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RESEARCH ARTICLE

# High Frequency Shoot Proliferation from Cotyledonary Node of *Lawsonia inermis* L. and Validation of their Molecular Finger Printing

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## Abstract

An efficient and reproducible protocol for *in vitro* plant regeneration was developed for *Lawsonia inermis* L. using cotyledonary node explant derived from axenic seedlings. Highest shoot proliferation frequency (ca 96.6%) was achieved on Murashige and Skoog's, 1962 (MS) basal medium supplemented with 8.88 μM 6-Benzyladenine (BA) + 2.68 μM Napthalene acetic acid (NAA). Up-scaling of shoots was carried out using *in vitro* nodes on MS medium supplemented with 4.44 μM BA. So overall, an average of 238 shoots was produced at 75 days. Of the four different forms of cotyledonary node explants evaluated, highest shoot multiplication was observed in cotyledonary node explant with two whole cotyledons. *In vitro* regenerated shoots were best rooted (ca 34.3 roots / shoot) on ½ MS medium devoid of any growth regulator. The plantlets were successfully acclimated in sand:soil :: 1:1and established in the garden soil. Random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) analysis revealed a homogeneous amplification profile for all micro-propagated plants validating the genetic fidelity of the *in vitro*-regenerated plants and supporting the regeneration protocol for economic commercial exploitation.

**Key words :** Cotyledonary node, genetic fidelity, ISSR, micropropagation, molecular markers, plant growth regulators, RAPD

## Introduction

*Lawsonia inermis* L. (syn. *Lawsonia alba* Lam.) of the family Lythraceae is commonly known as Henna or Mehendi exists in wild and cultivated forms (Sastri 1962). Henna has been widely used over the centuries for medical and cosmetic purposes (Al-Tufail et al. 1999; Charisty et al. 2012).The plant is cultivated generally in North Africa, India, SriLanka, and the Middle East etc. (Chung et al. 2002). In India, particularly in Sojat area of Pali district, Rajasthan, it is cultivated as cash crop (Ram and Sekhawat 2011). The plant contains mainly the active compound2-hydroxy-1,4-naphthoquinone (Lawsone; red-orange pigment) which makes it useful for coloring of palms, fingers, fingernails, soles as well as dyeing hair (Cartwright-Jones 2006; Hanna et al. 1998; Lekouch et al. 2001). Due to this property, the plant is also used for coloring wool, silk, leather, and dyeing industries (Nikfallah and Venugopal 2014). Along with its cosmetic property, the Henna plant is used to treat many diseases traditionally like oedema, bronchitis, menstrual disorder, rheumatism, haemorrhoids, jaundice, leprosy, headache, spleen enlargement, dysentery, skin problems, sore eyes, gonorrhoea, smallpox, veneral diseasese, spermatorohea, and herpes (Chaudhary et al. 2010; Rahmoun et al. 2010). Thus, henna claims its position as a multipurpose important medicinal plant with its important pharmacological properties.

To fulfil the requirement of cosmetic, pharmaceutical, and dyeing industry the production in India as well as in the world is not sufficient. Hence, there is an urgency to scale-up the production of the valuable multipurpose plants for com-

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mercial exploitation. Propagation through stem cuttings and seeds is a slow process and also not the preferred method due to pest and disease problems (Rout et al. 2001). On the other hand, propagation through tissue culture techniques has the potential for rapid multiplication of desired plant species in a short period of time.

There are only a few reports regarding micropropagation of *Lawsonia inermis* L. through nodal segments from mature plants (Moharana et al. 2016; Ram and Sekhawat 2011; Rout et al. 2001; Singh et al. 2012). These earlier micropropagation reports on *L. inermis* have their own limitations. Cotyledonary node, a juvenile meristematic explant, has been efficiently used for micropropagation of a number of plant species with medicinal importance namely *Punica granatum* (Naik et al. 2000), *Cassia sophera* (Parveen and Shahzad 2010), *Pongamia pinata* (Behera et al. 2013), *Cassia alata* (Ahmed et al. 2013), *Withania somnifera* (Nayak et al. 2013), etc. To date cotyledonary node-mediated plant propagation protocol has not been reported in *L. inermis*.

During tissue culture for large-scale propagation of commercially important plants, genotypic variation can be induced at any stage of development of plantlets due to culture environment and different culture conditions like pH, explant type, successive transfer of culture, and culture media type. It is therefore, imperative to establish genetic uniformity of micropropagated plants to confirm the quality of the plantlets for upholding certain important traits for its commercial utility (Moharana et al. 2016; Rai et al. 2012). Several molecular markers are used for evaluating genetic stability of micropropagated plants by different researchers like Lakshmanan et al. 2007; Martins et al. 2004; Palombi and Damiano 2002; Ray et al. 2006, etc. Among different molecular markers used for assessment of genetic fidelity of *in vitro*-regenerated plants, random amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSR) marker analysis are the simple, quick, and cost-effective methods (Lakshmanan et al. 2007). These two marker system have been successfully used to evaluate genetic stability among *in vitro*-regenerated plant species like *Withania somnifera* (Nayak et al. 2013), *Acacia auriculiformis* (Yadavet al. 2015), *Morus alba* (Saha et al. 2016), *Alpinia calcarata* (Bhowmik et al. 2016), etc.

In this paper, we developed a protocol for micropropagation with cotyledonary nodes of *L. inermis* and reported the genetic homogeneity between the regenerated and their mother plant.

# Materials and Methods

### Explants source and seed germination

Healthy mature capsules were selected and harvested from 5-year-old plant of *L. inermis* present in the campus of Ravenshaw University, Cuttack in the months of August-September. Capsules were sun dried for two days followed by three days of air drying (28-30°C) and stored at room temperature in an air-tight plastic jar. Seeds were separated from the capsules and washed thoroughly in running tap water for 30 min to remove the external adherents. Then a 10 min treatment with a liquid detergent 'Teepol' (1%; v/v; Reckitt Benckiser Ltd., India) followed by five rinses with distilled water for the proper removal of detergent from the seed surface. Thereafter, the seeds were surface sterilized with mercuric chloride ($HgCl_2$, Hi-Media, India) of 0.1% (w/v) for 15 min followed by five rinses with sterile double distilled water. The surface-sterilized seeds were inoculated in 150 ml Erlenmeyer flasks (Borosil, India) containing four different strengths (1/8, ¼, ½, and full) of Murashige and Skoog's, 1962 (MS) basal medium augmented with 3% sucrose, gelled with 0.6% agar (Hi-Media, India) without any plant growth regulators. The pH of the medium was maintained at 5.8 ± 0.1 and autoclaved for 17 min at 121°C and104 kPa. The cultures were maintained under light/dark (16/8) cycle provided by cool white fluorescent tubes (Phillips, India) and at 25 ± 1°C temperature.

### Multiple shoot induction and proliferation

The radical and plumule were removed from the 10-12-day-old axenic seedlings, then the cotyledonary node explants with two whole cotyledons were inoculated on full-strength MS medium fortified with different concentrations (0.44-17.76 µM) of 6-Benzyladenine (BA), Kinetin (Kin) and Thidiazuron (TDZ) individually. The cotyledonary nodal explants were also inoculated on MS +8.88 µM BA media supplemented with auxins [0.53-10.74] µM Naphthalene acetic acid (NAA) / [0.49-9.84] µM Indole-3-butyric acid (IBA)/ [0.57-11.42] µM Indole-3-acetic acid (IAA)] to check their influence for multiple shoot proliferation. The cotyledonary nodes with emerging shoot buds were sub-cultured to fresh media of same composition for further shoot multiplication at 15-day interval. Fifteen days after the 1st subculture, the same cotyledonary node explants were transferred to growth regulator free MS medium for elongation of shoots. Aiming at up-scaling of shoots, the primary shoots regenerated from cotyledonary node were harvested, cut into node pieces (0.9-1.0 cm) and the axenic nodes were cultured on MS supplemented with 4.44 µM BA for shoot multiplication. After harvest of *in vitro* shoots, the mother cotyledonary node explants were further repeatedly sub-cultured (four times) on shoot multiplication media [MS + BA (8.88 µM) + NAA (0.53-10.74 µM)] followed by growth regulator-free MS media.

Different forms of cotyledonary node explants (i.e. explant with two whole cotyledons, explant with two proximal halves of cotyledon, explant with single whole cotyledon with embryonic axis, and explant without cotyledons with intact embryonic axis) were evaluated in the best shoot multiplication medium. All the cultures were maintained under similar environments as for the seed germination experiment.

### Root induction of *in vitro*-regenerated shoots

*In vitro* shoots of 3.5-4.0 cm in length with fully expanded 3-4 leaves were excised from the clump and transferred to culture tubes (Borosil, India) of 60 ml containing 1/16 MS, 1/8 MS, ¼ MS, ½ MS, MS, and ½ MS + 0.57-11.42 µM IAA

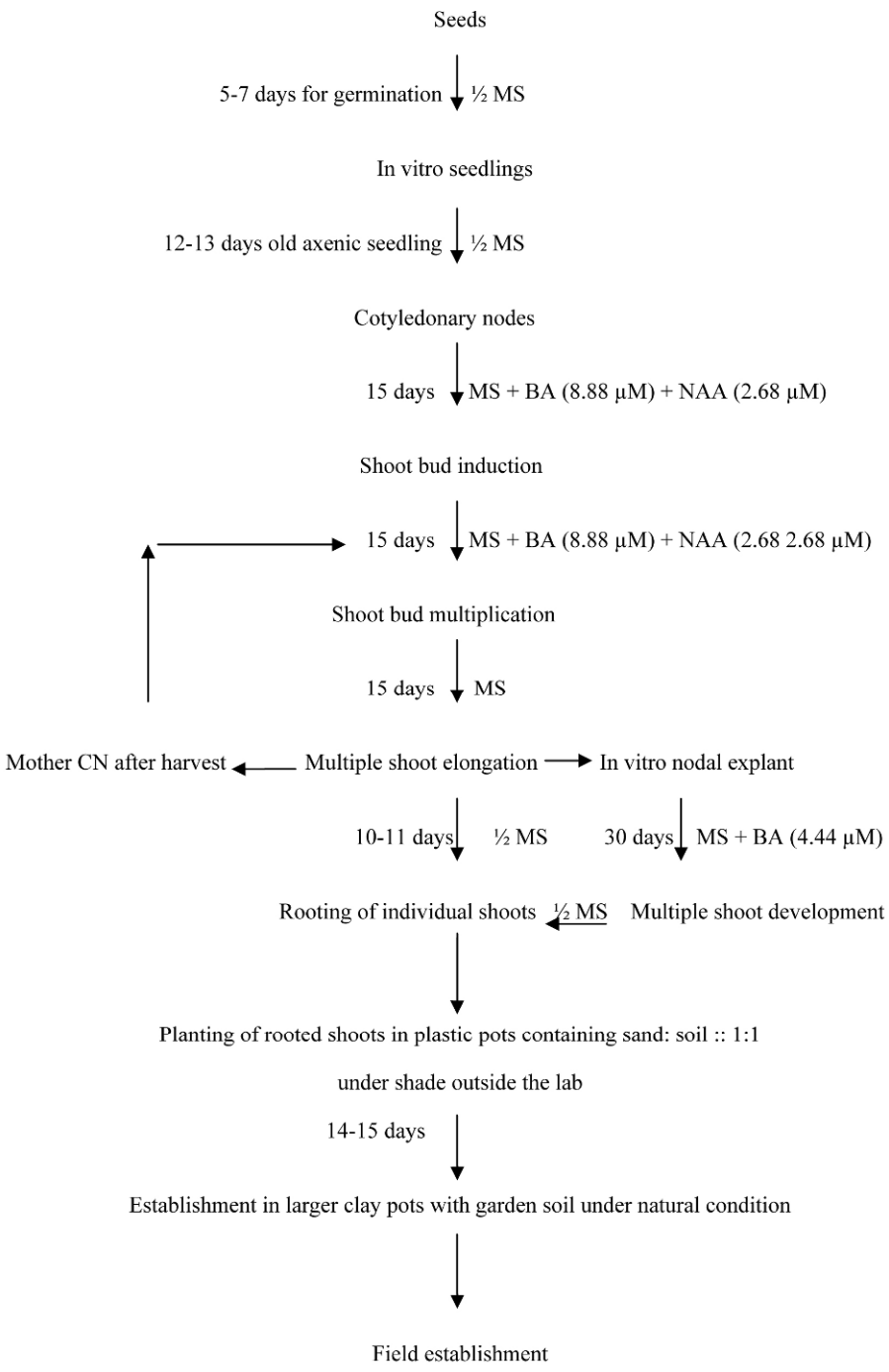


Fig. 1. A complete schematic representation of stepwise protocol of in vitro regeneration of *Lawsonia inermis* through cotyledonary node.

or 0.49-9.82 µM IBA, or 0.53-10.74 µM NAA medium gelled with 0.6% (w/v) agar for rooting.

## Acclimatization and soil establishment of plantlets

The perfectly rooted plantlets were carefully removed from the culture medium and the roots were gently washed in plastic tray with water to remove agar properly. The plantlets were then transferred to plastic glasses (3.0 cm dia.) containing different proportions of autoclaved sand: soil (1:1or 1:2 or 2:1) and moistened with normal tap water. The plantlets were kept outside the lab under shade (approx. 28-30°C) to avoid the direct contact of sunlight. The plantlets were dampened once in every day by spraying water to the leaves and surrounding of the plants for14-15 days after which they were transferred to larger earthenware pots containing garden soil under full sunlight with daily watering for its survival (Fig. 1).

### Statistical analysis of data

Seed viability was studied by taking the observations of germination percentage and days required for germination immediately after harvest up to one year with three-months intervals. For seed germination experiment each treatment consisted of two replicates (culture flasks) and the experimental unit was 25 seeds / flask. The experiment was repeated twice. The shoot proliferation experiment consisted of two explants / flask, five flasks per treatment, and repeated three times. Data on percent response, the number of shoots / explant, and shoot length were recorded after 70-75 days by visual observations. The experimental unit for rooting was set with one shoot per tube, five tubes per replica. This experiment was carried out thrice separately and data recorded after 15 days. Data were subjected to analysis of variance (ANOVA) for a completely randomized design (CRD) at 5% level of probability. Duncan's multiple range tests (DMRT; Gomez and Gomez 1984) was used to separate the means to determine significant effects. Some experimental data are presented as mean ± standard error (SE).

### Genetic fidelity analysis by RAPD and ISSR markers

#### *Plant materials for genomic DNA isolation*

Randomly 10 *in vitro*-raised plants were chosen from the regenerated plants derived from cotyledonary node to compare their clonal fidelity with the mother plant growing in natural habitat using RAPD and ISSR markers.

### Genomic DNA isolation from leaf

Modified cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle 1990) was used for extraction of total genomic DNA from the fresh young leaf tissue of both mother plant as well as 10 randomly selected micropropagated plants. The RNA was removed from the genomic DNA samples by treating them with RNase (10 mg/ml, Merk, India). Quantification of DNA samples were carried out by comparing band intensities on agarose gel (0.8%) and UV visible spectrophotometer (Sambrook and Russed 2001).

### RAPD and ISSR analysis

During the experiment a series of random RAPD decanucleotide primers consist of 25 primers of RPI series (RPI 1-RPI 25; GeNei$^{Tm}$, India), 19 primers of OPR (OPR1, OPR3-OPR20; Integrated DNA Technologies, USA), six of OPD (OPD 8-9, OPD 12, OPD 18-20; Operon Technoligies, USA), and two of OPN (OPN 18-19; Operon Technologies, USA) series were screened. PCR amplifications were carried out in total reaction mixture of 25 µl containing 1 µl (20 ng) of template DNA, 2.5 µl of (10x) *Taq* buffer (with15 mM $MgCl_2$), 0.2 µl of 25mM dNTPs, 1.0 µl of primer (5.0Pm/µl), and 0.17 µl of 3U/µl *Taq* DNA polymerase (GeNei$^{Tm}$, Mumbai), 20.13 µl nuclease-free distilled water (Integrated DNA Technologies, USA). DNA amplification was performed on a thermal cycler (Bio-Rad, USA). PCR was set for 42 cycles, for preliminary denaturation of template DNA at 94°C for 5 min, for denaturation at 92°C for 1 min, for primer annealing at 37 °C for 1 min and for primer extension at 72°C for 2 min. Seven minutes of an additional incubation at 72°C was given for the final extension cycle. At last the holding/soaking temperature was set and maintained at 4°C. On the basis of clear, bright, and scorable banding patterns of the amplicons, 14 of these were selected for analysis. Various primers, quantity of template DNA, annealing temperature, and buffer with $MgCl_2$ or without $MgCl_2$ were tested to determine the optimum result during the initial experiments.

For ISSR amplification 20 microsatellite primers (Sigma-Aldrich, India) which are anchored and non-anchored were randomly selected and used. Out of twenty primers, ten were chosen for the analysis because they produced highly reproducible and scorable bands. PCR for ISSR amplification was also performed in a final volume of 25 µl with same concentrations and volumes of all the reaction components as in RAPD. The optimized PCR conditions programmed for ISSR amplifications was same as that for RAPD, except the primer annealing temperature was set at 51°C.

### Data scoring and analysis

Resolutions of the amplified products were done by electrophoresis through 1.8% agarose gel (Lonza, USA) for RAPD and ISSR in (1x) TAE buffer (pH 8.0). The bands in gel were visualized and photographed using gel documentation system (Bio-Rad, USA). The amplified fragments sizes were estimated using Gene Ruler 1kb DNA ladder (Fermentas, Lithuania). Two independent amplification reactions were performed with all RAPD and ISSR primers to assess the consistency and accuracy of the reproducible bands. Independent of their intensity, amplified products with the same migration were considered homologous fragments. For accuracy all experiments were repeated twice.

## Results and Discussion

### Seed viability

Seed viability was evaluated on different concentrations of MS basal medium without any growth regulators at different interval of storage. Among four different strengths of basal MS tested, maximum 98.0% of seed germination was achieved on half-strength MS (½ MS) with 3% sucrose and 0.6% agar without any growth regulators after 5-6 days of inoculation (Fig. 2A). But previously Bakkali et al. (1997) germinated the Henna seed on ½ MS with 0.8% agar however he did not reported about the germination percentage, required time for germination etc. Half-strength MS was also found suitable for seed germination of another medicinal plant, *Withania somnifera* by Nayak et al. (2013).

In our experiment it was noticed that, germination was 98% when seeds were used immediately after harvest in the months of August-September. After three months of storage the ger-

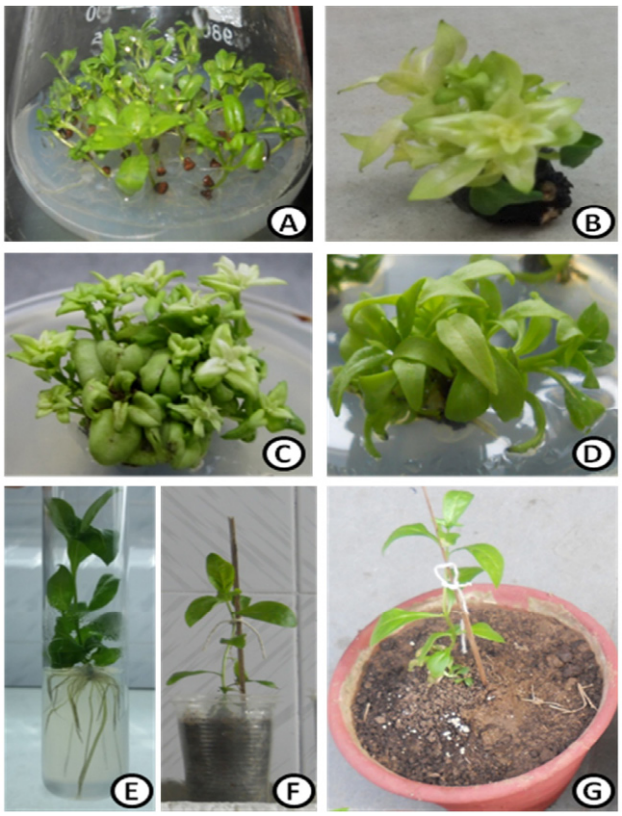


**Fig. 2.** (A) Seed germination on ½ MS (B) Initiation of multiple shoots from cotyledonary nodal explant cultured on MS + 8.88 µM BA + 2.68 µM BA NAA at day 20 (C) Elongation of multiple shoots on MS without any growth regulators at 45 days (D) Multiple shoots from an in vitro nodal explant culture on MS + 4.44 µM BA BA at day 30 (E) A rooted shoot on half-strength MS after 15 days of culture (F) A one week old acclimated plant in a plastic glass outside lab (G) A one month-old acclimated plant in a earthen ware pot.

mination rate reduced to 95%. Rate of seed germination declined gradually after subsequent storage and after six, nine, and twelve months the germination rate was recorded to be 85, 73, and 48%, respectively (Table 1). The decline of seed viability rate was observed with the passage of time. So, use of seeds within short period between collection and experimental use was advisable for maximum germination of *L. inermis*. The same was also reported by Nayak et al. 2013 while working with *Withania somnifera.*

## Shoot multiplication

10-12-day-old axenic seedlings were used as source of explants after the removal of primary shoot and root. The cotyledonary node explants developed only two shoots on growth regulator-free MS basal media which was enhanced when MS basal media supplemented with different concentrations of cytokinins (BA, Kin, and TDZ) separately or in combination with auxins (NAA, IAA, and IBA). Of the three cytokinins tested, BA was found superior than Kin and TDZ. Among the different concentrations of BA, 8.88 µM was found as optimum resulting 93.3% of shoot response, 7.1 shoots/explant with shoot length 3.4 cm. Addition of NAA at an optimal concentration of 2.68 µM to the above said shoot proliferation medium i.e. MS + 8.88 µM BA, increased the percentage of regeneration to 96.6 and number of shoots to 13.6 with average shoot length of 4.1 cm (Table 2, Fig. 2B and C). So the best result in our study was recorded on MS medium supplemented with BA (8.88 µM) + NAA (2.68 µM). However, to get the above optimum result (shoot number, percentage, and length) the whole explant was sub-cultured twice, first to the same shoot multiplication medium and second to the growth regulator-free MS medium for shoot elongation at 15-day intervals. Shekhawat et al. (1993) suggested that repeated transfer of explants on media resulted in rejuvenation of explant tissues which promoted activation and conditioning of meristems, while working on *Prosopis cineraria*. The synergistic effect of cytokinin-auxin combinations i.e. BA with NAA observed in our result was also reported by Faisal et al. 2006 (*Mucuna pruriens*), Parveen and Shahzad 2014 (*Senna sophera*), and Kumar et al. 2016 (*Salvadora oleoides*) while working with cotyledonary node explants. The requirement of higher concentration of cytokinin and lower concentration of auxin for the promotion of multiple shoot was also reported by Kohlenbach 1997 and Beena et al. 2003.

For the commercial scale-up purpose there is a necessity to accelerate the multiplication of shoots within less time. Thus, the mother cotyledonary node explant was sub-cultured on the above shoot multiplication medium after each harvest of *in vitro*-regenerated shoots. Each mother cotyledonary node explant was sub-cultured for three times after the 1$^{st}$ harvest, where we obtained 9-10 shoots, 7-8 shoots, and 2-3 shoots during 2$^{nd}$, 3$^{rd}$, and 4$^{th}$ harvest, respectively. A significant

**Table 1.** Assessment of seed viability with respect to storage time period of three months interval.

| Strength of MS | Immediately after harvest (Mean ± SE) | | After 03 months of harvest (Mean ± SE) | | After 06 months of harvest (Mean ± SE) | | After 09 months of harvest (Mean ± SE) | | After 12 months of harvest (Mean ± SE) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | PG | DG | PG | DG | PG | DG | PG | DG | PG | DG |
| 1/8 | 78.0±0.05 | 8-10 | 73.0±1.0 | 14-16 | 65.0±0.0 | 19-21 | 53.0±1.0 | 24-26 | 28.0±0.0 | 28-30 |
| 1/4 | 88.0±1 | 7-8 | 85.0±1.0 | 11-12 | 80.0±1.0 | 16-17 | 66.0±0.5 | 19-20 | 40.0±0.5 | 24-25 |
| 1/2 | 98±0.57 | 5-6 | 95.0±1.0 | 9-10 | 85.0±0.0 | 13-14 | 73.0±0.5 | 17-18 | 48.0±0.5 | 21-22 |
| Full | 92.0±0 | 5-6 | 90.0±1.0 | 9-10 | 82.0±1.0 | 13-14 | 71.0±0.5 | 17-18 | 47.0±0.5 | 21-22 |

PG = Mean percentage of germination, DG = Days for germination.

**Table 2.** Effect of plant growth regulators (PGRs) on shoot proliferation from axenic cotyledonary node of *L. inermis*

| MS + PGRs (µM) | Mean percentage of shoot development | Mean no. of shoots/explant | Mean shoot length (cm) |
|---|---|---|---|
| Control (MS) | 56.6$^{l}$ | 2.0$^{w}$ | 2.1$^{lmn}$ |
| MS + BA | | | |
| 0.44 | 66.6$^{i}$ | 2.7$^{tuv}$ | 2.1$^{lmn}$ |
| 1.11 | 66.6$^{i}$ | 2.7$^{tuv}$ | 2.2$^{klm}$ |
| 2.22 | 83.3$^{e}$ | 3.8$^{pqr}$ | 2.8$^{efgh}$ |
| 4.44 | 90.0$^{c}$ | 5.3$^{efg}$ | 2.9$^{defg}$ |
| 8.88 | 93.3$^{b}$ | 7.1$^{c}$ | 3.4$^{c}$ |
| 13.32 | 86.6$^{d}$ | 5.5$^{e}$ | 2.9$^{defg}$ |
| 17.76 | 63.3$^{j}$ | 6.0$^{d}$ | 2.6$^{ghij}$ |
| MS + Kin | | | |
| 0.46 | 56.6$^{l}$ | 2.0$^{w}$ | 2.1$^{lmn}$ |
| 1.16 | 60.0$^{k}$ | 2.0$^{w}$ | 2.1$^{lmn}$ |
| 2.33 | 60.0$^{k}$ | 2.7$^{tuv}$ | 2.5$^{kjih}$ |
| 4.67 | 70.0$^{h}$ | 3.1$^{rst}$ | 2.6$^{ghij}$ |
| 9.34 | 73.3$^{g}$ | 3.2$^{rs}$ | 2.8$^{efgh}$ |
| 14.01 | 63.3$^{j}$ | 2.9$^{stu}$ | 2.7$^{fghi}$ |
| 18.68 | 63.3$^{j}$ | 2.3$^{vw}$ | 2.3$^{jkl}$ |
| MS + TDZ | | | |
| 0.45 | 56.6$^{i}$ | 4.0$^{opq}$ | 2.0$^{lmno}$ |
| 1.13 | 56.6$^{l}$ | 4.0$^{opq}$ | 1.9$^{mnop}$ |
| 2.27 | 60.0$^{k}$ | 5.3$^{efg}$ | 1.7$^{opqr}$ |
| 4.55 | 70.0$^{h}$ | 5.3$^{efg}$ | 1.7$^{opqr}$ |
| 9.10 | 73.3$^{g}$ | 5.0$^{ghij}$ | 1.6$^{pqrs}$ |
| 13.6 | 63.3$^{j}$ | 4.8$^{jkl}$ | 1.5$^{qrst}$ |
| 18.2 | 56.6$^{l}$ | 4.5$^{lmn}$ | 1.5$^{qrst}$ |
| MS + BA + NAA | | | |
| 8.88 + 0.53 | 93.3$^{b}$ | 7.1$^{c}$ | 3.0$^{def}$ |
| 8.88 + 1.34 | 93.3$^{b}$ | 7.8$^{b}$ | 3.5$^{b}$ |
| 8.88 + 2.68 | 96.6$^{a}$ | 13.6$^{a}$ | 4.1$^{a}$ |
| 8.88 + 5.37 | 80.0$^{g}$ | 6.8$^{c}$ | 3.2d$^{c}$ |
| 8.88 + 10.74 | 73.3$^{g}$ | 5.3$^{efg}$ | 3.1$^{cde}$ |
| MS + BA + IBA | | | |
| 8.88 + 0.49 | 83.3$^{g}$ | 5.1$^{ghi}$ | 1.3$^{stu}$ |
| 8.88 + 1.23 | 83.3$^{g}$ | 5.4$^{ef}$ | 1.5$^{qrst}$ |
| 8.88 + 2.46 | 83.3$^{g}$ | 5.1$^{ghi}$ | 1.8$^{nopq}$ |
| 8.88 + 4.92 | 80.0$^{f}$ | 4.8$^{jkl}$ | 1.6$^{pqrs}$ |
| 8.88 + 9.84 | 63.3$^{j}$ | 4.3$^{no}$ | 1.4$^{rstu}$ |
| MS + BA + IAA | | | |
| 8.88 + 0.57 | 73.3$^{g}$ | 4.1$^{nop}$ | 1.6$^{pqrs}$ |
| 8.88 +1.42 | 83.3$^{g}$ | 5.2$^{fgh}$ | 1.6$^{pqrs}$ |
| 8.88 + 2.85 | 83.3$^{g}$ | 4.9$^{hijk}$ | 1.9$^{mnop}$ |
| 8.88 + 5.71 | 63.3$^{j}$ | 4.6$^{lm}$ | 1.8$^{nopq}$ |
| 8.88 + 11.42 | 56.6$^{l}$ | 4.1$^{nop}$ | 1.7$^{opqr}$ |

Means followed by the same letter within columns are not significantly different (P = 0.05) according to DMRT.

decline in the shoot number during the 4$^{th}$ harvest imposed us for no further subculture of the mother explants (Table 3). But contrary to our results, in *Acacia sanuata,* the maximum number of shoots reported in the 2$^{nd}$ harvest compared to 1$^{st}$ harvest and then subsequently it decreased gradually during 3$^{rd}$ and 4$^{th}$ harvest (Vengadesan et al. 2002). For further shoot multiplication, *in vitro* nodal segments derived from the primary shoots regenerated from mother cotyledonary node were used as explants source (Fig. 2 D; Moharana et al. 2016). This facilitated the production of ca 238 numbers of shoots after the 1$^{st}$ harvest and subsequent up-scaling with axenic nodes at 75 days, whereas ca 540 numbers of shoots were produced after 4$^{th}$ harvest of mother cotyledonary node at day 210.The shoot numbers in our case was higher than the other reports (Moharana et al. 2016; Ram and Shekhawat 2011; Rout et al. 2001; Singh et al. 2012) in *L. inermis* where they used mature node for multiple shoot regeneration.

The size and number of cotyledons affected the multiple

**Table 3.** Shoot multiplication during different harvest period from *L. inermis* cotyledonary node on MS + BA 8.88 µM and different concentrations of NAA.

| MS + PGRs (µM) | Percentage of explant responded (Mean ± SE) | Initial culture (1st harvest) | | I subculture (2nd harvest) | | II subculture (3rd harvest) | | III subculture (4th harvest) | |
|---|---|---|---|---|---|---|---|---|---|
| | | No. of shoots / explant (Mean ± SE) | Shoot length in (cm) (Mean ± SE) | No. of shoots / explant (Mean ± SE) | Shoot length in (cm) (Mean ± SE) | No. of shoots / explant (Mean ± SE) | Shoot length in (cm) (Mean ± SE) | No. of shoots / explant (Mean ± SE) | Shoot length in (cm) (Mean ± SE) |
| Control (MS) | 56.6±0.1 | 2.0±0.05 | 3.4±0.1 | 2±0.05 | 3.3±0.1 | 1.0±0.0 | 3.1±0.06 | 0±0.0 | 0±0.0 |
| BA + NAA | | | | | | | | | |
| 8.88+ 0.53 | 93.3±0.1 | 7.1±0.05 | 3.0±0.0 | 6.5±0.05 | 2.7±0.05 | 6.0±0.1 | 2.6±0.1 | 2.4±0.1 | 2.4±0.1 |
| 8.88+ 1.34 | 93.3±0.0 | 7.8±0.05 | 3.5±0.05 | 6.9±0.1 | 3.1±0.1 | 6.1±0.1 | 2.9±0.1 | 2.7±0.1 | 2.7±0.05 |
| 8.88+ 2.68 | 96.6±0.1 | 13.6±0.1 | 4.1±0.06 | 9.5±0.1 | 3.7±0.1 | 7.4±0.1 | 3.3±0.05 | 2.8±0.05 | 3.1±0.06 |
| 8.88+ 5.37 | 80.0±0.0 | 6.8±0.05 | 3.2±0.1 | 6.8±0.0 | 3.1±0.06 | 6.1±0.05 | 3.0±0.1 | 2.5±0.05 | 2.9±0.05 |
| 8.88+ 10.74 | 73.3±0.15 | 5.3±0.0 | 3.1±0.05 | 6.3±0.05 | 2.9±0.0 | 6.0±0.1 | 2.8±0.05 | 2.3±0.05 | 2.1±0.0 |

**Table 4.** Multiple shoot regeneration potency of different forms of cotyledonary node explant of *L. inermis* after 75 days of inoculation on MS + BA 8.88 µM + NAA 2.68 µM.

| Explant type (Different forms of cotyledonary node) | % of shoot development (Mean ± SE) | No. of shoots / explants (Mean ± SE) | Shoot length in cm (Mean ± SE) |
|---|---|---|---|
| Explants without cotyledons with intact embryonic axis | 20.0±0.05 | 2.2±0.05 | 3.28±0.01 |
| Explant with single whole cotyledon with embryonic axis | 80.0±1.0 | 5.3±0.05 | 3.39±0.005 |
| Explant with two whole cotyledons with embryonic axis | 95.0±0.5 | 13.6±0.5 | 4.1±0.05 |
| Explant with two proximal halves of cotyledon with embryonic axis | 90.0±0.5 | 8.1±0.5 | 3.51±0.01 |

shoot regeneration response during the culture of the cotyledonary node as explants. In our experiment it was observed that the different types of cotyledonary node explants influenced multiple shoot regeneration to a great extent on the optimum shoot regeneration medium, i.e. MS + BA (8.88 µM) + NAA (2.68 µM). Out of four different forms of explants used, highest shoot regeneration (13.6) were recorded with cotyledonary node having two whole cotyledons (Table 4). A similar result has been observed in *Lathyrus sativus* (Barik et al. 2004) where they also used the four different forms like us and best result was shown by cotyledonary node with two whole cotyledons. In *Lathyrus ochrus*, Saglam (2012) used a different form of cotyledonary node, i.e. longitudinally sliced, half-cotyledonary node for its plant regeneration. A delayed shoot regeneration response and production of few shoots were observed after complete removal of both the cotyledons. Barik et al. (2004) also reported about similar types of observations in *Lathyrus sativus*.

Nowadays juvenile explants have been extensively employed by workers for the clonal propagation of woody plants. According to Paiker and Kandir (2011), a beneficial, successful, and much rapid tissue culture protocol should be developed for commercially and medicinally valuable woody species such as *Lawsonia inermis*. The usefulness of axenic cotyledonary node is probably due to the high frequency multiplication rate of axillary meristematic tissue (Nayak et al. 2013). Besides the additional advantages of using the cotyledonary node meristems is that they are free from phenolic compounds whose accumulation inhibits shoot formation in micropropagation (Chiruvella et al. 2013) which is a common problem in micropropagation of *L. inermis* through *in vivo* nodal explants (Moharana et al. 2016).

## Root induction

For rooting, different strengths (1/16, 1/8, ¼, ½, full) of MS basal media and ½ MS supplemented with different auxins were tested. The best result was observed on half-strength MS medium devoid of any growth regulators (Table 5). The highest percentage of rooting (100%), highest root number/shoot (34.3), and highest average root length (3.9 cm) was achieved within 20 days on the said medium (Table 5, Fig. 2E). In addition, we also observed that auxins were not enhancing the rooting of *in vitro* regenerated shoots. Our result is in corroboration to Bakkali et al. (1997) where *in vitro* shoots were rooted on auxin-free ½ MS medium. Contrary to our results, Ram and Sekhawat (2011) failed to induce root in growth regulator free ¼ MS but with addition of IBA (24.6 µM) they were able to induce rooting in *L. inermis*. Further, they reported the requirement of IBA and activated charcoal for rooting. In *Cuphea aequipetala*, a genus belonging to same family of *L. inermis*, rooting was also observed on MS medium devoid of any growth regulators (Blanca et al. 2012). In medicinal shrub like *Cordia verbenacea*, Lameira and Pinto (2006) successfully rooted the *in vitro*-regenerated shoots on MS devoid of any auxins. Rooting on basal medium devoid of auxin was may be possible due to the presence of sufficient endogenous auxins in the *in vitro*-formed shoots (Daffalla et al. 2011).

**Table 5.** Influence of auxins on rooting of the *in vitro* derived shoots from the cotyledonary nodes of *L. inermis*.

| Media | Mean percentage of root development | Root / shoot Mean no. of roots / explant | Mean root length in (cm) |
|---|---|---|---|
| 1/16MS | 46.0$^{h}$ | 8.0$^{t}$ | 0.9$^{o}$ |
| 1/8 MS | 60.0$^{f}$ | 15.0$^{qp}$ | 1.4$^{klm}$ |
| ¼ MS | 86.6$^{b}$ | 21.7$^{k}$ | 3.1$^{c}$ |
| ½ MS | 100$^{a}$ | 34.3$^{a}$ | 3.9$^{a}$ |
| MS | 100$^{a}$ | 25.6$^{ed}$ | 3.6$^{b}$ |
| ½ MS + IBA | | | |
| 0.49 | 86.6$^{b}$ | 28.7$^{b}$ | 2.7$^{d}$ |
| 1.23 | 80.0$^{c}$ | 25.9$^{d}$ | 2.5$^{de}$ |
| 2.46 | 80.0$^{c}$ | 25.4$^{ef}$ | 2.3$^{efg}$ |
| 4.92 | 73.3$^{d}$ | 22.8$^{hi}$ | 2.2$^{fgh}$ |
| 9.82 | 66.6$^{e}$ | 20.3$^{m}$ | 2.1$^{ghi}$ |
| ½ MS + IAA | | | |
| 0.57 | 86.6$^{b}$ | 27.2$^{c}$ | 2.4$^{ef}$ |
| 1.42 | 73.3$^{d}$ | 25.1$^{fg}$ | 2.2$^{fgh}$ |
| 2.85 | 73.3$^{d}$ | 23.0$^{h}$ | 1.6$^{jk}$ |
| 5.71 | 66.6$^{e}$ | 21.6$^{kl}$ | 1.2$^{mn}$ |
| 11.42 | 53.3$^{g}$ | 17.8$^{o}$ | 0.6$^{p}$ |
| ½ MS + NAA | | | |
| 0.53 | 73.3$^{d}$ | 22.7$^{hij}$ | 2.3$^{efg}$ |
| 1.34 | 66.6$^{e}$ | 18.8$^{n}$ | 2.1$^{ghi}$ |
| 2.68 | 60.0$^{f}$ | 15.2$^{p}$ | 1.$^{9i}$ |
| 5.37 | 53.3$^{g}$ | 12.3$^{r}$ | 1.8$^{ij}$ |
| 10.74 | 46.0$^{h}$ | 9.7$^{s}$ | 1.5$^{kl}$ |

Auxins supplemented in µm; Means followed by the same letter within columns are not significantly different (P = 0.05) according to DMRT

## Acclimatization and soil establishment of plants

Perfectly rooted plantlets with fully expanded 5-7 leaves were successfully transferred directly to the plastic glass after gentle removal of agar from the roots (Fig. 2F). In this study, gradual steps for acclimatization was omitted which leads to time saving with 90% survival rate. Out of different planting substrate tried (sand: soil :: 2:1, 1:2, 1:1), the 1:1 ratio was most suitable. Thiyagarajan and Venkatachalam (2013) also used sand and soil as planting substrate for acclimatization of *in vitro*-raised plantlets of *Gymnema sylvestre*. However, they used sand and soil in 1:2 proportions. The ratio of sand with soil in the planting substrate was crucial for acclimatization process. Presence of appropriate proportion of sand may be responsible for proper aeration and water retention capacity of the plating substrate (Keng et al. 2009). Subsequently, we transferred the plantlets to the larger pots containing garden soil and then to field where 100% survival rate was noticed (Fig. 2G).

## Clonal homogeneity study

The production of clonal plants is one of the most important prerequisites for successful micropropagation and commercial exploitation of any crop species like *L. inermis.* In this study we have used a juvenile meristematic explant (cotyledonary node) for axillary shoot proliferation and plant regeneration. However, a number of factors during culture have the potential to induce genetic changes in the regenerants (Moharana et al. 2016). Thus to assess the genetic homogeneity among tissue culture derived plants and mother plant should be evaluated through morphological and molecular marker analysis. In this study the *in vitro*-raised plants were found to be phenotypically normal and essentially identical with their mother in field (natural environment) by visualization which partly suggest the minimal or absence of somaclonal variation in tissue culture-derived plants. However, morphological markers are greatly influenced by environment and age of the plant. But molecular markers are not subjective to environmental factors as well as age. In addition, molecular markers generate reproducible and reliable results (Kawiak and Lojkowska 2004; Rathore et al. 2011).

Thus in this study, two different molecular markers (RAPD and ISSR) were employed to screen the *in vitro*-regenerated plantlets of *L. inermis* for their clonal fidelity. Among the molecular marker systems, RAPD is becoming a widely employed method for detection of genetic fidelity since it has the advantage of being technically simple, quick to perform, and requires only small amounts of DNA (Williams et al. 1990). Many workers have reported the absence of genetic variation in micropropagated plants in a number of species including *Lilium bulblets* (Varshney et al. 2001), *Drosera binata* (Kawiak and Lojkowska 2004), and *Pongamia pinnata* (Satapathy et al. 2014) employing RAPD. At the same time, ISSR is an alternative technique to study polymorphism based on the presence of microsatellites throughout genomes (Zietkiewicz et al. 1994). The ISSR markers have also been useful in screening genetic stability of several micropropagated plants such as *Populus tremuloides* (Rahman and Rajora 2001),

**Table 6.** Details of primers used and amplification products obtained during RAPD analysis of mother and micropropagated plants of *L. inermis*.

| Primer | Sequence from 5'motif- 3'motif | No. of distinct scorable bands | Approximate range of amplifications (bp) |
|---|---|---|---|
| RPI-3 | AAG CGA CCT G | 7 | 650-1700 |
| RPI-4 | AAT CGC GCT G | 4 | 1000-2500 |
| RPI-5 | AAT CGG GCT G | 1 | 1750 |
| RPI-7 | ACA TCG CCC A | 2 | 700-1550 |
| RPI-9 | ACC GCC TAT G | 5 | 300-900 |
| RPI-12 | ACG GCA ACC T | 2 | 500-1100 |
| RPI-16 | AGG CGG GAAC | 2 | 650-1550 |
| RPI-17 | AGG CGG CAAG | 3 | 350-1000 |
| RPI-20 | AGT CCG CCT C | 4 | 600-1150 |
| RPI-21 | CAC GAA CCT C | 1 | 500 |
| RPI-24 | CCA GCC GAA C | 6 | 650-1600 |
| RPI-25 | GAG CGC CTTC | 2 | 700-1050 |
| OPN-16 | AAGCGACCTG | 4 | 650-1200 |
| OPN-18 | GGTGAGGTCA | 4 | 750-1300 |

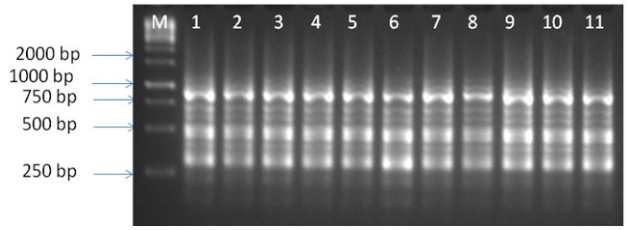


**Fig. 3.** Banding profile of both mother plant and tissue culture plant using RAPD marker RPI-9. Lane 1 mother plant, lane 2-6 in vitro regenerants of cotyledonary node prior to acclimatization in soil, 7-11 the other five were micropropagated field grown plants of 10-11 months old and M indicates the 1 Kb ladder.

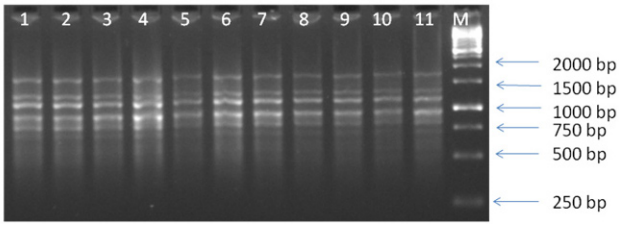


**Fig. 4.** ISSR banding profile of both micropropagated plants and mother plant regenerated by primer (GGA)4. Lane 1 mother plant, lane 2-6 in vitro regenerants of cotyledonary node prior to acclimatization in soil, 7-11 the other five were micropropagated field grown plants of 10-11 months old and M indicates the 1 Kb ladder.

*Ochreinauclea missionis* (Chandrika and Rai 2009), and *Psidium guajava* (Liu and Yang 2012). However, Marteins et al. (2004) suggested the use of two marker systems which amplifies different regions throughout the genomic DNA more perfectly and provide better information about the genetic variation in micropropagated clones. Genetic variations induced during tissue culture of *L. inermis* may lead to loss of important agronomic traits in the plant and thus, will hamper its commercial utility (Moharana et al. 2016). Thus, in this study we used two PCR-based molecular marker systems namely RAPD and ISSR to test the uniformity of the *in vitro*-regenerated plants of *L. inermis* at the genetic level. A total of 52 RAPD primers were used to screen somaclonal variations, if any, out of which only 14 primers were successful in amplifying the genomic DNA as they produce clear reproducible scorable bands ranging size from 300-2500 bp (Table 6). All these RAPD primers amplify separate regions which appear as distinct monomorphic bands (Fig. 3) ranging from 1-7 bands per primer and the 14 primers produced a total of 47 amplicons with average of 3.3 bands per primer. The number and size range of amplified scorable bands for RAPD profile has been depicted in Table 6 and Fig. 3. In this study, due to the absence of any polymorphic band, it was assumed that all the *in vitro* regenerants are genetically identical with that of the donor plant.

ISSR primers usually have better reproducibility and show higher polymorphism compared to RAPD may be due to the presence of more number of SSR regions (Ray et al. 2006). Further, ISSR markers are comparatively larger in size than the decamer RAPD primers and therefore have higher annealing temperature. Lower annealing temperature might produce artifact amplified products due to non-specific amplification but it was not the case in ISSR primers due to higher annealing temperature (Goyal et al. 2015). Thus, in addition to RAPD, we also used some anchored and non-anchored which were of di, tri and tetra-nucleotide repeats as ISSR marker. During ISSR finger printing, out of 20 preliminarily screened primers, 10 were selected which produced a total number of 45 clear and distinct bands, with an average of 4.5 bands per primer. A unique set of amplification products of size ranging from 300 to 2050 bp were generated by each ISSR primer which was depicted in the Table 7. The lowest number of bands, i.e. three amplified by ISSR primers namely $(AGGC)_6$, $(GTGC)_4$, and $(GATA)_4$. The highest number of bands, i.e. seven was exhibited by $(GAC)_5$. The uniformity in banding pattern using ISSR across all *in vitro*-raised plantlets and the parent as seen in RAPD revealed the genetic integrity of *in vitro*-regenerated plants (Table 7, Fig. 4). In our experiment, we found the average number of bands per primer was greater in ISSR (4.5) than RAPD (3.3). A similar trend was also observed in *Alpinia galanga* (Parida et al. 2011) and *Rhazya stricta* (Mohamed et

**Table 7.** Details of primers used and amplification products obtained during ISSR analysis of mother and *in vitro* regenerated plants of *L. inermis*.

| Primer | Sequence | Scorable bands (No.) | Approximate range of amplifications (bp) |
|---|---|---|---|
| (GA)9T | 5′GAGAGAGAGAGAGAGAGAT3′ | 6 | 400-1800 |
| T(GA)9 | 5′TGAGAGAGAGAGAGAGAGA3′ | 4 | 750-1750 |
| (GACA)4 | 5′GACAGACAGACAGACA3′ | 4 | 400-1400 |
| (AGG)6 | 5′AGGAGGAGGAGGAGGAGG3′ | 3 | 350-1250 |
| (GAC)5 | 5′GACGACGACGACGAC3′ | 7 | 300-1100 |
| (GTGC)4 | 5′GTGCGTGCGTGCGTGC3′ | 3 | 350-1500 |
| (GGA)4 | 5′GGAGGAGGAGGA3′ | 5 | 750-1650 |
| (CAA)5 | 5′CAACAACAACAACAA3′ | 5 | 700-2050 |
| (GTG)5 | 5′GTGGTGGTGGTGGTG3′ | 5 | 800-1650 |
| (GATA)4 | 5′GATAGATAGATAGATA3′ | 3 | 750-1250 |

al. 2014). Contradictory to our results bands per RAPD primer was greater than ISSR as reported in *Withania somnifera* (Nayak et al. 2013). Similar to our results both RAPD and ISSR were also used to check clonal fidelity in cotyledonary node based tissue culture plants in *Withania somnifera* (Nayak et al. 2013).

# Conclusion

The results of this study revealed that the micropropagated plants were genetically identical to that of the mother plant and no variation was induced during *in vitro* propagation. To the best of our knowledge, this is the first attempt which describes an efficient and cost-effective protocol for *in vitro* propagation of *Lawsonia* using cotyledonary node as an explant. This protocol could be used for commercial exploitation by pharmaceutical, cosmetic, and dyeing industries. The protocol could also be applied for the improvement of this medicinally and pharmaceutically important multipurpose plant by genetic engineering.

# Abbreviations

BA- 6-Benzyladenine, IAA- Indole-3-acetic acid, IBA- Indole-3-butyric acid, ISSR- Inter-simple sequence repeats, Kin- Kinetin, MS- Murashige and Skoog's (1962) basal medium, NAA- Napthalene acetic acid, PCR- Polymerase chain reaction, PGRs- Plant growth regulators, RAPD- Random amplified polymorphic DNA, TDZ-Thidiazuron.

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# Conflict of interest

The authors state that they have no conflict of interest.

# Author contributions

AM carried out all the experiments and wrote the first draft of the manuscript. AD helped in the RAPD and ISSR experiments. ES supervised the RAPD and ISSR experiments. AM and DPB examined and analyzed the data. SKN and DPB supervised the entire research work and edited the final manuscript. All authors read and approved the final version of manuscript.

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