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RESEARCH ARTICLE

An Improved Micropropagation and Assessment of Genetic Fidelity in Multipurpose Medicinal Tree, *Acacia auriculiformis*

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Abstract An efficient direct plant regeneration protocol has been developed for medicinal tree, *Acacia auriculiformis* that can be employed at a commercial scale. Bioactive properties of this tree attribute to spermicidal action and anti-HIV activity which has gained an increasing interest in world health sector. Among several media employed for present study, an utmost shoot regeneration frequency of 93 % with highest number 17.3 ± 0.61 of shoots per explant was obtained on MS medium supplemented with 2.0 mg l^{-1} KIN and 0.5 mg l^{-1} IAA. Inclusion of activated charcoal in media has effectively alleviated the browning problem of shoots. Maximum shoot length of 6.4 cm was attained on MS media supplemented with 0.25 mg l^{-1} GA_3 followed by rooting of individual microshoots on half strength MS media supplemented with 0.1 mg l^{-1} IAA. After successful hardening, rooted microshoots were maintained under greenhouse conditions. Genetic stability of micropropagated plants of *A. auriculiformis* have also been established via randomly amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) markers. A total of 83 (39 RAPD and 44 ISSR) clear, distinct and reproducible amplicons were produced. The banding patterns and similarity coefficients obtained by both RAPD and ISSR analysis have revealed no remarkable differences between tissue culture raised plants and the mother plant. This is the first report that evaluates the use of genetic markers to establish genetic fidelity of micropropagated *A. auriculiformis*. This method can be applied for the rapid clonal multiplication and true-to-type production of plant for attaining the ever increasing demand in pharmaceutical industries.

Keywords *Acacia auriculiformis* · Genetic fidelity · ISSR · Medicinal tree · RAPD

Introduction

Acacia auriculiformis (also known as earleaf acacia or black wittle) is a multipurpose, fast-growing and thorny leguminous tree (belongs to the family Fabaceae) with considerable medicinal and traditional values. It is native to India, Australia, Indonesia, and Papua New Guinea. This plant is a rich source of the bioactive compounds with good pharmacological values including microbicidal, anti-helminthic, and antifilarial activity [1, 2]. Saponins (pharmacological metabolite) isolated from this plant produce superoxide anions which can initiate lipid peroxidation and eventually trigger membrane damaging system [3]. Additionally, this tree species is an important source of pulpwood, fuel-wood, timber, and shelterbelts. A root decoction of *A. auriculiformis* is applied for treating pains, aches and sore eyes, whereas, a bark infusion of the plant is used for treating rheumatism in aborigines of Australia. Bark extracts of *A. auriculiformis* exhibit extensive pesticidal activity [4].

Acaciaside-A (Ac-A) and Acaciaside-B (Ac-B) are among those triterpenoid saponins which are obtained from seeds of this tree. A considerably low concentrations of Ac-B extracted from seeds of *A. auriculiformis* unveil

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spermicidal activity (anti-fertility) and reported to be safe for application on vaginal epithelia [5]. Moreover, Ac-B also inhibits transmission of HIV without any mutagenic effect [5, 6]. Such pharmacological properties of saponin Ac-B obtained from *A. auriculiformis* sustain its potential for being a future medicine of immense use.

Seeds are the product of meiotic events followed by the fusion of divergent set of alleles each coming from a distinct source. Since, saponin Ac-B has a tissue specific localization (seed) as well as stringent genetic regulation, a consequence of genetic recombination and segregation of alleles, loss of desirable traits can hinder or down-regulate its production mechanism especially in seeds [7]. Most recently, plant cell culture technologies are rapid alternative approach for obtaining disease-free plants which can be employed for exploitation of secondary metabolite production potential of the plants [8]. Although, in vitro culture and micropropagation of *A. auriculiformis* has earlier been reported from different explants [9–13], current report provide an effective protocol for high efficiency direct plant regeneration and rapid proliferation method for *A. auriculiformis* by using nodal segments.

Since, occurrence of mutational events in the somatic cells result in appearance of genetic variation in tissue culture-raised plants, production of true-to-type plants is extremely significant for retaining genotypic and phenotypic similarities of mother plant. Such absurdities are generally inherited and are therefore redundant in clonal propagation [14]. Genetic fidelity was also established via molecular marker based system for assurance of genetic stability of in vitro raised plants. Thus, present investigation not only undertakes high frequency direct plant regeneration in *A. auriculiformis* but also validates the genetic stability of tissue culture raised plants which is significant for high saponin-producing genotypes such as *A. auriculiformis*. This is the first report for genetic stability assessment of the medicinal tree species, *A. auriculiformis*.

Material and methods

Explant Preparation and Surface Sterilization

About 5-year-old adult plus tree of *A. auriculiformis* with newly growing canes and well spread canopy maintained in the campus fields of Guru Jambheshwar University of Science and Technology, Hisar was selected as the mother plant. Twigs of the tree were collected in the morning hours from fresh growing canes during the months of March–April. Explants consist of 8–15 cm long stem segments carrying 2–3 axillary buds with tweaked leaves were excised from the microcuttings collected from twigs. Initial diminution of heavy surface microflora was achieved by comprehensive washing of the explants under a tap-water-stream for 30 min followed by cleaning in 5 % (v/v) 'Laboline' solution along with 5 drops of Tween-20 for 5 min. The explants were soaked in 0.5 % Bavistin (50 % w/v Cabandazim) solution for 30 min. Eventually, the explants were surface-sterilized with 0.1 % (w/v) $HgCl_2$ solution for 10 min under aseptic conditions of Laminar Air Flow (NSW India, New Delhi) followed by a quick dip in rectified spirit for 10 s and rinsed four to five times with sterile distilled water to remove the traces of Bavistin, detergent and other surface sterilizing reagents. After surface sterilization, explants were dried onto sterile filter paper discs in order to absorb extra-moisture residing on their surface as a result of repeated washings.

Culture Media and Effect of Activated Charcoal

Following surface disinfestation, both terminals of each explant were trimmed. Clean explants were individually inoculated and cultured in sterilized 25 × 200 mm borosilicate culture tubes containing 10–15 ml liquid MS medium [15] supplemented with 2 mg l^{-1} BAP as shown in Fig. 1a. The pH of media was adjusted to 5.8 before autoclaving with 0.1 N NaOH/HCl. The medium was autoclaved at 1.06 kg cm^{-2} pressure and 121 °C temperature for 20 min. Cultures were placed in plant growth room at 25 ± 2 °C under a 16-h photoperiod. Light was equipped by fluorescent tubes at a photon flux density of 45 μ m^{-2} s^{-1}. After the bud break, cultures were transferred to semi-solid MS medium supplemented with same dose of 2 mg l^{-1} BAP and 0.8 % agar. Cultures were transferred to a fresh medium after every 3–4 weeks for adequate supply of nutrients.

In an experiment, individual nodal explants excised from 4 to 5 weeks old in vitro stem cultures growing in liquid MS media supplemented with 2 mg l^{-1} BAP were placed on MS medium containing 30 g l^{-1} sucrose, 0 or 1 g l^{-1} activated charcoal (AC) and 8 g l^{-1} agar. After the first series of experiments, remarkable proliferation rate of nodal explants was observed on AC medium. Thus, all media were further added with additional 1 g l^{-1} AC for the successful proliferation of shoots. AC was incorporated into the culture media before autoclaving.

Optimization of Plant Regeneration and Multiple Shoot Cluster Formation

The effect of plant growth regulators on plant regeneration and initiation of multiple shoot cluster formation was tested. Individual nodes and shoot tips were excised from 4 to 5 week old in vitro cultures of *A. auriculiformis*

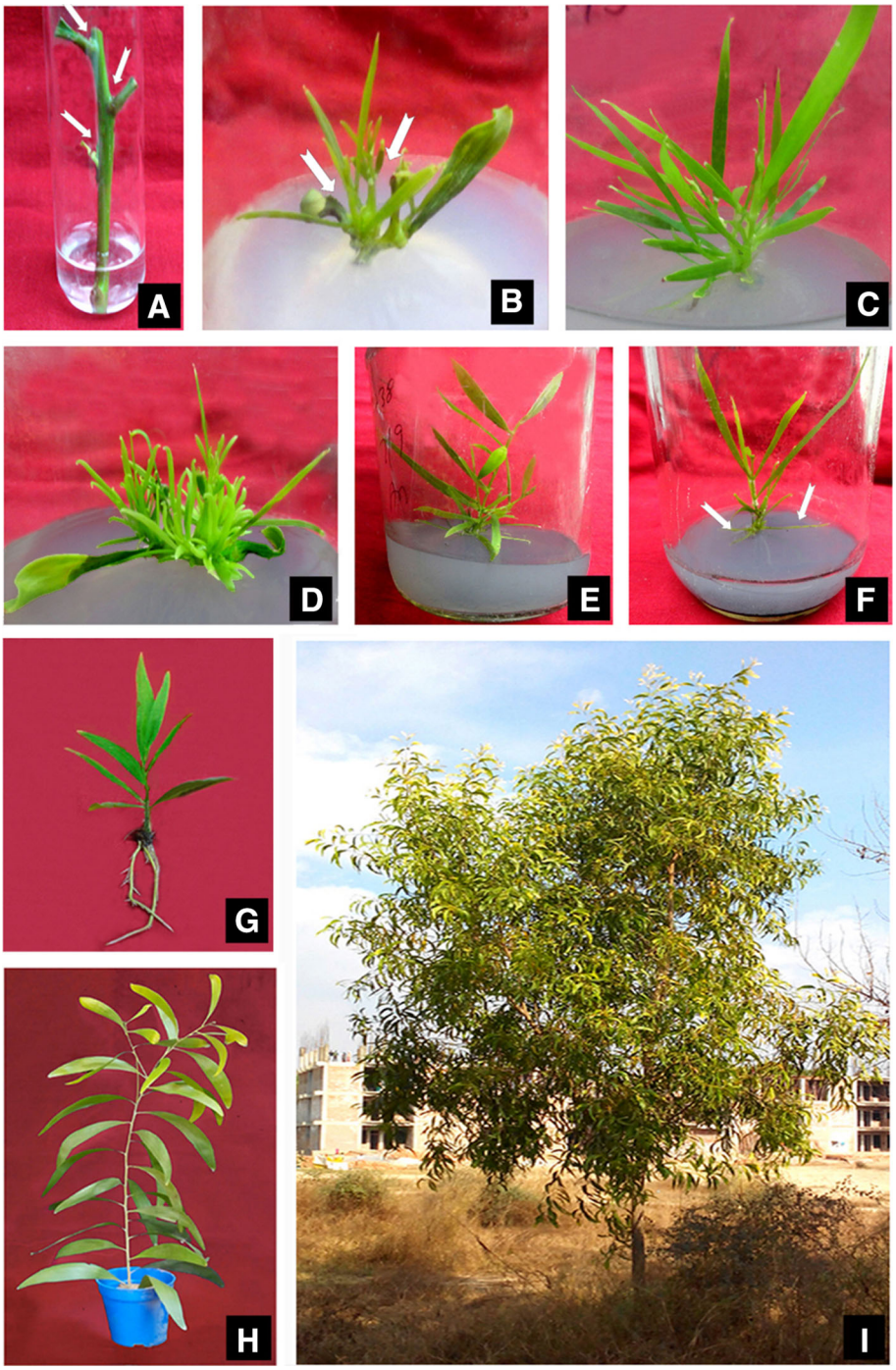

Fig. 1 High frequency direct plant regeneration, shoot bud induction and micropropagation of *Acacia auriculiformis*. **a** Initial culture of surface sterilized microcuttings on liquid MS medium supplemented with 2.0 mg l^{-1} BAP. *Arrows* represent axillary shoot buds; **b** sprouted shoot buds subcultured immediately after bud break onto MS medium supplemented with 2 mg l^{-1} BAP, 30 g l^{-1} sucrose, 10 mg l^{-1} ascorbic acid, 25 mg l^{-1} adenine sulphate, and 8 g l^{-1} agar bear brown leaves. *Arrows* represent the leaves which are turned brown; **c** similarly, shoot buds of same age and size were subcultured onto MS medium supplemented with 2 mg l^{-1} BAP, 30 g l^{-1} sucrose, 10 mg l^{-1} ascorbic acid, 25 mg l^{-1} adenine sulphate, 1 g l^{-1} AC and 8 g l^{-1} agar bear fresh green leaves; **d** direct plant regeneration and adeventitious shoot bud induction response of *A. auriculiformis* cultures on MS medium supplemented with 2.0 mg l^{-1} KIN and 0.5 mg l^{-1} IAA after 4 weeks of culture; **e** individual microshoot elongation on MS medium supplemented with 0.25 mg l^{-1} GA_3; **f** root induction in regenerated microshoots after elongation on half-strength liquid MS medium supplemented with 0.1 mg l^{-1} IAA. *Arrows* represent emergence of roots; **g** in vitro raised, rooted earleaf Acacia plant; **h** 8 weeks old earleaf Acacia plant transferred to pots containing a mixture of soil and vermi compost (1:1) in greenhouse for hardening and acclimatization; **i** eighteen months old, tissue culture raised *A. auriculiformis* tree standing in field

maintained on liquid MS media supplemented with 2 mg l^{-1} BAP and were transferred onto MS media supplemented with various concentrations of BAP, KIN and (Δ^2 isopentenyl) adenine (2iP) such as 0.1, 0.5, 1.0, 2.0, 5.0 mg l^{-1} both alone and the best optimized concentration of either BAP or KIN in combination with IAA at various concentrations of 0.1, 0.25, 0.5, 0.75 and 1.0 mg l^{-1}, resulting in a total of 25 different media. All media were supplemented with 30 g l^{-1} sucrose, 10 mg l^{-1} ascorbic acid, 25 mg l^{-1} adenine sulphate, 1 g l^{-1} activated charcoal (AC) and 8 g l^{-1} agar. Each axillary bud explant was placed in separate gem bottles and three replicates were prepared for each treatment. Observations on multiple shoot formation were recorded after a 4-week period of culture. Individual microshoots isolated from multiple shoot structures were sub-cultured onto various concentrations of GA_3 (0.1, 0.25, 0.5, 0.75 and 1.0 mg l^{-1}) for optimization of shoot elongation medium.

Rooting, Hardening and Transplantation to Greenhouse

Elongated healthy microshoots (about 3–4 cm in length) were excised and cultured onto half-strength MS medium supplemented with 0.1 mg l^{-1} IAA for rooting. Rooted microplants were removed from rooting medium and washed under running tap water to remove all the adherent traces of agar and media. The plants were kept overnight in tissue culture lab by submerging roots in tap water. Next day, the plants were transferred to plastic pots (6 cm diameter) containing mixture of vermiculite, soil and sand (1:1:1). The plastic pots were covered with polythene bags to maintain relative humidity. These pots were maintained at 24 ± 2 °C with a 16 h natural light photoperiod and watered every alternate day for first 2 weeks followed by once in a week for rest of the period in a greenhouse. Polythene bags were opened after 2 weeks so that plants were directly exposed to external climate for acclimatizing them in field conditions. After 8 weeks, acclimatized plants were transferred to soil (field) under natural conditions.

RAPD and ISSR Analysis

About 2 g of fresh leaf samples from mother (donor) and micropropagated plants were ground into fine powder in liquid nitrogen. Following this, gDNA was isolated by CTAB method with minor modification. The quantitative and qualitative analysis for quality and yield of gDNA was performed by gel electrophoresis and Nanodrop spectrophotometer (Nanodrop®, ND-1000, Nanodrop Technologies, Wilmington, Delaware, USA), respectively. DNA samples were diluted to 20 ng μl^{-1} with Tris EDTA buffer. A total of ten decamer arbitrary oligonucleotides (Operon Technologies Inc., Alameda, CA, USA) were used as single primer for the amplification of random amplified polymorphic DNA (RAPD) fragments according to [16, 17] with slight modifications. Similarly, ten inter simple sequence repeats (ISSR) primers (16–17 mer) were randomly selected and used for PCR amplification.

A PCR reaction mixture (20 µl) contained 30 ng gDNA, 1.5 mM $MgCl_2$, 0.4 mM each of the deoxynucleotide triphosphates (dATP, dGTP, dCTP, dTTP), 1.0 U Taq DNA polymerase, and 0.1 µM of primer. PCR amplifications were carried out in a *i*-cycler™ thermocycler (BioRad, USA), with the initial denaturation at 94 °C for 5 min followed by 45 cycles of 94 °C for 45 s, 37 °C for 60 s and 70 °C for 60 s and 1 final extension cycle of 72 °C for 10 min.

Amplicons of RAPD and ISSR analyses were resolved by electrophoresis on 1.2 % agarose gels run in 1× TAE buffer (40 mM Tris acetate, pH 8.2; 1 mM EDTA) buffer, stained with ethidium bromide. Separated DNA fragments were visualized and photographed with UV light on gel documentation system/bioimaging system (GeneGenius, Syngene, UK).

Data Recording and Statistical Analysis

The in vitro studies were performed in a complete randomized design model. Each treatment was repeated thrice with ten replicates. Data was inscribed every week after culturing both in liquid and agarified media. The frequency of shoot regeneration was calculated as the percent of nodal explants inoculated in a particular treatment. Recorded data was subjected to one way Analysis of Variance (ANOVA) with a 0.05 % significance level by assuming Duncan equal variance. These computational tests were conducted using the statistical algorithm/software package SPSS for windows (v. 13.0 SPSS Inc USA).

Each distinct and reproducible DNA band was considered as a binary character and was scored 1 (presence) or 0 (absence) for each sample and assembled in a data matrix. Three independent PCR reactions were carried out for all RAPD and ISSR primers included in the study. Data were subjected to the statistical and cluster analysis by running NTSys PC version 2.02j (http://www.exetersoftware.com) software. Similarity matrix was computed [18], and cluster analysis was performed via unweighted pair grouping method with arithmetic mean (UPGMA).

Results and Discussion

Sprouting of Nodal Cultures and Effect of Activated Charcoal

Nodal explants from the 5-year-old tree were cultured onto liquid MS supplemented with 2 mg l^{-1} BAP as shown in

Fig. 1a. Nodal explants were sprouted for producing buds within a week of culture. After 2–3 weeks, bud-break begun for developing shoot-primordia from the axils of nodal stem segment. Liquid MS media substantially promote bud-break process as compared to agarified media especially in tree species [19]. Therefore, initially stem cuttings were cultured onto liquid MS media for ease of nutrient absorption by the explants. Individual axillary buds of same age and size were excised and transferred to MS supplemented with 2 mg l^{-1} BAP, 30 g l^{-1} sucrose, 10 mg l^{-1} ascorbic acid, 25 mg l^{-1} adenine sulphate, 0 or 1 g l^{-1} AC and 8 g l^{-1} agar. Following 2 weeks of culturing, almost all cultured buds showed their initial response by producing adventitious shoots. Although, AC-free media have also initiated adventitious bud proliferation. AC media has induced vigorous proliferation of shoot buds for producing multiple shoot clusters with dark green color. A problem of yellowing and eventually browning of leaves with lowered growth was encountered during initial stages of nodal bud culture in AC-free media (Fig. 1b). The addition of AC into tissue culture media has a beneficial effect on growth, regeneration and development of the tissue as observed in Fig. 1c. Since, *A. auriculiformis* is a woody tree species with many bioactive secondary metabolites, the browning problem may be due to the excessive production of polyphenols or certain inhibitory substances in media. An alternative approach to manage the problem of deposition of inhibitory substances in media is frequent supply of fresh media. However, increasing the number of subcultures sometimes results in accumulation of mutations in cells and may cause loss of the ability of the affected cells to undergo organogenesis process. Inclusion of AC to culture media can efficiently alleviate this problem. AC prevented discoloration by adsorbing phenolics and rendering polyphenol oxidase and peroxidase inactive [20]. Reports are available for encouraged use of AC medium that can control browning and stimulated shoot growth problem in *Strelitzia reginae* and *Anemone oronaria* [21].

Exudation of phenolics by cultured explants in medium especially in the case of *Acacia* species led to browning of the medium and impairment of regeneration. AC was the commonly used anti-oxidants and adsorbents for prevention of browning in *Acacia auriculiformis* by removing inhibitory substances. It may adsorb toxic brown/black pigments and other unidentified colorless toxic compounds [20]. Therefore, by observing remarkable proliferation potential of nodal explants on AC supplemented medium as illustrated in Fig. 1c, all media were further added with 1 g l^{-1} AC for the efficient and healthy proliferation of organogenic shoots.

Adventitious Shoot Cluster Formation

For efficient shoot multiplication, prolific axillary shoot buds excised from liquid nodal cultures were transferred onto MS media supplemented with various combinations of BAP, KIN and 2-iP alone and best concentration of BAP and KIN in combination with various hormone levels of IAA. After 2 weeks of culturing, most axillary buds showed adventitious shoot proliferation and formation of multiple shoot structures known as clusters. All the media concentrations exhibited direct organogenesis without the formation of intermittent calli.

The data represents significant variations among the culture media supplemented with different types and doses of plant growth regulators. The overall frequency of shoot regeneration varied from 47.5 to 93 % as revealed in Table 1. BAP, KIN and 2iP alone has given a maximum shoot regeneration frequency of 87.5, 88.0 and 78.5 % respectively. 2iP could induce organogenic response and shoot formation but comparatively at lower efficiency. Out of five concentrations of IAA tested in combination with BAP or KIN, 0.1 and 0.5 mg l^{-1} IAA was excellent responsive with a maximum of 84.7 % shoot regeneration with 1 mg l^{-1} BAP and 93 % with 2 mg l^{-1} KIN, respectively. Higher doses of IAA than 0.5 mg l^{-1} resulted into lowered multiple shoot regeneration frequency and also influenced shoot proliferation potential of the explant. Although, BAP and KIN alone has produced good number of shoot clusters instead of 2iP, addition of IAA with BAP or KIN successfully triggered organogenic response of the explants up to the maximum enormity as depicted in Table 1. Girijashankar [12] reported highest percentage of multiple shoot induction on MS supplemented with 2 mg l^{-1} BAP and 0.1 mg l^{-1} NAA, whereas, the present results advocate for MS supplemented with 2.0 mg l^{-1} KIN and 0.5 mg l^{-1} IAA as most suitable combination of plant growth regulator for producing multiple shoot cluster from axillary bud culture as depicted in Fig. 1d. Highest average shoot length was 5.6 ± 0.07 on MS supplemented with 1.0 mg l^{-1} BAP and 0.1 mg l^{-1} IAA after multiplication stage. The combination of KIN and IAA was also reported excellent for high frequency direct plant regeneration in hardwood tree species, *Populus deltoides* [19]. Conclusively, instead of cytokinin alone, interaction of auxin and cytokinin in media can enhance organogenic and adventitious shoot proliferation response of the cultured explants.

Shoot Elongation

Profusely emerged microshoots after multiplication phase were diminutive in length. With the aim of elongation, individual microshoots obtained from multiple shoot clusters were transferred to MS medium containing various concentrations of GA_3. After 2–3 weeks of culture, the shoots got elongated to an average height of 5.5 cm on MS supplemented with 0.25 mg l^{-1} GA_3 (Fig. 1e). Several

Table 1 Effect of various plant growth regulators on multiple shoot regeneration from axillary shoot buds of *A. auriculiformis* after 12 weeks of culture

Plant growth regulators (mg l^{-1})				Percentage regeneration	No. of shoots	Shoot length (cm)
BAP	KIN	2-ip	IAA			
0.1	–	–	–	63.6	8.9 ± 0.27	3.9 ± 0.24
0.5	–	–	–	72.0	10.7 ± 0.30	4.5 ± 0.09
1.0	–	–	–	87.5	16.1 ± 0.73	5.1 ± 0.31
2.0	–	–	–	84.0	13.4 ± 0.45	5.3 ± 0.28
5.0	–	–	–	70.6	11.2 ± 0.81	3.6 ± 0.15
–	0.1	–	–	65.6	7.4 ± 0.37	2.7 ± 0.53
–	0.5	–	–	71.2	8.7 ± 0.15	3.4 ± 0.27
–	1.0	–	–	76.8	12.6 ± 0.86	5.4 ± 0.16
–	2.0	–	–	88.0	16.8 ± 0.49	5.0 ± 0.47
–	5.0	–	–	69.1	11.3 ± 0.70	3.2 ± 0.34
–	–	0.1	–	53.4	6.5 ± 0.12	2.8 ± 0.72
–	–	0.5	–	66.0	8.2 ± 0.51	3.1 ± 0.06
–	–	1.0	–	78.5	14.7 ± 0.75	4.3 ± 0.27
–	–	2.0	–	74.2	12.6 ± 0.23	4.8 ± 0.35
–	–	5.0	–	72.1	10.2 ± 0.94	3.7 ± 0.18
1.0	–	–	**0.1**	84.7	16.0 ± 0.11	**5.6 ± 0.07**
1.0	–	–	0.25	71.2	13.7 ± 0.36	5.2 ± 0.29
1.0	–	–	0.5	62.9	11.5 ± 0.23	4.6 ± 0.15
1.0	–	–	0.75	60.3	5.9 ± 0.44	4.9 ± 0.03
1.0	–	–	1.0	47.5	3.2 ± 0.56	3.2 ± 0.51
–	2.0	–	0.1	59.2	4.7 ± 0.32	3.9 ± 0.64
–	2.0	–	0.25	87.5	13.2 ± 0.37	4.4 ± 0.09
–	**2.0**	–	**0.5**	**93.0**	**17.3 ± 0.61**	4.6 ± 0.48
–	2.0	–	0.75	57.3	10.4 ± 0.48	5.3 ± 0.13
–	2.0	–	1.0	56.8	6.8 ± 0.12	2.9 ± 0.37

Data represent mean ± SE of three randomly selected readings of ten replicates per treatment. Bold characters denote significant values

researchers reported that inclusion of GA_3 in culture medium has triggered an increase in shoot length e.g. in *Albizia lebbeck* [22] in *Azadirachta indica* [23], in *Aegle marmelos* [24], *Acacia mangium* [25] and in *Acacia sinuate* [26].

GA_3, a natural growth regulator, is known to regulate several cell, tissue and organ developmental processes in plant. It is a major component in cell growth, division and differentiation events. Inclusion of GA_3 in tissue culture media can suppress callusing in cultures but promote healthier growth and differentiation even at very low concentration of 0.1 mg l^{-1} [27].

Rooting and Hardening

The elongated shoots (3–4 cm long) induced prominent rooting on half strength MS containing 0.1 mg l^{-1} IAA, 30 g l^{-1} sucrose, 1 g l^{-1} AC and 8 g l^{-1} agar (Fig. 1f, g) after 4 weeks of culture. IAA has been observed to induce better rooting response and has been extensively used to induce rooting in a wide range of plants especially in woody tree species [19]. Rooted plants were acclimatized by transferring them into plastic pots containing mixture of vermiculite, soil and sand (1:1:1) in green house condition as shown in Fig. 1h. The survival frequency of *A. auriculiformis* plants was about 85 %. Eventually, after 8 weeks, well adapted *A. auriculiformis* plants were transplanted to soil in the campus field (Fig. 1i). Both lower and higher concentrations of GA3 than 0.25 mg l^{-1} were no longer efficient for enhancing length of the microshoots as observed in Fig. 2.

RAPD and ISSR Analysis of Regenerated Microplants

Somaclonal variations are very common in tissue culture based micropropagation systems. Therefore, gDNA isolated from tissue culture-raised plants of *A. auriculiformis* was evaluated with the DNA of the mother plant for

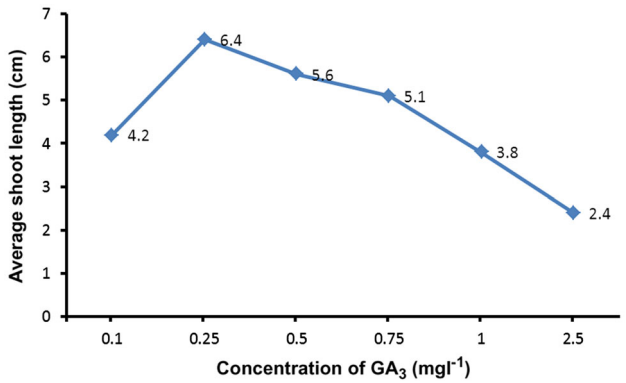

Fig. 2 Effect of various concentrations of GA_3 on shoot elongation of *A. auriculiformis*

verifying the genetic fidelity. Total 10 random RAPD primers were tested for the assessment of genetic stability. Out of these, only 7 primers produced 39 clear, scorable and reproducible amplified products. Two primers (RYR-03 and RYR-07) produced 5.12 % polymorphic bands, while all others were monomorphic in nature (Fig. 3a). The number of bands for each primer varied from 4 (RYR-01 and RYR-06) to 8 (RYR-07) and ranged between 120 and 1500 bp (Table 2). Lower frequency of polymorphic bands has also been reported in micropropagated plants of *Aconitum violaceum* [28] and in bulblets of *Lilium* sp. [14].

In contrast, out of 10 ISSR primers employed for genetic fidelity assessment, eight produced clear and scorable amplicons. Among 44 amplification products obtained from eight ISSR primers, only 2.72 % bands were polymorphic, whereas, the others were monomorphic (Fig. 3b). The number of bands for each primer varied from 3 (RYI-01) to 8 (RYI-04) in a size range of 80–2000 bp (Table 3). Virtually, among RAPD and ISSR analyses, higher number of amplification products from ISSR has been revealed. Moreover, the size range of amplicons was also higher in ISSR markers. This is because ISSR makers are widely distributed throughout the genome and yield larger number of amplification per primer in genomic DNA. The ISSR and RAPD markers have the ability to amplify different regions of genome thus result in better analysis of genetic affinity.

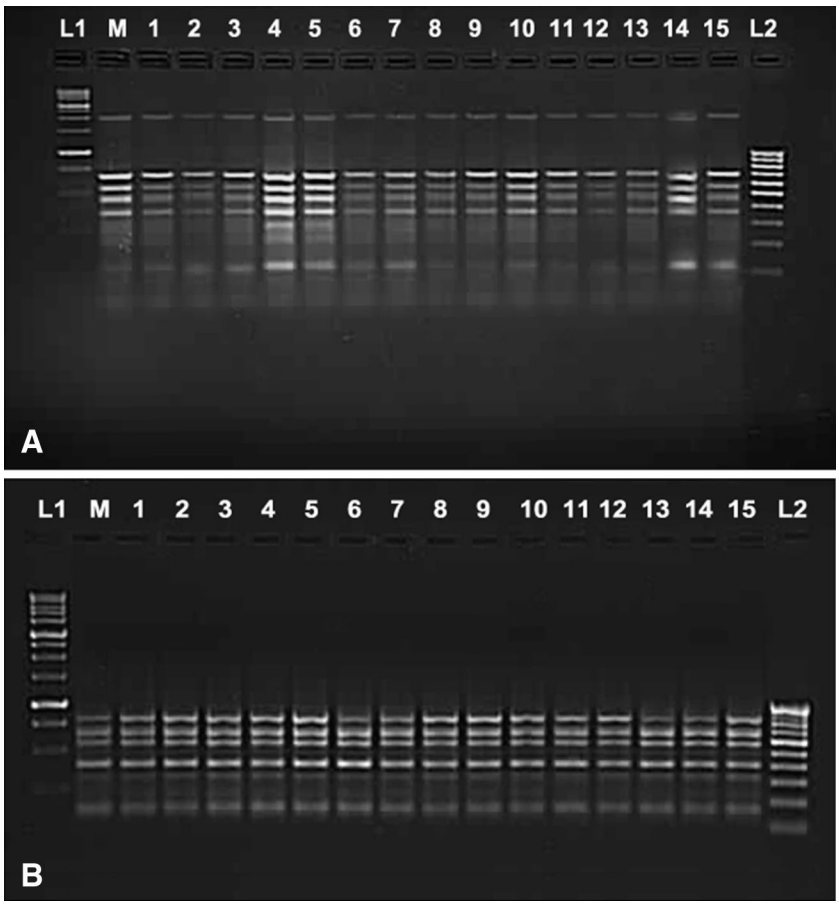

Fig. 3 Monomorphic gel profiles for DNA based molecular markers in tissue culture raised *A. auriculiformis* plants. **a** RAPD amplicon profile with RYR-04 primer; **b** ISSR amplicon profile with RYI-06 primer; *L1* and *L2*, Molecular size marker (1 kb and 100 bp DNA ladder respectively); *M* mother plant; *1–15*, fifteen randomly selected tissue culture raised plants

Table 2 List of RAPD primers used for verifying genetic fidelity in micropropagated plants of *A. auriculiformis* and their amplicons' profile

Primers code	Primer sequences (5′–3′)	No. of bands scored	No. of polymorphic bands	Size range (bp)	Polymorphic bands (%)
RYR-01	GGTCCTCTCA	4	0	300–1200	0
RYR-02	GGAGCGACTC	7	0	150–900	0
RYR-03	TCCACTACTA	5	1	250–1500	20.0
RYR-04	CACATAGGGT	6	0	120–1000	0
RYR-05	CGGTTTAGCA	–	–	–	–
RYR-06	CTAGGGCAGA	4	0	350–1000	0
RYR-07	GGAAGGCTGT	8	1	150–1200	12.5
RYR-08	AACGGCGATA	5	0	200–900	0
RYR-09	CACCAATGAG	–	–	–	–
RYR-10	TTCGACGCGA	–	–	–	–
Total		39	2	120–1500	5.12

Table 3 ISSR primers, their sequences, number and size range of the amplicons' generated by inter-simple sequence repeat (ISSR) primers in micropropagated plants of *A. auriculiformis*

Primers code	Primer sequences (5′–3′)	No. of bands scored	No. of polymorphic bands	Size range (bp)	Polymorphic bands (%)
RYI-01	$(GA)_8C$	3	0	150–800	0
RYI-02	$(GA)_8T$	5	0	350–1000	0
RYI-03	$(CT)_9A$	4	0	200–600	0
RYI-04	$(TC)_8G$	8	0	100–1200	0
RYI-05	$(CTC)_6$	–	–	–	–
RYI-06	$(AG)_8C$	7	0	150–1500	0
RYI-07	$(TC)_8C$	–	–	–	–
RYI-08	$(TG)_8G$	6	1	80–900	16.6
RYI-09	$(AC)_8G$	5	0	180–750	0
RYI-10	$(GACA)_4$	6	0	200–2000	0
Total		44	1	80–2000	2.72

Cluster analysis by the UPGMA clustering method was performed on the basis of similarity matrix calculated from the RAPD and ISSR results. UPGMA phenograms generated through similarity profile construct two major groups, which were clustered at similarity coefficients value of 0.936 and 0.982 in RAPD and ISSR analysis, respectively. These results clearly indicate that the different types of tissue culture-raised plants resembled the mother plant at the genomic level. As attributed in Fig. 3a, b, the regenerated progeny shared identical banding patterns to the donor plant, signified their genetical homology.

Conclusion

Rapid in vitro multiplication and true-to-type production of medicinal plant is important for attaining the ever increasing demand in pharmaceutical industries. Best KIN and IAA concentrations of 2.0 and 0.5 mg l^{-1} respectively, produced highest shoot regeneration frequency in *Acacia auriculiformis*. Further, GA_3 supplemented medium supported more elongation of regenerated shootlets. Arbitrary primers (RAPD and ISSR) were successfully employed for establishing genetic fidelity of micropropagated plants. These molecular markers have validated the genetic stability of tissue culture raised plants. Present study is significant for high saponin-producing medicinal plant species, *A. auriculiformis*.

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