyls explants; *Lanes 9–11*, regenerated plants from root node explants. *Red arrow* in (*c*) indicates polymorphic bands.

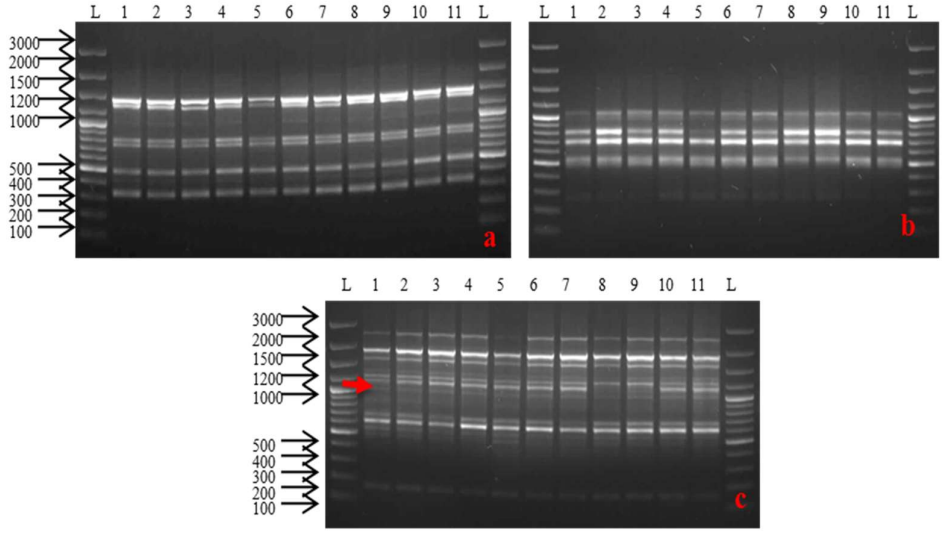

Fig. 72. ISSR profiles of *in vitro*-regenerated plants along with donor seedling of *S. glauca* obtained with different primers. (*a*) ISSR primer UBC-824, (*b*) ISSR primer UBC-813, (*c*) ISSR primer UBC-873. *Lane L*, molecular weight DNA marker; *Lane 1*, donor seedling; *Lanes 2–5*, regenerated plants from cotyledonary explants; *Lanes 6–8*, regenerated plants from hypocotyls explants; *Lanes 9–11*, regenerated plants from root node explants. *Red arrow* in (*c*) indicates polymorphic bands.

number (22) was observed with UBC-865; for both primers, all of the bands were monomorphic in the plants analyzed. The size of the amplicons produced from ISSR primers ranged from 250 to 2600 bp (Table 24). The 15 ISSR primers generated a total of 1104 bands across all 11 plants, of which 29 bands (2.6%) were found to be polymorphic.

The Jaccard similarity coefficient values obtained from combined RAPD and ISSR data revealed a high genetic similarity of the regenerated plants to the donor seedling (0.958–0.986; Table 25). The regenerated plants showed an average similarity coefficient of 0.971 with the donor seedling in combined RAPD and ISSR analysis.

Table 23. RAPD profiles of *in vitro* regenerated plants and donor seedling of *S. glauca.*

Primer name	Primer sequence (5′–3′)	Number of loci	Number of polymorphic loci	Total number of amplified bands across all plants	Total number of polymorphic bands across all plants	Size range (bp)
OPA-11	CAATCGCCGT	7	1	69	8	460–2100
OPA-16	AGCCAGCGAA	10	1	104	6	550–2400
OPA-17	GACCGCTTGT	12	1	131	1	280–2750
OPAK-14	CTGTCATGCC	7	1	76	1	750–1800
OPAL-08	GTCGCCCTCA	9	0	99	0	525–2100
OPB-07	GGTGACGCAG	3	0	33	0	950–2000
OPC-18	TGAGTGGGTG	13	0	143	0	220–2750
OPD-14	CTTCCCCAAG	9	1	96	3	575–2225
OPE-20	AACGGTGACC	4	0	44	0	800–2000
OPP-03	CTGATACGCC	7	0	77	0	375–2800
OPS-05	TTTGGGGCCT	6	0	66	0	200–2300
OPV-08	GGACGGCGTT	3	0	33	0	375–1400
OPV-17	ACCGGCTTGT	12	0	132	0	275–2600
OPZ-01	TCTGTGCCAC	11	1	120	1	250–2100
Total number of bands		113	6	1223	20	

Table 24. ISSR profiles of *in vitro* regenerated plants and donor seedling of *S. glauca.*

Primer name	Primer sequence (5′–3′)	Number of loci	Number of polymorphic loci	Total number of amplified bands across all plants	Total number of polymorphic bands across all plants	Size range (bp)
UBC-808	AGAGAGAGAGAGAGAGC	7	0	77	0	320–1000
UBC-810	GAGAGAGAGAGAGAGAT	8	0	88	0	320–850
UBC-812	GAGAGAGAGAGAGAGAA	6	0	66	0	250–700
UBC-813	CTCTCTCTCTCTCTCTT	5	0	55	0	280–1050
UBC-815	CTCTCTCTCTCTCTCTG	10	1	107	3	320–1900
UBC-818	CACACACACACACACAG	5	0	55	0	500–2000
UBC-824	TCTCTCTCTCTCTCTCG	6	0	66	0	320–1450
UBC-825	ACACACACACACACACT	7	0	77	0	350–1050
UBC-826	ACACACACACACACACC	10	1	102	8	250–1500
UBC-834	AGAGAGAGAGAGAGAGTT	5	1	48	7	400–1200
UBC-835	AGAGAGAGAGAGAGAGCC	10	0	110	0	320–1150
UBC-842	GAGAGAGAGAGAGAGAYG	5	2	46	9	280–1150
UBC-865	CCGCCGCCGCCGCCGCCG	2	0	22	0	720–950
UBC-873	GACAGACAGACAGACA	8	1	86	2	600–2600
UBC-880	GGAGAGGAGAGGAGA	9	0	99	0	350–1450
Total no. of bands		103	6	1104	29	

Table 25. Similarity coefficients for *in vitro* regenerated plants and donor seedling of *S. glauca* based on RAPD and ISSR analysis.

	Donor	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10
Donor	1.000										
R1	0.986	1.000									
R2	0.968	0.972	1.000								
R3	0.981	0.995	0.968	1.000							
R4	0.977	0.981	0.972	0.986	1.000						
R5	0.972	0.977	0.977	0.981	0.986	1.000					
R6	0.968	0.972	0.981	0.977	0.981	0.986	1.000				
R7	0.963	0.968	0.968	0.972	0.977	0.981	0.986	1.000			
R8	0.958	0.963	0.981	0.968	0.981	0.977	0.991	0.986	1.000		
R9	0.972	0.977	0.977	0.981	0.995	0.991	0.986	0.981	0.986	1.000	
R10	0.968	0.981	0.981	0.986	0.981	0.986	0.972	0.968	0.972	0.986	1.000

Regenerated plants derived from cotyledons are R1–R4, from hypocotyl are R5–R7, and from root node are R8–R10.

Discussion

5. DISCUSSION:

The present research work was focused mainly on studying the molecular genetic variability among the sexes of *S. glauca* and identifying the sex-linked molecular marker(s) for detection of sex at the seedling stage. The phytochemical constituents and antioxidant activities in extracts of different sexes of *S. glauca* were investigated to identify the sex-related metabolites, if any. The GC-MS technique was employed for qualitative phytochemical analysis of all genders. Studies were also undertaken to establish an efficient *in vitro* regeneration method for rapid multiplication of this tree species and ascertain the genetic similarity among the regenerated plants using RAPD and ISSR markers. The results obtained from the study are discussed below:

5.1. Genetic variability in different sexes of *S. glauca* was assessed by using RAPD and ISSR markers:

The genetic variability existing in any plant species provides the basis for selection and plant improvement in the breeding programmes. The information on genetic variation present in females and males increases the prospects of getting new genetic combinations besides knowing the adaptive and evolutionary potential of different sexes in dioecious plant species. The molecular markers are more informative in assessing the genetic variability and diversity in tree species (Fu 2015). In the present study, RAPD and ISSR markers that are PCR based were used as they are random and do not require prior knowledge of the genome sequence of the species under study. The assessment of genetic variability among female, male and andromonoecious plants of *S. glauca* is important, as it will provide an input in the management of its genetic resources for genetic improvement, repopulation, and conservation programmes.

5.1.1. ISSR primers detected higher genetic polymorphism in different sexes of *S. glauca* as compared to RAPD primers:

In the present study, the molecular analysis of genetic variability among female, male and andromonoecious genotypes using 61 RAPD and 24 ISSR primers produced 465 and 183 loci out of which 209 and 118 loci were polymorphic, resulting in 46.8% and 61.48% polymorphism with average loci of 7.62 and 7.63 loci per primer, respectively. ISSR primers (61.48%) showed higher polymorphism than RAPD primers

(46.8%). Two RAPD primers, OPA-11 and OPC-05, resulted in the highest polymorphism of 100% for all genotypes tested, whereas one ISSR primer, *i.e.* ISSR-14, resulted in 100% polymorphism. The higher polymorphism detected by ISSR primers than RAPD primers could be because the regions targeted by ISSR primers are more polymorphic. The polymorphism exhibited by RAPD and ISSR primers in our study was higher than previous reports of *S. glauca*. Choudhary (2014) reported that 67 RAPD and 22 ISSR primers generated 539 and 178 bands, of which 222 and 67 were polymorphic resulting in 42.37% and 39.35% average polymorphism, respectively. Kumar and Agrawal (2017) observed a low polymorphism of 14.13% in 48 genotypes of *S. glauca* using 14 ISSR markers. The present study revealed the superiority of ISSR primers over RAPD primers in detection of molecular DNA variability in *S. glauca*. The genetic variability in *S. glauca* could be the result of out-crossing, pollination, often through wind and dispersal, due its dioecious nature (Hilfiker *et al*. 2004).

The dissimilarity matrix based on Jaccard's dissimilarity index using DARwin software version 6 obtained for 36 genotypes of different sexes of *S. glauca* varied from 0.054 to 0.270 (0.73 to 0.946 similarity) with an average value of 0.139 for combined data for RAPD and ISSR markers. The dissimilarity values for females ranged from 0.054 to 0.245, males ranged from 0.0131 to 0.059, and for andromonoecious from 0.071 to 0.218 (0.782 to 0.929). The dissimilarity indices for both RAPD and ISSRs revealed less genetic variation among males compared to females and andromonoecious genotypes. Similarly, Srivashtav *et al*. (2013) reported highest Jaccard's coefficient similarity value between males of date palm based on RAPD and combined RAPD and ISSR data.

5.1.2. RAPD and ISSR markers revealed lesser genetic variability in males than other sexes of *S. glauca*:

The degree of polymorphism was evaluated by calculating the polymorphic information content (PIC) and genetic diversity parameters. The PIC values represent the allele frequency and diversity among the genotypes used in the study (Manimekalai and Nagarajan 2006). The PIC values for dominant markers like RAPD and ISSR primers ranged from 0.00 to a maximum value of 0.50, and markers used for a population that show equal distribution might exhibit higher PIC values (Chesnokov and Artemyeva 2015). In the present study, the PIC values for RAPD and ISSR primers

varied from 0.00 to 0.21 and 0.00 to 0.22 for all genotypes tested with a mean value of 0.09 and 0.13, respectively. Both RAPD and ISSR primers that were unable to detect polymorphism or detect less polymorphism resulted in zero PIC values. The RAPD primers exhibited mean PIC values of 0.09, 0.07 and 0.10 for female, male and andromonoecious genotypes, respectively. For ISSR primers, the mean PIC values for females, males and andromonoecious genotypes were 0.16, 0.08 and 0.13, respectively. In this study, the mean PIC values for both markers were found to be less in males compared to females and andromonoecious genotypes suggesting that there is less genetic variation in males. The mean PIC values obtained for female, males and andromonoecious genotypes based on both RAPD and ISSR markers were 0.11, 0.07, and 0.11, respectively, with an average PIC value of 0.10 for all genotypes. The low PIC values for RAPD and ISSR analysis in the present study were like earlier reports in *S. glauca.* This might be due to the reason that the source material of the plantation was derived from the population of a specific region having low genetic variability. The primers that could not detect polymorphism or detected less polymorphism might also result in low average PIC values. Similar observations were made in *S. glauca* by Kumar and Agrawal (2017) where the ISSR primers resulted in low mean PIC value of 0.10. On the contrary, in the genetic diversity study of *S. glauca,* Choudhary (2014) reported that the mean PIC values for RAPD and ISSR primers were 0.9879 and 0.9628, which were much higher than the values obtained in the present study.

The genetic diversity parameters using POPGENE software for different markers employed in this study were utilized for evaluating the genetic variation among the sexes of *S. glauca*. The mean values of na, ne, h, and I for RAPD primers were 1.45, 1.27, 0.16 and 0.24, whereas the mean values of na, ne, h, and I obtained for ISSR primers were 1.62, 1.36, 0.21 and 0.31, respectively. The mean values of na, ne, h, and I for combined RAPD and ISSR primers were 1.49, 1.29, 0.18, and 0.26, which were similar to values for RAPD data. These values obtained were slightly higher compared to the previous study using ISSR primers in *S. glauca* (Kumar and Agrawal 2017), which could be due to the reason that the genotypes analyzed as well as the number of primers used were different in these studies. The genetic diversity among female, male, bisexual and non-flowered, were carried out to compare the 'h' values, which resulted in similar values of 0.17 (h) for female, male and bisexual plants, and a very low value of h (0.11) was found in non-flowered plants (Kumar and Agrawal 2017). In the present

study, ISSRs exhibited higher values of genetic parameters compared to RAPD primers, and a similar result was observed by Medhi *et al*. (2014) in *Zanthoxylum* species where ISSR primers (na: 1.9802, ne: 1.6145, h: 0.3526, and I: 0.5230) exhibited higher genetic diversity than RAPD primers (na: 1.8182, ne: 1.5571, h: 0.3144, and I: 0.4610).

The comparison of both RAPD and ISSR markers used in the study revealed ISSRs to be more informative and efficient than RAPD markers as it resulted in a high polymorphism percentage. Similar findings with respect to diversity parameters were reported in *Phoenix dactylifera* (Mitra *et al*. 2011), *Moringa oleifera* (Saini *et al*. 2013) and *Memecylon* species (Ramasetty *et al*. 2016). In contrast, RAPD primers detected more polymorphism and were found to be efficient compared to ISSR primers in *Phoenix dactylifera* (Srivashtav *et al*. 2013).

5.1.3. Cluster and Principal coordinates pattern of different sexes of *S. glauca* revealed by RAPD and ISSR markers:

In this study, clustering patterns obtained for RAPD and ISSR data revealed some differences, which might be due to amplification of PCR products from different regions of the genomic DNA by these two methods. Furthermore, the HC and NJ exhibited a similar pattern of clustering for the combined data of RAPD and ISSR markers, and for RAPD markers alone, where males were grouped in a sub-cluster in the NJ tree. Similar results were reported by Bhardwaj *et al*. (2010) in jojoba where most of the female and male genotypes were grouped in distinct clusters, except for few female and male genotypes. In dioecious species jojoba, the genotypes resulted in grouping into small clusters based on sex for RAPD data, whereas for ISSR data male and female genotypes were interspersed and exhibited dendrogram with more complex genetic variation (Sharma *et al*. 2009). Choudhary (2014) reported that in *S. glauca*, the RAPD and ISSR data resulted in a dendrogram that did not show a clear pattern among sexes *i.e.,* female, male, and bisexual genotypes. In date palm, the dendrogram for ISSR markers showed two groups with all-male cultivars except a few, and the other group showed all-female cultivars and two male cultivars (MaleB1 and MaleB2) (Khankahdani and Bagheri 2019).

The maximum information from the data of molecular markers can be obtained by employing PCA or PCoA in combination with cluster analysis (Messmer *et al*.

1992). The ordination methods like PCA and PCoA have more advantages compared to cluster analysis as these methods are used in the detection of populations or individuals that exhibit some intermediation between any two groups (Lessa 1990; Mohammadi and Prasanna 2003). The principal coordinate analysis (PCoA) based on factorial analysis was done in this study to analyze the variation among the genotypes of different sexes based on RAPD, ISSR, and combined RAPD and ISSR binary data generated dissimilarity matrix using DARwin software. The PCoA for RAPD primers showed a similar pattern of grouping as in the NJ tree, where all males fell into one quadrant showing that among males, the dissimilarities were less and thus exhibit high genetic similarity. The genotypes F9 and A1 were distantly located, showing high genetic dissimilarity, *i.e.,* less similarity with other genotypes that was supported by NJ tree and hierarchical clustering. The PCoA for ISSR markers did not result in the grouping of males in one quadrant as M3 was grouped into the other quadrant, and all other genotypes of females and andromonoecious were interspersed in all four quadrants. The F9, A4 and A1 genotypes showed high dissimilarity compared to other genotypes used in the study. The PCoA for combined data of RAPD and ISSR analysis resulted in clear and separate grouping of all-male genotypes in quadrant IV, showing less dissimilarity within them and was similar to NJ tree clustering for combined RAPD and ISSR data. The clustering pattern for combined RAPD and ISSR data of 36 genotypes of *S. glauca* showed similarity with clustering pattern for RAPD data. Thus, the NJ tree and PCoA for RAPD and combined RAPD and ISSR data were highly similar. Similarly in the previous study, genetic polymorphism among different sexes in *S. glauca* using the combined data of RAPD and ISSR markers exhibited higher similarity with clustering pattern of RAPD data (Choudhary 2014). Thus, in this study, the factorial analysis generated by DARwin software was harmonious to dendrogram clustering obtained for combined RAPD and ISSR markers, where closely related individuals with less dissimilarity were grouped together than distinct groups.

Overall, the study revealed higher molecular genetic variability in females whereas it remained low in males of *S. glauca*. These results are in agreement with the previous reports in dioecious species where genetic variations between sexes were found to be different (Hilfiker *et al*. 2004). Although the number of male plants analyzed in the study was lower than other two sexes and the andromonoecious plants with predominance of male flowers and few bisexual flowers exhibited lower genetic

variation than females which justifies higher genetic variation in females compared to other sexes. The differences in genetic variations detected in different sexes of dioecious species could be because of several factors such as distribution patterns of individuals across the populations (de Jong and Klinkhamer 2005), sex ratio (Vandepitte *et al*. 2010), stochastic events (Engen *et al*. 2003) etc.

5.2. SCAR markers to differentiate sexes of *S. glauca* were developed and validated:

5.2.1. RAPD and ISSR markers for identification of sex in *S. glauca*:

S. glauca is a polygamodioecious tree species with female, male and andromonoecious plants segregating into 40-50%, 5%, and 40-50% respectively, which suggested that sex-determining genes might be present on the sex chromosomes (Xu *et al*. 2004; Simon *et al*. 2009). In most of the dioecious species where the determination of sex is genetically regulated, there are no heteromorphic chromosomes and therefore cannot be known by shape or size but can be identified by using genetic markers. With respect to *S. glauca,* there are no morphological and biochemical methods and due to its long vegetative phase, the identification of sex of the plant at early stage is difficult. Like *S. glauca*, in many dioecious plants, the identification of the sex of plants before the reproductive stage is not possible, and therefore for breeding programmes aimed at realizing economic potential, sex identification is of utmost importance.

Thus, the molecular markers would allow understanding the sexually dimorphic nature at the molecular level and help to select females and males/bisexuals at the seedling stage. Studies for sex identification using markers like RAPD and ISSR in *S. glauca* were done previously by other researchers (Savitha *et al*. 2008; Simon *et al*. 2009; Prasanthi *et al*. 2010; Vaidya and Naik 2014; Baratakke and Patil 2014; Choudhary 2014; Ghumatkar *et al*. 2015). In our research work, the above-reported RAPD and ISSR primers did not produce sex-associated markers in genotypes of *S. glauca* analyzed. Thus, we employed new RAPD and ISSR markers to determine their ability to produce sex-linked markers in *S. glauca*.

5.2.1.1. RAPD markers associated with sex were identified in *S. glauca*:

As RAPD primers are random, identification of molecular marker for sex depends on the chance and requires screening of the plants with few to many primers

(Hormaza *et al*. 1994). In the current research, 64 RAPD primers including previously reported random primers were tested initially on 3 bulk samples of *S. glauca* for sex identification using bulked segregant analysis before studying individual DNA samples. The bulk segregant analysis is useful, especially for screening many samples with a large number of primers. The bulk segregant analysis was used for identification of markers for sex type, diseases, species-specific, etc. in several dioecious plants (Nanda *et al*. 2013; Ghumatkar *et al.* 2015; Bortoloto *et al*. 2020). Out of 64 primers tested, 61 primers resulted in amplification in bulk samples of 3 sexes, of which 9 random primers (OPZ-10, OPC-18, OPA-12, OPS-06, OPG-08, OPK-10, OPC-08, OPA-08 and OPE-05) resulted in sex-specific amplification (Table 14). These random primers resulted in bands specific to bulks of female, male and andromonoecious genotypes. Nine random primers were further tested on 15 females, 6 males and 15 andromonoecious individual genotypes of *S. glauca*. Out of 9 primers, the primers OPA-12, OPK-10 and OPA-08 resulted in sex-specific amplification of ~2900 bp and ~1250 bp in females and andromonoecious genotypes, and ~350 bp in andromonoecious genotypes. Thus, OPA-12 and OPK-10 generated specific markers for female and andromonoecious genotypes, and OPA-08 resulted in marker specific to andromonoecious genotypes. The RAPD primers, OPA-12 and OPK-10 have proven useful for sex identification, and to the best of our knowledge, these primers were not used previously for sex identification in *S. glauca*. The primer RP-07 (OPA-08) was previously reported by Choudhary (2014) to produce sex-specific band of 900 bp (male and bisexual specific) in *S. glauca* but in the present study, the primer amplified 900 bp band exhibiting polymorphism without association to sex. The varied results could be due to different genotypes used in these studies. Thus, the present work demonstrated the usefulness of RAPDs in producing sex-specific markers in *S. glauca* as reported previously for other dioecious species.

5.2.1.2. ISSR markers were not effective in sex identification of *S. glauca*:

ISSR primers were successfully employed to determine the sex in dioecious species like jojoba (Heikrujam *et al*. 2014a; Jangra *et al.* 2014) and pointed gourd (Nanda *et al*. 2013; Adhikari *et al*. 2014). The previously reported ISSR primer UBC-814 by Choudhary (2014) when used in the present study exhibited polymorphism without any association with the sex type of *S. glauca*. Thus, studies were performed by screening the bulk DNA samples of *S. glauca* with unreported ISSR primers. Out of 24

ISSR primers screened on bulk DNA of *S. glauca* sexes, 3 primers UBC-899 (~800 bp), UBC-851 (~1600 bp and ~580 bp), and UBC-873 (~1300 bp and ~ 900 bp) produced bands specific to sex in bulk samples. These primers were further tested on individual genotypes of *S. glauca* to determine their ability to distinguish the sex of all genotypes analyzed. All 3 ISSR primers that resulted in sex-specific amplification in bulk samples were unable to detect the sex type in individuals tested. Similar results were observed in previous studies of *S. glauca* by Ghumatkar *et al*. (2015) where sex-associated bands were observed for primers ISSR-824 (female-specific 174 bp band and male-specific 410 bp band) and ISSR-864 (1319 bp and 1537 bp male-specific bands) with bulk DNA samples, but when tested on individual plants, these bands were not associated with sex. Out of 28 ISSR primers tested in *Garcinia indica,* only one primer UBC-881 resulted in amplification of putative sex-specific 1200 bp band which was present in all males except two plants and absent in most of the females (Thatte and Deodhar 2012). Dhawan *et al*. (2013) used RAPD and ISSRs to identify male and females of date palm where 6 RAPD and 10 ISSR primers resulted in sex-specific bands in bulk samples, and when screened on individuals, the RAPD primer OPA-02 alone resulted in the band (1000 bp) specific to males.

5.2.2. SCAR markers for sex identification from previous studies:

In previous studies, few SCAR markers were developed based on RAPD and ISSR primers for distinguishing the sexes. In the present study, these SCAR primers for markers RP-49 SCAR, UBC-815 SCAR, OPA-18 (MSSMS-01), and OPA-08 SCAR-1 & SCAR2 were tested in bulk and individual samples of *S. glauca* collected from PJTSAU location. The SCAR primers for RP-49 SCAR produced 353 bp in all bulk samples, and in individuals, it was randomly present in few individuals of three sexes, which was in contrast to the previous result by Choudhary (2014). The SCAR primers for marker UBC-815 SCAR resulted in amplification of 721 bp band in all bulk and individual genotypes tested. The SCAR primers for OPA-08, SCAR1-F/R showed no amplification and SCAR2-F/R resulted in amplification of ~950 bp band in all bulk samples at different SCAR-PCR conditions tested and therefore was not associated to sex. Likewise in papaya, previously reported 20-mer primers failed to distinguish males/hermaphrodites from female plants as they did not result in sex-specific amplification despite using many PCR conditions (Chaves-Bedoya and Nun˜ez 2007). Thus, SCAR markers developed in previous studies were unable to identify the sex of

genotypes of *S. glauca* used in the present work probably due to the reason that they are genotype specific, hence making it imperative to identify more molecular markers specific to sex in this species.

5.2.3. SCAR marker developed for sex identification:

5.2.3.1. Sequence analysis of sex-specific RAPD bands:

In several dioecious plant species with greater economic values for a specific sex type, the sex-specific RAPD bands generated were cloned, sequenced, and converted into more reliable SCAR markers with high specificity (Liao *et al*. 2009; Khadke *et al*. 2012; Zhou *et al*. 2018). In this study, three RAPD primers OPK-10, OPA-12 and OPA-08 resulted in sex-specific bands of sizes ~1250 bp, ~2900 bp, and ~350 bp, respectively. These bands were cloned, confirmed by digestion with *EcoR*I and *Hind*III enzymes, and their respective plasmids were sequenced. The size of the RAPD bands obtained was 1239 bp (OPK-10), ~1856 bp (OPA-12), and 341 bp (OPA-08), and for OPA-12, the full sequence was not obtained; only 1856 bp was obtained. The BLASTN homology search for 1239 bp, which is female and andromonoecious specific, resulted in no significant similarity with sequences of the database as the band might be unique. Similar results have been reported for date palm (Al-Qurainy *et al*. 2018), jojoba (Jangra *et al*. 2014), *Phoenix dactylifera* (Dhawan *et al*. 2013), *Garcinia gummi-gutta* (Joseph *et al*. 2014) where blast analysis of the cloned sex-specific bands did not exhibit homology with the known sequences in NCBI database. The ORF finder for 1239 bp sequence resulted in 6 ORF's and the protein BLAST analysis for a long protein of 180 aa has shown the presence of putative conserved domain for retrotran_gag_2 representing gag-polypeptide of LTR Copia-type which are present in plants and fungi. The sequence of protein showed homology of 38.06% with retrovirus-related pol polyprotein from transposon RE1 (*Vitis vinifera*). Similar findings have been reported in *Cannabis sativa* using RAPD marker which amplified 1500 bp band specific to males *i.e.* MADC3 (*C. sativa* male-associated DNA sequences) and MADC4, and these sequences show Copia-like retrotransposons encoded by gag/pol polyproteins (Sakamoto *et al*. 2005). In previous studies of *S. glauca* (Baratakke and Patil 2014), the blastx analysis for a male specific band (1100 bp) showed homology with complete sequence of *Oryza alta* isolate 1 retrotransposon Atlantys, and with the partial sequence of *Oryza officinalis* retrotransposon Atlantys whereas the analysis with tblastx revealed

homology of the sequence with unclassified, putative retrotransposon protein of *Oryza sativa*. In *Cannabis sativa*, a DNA sequence associated with males (MADC1) along with its flanking region encoding reverse transcriptase exhibited high homology with LINE-like retrotransposons and ORFs in several plants (Sakamoto *et al*. 2000). In a similar study, Gangopadhyay *et al*. (2007) showed that the RAPD based male-specific SCAR marker exhibited homology with *Ginkgo biloba* reverse transcriptase and tracked by putative retroelement pol polyprotein of *Arabidopsis thaliana* and *Oryza sativa*. They considered that this retroelement sequence of males in future helps to isolate full-length nucleotide sequences and can be used as a male-specific marker. In *Asparagus officinalis*, the female-specific 928 bp RAPD band showed limited homology with repetitive regions of retrotransposon sequences, which might be present in the non-coding genomic region (Gao *et al*. 2007). Li *et al*. (2017) reported that out of 17 sex-linked nucleotide sequences in males of *Humulus scandens*, 7 showed high similarities with retrotransposons. Thus, in the present study, the female and andromonoecious specific band (1239 bp) exhibiting homology for retroelements might also have an association with the sex of female and andromonoecious plants of *S. glauca*.

The primer OPA-12 resulted in female and andromonoecious specific amplification of ~2900 bp band, and homology search for forward and reverse sequences for this band resulted in high similarity of 98.91% with *Phoenix dactylifera* mitochondrion, complete genome, and no ORF's were found. The mitochondrial genome in recent days has gained significance because of its practical use among various species as a basis for genetic variation and possesses a vital role in the growth and development of plants (Ogihara *et al*. 2005). The genomes of mitochondria are much complicated in plants compared to the rest of the eukaryotes (Li *et al*. 2009; Liu *et al*. 2011). Sequence analysis of another male and andromonoecious sex-specific RAPD band, 341 bp (OPA-08) revealed no sequence similarity with the existing database, and ORF's were not detected as the band produced might be unique. The lack of significant similarity of the cloned DNA fragments for the markers obtained in the study might be because they are generally from regions of genomes that are linked to the associated trait and not a part of that gene or genes as suggested previously (Dhawan *et al*. 2013).

5.2.3.2. Effective SCAR markers were identified and validated in *S. glauca*:

The SCAR markers in the present study were developed from three RAPD bands that were specific to sex in *S. glauca* based on the sequences obtained from the amplified bands. SCAR primers are known to be independent of the conditions of PCR reactions and are co-dominant markers that detect single locus and were thus found to be more specific and reproducible compared to RAPD and ISSR primers that are more sensitive to reaction conditions (Paran and Michelmore 1993). For SCAR marker amplification for sex identification, high annealing temperatures were used to avoid non-specific amplification and amplification of other sex types (Joseph *et al*. 2014). The RAPD based SCAR markers were developed for several dioecious species like *Hippophae rhamnoides* (Zhou *et al*. 2018), *Phoenix dactylifera* (Dhawan *et al*. 2013; Al-Qurainy *et al*. 2018), *Garcinia gummi-gutta* (Joseph *et al*. 2014) etc. The markers developed could effectively identify the sex of plants at the vegetative stage before field transfer and further helped to study the basis of sex chromosome evolution, reduce the cost of labor, save time and energy to the farmers, which in turn resulted in highly productive desired plants. In the present study, SCAR markers, Sg SCAR1 (OPK-10 primer) and Sg SCAR2 (OPA-08) have effectively identified the sex of the *S. glauca* plants as they resulted in sex-specific amplification of sizes 1063 bp and 341 bp, respectively in both bulk and individual genotypes of female and andromonoecious samples tested. But the Sg SCAR3 (OPA-12) failed to identify sex in *S. glauca* as it resulted in amplification in bulk and individual samples of all sexes. Similar observations have been recorded in papaya where out of three sex-linked SCAR markers SCAR T1, SCAR T12, and SCAR W11, only SCAR T1 resulted in amplification of 1300 bp in all-female and hermaphrodite individuals regardless of the sex of flowers, and the other two markers exhibited co-segregation of sex-linked markers based on sex (Deputy *et al*. 2002).

The SCAR markers generated in the present study were validated by testing the primers on the genomic DNA from mature trees of *S. glauca* genotypes of known sexes obtained from the IIOR, Narkhoda region and unknown sexes of *in vitro* raised seedlings and plants growing at the University of Hyderabad campus. Recently, Al-Qurainy *et al*. (2018) successfully validated the male-specific SCAR marker using known genders of males and females and also seedlings to differentiate the sex of date palm plants. Similarly, Simon *et al*. (2009) confirmed the efficiency of the marker OPD-

20 (900 bp) linked to sex of *S. glauca* using parental trees, sibling and half-siblings. Gao *et al*. (2007) also verified the female-specific SCAR marker ($SCAR_{928}$) developed based on RAPD fragment on other plants of *Asparagus officinalis* population. In papaya, the hermaphrodite-specific SCAR marker T12 was validated using F_1 hybrids and a large population of seedlings prior to the plantation to predict the plant sex (Deputy *et al*. 2002). In this study, the SCAR markers Sg SCAR1 (OPK-10 primer) and Sg SCAR2 (OPA-08 primer) revealed their ability to identify the sex of *S. glauca* by producing sex-specific bands of 1063 bp (female & andromonoecious specific) and 341 bp (male & andromonoecious specific) in bulk and individuals tested. This was further confirmed by sequencing the 1063 bp and 341 bp bands amplified in Narkhoda samples which resulted in the same sequence as obtained for PJTSAU genotypes. These SCAR markers were able to effectively identify the sex of unknown seedlings based on the amplification obtained for two SCAR markers. Thus, for a particular DNA sample when 1063 bp and 341 bp bands are present in both SCAR-PCR results, then the sex of seedling is andromonoecious; when 1063 bp band is present and 341 bp band absent then it is female type; and when 1063 bp band is absent and 341 bp band is present, then it is male type. Thus, the two-level screening developed in the present study was efficient in identifying the sex of seedlings and eliminates the cultivation of undesired sexes and helps farmers to have female types in the desired ratio, which are having greater commercial value for oil-yielding seeds.

5.2.4. Sequence analysis of RAPD and ISSR polymorphic bands of *S. glauca*:

A few informative polymorphic RAPD and ISSR bands were cloned and sequenced to understand the genome sequence information related to genetic variability and sex determination mechanisms at the molecular level in *S. glauca,* whose complete genome data is not available in the database. Thus, the bands 972 bp (OPD-20), 1201 bp (OPD-12), 1130 bp (OPC-06), 409 bp (ISSR-19) and 414 bp (UBC 873) were selected in the present study for obtaining information about polymorphic bands in *S. glauca*. The primer OPD-20/RP-49 was previously reported to produce sex-specific amplification of 900 bp band in females of *S. glauca* by Simon *et al*. (2009) and Choudhary (2014) but in the present study, it exhibited only polymorphism in all sexes and therefore was cloned and sequenced. The sequencing analysis showed a high similarity of 98.76% with the female-specific SCAR marker (SGS1) of *S. glauca* with accession no. KJ572402.1 (Sarkar and Sikdar 2014, unpublished data). The sequence

analysis of 1201 bp (OPD-12) did not show any significant similarity with BLASTN analysis, and the BLASTP analysis resulted in identification of very short ORF's of which amino acid sequences of 3 ORF's exhibited minimal similarity. The BLASTP analysis of 179 aa sequence showed the presence of putative conserved domain for mule transposase domain of DDE_Tnp_ISL3 superfamily which were previously reported to be sex-associated in *Cannabis sativa* (Sakamoto *et al*. 2000; Sakamoto *et al*. 2005; Li *et al*. 2017) and exhibited homology of 46.59% and 44.91% with PKS-NRPS hybrid synthetase CHGG_01239-like (*Malus domestica*) and Squalene synthase 1 (*Vitis vinifera*), respectively. The enzyme squalene synthase is a membrane-bound protein that catalyzes the first step of the biosynthetic pathway of sterols (Yan *et al*. 2003; Ali *et al*. 2016) to produce squalene (triterpene in plants) from farnesyl diphosphate (FPP) (Zhao *et al*. 2010; Liu *et al*. 2018). The squalene finally results in sterol formation by series of reactions catalyzed by enzymes, and thus it has an essential role in the sterol pathway in eukaryotes. In this study, the BLASTN analysis of 1130 bp (OPC-06) resulted in 88.73% and 79.96% similarity with *Pistacia vera* DNA mismatch repair protein MSH6 (LOC116146751), mRNA, and *Gossypioides kirkii* chromosome K1_07, whereas 54 amino acid sequence of ORF exhibited 50% similarity with hypothetical protein Ahy_B06g080669 isoform B (*Arachis hypogaea*). The BLASTN and BLASTP analysis of 409 bp (ISSR-19) and 414 bp (UBC 873) resulted in no similarity with sequences in the database. Similar results of no similarity with BLASTN analysis have been reported in *Garcinia gummi-gutta* (Joseph *et al*. 2014).

5.3. Phytochemical analysis in dioecious plants to differentiate the genders:

The studies on medicinal plants gained importance in recent years due to increased demand for the natural products in the production of nutraceuticals and drugs. Even though majority of medicinal plants are monoecious, there are also dioecious plants (about 7%) that exhibited medicinal properties (Renner and Ricklefs 1995). The metabolomic approach will lead to understanding the condition of dioecy, which is an adaptation for out-breeding. The chemo- or biodiversity based on gender is beyond and over the chemical diversity noticed within a species based on the seasons, location, and developmental stages of the plants. There might be differences at the metabolite level both qualitatively and quantitatively among the different genders which can benefit to identify the sex of dioecious plants at early stage.

5.3.1. Detection of phytochemical differences from leaf and shoot apex extracts of different genders:

The differences in the metabolites (primary and secondary) within the species based on gender were reported for many dioecious plants. These metabolites exhibited sex-related differences at the level of activity of enzymes like guaiacol-peroxidase and polyphenol-oxidase which are involved in several metabolic pathways responsible for biotic factors (Ruuhola *et al*. 2013). The abiotic factors like drought, salinity, low temperatures, etc. also resulted in varied responses to enzymes activities, chlorophyll content, growth, etc. among females and males (Chen *et al*. 2010; Zhang *et al*. 2011).

The chemical constituents present in plants exhibit different polarities and properties, and therefore, solvents of different polarities were frequently used for the isolation of phytochemical compounds (Arif *et al*. 2016). The yield of different extracts varies depending on the nature of solvents used for the extraction of phytochemicals (Shah *et al*. 2014). The phytochemicals like phenolic compounds and the other bioactive compounds were generally known to be more soluble in polar solvents compared to non-polar solvents (Koffi *et al*. 2010). The preliminary screening of phytochemicals was done in this study using different solvents for three genders (female, male and andromonoecious plants) of *S. glauca* to identify the differences in phytochemical constituents among them. This is the first study to detect the phytochemical differences among genders of *S. glauca*. The solvents of different polarities from polar to non-polar, *i.e.*, methanol, acetone, and hexane, were used for extraction of phytochemicals from leaves and shoot apices of *S. glauca* genotypes. Phenols, tannins and flavonoids were detected in methanolic and acetone extracts of leaf and shoot apex samples and absent in hexane extracts. Thus, phytochemical differences were observed for the solvents used for extraction regardless the sex type. In the present study, the methanolic extracts of both leaves and shoot apices showed strong presence of phytochemicals tested and was consistent with the results reported in other studies (Dhawan *et al*. 2017; Batool *et al*. 2019) and these phytochemicals are known for their health benefits to humans.

5.3.2. Total content of polyphenols, flavonoids, and tannins varied among genders:

Here, the phytochemical differences among genders were interpreted based on the genetic variation with respect to gender but not the environmental factors as the plants studied were from the same environmental conditions. The content of phenols,

flavonoids, and tannins was determined only in methanolic and acetone extracts of leaf and shoot apex of three genders due to the presence of most of the phytochemicals in these extracts. The total contents of phenolics and flavonoids were higher in methanolic extracts of leaves of all 3 genders than in acetone extracts. Among methanolic leaf extracts, males exhibited higher content of phenols (267.28 mg GAE/gDW) compared to females (236.65 mg GAE/gDW) and andromonoecious (257.52 mg GAE/gDW) extracts, and *vice versa* in acetone leaf extracts, where females and andromonoecious extracts exhibited higher phenolic content than male extracts. Nybakken and Julkunen-Tiitto (2013) reported that the content of phenols was high both in males and females of *Salix myrsinifolia*. Similarly, Yosr *et al*. (2018) noticed higher content of polyphenols in vegetative organs of male *P. lentiscus* plants compared to females. In dioecious plants, the sexual differences like high content of phenolic compounds in males or females could not only be related to the reproductive attributes but may also be attributed to putative defense responses to protect from herbivores (Palumbo *et al*. 2007), pathogens, and obtaining resistance for drought or cold conditions (Yosr *et al*. 2018). Makhlouf *et al*. (2016) observed higher total phenolic content in males of date palm as compared to females. The differential biochemical and physiological activities in females (Juvany *et al*. 2014) or higher reproductive and photosynthetic rates mainly in winter in females than in males was linked to lower content of these phenolic compounds in females (Rozas *et al*. 2009). As far as solvents used for extracting phytochemicals, Umesh *et al*. (2015) observed higher content of phenols in water extracts of leaves compared to methanolic and ethanolic extracts in *S. glauca*. Sajeeda *et al.* (2019) reported high content of phenols and carbohydrates in methanolic extracts of leaves of *S. glauca* compared to other extracts used.

The higher content of flavonoids in our work was observed in leaf methanolic extracts of males compared to other genders and extracts used. The flavonoids content was higher in our studies compared to the previous studies for methanolic extracts of *S. glauca* leaves by Umesh *et al*. (2015). In contrast, the methanolic extract of leaves of *S. glauca* in previous studies resulted in the absence of flavonoids but present in ethanolic extracts (Sajeeda *et al*. 2019). The vegetative organs of all periods of the growth cycle of male *Pistacia lentiscus* exhibited significantly higher content of flavonoids, and tannins compared to females (Yosr *et al*. 2018). In *Pistacia atlantica,* significant variations were not detected among the leaf methanolic extracts of male and

female genders for the total content of polyphenols and flavonoids (Amri *et al*. 2018). Zhang *et al*. (2016) observed that the content of total phenolics and flavonoids either increased or decreased among male and female trees of *Pistacia chinensis* Bunge due to different evolutionary pathways involved in them.

The total condensed tannins in the present study showed low content in methanolic and acetone extracts of leaves of all three genders tested. In relation to the sex, the tannin content was found to be relatively higher in acetone extracts of females than other sexes. On the contrary, Umesh *et al*. (2015) reported higher content of condensed tannins in methanolic extracts of leaves of *S. glauca* than detected in our study. The varied observations in the total content of phytochemicals could be because of different factors such as the climatic effects and the genetic variations among the plants growing in different geographic regions as suggested previously in *Pistacia atlantica* (Amri *et al*. 2018).

The methanolic and acetone extracts of shoot apex exhibited a similar pattern as that of polyphenol content in leaf extracts, with highest polyphenol content detected in methanolic extracts of males. The total flavonoids were found to be higher in acetone extracts of andromonoecious genotypes than in other genders. In methanolic and acetone shoot apex extracts, the total content of tannins was low as recorded for leaf extracts. The polyphenols, flavonoids, and tannins were considered as bioactive constituents of plants and act as biological antioxidants by scavenging free radicals and exhibit activity to prevent or inhibit cancer (Ruch *et al*. 1989; Rice-Evans *et al*. 1997; Bravo 1998; Hagerman *et al*. 1998; Fresco *et al*. 2006).

5.3.3. Antioxidant activities of different extracts varied among genders:

Various metabolic processes in the biological systems result in the generation of free radicals that causes damage extensively to the biomolecules and tissues and thereby leads to severe health complications such as chronic inflammation, cancer, diabetes mellitus, and neurodegenerative diseases (Frankel and German 2006; Yazdanparast *et al*. 2008). The phenolics are the known natural antioxidants that exhibit antioxidant activity and thereby contribute to different biological activities (Lee *et al*. 2002). In the present study, the antioxidant activity of different extracts of *S. glauca* was estimated by calculating DPPH free radical scavenging activity and the total antioxidant activity. Our study revealed a strong correlation of total phenolic content with the antioxidant

activity. The total antioxidant activity of male methanolic extracts of leaves and shoot apex samples was high which correlated with the high phenolics present in these extracts that could be responsible for the high antioxidant activity. Among different extracts used in the study, methanolic shoot apex extracts exhibited high total antioxidant activity. Similarly in earlier studies the total antioxidant values were correlated with the content of total polyphenols (Zhu *et al*. 2004; Li *et al*. 2008; Gul *et al*. 2011).

DPPH free radical scavenging assay that exhibits the capacity to quench the free radicals is the most commonly used method for estimating the antioxidant potential of the extracts. The method involves the electron/hydrogen transfer from the extracts with antioxidants to DPPH radicals (Prathapan *et al*. 2010). In the present work, the radical scavenging activity of methanolic and acetone extracts of leaf and shoot apex samples were evaluated among the genders. The phytochemical compounds and antioxidant activity of *S. glauca* leaf extracts were previously reported by researchers (Lakshmi *et al*. 2014; Osagie-Eweka *et al*. 2016; Sridevi *et al*. 2017), but there are no reports for shoot apex extracts as well as no studies were performed to evaluate the differences in phytochemicals and antioxidant activities among the different genders of *S. glauca*. The extracts used in the study displayed the ability to scavenge free radicals in a concentration-dependent way. The maximum scavenging activity was exhibited by methanolic shoot apex extracts compared to leaf extracts and showed correspondence with the total content of polyphenols in the extracts. Interestingly, the methanolic shoot apex extracts that exhibited potent antioxidant activity, resulted in significantly closer values like that of the ascorbic acid standard. The highest DPPH radical scavenging activity was observed for methanolic extracts of shoot apices of males with the IC_{50} value closer to ascorbic acid. With the increase in the total content of polyphenols, there was an increase in the antioxidant activity in the DPPH method, except for male leaf acetone extracts, which resulted in a low IC_{50} value despite having low polyphenol content as compared to leaf methanolic extracts of all genders. Sajeeda *et al*. (2019) reported high scavenging activity for the bark compared to leaves and flower in an aqueous solution. The antioxidant activity for the root bark of *S. glauca* has been recently reported (Osagie-Eweka and Okosun 2019).

5.3.4.1. GC-MS analysis of leaf and shoot apex extracts:

The higher plants comprise metabolites that belong to different classes of phytochemical compounds. In this study, the solvent methanol was used for extraction, followed by heat treatment for GC-MS analysis to identify most of the metabolites of *S. glauca*. The methanol and high temperature (°C) combination results in the inactivation of enzymes in several pathways and prevents changes in the composition of metabolites (Katona *et al*. 1999; Roessner *et al.* 2000). The derivatization reagent N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) used is more efficient to volatilize a wide range of phytochemical compounds in the study and make them accessible for GC analysis (Katona *et al*. 1999). In this study, GC-MS analysis of leaf and shoot apex extracts of genders of *S. glauca* have revealed the differences in the metabolites as evident by the spectra obtained in the chromatogram.

Metabolites differences relating to carboxylic acids, sugars and their derivatives, lipids, organic acids, carbohydrates, organic compounds and amino acids were observed in leaf and shoot apex extracts of different genders. Among all the metabolites analyzed, carbohydrates (including sugar derivatives) and carboxylic acids were found in the highest percentage in all genders of leaf extracts compared to other kinds of metabolites. The leaf and shoot apex extracts of males revealed high carboxylic acid percentage (26% and 21%, respectively) compared to females (22% and 17%) and andromonoecious (13% and 19%) extracts. The leaf extracts of males revealed high carbohydrate percentage (28%) than female (22%) and andromonoecious (26%) extracts. Carbohydrates, which were considered as a basic energy sources in plants, also exhibit a significant role in pollen development under *in vivo* and *in vitro* conditions (Pacini 1996; Clément and Audran 1999; Rolland *et al*. 2002). In *Lilium,* normal microspores development was favoured by the transport of carbohydrates to floral organs during the reproductive phase (Clément and Audran 1999; Castro and Clément 2007). In this study, the carbohydrates content in leaves was in contrast to female sap of date palm, which exhibited higher content of sugar as compared to males (Makhlouf *et al.* 2016). In male and female dioecious plants of *Pistacia chinensis*, the LC/MS and GC/MS analysis revealed 235 metabolites; of which female plants exhibited 20 metabolites in higher content compared to males, and regarding other 36 kinds of metabolites, males exhibited higher content than females which might be attributed to the biological differences between female and male plants (Li *et al*. 2016). Bajpai *et al.*

(2012) observed differences in the spectra obtained for male and female plants of dioecious *Piper betle* and the metabolites of different sexes, resulted in two metabolic types. In this study, the metabolite analysis of leaf and shoot apex of *S. glauca* for amino acids exhibited a higher percentage in andromonoecious extracts (14% and 19%) than females (10% and 16%) and comparably less in males (8% and 11%), respectively. These results corroborate the findings of the previous reports (Khan *et al.* 1982; Dutta and Mazumdar 1989), where leaves showed higher content of amino acids in female plants than in male plants of papaya. El-Yazal (2018) reported a higher concentration of total free amino acids in leaves of female trees of date palm compared to male trees. The reasons for differences in specific metabolites observed in different sexes and their biological significance remain unknown and further research is needed to clarify their relationship with sex.

5.3.4.2. GC-MS analysis revealed the presence of sex hormones in *S. glauca*:

The GC-MS analyzes in the present study revealed characteristic differences of few sex-related metabolites in leaf and shoot apex extracts which are well-known sex hormones in animals. Interestingly in the present study, the secondary metabolite testosterone (testosterone, 17-O-(t-butyldimethylsilyl)-) was present in leaf extracts of male genotypes and not detected in other genders as well as in all genders of shoot apex extracts. Another metabolite epiandrosterone (o-tetramethylen (tert butyl) silylepiandrosterone), which acts as a precursor for testosterone was present in shoot apex extract of andromonoecious genotypes and not detected in other genders and leaf extracts of all 3 genders. Ramesh *et al.* (2017) showed the presence of 5alpha-androatane-3, 17-dione 17-monooxime, androstane-3,17-dione 17-oxime in the leaf ethanolic extracts of *S. glauca* by GC-MS analysis which were known to be endogenous androgen metabolites. In plants, testosterone and other androgens, unlike in animals where they act as only sex hormones, are known to affect not only reproductive development such as floral sex determination and flowering but also vegetative development (Janeczko and Skoczowski 2005; Janeczko 2012).

The GC-MS analysis in the present study also showed the presence of estra-1,3,5 (10)-trien-4-amine in *S. glauca*, which is a steroid sex hormone in mammals. It was detected in leaf extracts of female and andromonoecious genotypes and absent in other extracts. The various scientific reports have suggested that estrogens could affect

growth, flowering, reproduction, stimulate the growth of embryos and seedlings of pea, sunflower, and tomato (Helmkamp and Bonner 1953; Kopcewicz 1969; Sláma 1980; Bhattacharya and Gupta 1981; Guan and Roddick 1988; Janeczko and Skoczowski 2005) and the estrogen application in dioecious plants have affected the sex determination of male and female flowers (Kopcewicz 1971b).

The secondary metabolite, prostaglandins (prostaglandin D(2), O,O'-bis(trimethylsilyl)-, trimethylsilyl ester) was also detected in GC-MS analysis and present in shoot apex extracts of all 3 genders exhibiting no differences among genders, whereas present in leaf extracts of male and andromonoecious genotypes and absent in females. In higher plants, the prostaglandin-like compounds known as phytoprostanes were discovered by Imbusch and Muller (2000) which were produced from α-linolenic acid by auto-oxidation, through a non-enzymatic process. The functions of prostaglandins in short-day plants include flowering, seed germination, protein synthesis, and nucleic acid metabolism, photosynthesis, responses controlled by gibberellic acid, cyclic-AMP-related responses, membrane permeability, photomorphogenesis, and stomata closure (Groenewald and van der Westhuizen 1997 & 2005). The presence of these metabolites as plant hormones was thus supported by previous literature, and further, the mechanism of action of these plant hormones can be understood by using the inhibitors of biosynthesis pathways and by employing mutant studies in plants.

Thus, the present study unravelled the presence of few metabolites specifically in males and females, respectively suggesting that they could be involved in sex determination in *S. glauca*. There is a need to investigate further the underlying mechanisms of differential synthesis in different sexes as well as their roles in sex determination in *S. glauca*.

5.4. *In vitro* plant regeneration was successfully achieved from seedling explants and genetic stability of regenerated plants of *S. glauca*:

5.4.1. *In vitro* regeneration from nodal and shoot apex explants of *S. glauca*:

The nodal explants collected from mature trees exhibited browning due to phenolic exudation whereas the extent of browning was relatively lower from shoot tip explants cultured on MS medium with BAP. The surviving nodal explants showed

slight callus proliferation without any shoot induction, whereas shoot tip explants developed into single shoots without any shoot multiplication after subculture on MS medium with 2 mg/l BAP. Thus, the explants derived from mature trees presented problems of shoot induction in case of nodal explants, and shoot multiplication in shoot tip explants. In *Terminalia bellerica*, Roy *et al*. (1987) used mature nodes for micropropagation whereas Bilochi (2002) used seedling nodes to establish the cultures, both of which resulted in significantly low multiplication efficiency. Rathore *et al*. (2008) achieved micropropagation when they used explants derived from aseptically produced seedlings of *Terminalia bellerica* Roxb. Thus, further studies were conducted to induce seed germination and utilize explants from *in vitro* germinated seedlings for optimizing plant regeneration.

5.4.2. GA_3 enhanced *in vitro* seed germination of *S. glauca*:

The explants derived from seedlings are juvenile and therefore used for *in vitro* regeneration frequently as they have the ability to establish cultures easily (Aitken-Christie and Connet 1992). In this study, *in vitro* germination experiments showed a significant enhancement of seed germination (45.5–88.8%) with the addition of GA_3, whereas the germination frequency was low (3.4–9.2%) without GA_3. The highest germination percentage (88.8%) was achieved on MS full-strength medium with 2.0 mg/l GA_3, while it remained comparatively low (64.7–69.0%) on half-strength MS or half-strength WP medium containing 2 mg/l GA_3. These results signify the importance of GA_3 to germinate *S. glauca,* as reported by Prasanthi *et al*. (2009). Similarly, Mahender *et al*. (2014) reported that half-strength medium constituents (either MS or WP) failed to give optimal *in vitro* seed germination response as compared to MS full-strength medium supplemented with BAP in *Butea monosperma*. The enhancement of seed germination in the presence of GA_3 could be due to increased metabolic activity and growth potential of the embryos, thus causing endosperm weakening (Taiz and Zeiger 2002).

5.4.3.1. Efficient shoot bud induction and elongation in different explants were achieved by altering the BAP and GA_3 concentrations during subculturing:

Efficient *in vitro* shoot multiplication is critical for rapid propagation and depends on the selection of the appropriate explants, media, and growth regulators. BAP has been the most commonly used cytokinin for shoot bud induction and multiplication

in many woody plant species (George 1993; Rathore *et al*. 2004). In this study, 2 mg/l BAP proved to be superior in inducing efficient shoot bud formation, which ranged from 30.8 to 76.2% among the different explants and media tested. The combination of 2 mg/l BAP and 0.2 mg/l NAA was less effective than BAP alone at both induction and proliferation stages. This was evident by the decrease in shoot regeneration frequency (15.5–51.7%) and limited shoot elongation associated with yellowing of leaves during subculturing. Similarly, Malik *et al*. (2005) reported that BAP alone was more effective at promoting multiple shoot formation than the combination of BAP and NAA in *Garcinia indica*. A previous study revealed that the combination of BAP and NAA induced better shoot regeneration than BAP alone from nodal explants of mature trees of *S. glauca* (Shukla and Padmaja 2012); the difference between the previous findings and those in the present study could be due to the different explant source.

TDZ is a potent cytokinin that has been selected for micropropagation of several species of woody plants because of its tremendous ability to stimulate shoot proliferation (Huetteman and Preece 1993). The effectiveness of TDZ (0.5 mg/l) in inducing shoot bud formation in different explants of *S. glauca* as evaluated in this study showed relatively low induction frequencies (10.8–52%) in all explants in comparison to BAP (30.8–76.2%). Similar findings have been reported by Ružić and Vujović (2008), who found that TDZ was less effective than BAP on shoot multiplication of sweet cherry cv. Lapins (*Prunus avium* L.). The superiority of BAP for shoot organogenesis could be due to the ability of the plant tissues to metabolize BAP more readily than other growth regulators (Zaerr and Mapes 1982).

In the present work, the explants displayed a higher capacity for shoot bud formation on MS medium (15.6–76.2%) than on WP medium (10.8–63.8%) regardless of the growth regulators used. Thus, it can be suggested that higher concentrations of ions along with 2 mg/l BAP are optimal for shoot bud induction in *S. glauca*, as total ionic concentrations of MS medium are higher than in WP medium.

Dudhare *et al*. (2014) reported that *in vitro* shoot multiplication response of *Simarouba* was relatively low. In the present work, similar difficulties were encountered in the initial shoot multiplication experiments due to the limited capability of the shoot buds to elongate and form shoots. Repeated experiments using subculture manipulations helped to overcome the problem of poor shoot elongation. This approach for obtaining

enhanced proliferation of shoot buds involved at least three passages on medium with 2 mg/l BAP. The elongation of shoots and their continued growth from all explants except epicotyls were promoted by the gradual reduction of the BAP level (from 1.0 down to 0.2 mg/l) during subculturing and the subsequent inclusion of GA_3 (0.2–0.5 mg/l) in the later stages of regeneration. Similarly, Kaushik *et al*. (2015) reported that the addition of GA_3 to medium supplemented with BAP and kinetin enhanced the shoot elongation by 2.33-fold in *Ophiorrhiza mungos* Linn.

In the present study, the explants differed in their capability for shoot bud induction, proliferation, and shoot elongation, with the superior response observed from cotyledon explants in terms of shoot bud induction frequency (76.2%) and the number of elongated shoots (9.1 shoots per explant). Although higher numbers of shoot buds proliferated per explant from root nodes (28.5) and hypocotyls (27.5), the shoot elongation response remained lower (5.1–5.7 elongated shoots per explant) than that of cotyledons. Quoirin *et al*. (1998) opined that the requirements for exogenous plant growth regulators vary with explant type and apparently depend on the endogenous level of plant growth regulators. As the shoot proliferation rate from shoot tips remained low in this study, further investigations are needed to improve the shoot multiplication rate as it would allow the recycling of shoots for long periods during *in vitro* culture.

5.4.3.2. IBA pulse treatment enhanced *in vitro* root induction:

One of the difficulties encountered in the micropropagation of tree species is the induction of adventitious roots from shoots (Cha-um *et al*. 2011). In the present study, pulse treatment of the regenerated shoots with IBA (10 mg/ml for 5 min) resulted in better root induction than untreated shoots on the media tested. The untreated shoots failed to root on full-strength WP or MS media, whereas root induction varied from 10.4 to 33.3% on half-strength WP or MS media, with or without IBA. Furthermore, pulse-treated shoots exhibited higher root induction (54.3%) on half-strength WP medium as compared to those on full-strength WP or either strength of MS media without IBA. Overall, the highest root induction frequency (66.7%), the highest number of roots per shoot (11.3), and maximum root length (3.4 cm) were observed from the pulse-treated shoots on a half-strength WP medium containing 0.2 mg/l IBA. Similarly, a low salt concentration in the medium in combination with IBA resulted in better root induction from shoots in different plant species (Rai *et al*. 2010; Phulwaria *et al*. 2012).

Rout *et al*. (1999) reported that a high concentration of IBA was necessary with a full-strength MS medium for *in vitro* rooting of *S. glauca*. Warakagoda and Subasinghe (2013) reported root induction from micro shoots of *Pterocarpus santalinus* after exposure to pulse treatment with 0.05 mg/ml IBA for 12 h and subsequent culture on half-strength MS medium containing 0.1 mg/l IBA. Woodward and Bartel (2005) suggested that root induction achieved by IBA treatment could be due to an endogenous increase in IAA concentration. In the present study, the *in vitro* regenerated plants were acclimatized and established in the glasshouse with a high success rate of 80%, indicating the suitability of the protocol for micropropagation of *S. glauca*.

5.4.4. RAPD and ISSR markers revealed high genetic similarity of the regenerated plants with the donor seedling:

Regeneration of plants through direct shoot organogenesis without any intermediary callus formation is generally expected to give rise to true-to-type plants, but occasionally, genetic variations arise at the phenotypic, cytological, and DNA sequence levels (Brown *et al*. 1993; Chen *et al*. 1998). It is therefore essential to ascertain the suitability of a regeneration protocol in the production of true-to-type plants in tree species where quality planting material is the primary consideration. In most of the reports with other plant species, the genetic stability analysis of micropropagated plants *vs*. donor using molecular markers revealed 100% monomorphic bands. In this work, the percentage of polymorphic loci generated by ISSR primers was slightly higher (5.8%) than RAPD primers (5.3%) between donor seedling and regenerated plants. The 14 RAPD and 15 ISSR primers employed in the study resulted in many monomorphic bands (1203 and 1075, respectively), with a low percentage (1.6–2.6%) of polymorphic bands in the regenerated plants. Overall, 6 RAPD and 5 ISSR primers generated one to two polymorphic bands absent from a few regenerated plants or the donor seedling. The appearance or disappearance of RAPD and ISSR bands in the plants analyzed could be the result of changes in the DNA sequences of the primer binding sites or changes that could have altered the sizes or prevented the amplification of DNA fragments. Similarly, polymorphisms between the tissue culture products (calluses, adventitious shoots, and regenerated plants) and seedlings were detected by RAPD and ISSR analysis in *S. involucrata* (Yuan *et al*. 2009). Modgil *et al*. (2005) detected a high level of polymorphism (23.2%) in 10 micropropagated plants regenerated through axillary buds of clonal apple (*Malus pumila*

Mill.) rootstock using RAPD markers. In the study by Sharma *et al*. (2011), the proliferation of axillary buds from nodal explants was done, and the micropropagated plantlets exhibited 4 polymorphic bands out of 177 total bands in the 2nd subculture, whereas no polymorphic bands were detected in 8th as well as 16th subcultures by RAPD primers in *Jatropha curcas*. Thus, the present study showed that the regeneration procedure employed in the study does not cause major genetic changes in the RAPD- and ISSR-amplified DNA regions in the regenerated plants. In the present study, the low level of genetic variation detected in the regenerated plants of *S. glauca* using RAPD and ISSR markers could be mainly due to the use of BAP, which has been implicated in inducing genetic changes during tissue culture in other plant species (Hossain *et al*. 2003; Kaushal *et al*. 2004). As the genetic stability of the regenerated plants derived from a single donor seedling was analyzed in this study, further studies with molecular markers using a greater number of regenerated plants obtained from different seedlings and explants would be useful in analyzing the extent of genetic similarities and variations among the regenerated plants and seedlings before practical utilization.

Summary and Conclusions

6. SUMMARY AND CONCLUSIONS:

S. glauca, a polygamodioecious tree species is of great economic importance valued for seed oil with industrial applications and for its medicinal properties. The plant shows three different sexes, *i.e.* females, males and andromonoecious types, with females having more economic importance because of seeds. The knowledge of genetic variability present in different sexes would be helpful for its breeding programmes aimed at genetic improvement. There are no morphological characteristics that can be used for differentiating the sex at the juvenile stage and hence molecular markers hold promise for early identification of sex. Studies on phytochemical differences in relation to sex have not yet been explored in this species. There is also a need to develop efficient *in vitro* regeneration methods for different applications. The present study was aimed at analyzing the molecular genetic variability in different sexes and identification of molecular marker(s) linked to sex. The phytochemical constituents in leaf and shoot apex extracts of different sexes were analyzed to identify differences, if any related to sexes of *S. glauca*. Additionally, the study aimed at developing efficient *in vitro* regeneration system using explants obtained from mature plants and seedlings which can be used for rapid multiplication, conservation and genetic transformation of the species.

6.1. RAPD and ISSRs revealed molecular genetic variability in different sexes of *S. glauca:*

In the present study, 61 RAPD and 24 ISSR primers were used to assess the molecular genetic variability among 36 genotypes of different sexes in *S. glauca*. The extent of genetic polymorphism revealed among different sexes remained higher for ISSR markers than RAPD markers which could be due to different genomic regions targeted by these markers. Two RAPD primers (OPA-11 and OPC-05) and one ISSR primer detected 100% polymorphism. The genetic variability was assessed based on PIC and genetic diversity parameters such as the observed number of alleles (na), effective number of alleles (ne), Shannon's information index (I), and Nei's gene diversity (h). The RAPD markers for female, male and andromonoecious genotypes exhibited mean PIC values of 0.07, 0.09, and 0.10, respectively. For ISSR markers, the mean PIC values for females, males and andromonoecious genotypes were 0.16, 0.08 and 0.13, respectively. The mean values of genetic diversity parameters such as na, ne,

h, and I for RAPD primers were 1.45, 1.27, 0.16, and 0.24, and for ISSR primers were 1.62, 1.36, 0.21, and 0.31, respectively. The mean values of combined RAPD and ISSR primers for na, ne, h, and I were 1.49, 1.29, 0.18, and 0.26, respectively, which were closer to the values detected for RAPD primers.

Based on the dissimilarity matrix, the NJ tree for RAPD markers resulted in grouping of all females, males and andromonoecious genotypes into sub-clusters except for a few females and andromonoecious genotypes, and the PCoA analysis supported NJ clustering. The HC was almost similar except for all males that grouped under two sub-clusters. The combined RAPD and ISSR data for NJ, HC and PCoA resulted in similar clustering as for RAPD markers. The HC and NJ tree for ISSR markers exhibited a similar pattern of grouping, with grouping of males into sub-cluster whereas females and andromonoecious genotypes were interspersed. The PCoA for ISSR markers did not support the HC and NJ pattern of clustering for males as they were found interspersed along with other genotypes in the study. Overall, few differences were observed in clustering patterns of RAPD and ISSR markers, but the study showed the specific grouping of males together as they exhibited less genetic variation within them compared to other genotypes that was evident by PIC values.

6.2. Molecular markers developed for identification of sex in *S. glauca*:

S. glauca has a long vegetative phase, and there are no morphological and biochemical characteristics that can be used for the identification of sex at the juvenile stage. Usually, in most of the dioecious species, the male and female genders possess sex-specific commercial importance. Therefore, there is a need for distinguishing the gender at the juvenile stage in *S. glauca* which has sex-specific economic value to avoid unnecessary expenditure, minimize overall cost for farmers and facilitate large-scale plantations of *S. glauca* with the desired sex ratio of female and male plants. Nine RAPD primers (OPZ-10, OPC-18, OPA-12, OPS-06, OPG-08, OPK-10, OPC-08, OPA-08, and OPE-05) showed sex-specific bands in bulk samples of 3 sexes. When these RAPD primers were further tested on individual plants of different sexes, only 3 RAPD primers, namely OPA-12, OPK-10, and OPA-08, resulted in sex-specific amplification of ~2900 bp and ~1250 bp bands in females and andromonoecious genotypes, and ~350 bp band in andromonoecious genotypes, respectively. Among 24 ISSR primers used, 3 primers, namely, UBC 899 (~800 bp), UBC 851 (~1600 bp and ~580 bp), and UBC 873

(~1300 bp and ~900 bp), amplified sex-specific bands in bulk samples of female, male and andromonoecious genotypes. However, these 3 ISSR primers failed to produce sex-specific bands when screened on individual samples of all 3 sexes.

The sex-specific bands produced by RAPD primers were cloned, sequenced, and converted into more reliable SCAR markers based on those sequences. The sequencing of the cloned fragments of 1239 bp (OPK-10), and 341 bp (OPA-08) resulted in complete sequence whereas partial sequence of 1856 bp was obtained for 2900 bp cloned fragment of OPA 12. The BLASTN homology search for 1239 bp and 341 bp resulted in no significant similarity with sequences of the database as the band might be unique. The protein BLAST for 1239 bp band resulted in 180 amino acids showing the presence of putative conserved domain for retrotran_gag_2 representing gag-polypeptide of LTR Copia-type, which are present in plants and fungi. For ~2900 bp band, the homology search for forward and reverse sequences showed a high similarity of 98.91% with *Phoenix dactylifera* mitochondrion, complete genome, and no ORF's were found. The SCAR markers were developed based on sequencing and validated on 51 genotypes of known sex types from two locations (PJTSAU, IIOR), unknown sexes of *in vitro* raised seedlings, and 2-3-year-old plants growing in the University of Hyderabad campus. The SCAR markers, Sg SCAR1 (OPK-10 primer) and Sg SCAR2 (OPA-08 primer) revealed their ability to identify the sex and showed 1063 bp band in female and andromonoecious genotypes and 341 bp band in male and andromonoecious genotypes, respectively. Thus, the present study led to development of 2 SCAR markers that were effective in differentiating the sex at the juvenile stage.

6.3. Differences in phytochemicals and antioxidant enzymes detected among genders of *S. glauca*:

S. glauca is a tree species of high economic importance valued for seed oil and medicinal properties. The phytochemical differences with respect to gender have been reported in few dioecious species. In this study, the phytochemical constituents of leaf and shoot apex of different sexes were investigated to identify the differences related to sex. The phytochemical analysis revealed differences based on the polarity and gender of the extracts. The total antioxidant activity of methanolic extracts was higher for shoot apex than leaves, with higher activity observed in males than females in *S. glauca*.

The GC-MS analysis revealed differences in the number and percentage of metabolites among genders for leaf and shoot apex extracts. A few metabolites in leaf and shoot apex extracts were found to be sex related in *S. glauca*. The metabolites detected in the present study are the well-known sex hormones in humans, such as testosterone (leaf extracts of males), epiandrosterone (andromonoecious shoot apex extracts), estrogen (female and andromonoecious leaf extracts), and prostaglandin (shoot apex extracts of all three genders and leaf extracts of male and andromonoecious genotypes only). The earlier studies by several researchers also reported the presence of these animal hormones in plants that naturally occur as integral plant compounds and might involve cell-cell communication regulation and exhibit a physiological role in the growth, development, and reproduction of plants. Further studies are needed to confirm the presence of animal hormones in other plants and know their relationship with the sex.

6.4. Efficient *in vitro* plant regeneration system was established for propagation of *S. glauca* from seedling-derived explants and regenerated plants exhibited high genetic similarity:

In vitro experiments carried out initially with nodal and shoot tip explants obtained from mature trees have not yielded favourable results due to lack of shoot induction from nodal explants and production of single shoots (47.36%) without any shoot multiplication from shoot tip explants. Subsequently, explants obtained from *in vitro* raised seedlings were used for achieving *in vitro* regeneration which could be utilized for rapid multiplication of desired sex after it is identified at the seedling stage with the molecular markers developed in the study. The study was successful in establishing efficient regeneration method from different explants of *S. glauca*. Shoot bud induction and proliferation in different explants were best achieved on full-strength MS medium with 2 mg/l BAP, while a step-wise reduction in BAP levels (1.0, 0.5, and 0.2 mg/l) along with the addition of GA_3 (0.2 and 0.5 mg/l) during subculturing resulted in shoot elongation, with the highest number of shoots being produced from cotyledon explants compared to other explants. The IBA pulse treatment of the shoots proved to be effective for root induction, with the highest frequency of root induction achieved on half-strength WP medium with 0.2 mg/l IBA than the untreated shoots. The *in vitro* regenerated plants were acclimatized in the culture room and later established with a high success rate of 80% in the glasshouse, signifying the utility of protocol for

micropropagation of *S. glauca*. Furthermore, the RAPD and ISSR primers used in the study revealed high genetic similarity in regenerated plants and donor seedling.

Major findings from the present study:

- The RAPD and ISSR markers used in the present study for genetic variability analysis among different sexes of *S. glauca* revealed that ISSRs (61.48%) were more efficient than RAPD (46.8%) markers as it resulted in a higher polymorphism percentage, higher average number of polymorphic loci per primer, higher average PIC value and genetic diversity parameter (na, ne, h, and I) values.
- The values of dissimilarity indices for both RAPD and ISSR markers revealed that genetic variation was lesser in males compared to females, and andromonoecious genotypes. Thus, males are closely related compared to other sexes as supported by PIC values, cluster analysis, and PCoA.
- The neighbor-joining tree (UnWeighted Neighbor-Joining), hierarchical clustering (UPGMA) and PCoA (Factorial analysis) for RAPD markers and combined RAPD and ISSR data exhibited a similar pattern of clustering except for a few differences and showed grouping of all males in one sub-cluster due to fewer dissimilarities among males.
- Out of 61 RAPD and 24 ISSR primers tested, 3 random primers resulted in sex-specific amplification of 1239 bp, 341 bp, and ~2900 bp in bulk and individual DNA samples. The ISSR primers, even though they were efficient in detecting higher polymorphism, they failed to generate sex-specific amplification.
- Cloning and sequencing of the 1239 bp band showed the presence of putative conserved domain of retrotran_gag_2, ~2900 bp band showed 98.91% similarity with *Phoenix dactylifera* mitochondrion, complete genome, and 341 bp band showed no significant similarity.
- Two SCAR markers (Sg SCAR1 and Sg SCAR2) were successfully developed and proved to be effective in differentiating female, male and andromonoecious genotypes of *S. glauca* using the two-level screening approach developed in the study.
- The leaf and shoot apex methanolic extracts of *S. glauca* showed good antioxidant activities. The highest total antioxidant activity was observed for methanolic extracts of shoot apex followed by leaves in males as compared to

other sexes as evident by total antioxidant and DPPH free radical scavenging activities.

- The leaf and shoot apex extracts showed differences with respect to carboxylic acids, sugars and their derivatives, lipids, organic acids, carbohydrates, and amino acids in different sexes.
- The GC-MS analysis also showed differences in sex-related metabolites like testosterone, epiandrosterone, estrogens and prostaglandins among genders.
- The seedling derived explants differed in their capability for shoot bud induction, proliferation, and shoot elongation, with the superior response observed from cotyledon explants in terms of shoot bud induction frequency (76.2%) and the number of elongated shoots (9.1 shoots per explant) per explant. The step-wise reduction in BAP levels (1.0, 0.5, and 0.2 mg/l) along with the addition of GA_3 (0.2 and 0.5 mg/l) during subculturing helped in overcoming the shoot elongation problem in *S. glauca*.
- The pulse treatment of the regenerated shoots with IBA (10 mg/l for 5 min) resulted in better root induction than untreated shoots on the media tested with the highest root induction frequency (66.7%), the number of roots per shoot (11.3), and maximum root length (3.4 cm) on a half-strength WP medium containing 0.2 mg/l IBA.
- The RAPD and ISSR primers employed for genetic stability analysis produced high number of monomorphic bands with only 1.6-2.6% bands being polymorphic among regenerated plants and donor seedling indicating the efficiency of the system in producing a large number of genetically similar plants.

Conclusions and future perspectives of the study:

The genetic variability among different sexes revealed in the study using RAPD and ISSRs would be useful in breeding programmes of this tree species aimed at its improvement. The study of molecular marker analyzes for sex identification has successfully identified and produced two SCAR markers linked to sex which were able to differentiate female, male and andromonoecious genotypes effectively. The genetic variability and sex-identification studies facilitate in future to understand the sex determining mechanisms in this polygamodioecious tree species, *S. glauca*. The study revealed differences in metabolite profiles and antioxidant activities and most

importantly sex-related metabolites that are similar to animal hormones were identified for the first time in *S. glauca*. Thus, the present study helped to understand the nature of metabolites present in leaves and shoot apex of *S. glauca*. It would be of interest to confirm the presence of these animal hormones in other dioecious plants which can lead to identification of novel pathways involved in the synthesis of sex-related metabolites and also would pave way for determining the sex based on metabolites in plants. Studies on relationship between the gender and the medicinal properties of the plant based on metabolites are also worth pursuing. The *in vitro* regeneration from seedling derived explants established in the study could be utilized for rapid multiplication, conservation, genetic transformation and secondary metabolite production of this species.

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PLANT TISSUE CULTURE

Efficient in vitro plant regeneration from seedling-derived explants and genetic stability analysis of regenerated plants of *Simarouba glauca* DC. by RAPD and ISSR markers

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Abstract *Simarouba glauca* DC. is a multipurpose tree species known for oil, timber, and medicinal properties. The application of biotechnological methods for genetic improvement of this species depends on the availability of an efficient plant regeneration system. In this study, the shoot regeneration potential of various seedling-derived explants was assessed after culturing on Murashige and Skoog (MS) and woody plant (WP) medium containing different growth regulators. The explants differed in their capacity for shoot bud formation and subsequent shoot elongation on the media tested. Shoot bud induction was achieved at a high frequency (44.8–76.2%) from different explants on MS medium with 2 mg L^{-1} 6-benzylaminopurine (BAP) as compared to other media tested. Cotyledons exhibited the highest capacity for shoot bud induction (76.2%) and shoot elongation (9.1 elongated shoots per explant). The *in vitro*-regenerated shoots rooted at a frequency of 66.7% after pulse treatment in 10 mg mL^{-1} indole-3-butyric acid (IBA) solution for 5 min followed by culture on half-strength WP medium with 0.2 mg L^{-1} IBA. The regenerated plants were acclimatized and established in the glasshouse with a survival rate of 80%. Molecular characterization of regenerated plants using 14 random amplified polymorphic DNA (RAPD) and 15 intersimple sequence repeat (ISSR) primers revealed a high number of monomorphic bands, with only 1.6–2.6% of the bands being polymorphic. The regeneration system established in the study has the potential to be used for rapid multiplication, conservation, and genetic transformation of this species.

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Introduction

Simarouba glauca DC. (Simaroubaceae) is a multipurpose fast-growing tree grown for oil, wood, and medicinal properties. It grows well in tropical and subtropical climates, with high adaptability to different soil types. It is native to South and Central American countries and was introduced into India (state of Orissa) during the 1960s. Subsequently, its cultivation was extended into the states of Gujarat, Maharashtra, Tamil Nadu, Karnataka, Andhra Pradesh, and Telangana. The seeds of *Simarouba* contain 60–75% oil, and the refined oil is used in the manufacture of vegetable fat or margarine (Armour 1959; Joshi and Hiremath 2001). The seed oil of *S. glauca* is considered to be a potential source of biodiesel and also used in the manufacture of soaps, lubricants, paints, polishes, and pharmaceuticals. Several medicinal properties such as antidysenteric, antiherpetic, antihelminthic (Roig 1974), antiprotozoal (Franssen *et al.* 1997), antibacterial (Caceres *et al.* 1990), antitumor (Rivero-Cruz *et al.* 2005), antimalarial (Rodriguez *et al.* 2006), and skin depigmentation (Bonte *et al.* 1997) activities have been attributed to extracts, fractions, and isolated constituents of *S. glauca*.

S. glauca is mainly propagated through seeds. However, the loss of seed viability within 2–3 mo at ambient conditions, physical dormancy imposed by its hard seed coat, and germination problems are the limiting factors for mass propagation (Orwa *et al.* 2009; Patil and Awate 2014). Previous studies showed that mechanically broken seeds germinated faster than intact seeds and that plant growth regulators such as gibberellic acid (GA_3), 6-benzyl aminopurine (BAP), and salicylic acid (SA) are involved in breaking physical

dormancy in *Simarouba* (Patil and Gaikwad 2011). Vegetative propagation through grafting, air layering, and cutting has been reported previously (Joshi and Joshi 2002) but has practical limitations. *In vitro* propagation offers several advantages over conventional propagation methods such as rapid multiplication, season-independent production of plants, production of disease-free plants, and germplasm conservation (George and Debergh 2008).

The best source of explants for micropropagation of tree species are nodal explants or shoot tips obtained from mature trees, as they are expected to produce true-to-type plants. However, micropropagation using explants from mature trees is generally difficult due to problems of phenolic exudation, microbial contamination, browning of media and explants, and recalcitrance to *in vitro* regeneration (Amin and Jaiswal 1987). Alternatively, micropropagation of plant species can be carried out using explants of germinated seedlings, which are easily available and exhibit high regeneration capacity. However, the seedlings of outcrossed plant species are genetically heterogeneous and heterozygous and might not retain the desirable characteristics of the donor plant, thus limiting their use for micropropagation and genetic transformation. The disadvantages of using seedlings of unknown genotypes for micropropagation and genetic transformation can be minimized by using seeds produced by controlled pollination of plants of known genetic background or apomictic seeds. The regenerated plants derived from heterozygous seedlings, however, could be used for purposes of reforestation and plantation programs where genetic variation is required.

There are relatively few published papers on *in vitro* propagation of *S. glauca* using juvenile or mature explants. Regeneration of plants through somatic embryogenesis and organogenesis has been achieved in *S. glauca* by various research groups and using different explants. Rout and Das (1994a) first reported somatic embryogenesis in callus cultures derived from immature cotyledons of *S. glauca* using BAP and α-naphthaleneacetic acid (NAA). From those cultures, 20–25% of the somatic embryos germinated and regenerated into plants. Subsequently, Rout and Das (1994b) showed an enhancement in the frequency of somatic embryogenesis to 80% from immature-cotyledon-derived callus cultures using BAP, NAA, and ascorbic acid in Murashige and Skoog (MS) medium. In later studies, somatic embryos were also produced from immature zygotic embryos using BAP and NAA, of which 25% germinated and developed into plants (Das 2011). Jyothi *et al.* (2014) reported that a combination of NAA and 2,4-dichlorophenoxyacetic acid (2,4-D) was effective in induction of friable embryogenic callus from leaf explants of *S. glauca*.

In vitro shoot multiplication using nodal explants of *S. glauca* has been reported by Rout and Das (1995), with induction of 5.83 shoots per explant in MS medium with BAP and NAA. Rout *et al.* (1999) showed that microshoots produced from nodal explants could be rooted on MS medium with IBA and sucrose. Shukla and Padmaja (2012) reported better regeneration capacity from nodal explants than from shoot tip explants on MS medium with BAP and NAA. Recently, Dudhare *et al.* (2014) attempted *in vitro* propagation of *S. glauca* using shoot tips and axillary buds and observed that the shoot multiplication response from both explants was low on MS medium with BAP and indole-3-acetic acid (IAA). Thus, there is still a need to develop an efficient regeneration system that can be utilized for large-scale plant propagation, genetic transformation, conservation, and phytochemical production in this plant species.

Tissue culture-induced variation (somaclonal variation) is quite common and can limit the utility of an *in vitro* regeneration system for various applications (Salvi *et al.* 2001). In tree species with long generation times, assessing genetic variation by morphological, karyotypic, and isozyme analysis has several limitations (Gupta *et al.* 2009), and therefore, DNA-based markers are being increasingly used for this purpose. Among DNA markers, random amplified polymorphic DNA (RAPD; Williams *et al.* 1990) and intersimple sequence repeat (ISSR; Zietkiewicz *et al.* 1994) markers have been widely used in the genetic analysis of micropropagated plants in many plant species. Several researchers reported that regenerated plants were found to be genetically stable using RAPD and ISSR analysis in banana (*Musa* spp.; Venkatachalam *et al.* 2007), *Sapindus trifoliatus* (Asthana *et al.* 2011), *Dendrocalamus strictus* (Goyal *et al.* 2015), and *Ceropegia evansii* (Chavan *et al.* 2015). In contrast, genetic variations have been reported in regenerated plants in comparison to the donor seedling using RAPD and ISSR markers in almond (*Prunus dulcis*; Martins *et al.* 2004) and *Saussurea involucrata* (Yuan *et al.* 2009). It has been suggested that the presence or absence of genetic variation depends on several factors such as the genotype, type of explant, mode of regeneration, growth regulators, and number and duration of subcultures (Goto *et al.* 1998; Martins *et al.* 2004). Thus, it is essential to ascertain the genetic uniformity of *in vitro* regenerants of any plant species before commercial exploitation.

The present study aimed at the development of a method for rapid *in vitro* propagation of *S. glauca* from seedling-derived explants along with the analysis of genetic stability of the regenerants using RAPD and ISSR markers. The results reported in this study differ from previously published papers in terms of demonstrating the shoot regeneration potential of seedling-derived explants as well as describing a method for achieving high rates of shoot bud induction, shoot elongation and rooting of regenerated shoots, and plant establishment in the greenhouse. This is also the first report to assess the genetic stability of the regenerated plants from different explants by using RAPD and ISSR primers.

Materials and Methods

Plant material and *in vitro* seed germination Mature seeds of *S. glauca* were collected from 6- to 7-yr-old trees growing at Professor Jayashankar Telangana State Agricultural University, Rajendranagar, Hyderabad, and used for *in vitro* seed germination. The seeds used in this study are expected to be heterozygous as they were collected from open-pollinated trees. The seeds were initially treated with 2% Bavistin (BASF India Ltd., Mumbai, India) for 10 min and rinsed two to three times with double-distilled water. Further sterilization was carried out in the laminar flow chamber with 70% (*v*/*v*) ethanol (Hayman, Witham, UK) for 5 min, 0.4% (*v*/*v*) sodium hypochlorite (Fisher Scientific, Mumbai, India) for 5 min, and 0.1% (*w*/*v*) mercuric chloride (Sisco Research Laboratories Pvt. Ltd., Mumbai, India) with 50 μL (for 100-mL solution) of Tween 20 (Sigma-Aldrich®, St. Louis, MO) for 10 min; seeds were rinsed two to three times thoroughly with double-distilled water after each sterilization step. The seeds were inoculated on full-strength or half-strength MS (Murashige and Skoog 1962) or woody plant (WP) (Lloyd and McCown 1981) medium containing 3% (*w*/*v*) sucrose (Sisco Research Laboratories Pvt. Ltd.) and 0.8% (*w*/*v*) phyto agar. All the growth regulators including phyto agar used in the study were procured from Duchefa Biochemie, Haarlem, The Netherlands. The pH of the media was adjusted to 5.6–5.8 with 1 N NaOH or 1 N HCl before adding 0.8% (*w*/*v*) phyto agar and autoclaved at 121°C for 15 min. Different concentrations of GA_3 (0.5, 1, and 2 mg L^{-1}) were added to the media to study their effects on seed germination. GA_3 (10 mg mL^{-1} stock with ethanol as solvent) was filter sterilized using a Millex-GV filter unit (Millipore, Carrigtwohill, Ireland) and added to the autoclaved medium (at about 50°C) in the laminar flow chamber. Two seeds were inoculated into each culture bottle (60 × 110 mm) containing about 30 mL of medium. The cultures were incubated in the dark for 1 wk and then moved to culture racks under light provided by white fluorescent tubes (65 μmol photons m^{-2} s^{-1}; OSRAM Lighting India Private Limited, Haryana, India) for a 16-h photoperiod at 25 ± 2°C. The observations on duration for germination, percentage of germination, and length of the seedlings (cm) were recorded after 4 wk of culture.

***In vitro* shoot regeneration from explants derived from aseptically germinated seedlings** Various explants (cotyledons, cotyledonary nodes, hypocotyls, epicotyls, root nodes, shoot tips, leaves, and petioles) obtained from 25-d-old *in vitro*-germinated seedlings of *S. glauca* were used for regeneration studies. In preliminary experiments, the cotyledons were cultured on MS medium with BAP (0.5, 1.0, 2.0, and 3.0 mg L^{-1}) or thidiazuron (TDZ; 0.1, 0.5, and 1.0 mg L^{-1}), either individually or in combination with NAA (0.1, 0.2, and 0.5 mg L^{-1}). MS medium with 3.0% sucrose and 0.8% phyto agar was prepared as described previously for *in vitro* seed germination. The growth regulators were added to the autoclaved media and then dispensed into culture bottles in the laminar flow chamber. The stock solution of TDZ (5 mg mL^{-1}) was prepared in DMSO (Sigma-Aldrich®), whereas BAP and NAA stocks (5 mg mL^{-1}) were prepared using 1 N NaOH as a solvent and sterile double-distilled water as diluent. The optimal concentrations of BAP, TDZ, and these cytokinins combined with NAA were selected for further studies based on shoot regeneration frequency, number of shoot buds, low callusing, and lack of yellowing of shoot buds (data not shown). The optimized concentrations of growth regulators, i.e., 2 mg L^{-1} BAP alone or in combination with 0.2 mg L^{-1} NAA, or 0.5 mg L^{-1} TDZ alone, were used in MS or WP medium to evaluate the shoot regeneration response from different explants. During the initial experiments, the responding explants with shoot buds were repeatedly subcultured on MS medium with BAP (0.5–2.0 mg L^{-1}) alone or in combination with 0.2 mg L^{-1} NAA for shoot bud proliferation or multiplication. In later studies, clusters of shoot buds/shoots produced from explants on MS medium with 2.0 mg L^{-1} BAP were divided into two to three pieces and subcultured on medium containing different concentrations of BAP (1.0, 0.5, and 0.2 mg L^{-1}) and GA_3 (0.2 and 0.5 mg L^{-1}) for promoting shoot elongation. The number of explants placed in each culture bottle (60 × 110 mm) varied from 1 to 4 depending on the explant size (1–2 cm), stage of culture (initiation, shoot multiplication, or shoot elongation), and number of shoot buds per clump.

***In vitro* root induction from regenerated shoots and establishment of plants in soil** The regenerated shoots (3–4 cm) derived from different explants were excised and treated by dipping the base of the shoots in 10 mg mL^{-1} IBA for 5 min. The IBA-treated and IBA-untreated shoots were cultured on full-strength and half-strength MS or WP media with or without 0.2 mg L^{-1} IBA. The half-strength and full-strength media consisted of 3% sucrose and 0.8% phyto agar. The media were prepared as described previously, and 0.2 mg L^{-1} of IBA (10 mg mL^{-1} stock was prepared with 1 N NaOH as a solvent and sterile double-distilled water as diluent) was added to the autoclaved media. The duration for root induction was recorded for different treatments. The observations on the percentage of root induction, average number of roots per shoot, and average length of roots (cm) were taken after 6 wk of culture on different rooting media.

For all the experiments on *in vitro* shoot bud induction, shoot elongation, and root induction from the regenerated shoots, the cultures were placed in culture racks and incubated in light as described previously for *in vitro* seed germination. The rooted plants with well-differentiated leaves were carefully removed from the bottles and washed in water to remove any agar sticking onto the roots. Then, the rooted plants were

transferred to plastic pots containing autoclaved soil, manure, and Soilrite (3:1:1 *v*/*v*/*v*; Keltech Energies Ltd., Bangalore, India) and acclimatized for 4 wk in the culture room before moving to the glasshouse. Regenerated plants were maintained in the glasshouse for 4 wk before repotting to larger pots (15 cm × 17 cm) containing soil and manure (3:1 *v*/*v*). Plant height and survival percentage were recorded 8 wk after transfer to larger pots in the glasshouse.

Statistical analysis The seed germination and shoot regeneration experiments were carried out each time with a minimum of 20 seeds or explants per treatment. The explants obtained from 20 to 25 different seedlings were used in each treatment. The root induction experiments were performed using at least 10 regenerated shoots per treatment. All of the experiments were performed three times for each treatment. The data scored are presented as means ± SE of three experiments. Treatment means were statistically analyzed by one-way analysis of variance (ANOVA), and mean comparisons were made by the Newman–Keuls multiple comparison test at the 5% probability level using SigmaPlot for Windows (Systat Software, Inc., Bangalore, India).

Genetic stability analysis of the regenerated plants using RAPDs and ISSRs The banding patterns of *in vitro*-regenerated plants from cotyledon, hypocotyl, and root node explants originating from a single seedling were studied using RAPDs and ISSRs. The *in vitro*-multiplied shoots originating from cotyledons, hypocotyl, and root node explants after 10 passages on regeneration medium and the shoot excised from the donor seedling were pulse-treated with IBA (10 mg mL^{-1}) for 5 min and placed on half-strength WPM with 0.2 mg L^{-1} IBA for root induction. A total of 11 plants, comprising 4 from cotyledons (R1–R4), 3 each from hypocotyl (R5–R7) and root node (R8–R10), and the donor seedling, were subjected to RAPD and ISSR analysis. The leaves of the *in vitro*-rooted plants were quickly frozen in liquid nitrogen and stored at −70°C until further use. Genomic DNA was extracted from leaves using a plant DNA extraction kit (Bioserve Biotechnologies (India) Pvt. Ltd., Hyderabad, India) following the manufacturer's instructions. Quantitative and qualitative assessment of genomic DNA was carried out with a NanoDrop™ ND-1000 spectrophotometer (Thermo Fisher, Wilmington, DE) and 1% (*w*/*v*) agarose (Sigma-Aldrich®) gel electrophoresis, respectively. Equal concentrations of DNA were used for PCR for all the samples analyzed. RAPD and ISSR reactions were performed in an Eppendorf™ Mastercycler™ (Hamburg, Germany). The reaction mixture of 25 μL contained 2.5 μL of 10× PCR buffer with 15 mM magnesium chloride (Bioserve), 0.5 μL of 10 mM dNTP (Bioserve), 2 μL of 10 pmol μL^{-1} primer (Bioserve), 15 ng of template DNA, 0.5 μL of Taq polymerase (5 U μL^{-1}, Bioserve) and sterile Milli-Q water. Sixteen RAPD primers and 20 ISSR primers were initially screened on the genomic DNA of a single plant to determine their ability to produce amplified products. Subsequently, 14 RAPD and 15 ISSR primers that produced polymorphic bands on three different accessions (male, female, and andromonoecious) were selected for analyzing the genetic stability of the regenerated plants. PCR conditions used for RAPD reactions consisted of an initial denaturation step at 94°C for 3 min; 42 cycles of 30 s at 94°C, primer annealing at 37°C for 1 min, and primer extension at 72°C for 2 min; followed by final extension of 5 min at 72°C.

ISSR reactions were carried out in an Eppendorf™ Mastercycler™ with an initial denaturation step of 94°C for 5 min; 42 cycles of 1 min at 94°C, primer annealing at 50°C for 1 min, and primer extension at 72°C for 2 min; followed by final extension for 10 min at 72°C. The amplified products were separated on 1.2% (*w*/*v*) agarose gels, stained with ethidium bromide (Sigma-Aldrich®), and photographed in a gel documentation system (Syngene, Frederick, MD). The sizes of the amplification products were estimated with a 100-bp DNA ladder (MBI Fermentas Vilnius, Lithuania). All PCR reactions for RAPD and ISSRs were performed at least twice to check for reproducibility, and only clear and reproducible bands were scored. The data were scored as "1" for the presence and "0" for the absence of a band for each plant analyzed. Bands that were present in all plants were considered as monomorphic, whereas those that were absent in one or more regenerated plants or donor seedling were considered as polymorphic. The percentage of polymorphic bands was calculated based on the total number of polymorphic bands and the total number of amplified bands produced by all primers across the plants analyzed.

A similarity matrix was constructed based on the combined data from RAPD and ISSR analysis using the NTSYSpc software package (Rohlf 2000). The genetic similarities between samples were calculated by the Jaccard similarity coefficient (Jaccard 1908) with SIMQUAL format.

Results

***In vitro* seed germination** Seed germination frequency was influenced by the type of medium and the concentration of GA_3 used. The germination frequency of the seeds remained low (3.4–9.2%) on full-strength or half-strength MS or WP medium without GA_3. The addition of GA_3 enhanced the germination rate, with significantly higher response (64.3–88.8%) being observed on full-strength MS medium than on half-strength MS or on either strength of WP medium. Maximum germination (88.8%) was achieved on full-strength MS medium with 2 mg L^{-1} GA_3 (Fig. 1), with radicle emergence in 8–10 d and development of seedlings of 5–6-cm length after 4 wk of culture.

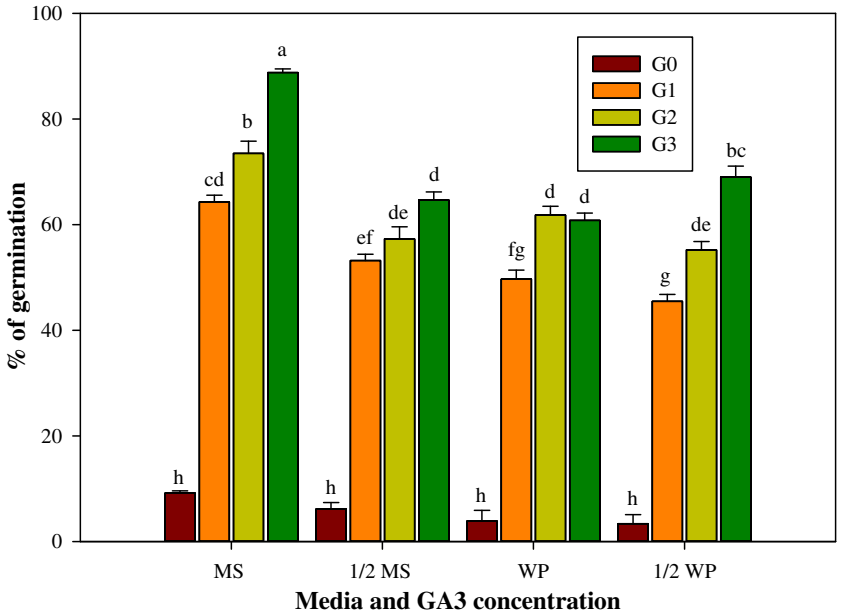

Figure 1. Germination response from seeds of *Simarouba glauca* on different media. Media used were either full-strength or half-strength Murashige and Skoog (MS) or woody plant (WP). *G0* basal medium (without GA_3), *G1* 0.5 mg L^{-1} GA_3, *G2* 1.0 mg L^{-1} GA_3, *G3* 2.0 mg L^{-1} GA_3. The values presented are percentages of germination (number of seeds germinated per total number of seeds cultured × 100) for different treatments. Values represent means ± SE (standard error) of three experiments. Means marked with the *same letter* are not significantly different at the 5% probability level by one-way analysis of variance (ANOVA) followed by the Newman–Keuls multiple comparison test.

Shoot bud proliferation and elongation in explants derived from *in vitro*-germinated seedlings Shoot buds were induced directly without any callus phase in all explants except leaves and petioles after culture on MS or WP medium with different growth regulators. Shoot bud initiation was observed in the explants after 15–20 d of culture. The frequency of shoot bud induction in different explants varied from 10.8 to 76.2% on the media tested (Fig. 2). MS medium with 2 mg L^{-1} BAP induced shoot buds in most explants at a higher frequency (44.8–76.2%) than did WP medium containing a similar concentration of BAP (30.8–63.8%). A decrease in shoot regeneration frequency (down to 10.8–52.0%) was observed in all explants when they were cultured on MS or WP medium either with BAP and NAA or with TDZ alone. Among the different explants, cotyledons exhibited a higher frequency of shoot regeneration (76.2%) than other explants on MS medium with 2.0 mg L^{-1} BAP (Fig. 2).

To induce shoot multiplication, a series of experiments was conducted in which explants were subcultured on medium with different concentrations of BAP (0.5–2.0 mg L^{-1}) alone or in combination with 0.2 mg L^{-1} NAA. However, only one to two shoots continued to elongate after repeated subcultures on medium with BAP (0.5–2.0 mg L^{-1}) and 0.2 mg L^{-1} NAA, and the shoots that formed exhibited retarded growth associated with leaf yellowing and dropping at the end of subcultures (data not shown). This difficulty was successfully overcome by gradually reducing the concentration of BAP (1.0, 0.5, and 0.2 mg L^{-1}) along with the addition of GA_3 (0.2 and 0.5 mg L^{-1}) in the medium during subcultures. To achieve higher shoot bud induction, the explants were subjected to at least three passages (2 wk each) on MS medium with 2 mg L^{-1} BAP before transferring to medium with 1 mg L^{-1} BAP (Fig. 3*a*). Significantly higher numbers of shoot buds per explant were induced in root nodes (28.5) and hypocotyls (27.5) followed by cotyledons (14.5) and cotyledonary nodes (11.4), whereas the lowest values were obtained for epicotyl (7.9) and shoot tip (5.2) explants (Table 1). A shoot bud proliferation rate of 3- to 5-fold was achieved per cycle in root nodes, hypocotyls, and cotyledonary explants, without any decrease over the six passages examined in this study. The induced shoot buds started to elongate upon transfer to MS medium with 1 mg L^{-1} BAP (no GA_3), regardless of explant type. Further elongation of the shoots was achieved after subcultures on MS medium with 1 mg L^{-1} BAP and 0.2 mg L^{-1} GA_3, followed by MS medium with 0.5 mg L^{-1} BAP and 0.2 mg L^{-1} GA_3 (Fig. 3*b*), followed by MS medium with 0.2 mg L^{-1} BAP and 0.5 mg L^{-1} GA_3, with each subculture lasting for 3 wk (Fig. 3*c*). This approach yielded consistent results in inducing proliferation and elongation of shoots from all the explants except for those induced in epicotyl explants, which failed to elongate (Table 1).

The shoot buds induced in different explants exhibited differences in their capability for shoot elongation and development. The maximum shoot elongation response was observed from cotyledon explants, with induction of 9.1 elongated shoots per explant and an average shoot length of 3.3 cm at

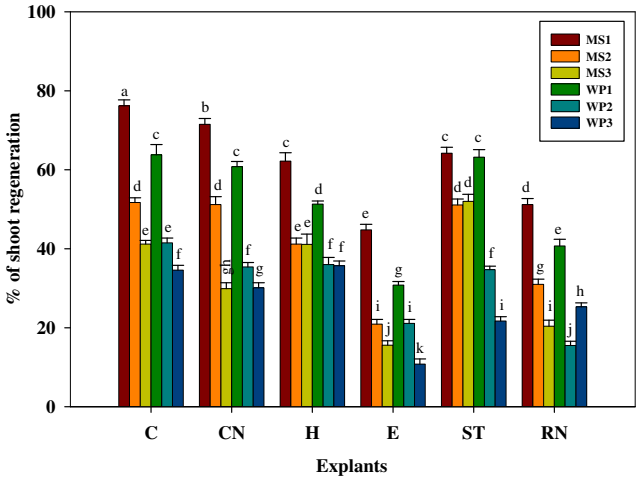

Figure 2. Shoot regeneration frequency from different explants of *S. glauca* cultured on different media. The explants used for regeneration were cotyledons (C), cotyledonary nodes (CN), hypocotyls (H), epicotyls (E), shoot tips (ST), and root nodes (RN). Media used were Murashige and Skoog (MS) and woody plant (WP). *MS1* MS with 2 mg L^{-1} BAP, *MS2* MS with 2 mg L^{-1} BAP and 0.2 mg L^{-1} NAA, *MS3* MS with 0.5 mg L^{-1} TDZ, *WP1* WP with 2 mg L^{-1} BAP, *WP2* WP with 2 mg L^{-1} BAP and 0.2 mg L^{-1} NAA, *WP3* WP with 0.5 mg L^{-1} TDZ. Values are percentages of shoot regeneration (number of explants with shoot buds per total number of explants × 100) for different treatments. Values represent means ± SE of three experiments. Means marked with the *same letter* are not significantly different at the 5% probability level by one-way ANOVA followed by the Newman–Keuls multiple comparison test.

the end of the last passage (medium with 0.2 mg L^{-1} BAP and 0.5 mg L^{-1} GA_3) (Table 1). Although a large number of shoot buds proliferated from the root node and hypocotyl explants, the shoot elongation response remained relatively low, with induction of 5.1 and 5.7 elongated shoots per explant, respectively (Table 1). About 50% of shoots induced from the root node and cotyledonary node explants exhibited stunted growth with premature differentiation of leaves and without subsequent shoot elongation. The shoot proliferation rate from shoot tip explants remained low with induction of 5.2 shoot buds per explant, from which only 1.8 shoots elongated and formed well-developed shoots.

Root induction and acclimatization In initial experiments, the explant source from which the shoots were regenerated did not have a marked effect on root induction (data not shown). Thus, rooting experiments were performed using the regenerated shoots from different explants. Rooting was achieved more efficiently from IBA pulse-treated shoots than from untreated shoots regardless of medium type (Table 2). Pulse treatment of shoots with IBA resulted in high root induction frequency (66.7%), with induction of 11.3 roots per shoot of 3.4-cm average length, after culture on half-strength WP medium containing 0.2 mg L^{-1} IBA (Table 2 and Fig. 3*d*, *e*). In contrast, the untreated shoots grown on the same medium rooted at a low frequency (33.3%), with induction of 4.7 roots per shoot of 1.7-cm average length (Table 2). There were no significant differences in root induction from IBA-treated shoots whether cultured on full-strength MS or full-strength WP basal medium. However, the root induction frequency from IBA-treated shoots was higher on half-strength WP medium (54.3%) than on half-strength MS medium (42.2%). Roots were initiated within 12–20 d from IBA-treated shoots on half-strength WP medium with or without IBA, whereas it varied from 15 to 30 d from treated or untreated shoots cultured on other media.

A total of 30 regenerated plants derived from explants of different seedlings were transferred to soil, out of which 24 plantlets were successfully acclimatized and established in soil in 7–8 mo with a survival rate of 80% (Table 3). The plants exhibited normal growth and reached a height of 10–11-cm with well-developed leaves after 12 wk of growth in the glasshouse (Fig. 3*f*). The regeneration procedure established in this study resulted in the recovery of about 25 plants from different explants of a single seedling (Table 3 and Fig. 4).

Genetic stability analysis of the regenerated plants using RAPD and ISSR primers Fourteen RAPD and 15 ISSR primers that resulted in clear amplification products in a preliminary experiment (data not shown) were employed for assessment of the genetic stability of plantlets regenerated from cotyledons, hypocotyl, and root node explants derived from a single seedling. The number of scorable loci for each RAPD primer varied from 3 (OPB-07 and OPV-08) to 13 (OPC-18), with an average of 8.1 loci per primer (Table 4). Eight RAPD primers produced monomorphic bands across the regenerated plants and the donor seedling. The number of amplified fragments generated from the RAPD primers varied from 33 (OPB-07 and OPV-08) to 143 (OPC-18), with size ranging from 200 to 2800 bp. Out of 113 loci generated by RAPD primers, 6 loci (5.3%) were found to be polymorphic. A total of 1223 bands were amplified in the regenerated plants (across 11 plants analyzed with 14 RAPD primers), of which 20 bands (1.6%) were found to be polymorphic (Fig. 5*a*, *b*).

The ISSR primers used in this study produced an average of 6.9 loci per primer, with the lowest number (2 loci) for UBC-865 and the highest number (10 loci) from three primers, viz., UBC-815, UBC-826, and UBC-835. A total of 103 loci were generated by 15 ISSR primers, of which 6 loci (5.8%) were found to be polymorphic (Fig. 5*c*, *d*). Out of 15 ISSR primers, 10 produced monomorphic bands. The UBC-835 primer generated the highest number of amplified fragments (110), whereas the lowest number (22) was observed with UBC-865; for both primers, all of the bands were monomorphic in the plants analyzed. The size of the amplicons produced from ISSR primers ranged from 250 to 2600 bp (Table 5). The 15 ISSR primers generated a total of 1104 bands across all 11 plants, of which 29 bands (2.6%) were found to be polymorphic.

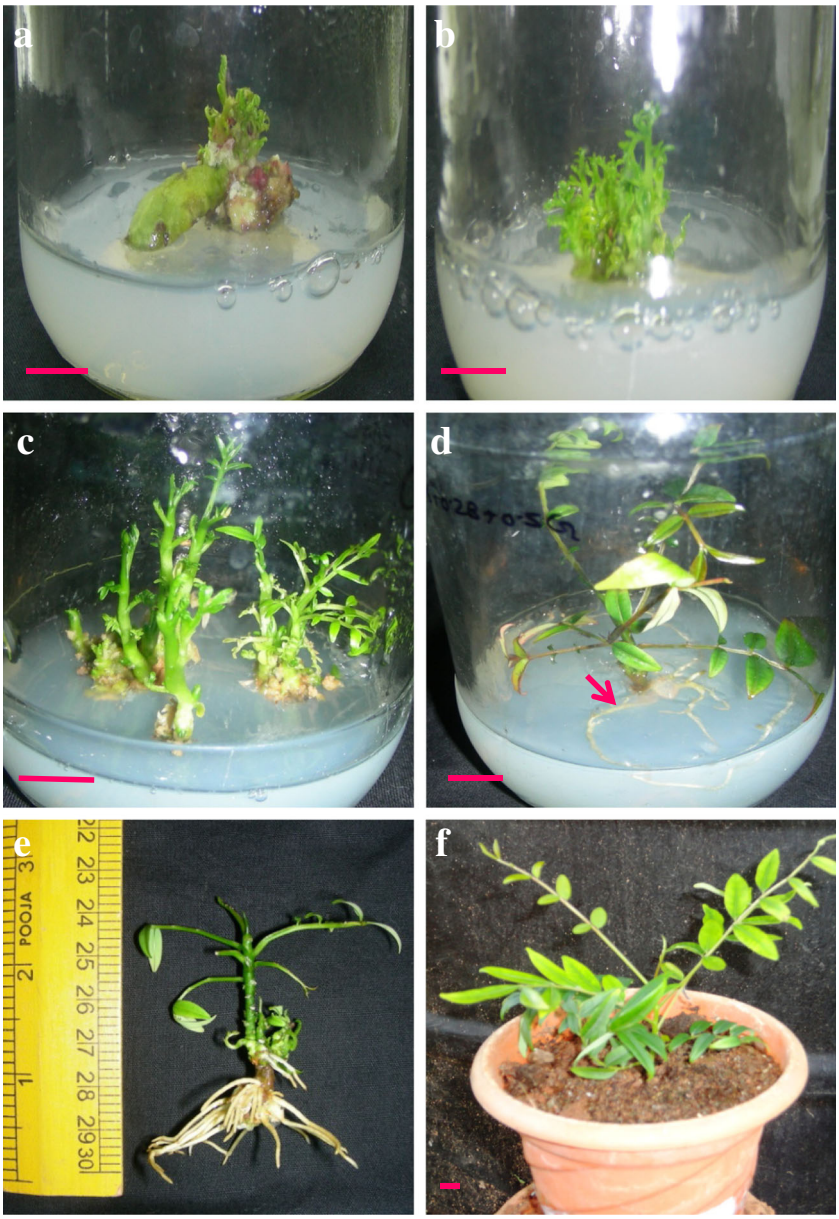

Figure 3. Regeneration of plants from cotyledon explants of *S. glauca* and establishment in soil. (*a*) Induction of multiple shoot buds from cotyledon explants after three passages (2 wk each) on MS medium with 2.0 mg L^{-1} BAP. (*b*) Development of multiple shoots after 3 wk of subculture on MS medium with 0.5 mg L^{-1} BAP and 0.2 mg L^{-1} GA_3. (*c*) Elongation of shoots after the last subculture on MS medium with 0.2 mg L^{-1} BAP and 0.5 mg L^{-1} GA_3. (*d*) Root induction (*arrow*) from pulse-treated shoots (10 mg mL^{-1} IBA for 5 min) after 4 wk of culture on half-strength WP medium with 0.2 mg L^{-1} IBA. (*e*) Pulse-treated shoot with well-developed roots taken out of culture medium. (*f*) Regenerated plant established in soil. *Bars* = 1 cm (*a–d*, *f*)

The Jaccard similarity coefficient values obtained from combined RAPD and ISSR data revealed a high genetic similarity of the regenerated plants to the donor seedling (0.958–0.986; Table 6). The regenerated plants showed an average similarity coefficient of 0.971 with the donor seedling in combined RAPD and ISSR analysis.

Discussion

GA_3 enhanced *in vitro* seed germination of *Simarouba* The present studies were conducted to induce seed germination and utilize explants from *in vitro*-germinated seedlings for optimizing plant regeneration. The results revealed a significant enhancement of seed germination (45.5–88.8%) with the addition of GA_3, whereas the germination frequency was low (3.4–9.2%) without GA_3. The highest germination percentage (88.8%) was achieved on MS full-strength medium with 2.0 mg L^{-1} GA_3, while it remained comparatively low (64.7–69.0%) on half-strength MS or half-strength WP medium containing 2 mg L^{-1} GA_3. These results verify the importance of GA_3 to germinate *S. glauca* as reported by Prashanthi *et al.* (2009). Similarly, Mahender *et al.* (2014) reported that half-strength medium constituents (either MS or WP) failed to give optimal *in vitro* seed germination response as compared to MS full-strength medium supplemented with BAP in *Butea monosperma*. The promotion of seed germination in the presence of GA_3 could be due to increased

Table 1. Response of shoot proliferation and elongation from shoot buds induced from different explants of *S. glauca*

Explant type	Average number of shoot buds/explant[z]	Average number of elongated shoots/explant[y]	Average length of shoots (cm)[y]
Cotyledons	14.5 ± 0.5 b	9.1 ± 0.3 a	3.3 ± 0.2 a
Cotyledonary nodes	11.4 ± 0.7 b	5.2 ± 0.3 b	1.9 ± 0.2 b
Hypocotyls	27.5 ± 3.3 a	5.7 ± 0.4 b	3.0 ± 0.1 a
Epicotyls	7.9 ± 0.8 c	0.0 ± 0.0 d	0.0 ± 0.0 d
Shoot tips	5.2 ± 0.5 c	1.8 ± 0.2 c	0.7 ± 0.1 c
Root nodes	28.5 ± 1.2 a	5.1 ± 0.5 b	1.8 ± 0.1 b

Values are means ± SE of three experiments, and in each experiment, 20 explants were used per treatment. Means followed by the same *letter* in a *column* are not significantly different at the 5% probability level by one-way ANOVA followed by the Newman–Keuls multiple comparison test

[z] Data were scored after three passages (2 wk each) on MS medium with 2.0 mg L^{-1} BAP

[y] Data were scored after three passages (2 wk each) on MS medium with 2.0 mg L^{-1} BAP, followed by subcultures on MS medium with 1.0 mg L^{-1} BAP, MS medium with 1.0 mg L^{-1} BAP and 0.2 mg L^{-1} GA_3, MS medium with 0.5 mg L^{-1} BAP and 0.2 mg L^{-1} GA_3, and MS medium with 0.2 mg L^{-1} BAP and 0.5 mg L^{-1} GA_3, with each subculture lasting for 3 wk

metabolic activity and growth potential of the embryos, thus causing endosperm weakening (Taiz and Zeiger 2002).

Efficient shoot bud induction and elongation in different explants were achieved by altering the BAP and GA_3 concentrations during subculturing Efficient *in vitro* shoot multiplication is critical for rapid propagation and depends on the selection of the appropriate explants, media, and growth regulators. BAP has been the most commonly used cytokinin for shoot bud induction and multiplication in many woody plant species (George 1993; Rathore *et al.* 2004). In this study, 2 mg L^{-1} BAP proved to be superior in inducing efficient shoot bud formation, which ranged from 30.8 to 76.2% among the different explants and media tested. The combination of 2 mg L^{-1} BAP and 0.2 mg L^{-1} NAA was less effective than BAP alone at both induction and proliferation stages. This was evident by the decrease in shoot regeneration frequency (15.5–51.7%) and limited shoot elongation associated with yellowing of leaves during subculturing. Similarly, Malik *et al.* (2005) reported that BAP alone was more effective at promoting multiple shoot formation than the combination of BAP and NAA in *Garcinia indica*. A previous study revealed that the combination of BAP and NAA induced better shoot regeneration than BAP alone from nodal explants of mature trees of *S. glauca* (Shukla and Padmaja 2012); the difference between the previous findings and those in the present study could be due to the different explant source.

TDZ is a potent cytokinin that has been selected for micropropagation of several woody plant species because of its tremendous ability to stimulate shoot proliferation (Huetteman and Preece 1993). The effectiveness of TDZ (0.5 mg L^{-1}) in inducing shoot bud formation in different explants of *S. glauca* as evaluated in this study showed relatively low induction frequencies (10.8–52%) in all explants in comparison to BAP (30.8–76.2%). Similar findings have been reported by Ružić and Vujović (2008), who found that TDZ was less effective than BAP on shoot multiplication of sweet cherry cv. Lapins (*Prunus avium* L.). The superiority of BAP for shoot organogenesis could be due to the ability of the plant tissues to metabolize BAP more readily than other growth regulators (Zaerr and Mapes 1982).

In the present work, the explants displayed higher capacity for shoot bud formation on MS medium (15.6–76.2%) than on WP medium (10.8–63.8%) regardless of the growth regulators used. Thus, it can be suggested that higher concentrations of ions along with 2 mg L^{-1} BAP are optimal for shoot bud induction in *S. glauca*, as total ionic concentrations of MS medium are higher than in WP medium.

Recently, Dudhare *et al.* (2014) reported that *in vitro* shoot multiplication response of *Simarouba* was relatively low. In the present study, similar difficulties were encountered in the initial shoot multiplication experiments due to the limited capability of the shoot buds to elongate and form shoots. Repeated experiments using subculture manipulations helped to overcome the problem of poor shoot elongation. This approach for obtaining enhanced proliferation of shoot buds involved at least three passages on medium with 2 mg L^{-1} BAP. The elongation of shoots and their continued growth from all explants except epicotyls were promoted by the gradual reduction of the BAP level (from 1.0 down to 0.2 mg L^{-1}) during subculturing and the subsequent inclusion of GA_3 (0.2–0.5 mg L^{-1}) in the later stages of regeneration. Similarly, Kaushik *et al.* (2015) reported that addition of GA_3 to medium supplemented with BAP and kinetin enhanced the shoot elongation by 2.33-fold in *Ophiorrhiza mungos* Linn.

In the present study, the explants differed in their capability for shoot bud induction, proliferation, and shoot elongation, with the superior response observed from cotyledon explants

Table 2. Root induction from regenerated shoots of *S. glauca* on different media

Medium and concentration of IBA (mg L^{-1})	With IBA pulse treatment					Without IBA pulse treatment				
	No. of shoots cultured	No. of shoots rooted	Rooting frequency (%)	Average no. of roots per shoot	Average root length (cm)	No. of shoots cultured	No. of shoots rooted	Rooting frequency (%)	Average no. of roots per shoot	Average root length (cm)
WPM	30	10	33.2 ± 1.8 d	6.3 ± 0.3 cd	1.2 ± 0.1 d	30	0	0.0 ± 0.0 c	0.0 ± 0.0 d	0.0 ± 0.0 d
WPM + 0.2 IBA	30	13	43.3 ± 1.7 c	7.3 ± 0.3 bc	2.2 ± 0.2 c	30	0	0.0 ± 0.0 c	0.0 ± 0.0 d	0.0 ± 0.0 d
Half-strength WPM	33	18	54.3 ± 2.3 b	9.3 ± 0.3 b	2.8 ± 0.1 b	27	3	11.2 ± 0.7 b	3.3 ± 0.3 c	1.1 ± 0.1 c
Half-strength WPM + 0.2 IBA	30	20	66.7 ± 3.3 a	11.3 ± 0.9 a	3.4 ± 0.1 a	30	10	33.3 ± 3.3 a	4.7 ± 0.3 a	1.7 ± 0.1 a
MS	29	10	34.4 ± 1.0 d	5.0 ± 0.6 c	1.2 ± 0.2 d	30	0	0.0 ± 0.0 c	0.0 ± 0.0 d	0.0 ± 0.0 d
MS + 0.2 IBA	32	14	44.0 ± 1.0 c	8.0 ± 0.6 b	2.4 ± 0.1 c	30	0	0.0 ± 0.0 c	0.0 ± 0.0 d	0.0 ± 0.0 d
Half-strength MS	33	14	42.2 ± 1.7 c	7.0 ± 0.6 b	2.9 ± 0.1 b	29	3	10.4 ± 0.4 b	2.3 ± 0.3 b	0.5 ± 0.0 d
Half-strength MS + 0.2 IBA	32	17	53.0 ± 1.5 b	9.0 ± 0.6 b	3.0 ± 0.1 ab	28	3	10.7 ± 0.4 b	4.3 ± 0.3 a	1.4 ± 0.1 b

Shoots elongated on MS medium with 0.2 mg L^{-1} BAP and 0.5 mg L^{-1} GA_3 with pulse treatment (10 mg mL^{-1} solution for 5 min) or without treatment were cultured on different rooting media, and the data were scored after 45 d of culture. Values are means ± SE of three experiments. Means followed by the same letter in a column are not significantly different at the 5% probability level by one-way analysis of variance (ANOVA) followed by the Newman–Keuls multiple comparison test

Table 3. Regeneration of plants from different explants of *S. glauca* and their establishment in soil

Explant	Average no. of regenerated plants per seedling[z]	Total no. of regenerated plants transferred to soil[y]	Total no. of regenerated plants established in soil[y]
Cotyledons	12.3 ± 0.2 a	13	11
Cotyledonary nodes	3.7 ± 0.1 b	5	4
Hypocotyls	3.8 ± 0.1 b	6	5
Shoot tips	1.6 ± 0.1 c	3	2
Root nodes	3.8 ± 0.1 b	3	2

[z] Values are means ± SE of three experiments. Means followed by the same *letter* in a *column* are not significantly different at the 5% probability level by one-way ANOVA followed by the Newman–Keuls multiple comparison test

[y] Regenerated plants from different seedlings

in terms of shoot bud induction frequency (76.2%) and the number of elongated shoots (9.1 shoots per explant) per explant. Although higher numbers of shoot buds proliferated per explant from root nodes (28.5) and hypocotyls (27.5), the shoot elongation response remained lower (5.1–5.7 elongated shoots per explant) than that of cotyledons. Quoirin *et al.* (1998) opined that the requirements for exogenous plant growth regulators varies with explant type and apparently depends on the endogenous level of plant growth regulators. As the shoot proliferation rate from shoot tips remained low in this study, further investigations are needed to improve the shoot multiplication rate as it would allow recycling of shoots for long periods during *in vitro* culture.

IBA pulse treatment enhanced *in vitro* root induction One of the difficulties encountered in the micropropagation of tree species is the induction of adventitious roots from shoots (Cha-um *et al.* 2011). In the present study, pulse treatment of the regenerated shoots with IBA (10 mg mL^{-1} for 5 min) resulted in better root induction than untreated shoots on the media tested. The untreated shoots failed to root on full-strength WP or MS media, whereas root induction varied from 10.4 to 33.3% on half-strength WP or MS media, with or without IBA. Furthermore, pulse-treated shoots exhibited higher root induction (54.3%) on half-strength WP medium as compared to those on full-strength WP or either strength of MS media without IBA. Overall, the highest root induction frequency (66.7%), highest number of roots per shoot (11.3), and maximum root length (3.4 cm) were observed from the pulse-treated shoots on half-strength WP medium containing 0.2 mg L^{-1} IBA. Similarly, a low salt concentration in the medium in combination with IBA resulted in better root induction from shoots in different plant species (Rai *et al.* 2010; Phulwaria *et al.* 2012).

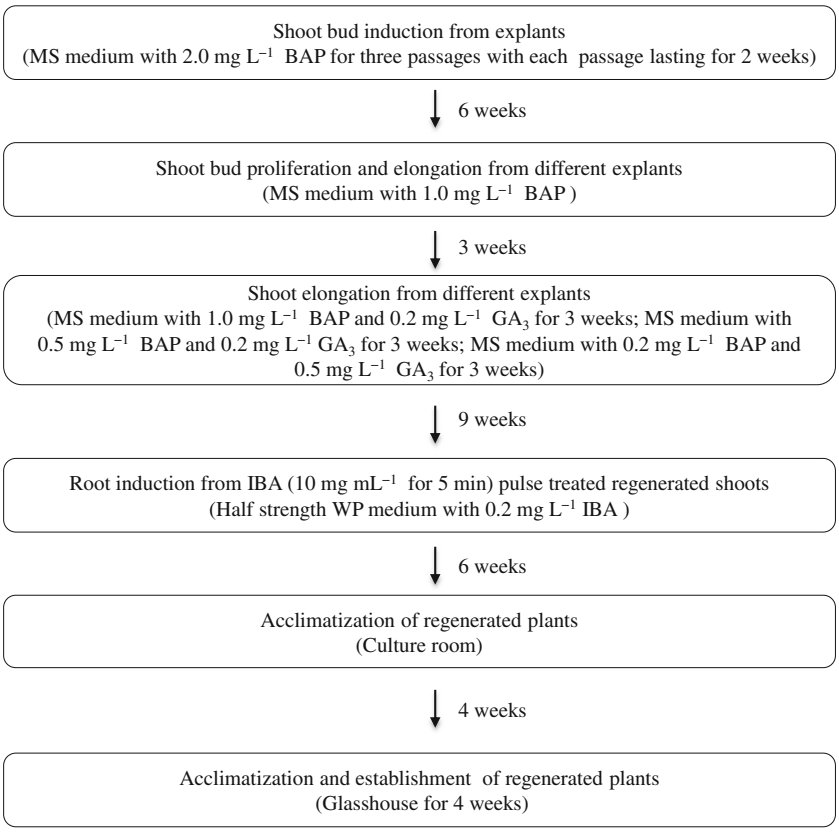

Figure 4. Schematic representation of plant regeneration from seedling-derived explants of *S. glauca*.

Rout *et al.* (1999) reported that a high concentration of IBA was necessary with full-strength MS medium for *in vitro* rooting of *S. glauca*. Warakagoda and Subasinghe (2013) reported root induction from microshoots of *Pterocarpus santalinus* after exposure to pulse treatment with 0.05 mg mL^{-1} IBA for 12 h and subsequent culture on half-strength MS medium containing 0.1 mg L^{-1} IBA. Woodward and Bartel (2005) suggested that root induction achieved by

Table 4. RAPD profiles of *in vitro*-regenerated plants and donor seedling of *S. glauca*

Primer name	Primer sequence (5′–3′)	Number of loci	Number of polymorphic loci	Total number of amplified bands across all plants	Total number of polymorphic bands across all plants	Size range (bp)
OPA-11	CAATCGCCGT	7	1	69	8	460–2100
OPA-16	AGCCAGCGAA	10	1	104	6	550–2400
OPA-17	GACCGCTTGT	12	1	131	1	280–2750
OPAK-14	CTGTCATGCC	7	1	76	1	750–1800
OPAL-08	GTCGCCCTCA	9	0	99	0	525–2100
OPB-07	GGTGACGCAG	3	0	33	0	950–2000
OPC-18	TGAGTGGGTG	13	0	143	0	220–2750
OPD-14	CTTCCCCAAG	9	1	96	3	575–2225
OPE-20	AACGGTGACC	4	0	44	0	800–2000
OPP-03	CTGATACGCC	7	0	77	0	375–2800
OPS-05	TTTGGGGCCT	6	0	66	0	200–2300
OPV-08	GGACGGCGTT	3	0	33	0	375–1400
OPV-17	ACCGGCTTGT	12	0	132	0	275–2600
OPZ-01	TCTGTGCCAC	11	1	120	1	250–2100
Total number of bands		113	6	1223	20	

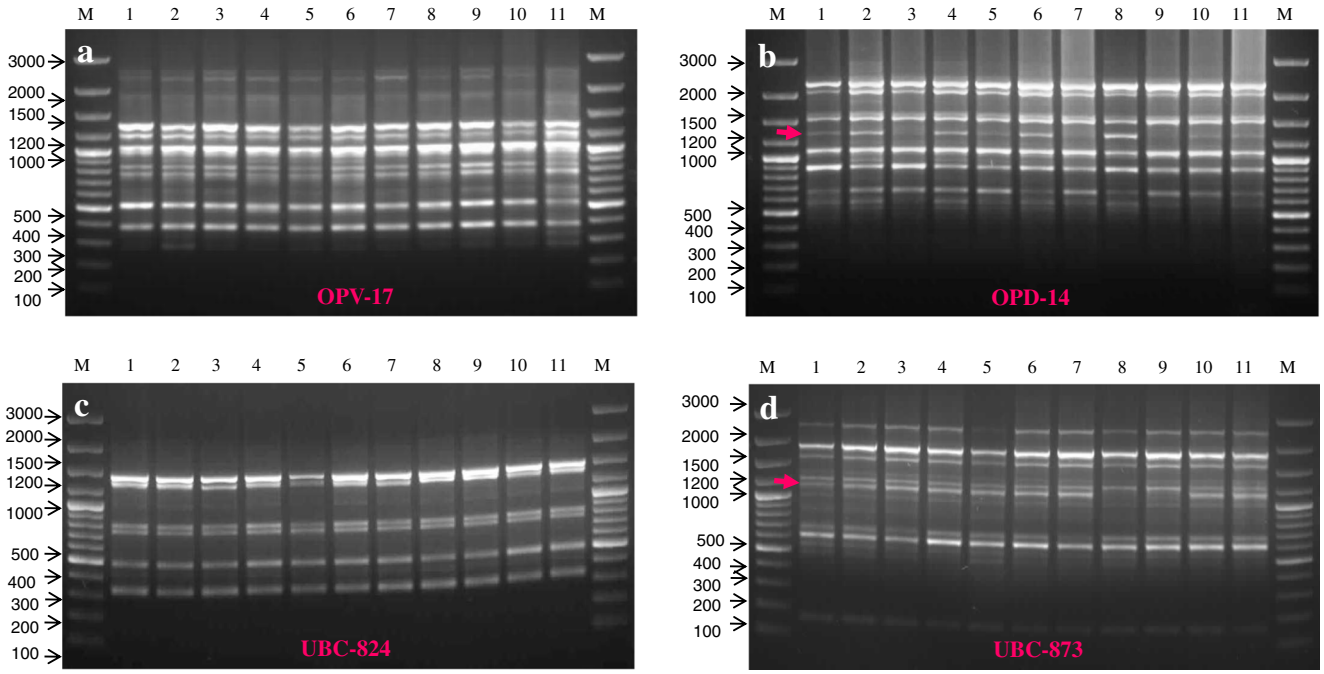

Figure 5. RAPD and ISSR profiles of *in vitro*-regenerated plants along with donor seedling of *S. glauca* obtained with different primers. (*a*) RAPD primer OPV-17. (*b*) RAPD primer OPD-14. (*c*) ISSR primer UBC-824. (*d*) ISSR primer UBC-873. *Lane M*, molecular weight DNA marker. *Lane 1*, donor seedling. *Lanes 2–5*, regenerated plants from cotyledonary explants. *Lanes 6–8*, regenerated plants from hypocotyl explants. *Lanes 9–11*, regenerated plants from root node explants. *Pink arrows* in (*b*) and (*d*) indicate polymorphic bands.

IBA treatment could be due to an endogenous increase in IAA concentration. In the present study, the *in vitro*-regenerated plants were acclimatized and established in the glasshouse with a high success rate of 80%, indicating the suitability of the protocol for micropropagation of *S. glauca*.

RAPD and ISSR markers revealed high genetic similarity of the regenerated plants with the donor seedling

Regeneration of plants through direct shoot organogenesis without any intermediary callus formation is generally expected to give rise to true-to-type plants, but occasionally, genetic

Table 5. ISSR profiles of *in vitro*-regenerated plants and donor seedling of *S. glauca*

Primer name	Primer sequence (5′–3′)	Number of loci	Number of polymorphic loci	Total number of amplified bands across all plants	Total number of polymorphic bands across all plants	Size range (bp)
UBC-808	AGAGAGAGAGAGAGAGC	7	0	77	0	320–1000
UBC-810	GAGAGAGAGAGAGAGAT	8	0	88	0	320–850
UBC-812	GAGAGAGAGAGAGAGAA	6	0	66	0	250–700
UBC-813	CTCTCTCTCTCTCTCTT	5	0	55	0	280–1050
UBC-815	CTCTCTCTCTCTCTCTG	10	1	107	3	320–1900
UBC-818	CACACACACACACACAG	5	0	55	0	500–2000
UBC-824	TCTCTCTCTCTCTCTCG	6	0	66	0	320–1450
UBC-825	ACACACACACACACACT	7	0	77	0	350–1050
UBC-826	ACACACACACACACACC	10	1	102	8	250–1500
UBC-834	AGAGAGAGAGAGAGAGTT	5	1	48	7	400–1200
UBC-835	AGAGAGAGAGAGAGAGCC	10	0	110	0	320–1150
UBC-842	GAGAGAGAGAGAGAGAYG	5	2	46	9	280–1150
UBC-865	CCGCCGCCGCCGCCGCCG	2	0	22	0	720–950
UBC-873	GACAGACAGACAGACA	8	1	86	2	600–2600
UBC-880	GGAGAGGAGAGGAGA	9	0	99	0	350–1450
Total no. of bands		103	6	1104	29	

Table 6. Similarity coefficients for *in vitro*-regenerated plants and donor seedling of *S. glauca* based on RAPD and ISSR analysis

	Donor	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10
Donor	1.000										
R1	0.986	1.000									
R2	0.968	0.972	1.000								
R3	0.981	0.995	0.968	1.000							
R4	0.977	0.981	0.972	0.986	1.000						
R5	0.972	0.977	0.977	0.981	0.986	1.000					
R6	0.968	0.972	0.981	0.977	0.981	0.986	1.000				
R7	0.963	0.968	0.968	0.972	0.977	0.981	0.986	1.000			
R8	0.958	0.963	0.981	0.968	0.981	0.977	0.991	0.986	1.000		
R9	0.972	0.977	0.977	0.981	0.995	0.991	0.986	0.981	0.986	1.000	
R10	0.968	0.981	0.981	0.986	0.981	0.986	0.972	0.968	0.972	0.986	1.000

Regenerated plants derived from cotyledons are R1–R4, from hypocotyl are R5–R7, and from root node are R8–R10

variations arise at the phenotypic, cytological, and DNA sequence levels (Brown *et al.* 1993; Chen *et al.* 1998). It is therefore important to ascertain the suitability of a regeneration protocol in production of true-to-type plants in tree species where quality planting material is the main consideration. In most of the reports with other plant species, the genetic stability analysis of micropropagated plants vs. donor using molecular markers revealed 100% monomorphic bands. In this study, the percentage of polymorphic loci generated by ISSR primers was slightly higher (5.8%) than that generated by RAPD primers (5.3%) between donor seedling and regenerated plants. The 14 RAPD and 15 ISSR primers employed in the study resulted in a large number of monomorphic bands (1203 and 1075, respectively), with a low percentage (1.6–2.6%) of polymorphic bands in the regenerated plants. Overall, six RAPD and five ISSR primers generated one to two polymorphic bands absent from a few regenerated plants or the donor seedling. The appearance or disappearance of RAPD and ISSR bands in the plants analyzed could be the result of changes in the DNA sequences of the primer binding sites or changes that could have altered the sizes or prevented the amplification of DNA fragments. Similarly, polymorphisms between the tissue culture products (calluses, adventitious shoots, and regenerated plants) and seedlings were detected by RAPD and ISSR analysis in *S. involucrata* (Yuan *et al.* 2009). Modgil *et al.* (2005) detected a high level of polymorphism (23.2%) in 10 micropropagated plants regenerated through axillary buds of clonal apple (*Malus pumila* Mill.) rootstock using RAPD markers. Thus, the present study showed that the regeneration procedure employed in the study does not cause major genetic changes in the RAPD- and ISSR-amplified DNA regions in the regenerated plants. In the present study, the low level of genetic variation detected in the regenerated plants of *S. glauca* using RAPD and ISSR markers could be mainly due to the use of BAP, which has been implicated in inducing genetic changes during tissue culture in other plant species (Hossain *et al.* 2003; Kaushal *et al.* 2004). As the genetic stability of the regenerated plants derived from a single donor seedling was analyzed in this study, further studies with molecular markers using a greater number of regenerated plants obtained from different seedlings and explants would be useful in analyzing the extent of genetic similarities and variations among the regenerated plants and seedlings before practical utilization.

Conclusions

In conclusion, this paper describes an efficient *in vitro* method for regeneration of plants from *S. glauca* using seedling-derived explants, including the establishment of the regenerated plants in the soil. Shoot bud induction and proliferation in different explants were best achieved on full-strength MS medium with 2 mg L^{-1} BAP, while stepwise reduction in BAP levels (1.0, 0.5, and 0.2 mg L^{-1}) along with addition of GA_3 (0.2 and 0.5 mg L^{-1}) during subculturing resulted in shoot elongation, with the highest number of shoots being produced from cotyledon explants. IBA pulse treatment of the shoots proved to be effective for root induction, with the highest frequency of root induction achieved on half-strength WP medium with 0.2 mg L^{-1} IBA. Furthermore, the RAPD and ISSR primers used in the study revealed a large number of monomorphic bands in the regenerated plants. Thus, the study generated valuable information with regard to the utility of RAPD and ISSR primers in analyzing the genetic stability of regenerated plants of *S. glauca*, which will be very useful in the future for screening *in vitro*-multiplied or *in vitro*-conserved plants. The *in vitro* regeneration system established in this study offers a rapid multiplication protocol for the conservation of elite germplasm and their genetic transformation for the transfer of desirable genes into this tree species.

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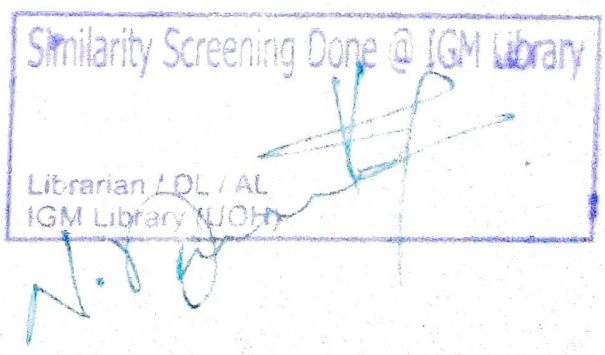

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