



# Micropropagation of commercially cultivated Henna (*Lawsonia inermis*) using nodal explants

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**Abstract** *Lawsonia inermis* Linn. (Mehandi) is cultivated as cash crop in India particularly in Sojat area of Pali district, Rajasthan. Present investigation describes an efficient regeneration system for elite genotype of *L. inermis* using nodal segments. Optimum response in terms of percent cultures responding, days to bud break and average shoot length was observed on MS medium supplemented with 6-benzylaminopurine (BA;  $2.0 \text{ mg l}^{-1}$ ). Shoot multiplication was influenced by plant growth regulators, repeated transfer of explants and addition of ammonium sulphate. Maximum shoots were regenerated on MS medium supplemented with BA ( $0.25 \text{ mg l}^{-1}$ ), kinetin (Kn;  $0.25 \text{ mg l}^{-1}$ ), indole-3-acetic acid (IAA;  $0.1 \text{ mg l}^{-1}$ ) and ammonium sulphate ( $150 \text{ mg l}^{-1}$ ). To reduce resources, time and labours costs, we have also attempted ex vitro rooting of shoots. About 95 % shoots were rooted ex vitro on soilrite after treatment with indole-3-butyric acid (IBA;  $300 \text{ mg l}^{-1}$ ) and 2-naphthoxy acetic acid (NOA;  $100 \text{ mg l}^{-1}$ ) and establishment in soil successfully.

**Keyword** Ex vitro rooting · *Lawsonia inermis* · Plant growth regulator · In vitro propagation · Repeated transfer

## Abbreviation

Ac	Activated charcoal
BA	6-Benzylaminopurine
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
Kn	Kinetin

NOA	2-Naphthoxy acetic acid
PPFD	Photosynthetic photon flux density
RH	Relative Humidity

## Introduction

*Lawsonia inermis* Linn (family-Lythraceae), commonly called as Henna (Persian) or Mehendi (Hindi/Urdu), is cultivated in the Middle East and along the African Coast of the Mediterranean Sea. In India, it is cultivated as cash crop particularly in Sojat area of Pali district, Rajasthan (Chand and Jangid 2007). Many pharmacological studies revealed that Henna has been reported to have analgesic, hypoglycemic, hepatoprotective, immunostimulant, anti-inflammatory, antibacterial, antimicrobial, antifungal, antiviral, antiparasitic, antitrypanosomal, antidermatophytic, antioxidant, antifertility, tuberculostatic and anticancer properties (Mikhaeil et al. 2004; Syamsudin and Winarno 2008; Chaudhary et al. 2010). *L. inermis* is also well known for its use as cosmetic for staining hair and hands and popular among women/girls who use it for beautifying their hands and feet in an artistic way during auspicious occasion like wedding and festivals. The dying property of this plant is attributed to the presence of lawsone (2-hydroxy-1, 4-naphthoquinone, 0.5–1.5 %), which is commonly extracted from the aerial part of the plant (Bakkali et al. 1997). Phytochemical analysis of Henna reveals that powdered leaves contain lawsone, mannite, tannic acid, mucilage, gallic acid and naphthoquinone (Chaudhary et al. 2010). Oyedeji et al. (2005) reported that leaves of this plants contains of ethyl hexadecanoate (24.4 %), (E)-methyl cinnamate (11.4 %), isocaryophyllene (8.1 %), (E)- $\beta$ -ionone (5.8 %) and methyl lenolenate (4.1 %).

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The worldwide increasing demand of mehandi leaf encourages many countries to adopt commercial farming of the plant. The farmers applied their own indigenous technology in every aspect of the farming, using branch cuttings as well as seed propagation material (Chowdhury et al. 2010). However, propagation through seed is unreliable because of short viability and infestation of seed with diseases and pests. Also seed is product of cross pollination and as such genetic fidelity of selected clone/variety cannot be maintained. Propagation through stem cutting restricts their multiplication on a large scale (Rout et al. 2001). Cosmetics and pharmaceutical companies largely depend on leaves of *L. inermis* procured from naturally cultivated plants. Selected plants for development of in vitro techniques for mass propagation for fulfill requirement of these companies. In recent years, in vitro culture of cell, tissue and organ has been used as an efficient tool for the large-scale propagation of many commercially important plants (Phulwaria et al. 2011). Plant tissue culture technology not only fulfills the demand but also involved in crop improvement programs and production of secondary metabolites at commercial level (Shekhawat and Shekhawat 2011). During past year, Rout et al. (2001) reported a micropropagation protocol for *L. inermis* using apical/axillary meristem of explant. However, the no. of shoots regenerated in this protocol is very low (3–4 shoots per explants). Therefore, there is a great need to develop an efficient and reliable in vitro regeneration protocol for this commercially important plant.

In the present paper, we describe rapid and efficient plant regeneration of *L. inermis* using nodal explants obtained from mature plant. The improved method that we have established for plant regeneration in *L. inermis* could be applied for large scale propagation and also ensure a continuous supply of plants produced in limited time and space through ex vitro rooting.

## Materials and methods

### Site for collection and selection of plant material

Field surveys and studies were conducted in Sojat city (Pali district) of Rajasthan province of India. Two types i.e. one with spines and small leaves and other spineless with broad leaves, of plants of *L. inermis* were identified in agricultural field. Due to their superiority, plants with spineless and broad leaves were marked and maintained in the field and used as source of plants for explants collection (Fig. 1a,b). Explants were collected from field selected plants during different seasons. Various explants were used for culture establishment on include apical shoot tip, below apical nodal segment, fresh hard nodal

segments and woody nodal segment. The harvested shoots were brought to laboratory and properly trimmed and used as source of explants for establishment of cultures and subsequent micropropagation.

### Surface sterilization of explants

Properly trimmed explants were cut into nodal segment (3–5 cm length×0.3–0.5 cm diameter) and pretreated with 0.1 % bavistin (*w/v*) for 16 min, washed with autoclaved water for 7 times. Surface sterilization was carried out with 0.1 % HgCl<sub>2</sub> (*w/v*) for 5 min and were washed thoroughly with sterilized water for 7 times.

### Shoot multiplication and culture conditions

Surface sterilized explants were inoculated horizontally or vertically on MS (Murashige and Skoog 1962) medium with additives (50 mg l<sup>-1</sup> ascorbic acid, 25 mg l<sup>-1</sup> of each of adenine sulphate, arginine and citric acid). Various concentrations (0.5–4.0 mg l<sup>-1</sup>) of cytokinins such as 6-benzylaminopurine (BA) or Kinetin (Kn) either alone or combination of BA with 0.1 mg l<sup>-1</sup> Kn were incorporated in culture media for multiple shoot induction. Explants were incubated at 28±2 °C under a 16 h photoperiod at a PPF of 50 to 70 μmol m<sup>-2</sup> s<sup>-1</sup> from cool white fluorescent tubes (Philips, India)

The shoots were further multiplied through a two step process:—(1) Repeated transfer of original explants along with in vitro produced shoots, (2) Subculture of excised shoot segments differentiated and shoot clumps generated in vitro. In repeated transfer, explants were cultured onto MS medium with various concentrations (0.25–2.0 mg l<sup>-1</sup>) of cytokinins (BA or Kn alone) or combination of BA with Kn (0.25 mg l<sup>-1</sup>) for 4 passages, each passage with interval of 4-week. After third passage of repeated transfer, induced multiple shoots were excised into clumps (2–4 shoots) and transferred to MS medium containing various concentrations of BA (0.10–1.5 mg l<sup>-1</sup>) in combination with either Kn (0.25 mg l<sup>-1</sup>) alone or Kn and IAA (0.1 mg l<sup>-1</sup>) for further shoot amplification. To evaluate the effect of ammonium sulphate on shoot multiplication, shoot clumps generated in vitro on multiplication medium (MS Medium supplemented with 0.25 mg l<sup>-1</sup> BA, 0.25 mg l<sup>-1</sup> Kn, 0.1 mg l<sup>-1</sup> IAA) were excised and subcultured in medium containing different concentrations (50–200 mg l<sup>-1</sup>) of ammonium sulphate.

### In vitro root induction of micropropagated shoots

Well developed shoots regenerated on multiplication medium were individually excised and transferred to full, half or quarter strength MS medium with additives

**Fig. 1** Micropropagation of *L. inermis*: **a** and **b** Explants source of *Lawsonia inermis* in cultivated field, **c** Bud break from nodal explants on MS medium + 2.0 mg l<sup>-1</sup> BA, **d** Repeated transfer of mother explant on MS medium + 0.75 mg l<sup>-1</sup> BA+0.25 mg l<sup>-1</sup> Kn, **e** Multiplication of shoots on MS medium + 0.25 mg l<sup>-1</sup> BA + 0.25 mg l<sup>-1</sup> Kn + 0.1 mg l<sup>-1</sup> IAA + 150 mg l<sup>-1</sup> (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, **f** In vitro rooted shoots on one-fourth strength MS + 5 mg l<sup>-1</sup> IBA, **g** Ex vitro rooted plantlets with pulse treatment of 300 mg l<sup>-1</sup> IBA + 100 mg l<sup>-1</sup> NOA, **h** Cloned and hardened plants of *L. inermis* in green house, **i** A well acclimatized plant of *L. inermis* in clay pot



and different concentrations (1.0–6.0 mg l<sup>-1</sup>) of IBA or NOA alone or combination of IBA with NOA (1.0 mg l<sup>-1</sup>) and activated charcoal (200 mg l<sup>-1</sup>) for root induction. The cultures were initially incubated under dark for 3–4 days and then incubated in growth room for induction of roots.

#### Ex vitro root induction of micropropagated shoots

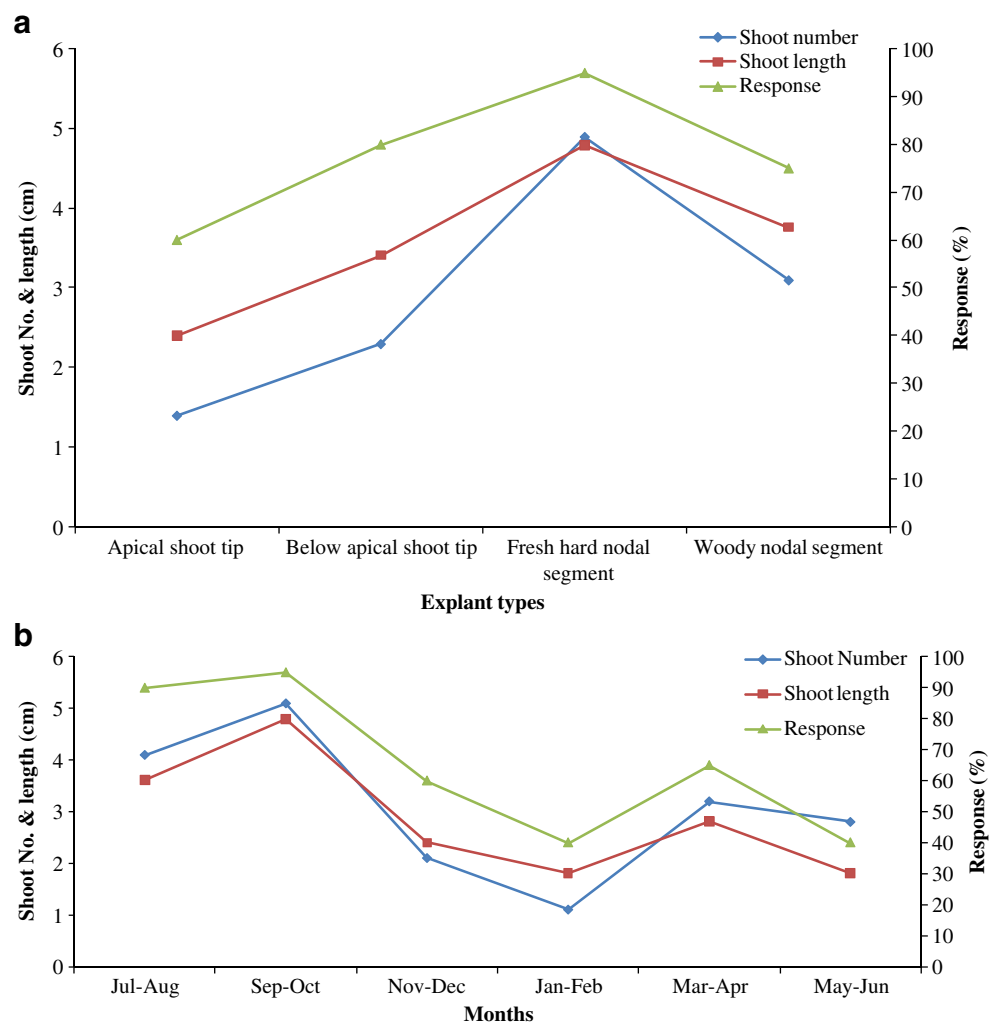
To induce root under ex vitro conditions, base (3–6 mm) of shoots excised individually from multiple shoots and treated with different concentrations (100–700 mg l<sup>-1</sup>) of IBA or NOA alone or combination of IBA (100–700 mg l<sup>-1</sup>) and NOA (100 mg l<sup>-1</sup>) for 5 min. The shoots treated with auxins were transferred to bottles containing autoclaved soilrite and moistened with 1/4<sup>th</sup> strength of MS macro salts. The bottles were capped and

were placed in greenhouse initially; near pad section with relatively high humidity (70–80 %) and low temperature (26–28±2 °C).

#### Hardening and acclimatization of in vitro and ex vitro rooted plantlets

In vitro rooted plantlets were removed from culture vessels and washed under tap water to remove adhered traces of nutrient agar. Plants were carefully transferred to bottles containing autoclaved soilrite moistened with 1/4 strength of MS macro salt and placed in the greenhouse near pad section. After rooted plantlets established in soilrite containing capped bottle, caps were gradually loosened and finally removed within 2 weeks. After 3–4 weeks, the in vitro and ex vitro rooted plantlets were transferred into polybags (12 cm length×6 cm diameter) containing mixture

**Fig. 2 a** Responses of different explants in terms of shoot number and shoot length, **b** Responses of nodal segment in terms of bud break in different months of the year



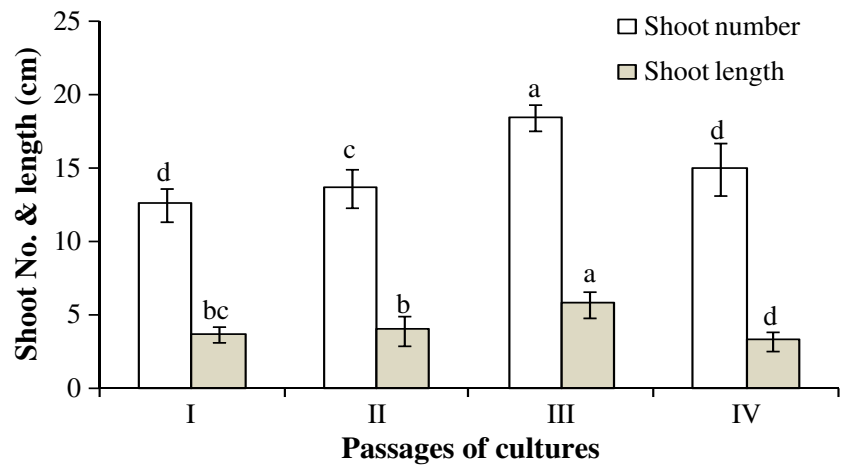
**Table 1** Effect of two cytokinins (BA and Kn) alone or in combination with auxin (IAA) on bud break from nodal explants of *Lawsonia inermis*

BA conc. (mg l <sup>-1</sup> )	Kn conc. (mg l <sup>-1</sup> )	IAA conc. (mg l <sup>-1</sup> )	Response (%)	Mean Shoot No ± SD	Mean Shoot length (cm) ± SD
0.0	0.0	0.0	00	0.00±0.00 <sup>i</sup>	0.00±0.00 <sup>i</sup>
0.5	–	–	40	1.3±0.47 <sup>h</sup>	2.37±0.50 <sup>h</sup>
1.0	–	–	65	2.4±1.13 <sup>ef</sup>	4.07±1.45 <sup>bc</sup>
2.0	–	–	95	4.9±0.85 <sup>a</sup>	4.79±0.72 <sup>a</sup>
3.0	–	–	90	3.8±0.76 <sup>b</sup>	3.92±0.64 <sup>bcd</sup>
4.0	–	–	80	2.5±0.82 <sup>ef</sup>	3.24±0.10 <sup>fg</sup>
–	0.5	–	0	0.00±0.00 <sup>i</sup>	0.00±0.00 <sup>i</sup>
–	1.0	–	30	1.8±0.76 <sup>g</sup>	2.44±0.83 <sup>h</sup>
–	2.0	–	60	3.2±0.99 <sup>c</sup>	3.62±0.71 <sup>de</sup>
–	3.0	–	65	2.5±0.50 <sup>ef</sup>	3.44±0.72 <sup>ef</sup>
–	4.0	–	60	2.1±0.71 <sup>fg</sup>	3.06±0.50 <sup>fg</sup>
0.5	–	0.1	30	1.4±0.50 <sup>h</sup>	2.5±0.33 <sup>h</sup>
1.0	–	0.1	60	2.4±0.81 <sup>ef</sup>	3.21±0.41 <sup>fg</sup>
2.0	–	0.1	85	3.7±0.65 <sup>b</sup>	4.15±0.58 <sup>b</sup>
3.0	–	0.1	80	2.9±0.71 <sup>cd</sup>	3.80±0.33 <sup>b</sup>
4.0	–	0.1	70	2.8±0.88 <sup>de</sup>	3.72±0.33 <sup>de</sup>

Values represent means ± SD of three independent experiments. Values followed by different letters are significantly different at 5 % level as determined by Duncan's test (DMRT)



**Fig. 3** Effect of passages with interval of 4 weeks of repeated transfer of cultures on MS medium with additives, 0.75 mg l<sup>-1</sup> BA + 0.25 mg l<sup>-1</sup> Kn. Values followed by different letters are significantly different at 5 % level as determined by Duncan's test (DMRT)



of organic manure, garden soil and sand (1:1:1). The polybags plantlets were shifted after regular interval of 15–18 days from low temperature (26–28±2 °C) and high humidity zone (70–80 %) (Pad section) towards high temperature (30–32±2 °C) and low humidity zone (55–65 %) in a greenhouse near the fan section. Near fan section acclimatized and hardened plants were kept for 35–40 days in black polybags, then shifted to nursery and finally transfer to the field for evaluation.

**Experimental design, data collection and statistical analysis**

All the experiments were conducted with a minimum of 20 replicates per treatment. Growth regulator free MS medium is used as control in all experiments. The experiments were repeated three times. Observations were recorded after 4 weeks of interval. The results are expressed as mean ± SD of three experiments. The data

was analyzed statistically using SPSS ver 17 (SPSS Inc., Chicago, USA). The significance of differences among mean values was carried out using analysis of variance (ANOVA) at P<0.05.

**Results and discussion**

**Explants selection and surface sterilization**

Among different types of explants evaluated, fresh hard nodal segment collected after rainy season (August–October) was found best for the culture initiation (Fig. 2a,b). Woody nodal segment was not suitable for culture establishment owing to high phenolic exudation. Phenolic exudation is one the major problem in culture establishment when explants taken from woody tissues (Rai et al. 2010). Furthermore, old branches shoots carry

**Table 2** Effect of various types of cytokinins (BA, Kn) on shoot amplification through repeated transfer, explants of *Lawsonia inermis* with 3<sup>rd</sup> passage of cultures

BA conc. (mg l <sup>-1</sup> )	Kn conc. (mg l <sup>-1</sup> )	Mean Shoot No ± SD	Mean Shoot length (cm) ± SD
0.00	0.0	0.0±0.0 <sup>k</sup>	0.0±0.0 <sup>j</sup>
0.25	–	4.6±0.93 <sup>i</sup>	3.1±0.55 <sup>h</sup>
0.50	–	6.4±0.93 <sup>h</sup>	3.74±0.82 <sup>ef</sup>
0.75	–	11.8±0.88 <sup>e</sup>	4.47±0.09 <sup>c</sup>
1.00	–	15±1.11 <sup>c</sup>	4.88±0.35 <sup>b</sup>
2.00	–	11.8±1.91 <sup>e</sup>	3.74±0.17 <sup>ef</sup>
–	0.25	2.7±1.11 <sup>j</sup>	2.14±0.73 <sup>i</sup>
–	0.50	4.2±1.56 <sup>i</sup>	2.56±0.88 <sup>h</sup>
–	0.75	7.5±0.82 <sup>g</sup>	3.61±0.17 <sup>f</sup>
–	1.00	12.3±1.44 <sup>e</sup>	4.43±0.33 <sup>c</sup>
–	2.00	8.9±1.15 <sup>f</sup>	4.04±0.23 <sup>de</sup>
0.25	0.25	6.5±0.68 <sup>h</sup>	4.06±0.31 <sup>de</sup>
0.50	0.25	13.2±0.76 <sup>d</sup>	4.93±0.50 <sup>b</sup>
0.75	0.25	18.5±0.94 <sup>a</sup>	5.78±0.92 <sup>a</sup>
1.00	0.25	17.1±1.32 <sup>b</sup>	5.14±0.91 <sup>b</sup>
2.00	0.25	15.3±1.44 <sup>c</sup>	4.2±0.65 <sup>cd</sup>

Values represent means ± SD of three independent experiments. Values followed by different letters are significantly different at 5 % level as determined by Duncan's test (DMRT)

**Table 3** Effect of various types of cytokinins with auxin (IAA) on multiplication of cultures of *Lawsonia inermis*

BA conc. (mg l <sup>-1</sup> )	Kn conc. (mg l <sup>-1</sup> )	IAA conc. (mg l <sup>-1</sup> )	Mean Shoot No ± SD	Shoot length (cm) ± SD
0.00	0.00	0.0	0.0±0.0 <sup>i</sup>	0.0±0.0 <sup>h</sup>
0.10	0.25	–	14.1±1.84 <sup>g</sup>	4.8±0.59 <sup>e</sup>
0.25	0.25	–	21.8±1.86 <sup>c</sup>	5.35±1.53 <sup>bcd</sup>
0.50	0.25	–	19.8±1.35 <sup>d</sup>	5.06±1.06 <sup>cde</sup>
0.75	0.25	–	18.7±2.08 <sup>e</sup>	4.89±0.75 <sup>de</sup>
1.00	0.25	–	14.7±1.57 <sup>g</sup>	4.32±0.78 <sup>f</sup>
1.50	0.25	–	12.9±1.84 <sup>h</sup>	3.9±0.65 <sup>g</sup>
0.10	0.25	0.1	14.5±1.79 <sup>f</sup>	5.32±0.22 <sup>bcd</sup>
0.25	0.25	0.1	25.1±1.15 <sup>a</sup>	6.09±1.56 <sup>a</sup>
0.50	0.25	0.1	22.7±0.91 <sup>b</sup>	5.82±1.20 <sup>ab</sup>
0.75	0.25	0.1	20.2±1.62 <sup>d</sup>	5.52±0.79 <sup>bc</sup>
1.00	0.25	0.1	16.4±1.45 <sup>f</sup>	5.08±0.81 <sup>cde</sup>
1.50	0.25	0.1	14.6±0.93 <sup>g</sup>	3.98±0.62 <sup>fg</sup>

Values represent means ± SD of three independent experiments. Values followed by different letters are significantly different at 5 % level as determined by Duncan's test (DMRT)

recalcitrant microbes in the tissues and these affect the process of surface sterilization and subsequent culture, behavior and growth of shoots (Bonga et al. 2010). Apical juvenile or below apical nodal segments were sensitive to surface sterilization (Fig. 2b). The explant i.e. hard nodal segment, pretreated with 0.1 % of bavistin for 16 min and surface sterilization with 0.1 % HgCl<sub>2</sub> for 5 min was sufficient for the eliminating fungal and bacterial contamination up to 90 %.

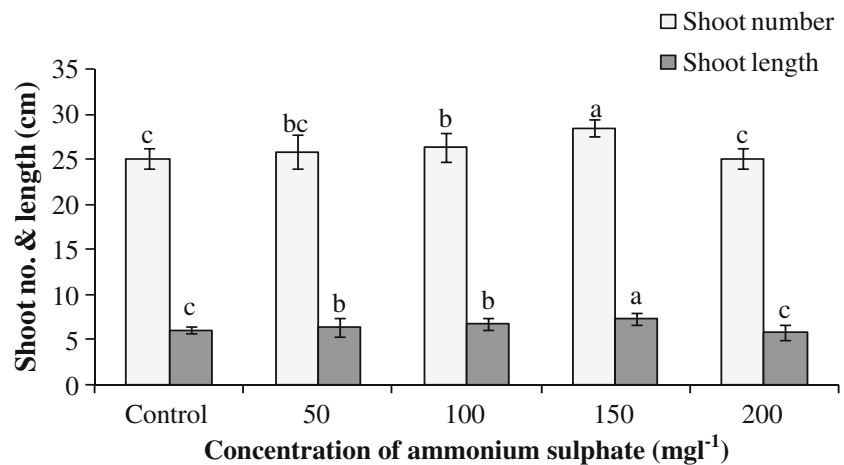
#### Effect of plant growth regulator on bud break

Nodal segments cultured on MS medium containing different concentrations of cytokinin (BA or Kn) responded in terms of bud break within 8–10 days of inoculation. MS medium supplemented with 2.0 mg l<sup>-1</sup> BA with additives was found to be the most effective for the shoot bud induction (Fig. 1c). Upon this medium, maximum shoot numbers (4.9±0.85) and highest shoot length (4.79±0.72 cm) were achieved after 3 week. At higher concen-

trations (3.0–4.0 mg l<sup>-1</sup>) of BA, a mass of callus was formed at base of explants resulting in reduced average shoot number. As compared to BA alone, number of shoots per explant was decreased significantly on medium containing Kn alone or combination of BA with IAA (0.1 mg l<sup>-1</sup>) (Table 1). BA is one of the most commercial cytokinin to causes reinvigoration of mature/old tissues and causes bud induction, a prerequisite for cloning of mature trees (Bonga and von Aderkas 1992; Zhang et al. 2010; Rathore et al. 2010).

#### Influence of repeated transfer of mother explants and subculture of excised shoot clumps on shoot multiplication

Shoot multiplication was significantly influenced by repeated transfer of mother explants on different concentrations and combinations of cytokinin for 4 passages each with 4 weeks of intervals (Fig. 3). Maximum number of shoots (18.5±0.97) was regenerated on MS medium with

**Fig. 4** Effect of ammonium sulphate concentration in terms of shoot number and shoot length on MS medium + 0.25 mg l<sup>-1</sup> BA + 0.25 mg l<sup>-1</sup> Kn + 0.1 mg l<sup>-1</sup> IAA. Values followed by different letters are significantly different at 5 % level as determined by Duncan's test (DMRT)

**Table 4** Effect of various types of auxins (IBA, NOA) on in vitro root induction from shoots of *Lawsonia inermis* on 1/4 strength MS salt with 200 mg l<sup>-1</sup> of activated charcoal

IBA conc. (mg l <sup>-1</sup> )	NOA conc. (mg l <sup>-1</sup> )	Response (%)	Mean Root No ± SD	Mean Root length (cm) ± SD
0.0	–	00	0.0±0.0 <sup>h</sup>	0.0±0.0 <sup>i</sup>
1.0	–	00	0.0±0.0 <sup>h</sup>	0.0±0.0 <sup>i</sup>
2.0	–	20	1.6±0.67 <sup>g</sup>	2.13±0.51 <sup>h</sup>
3.0	–	40	2.8±1.18 <sup>ef</sup>	3.08±0.36 <sup>e</sup>
4.0	–	70	4.3±1.20 <sup>b</sup>	3.78±0.19 <sup>c</sup>
5.0	–	90	5.8±0.87 <sup>a</sup>	4.48±0.60 <sup>a</sup>
6.0	–	80	3.7±0.92 <sup>c</sup>	4.17±0.42 <sup>b</sup>
–	1.0	00	0.0±0.0 <sup>h</sup>	0.0±0.0 <sup>i</sup>
–	2.0	20	1.7±0.65 <sup>g</sup>	2.48±0.25 <sup>g</sup>
–	3.0	35	2.4±0.67 <sup>f</sup>	3.08±0.20 <sup>e</sup>
–	4.0	60	2.8±0.76 <sup>ef</sup>	4.19±0.35 <sup>b</sup>
–	5.0	80	4.3±1.12 <sup>b</sup>	4.39±0.92 <sup>ab</sup>
–	6.0	70	3.7±0.91 <sup>c</sup>	4.43±0.28 <sup>a</sup>
0.5	1.0	20	1.5±0.51 <sup>g</sup>	2.81±0.28 <sup>f</sup>
1.0	1.0	30	2.5±0.82 <sup>f</sup>	3.24±0.50 <sup>e</sup>
2.0	1.0	45	3±0.91 <sup>de</sup>	3.13±0.37 <sup>e</sup>
3.0	1.0	65	3.2±1.0 <sup>de</sup>	3.9±0.33 <sup>c</sup>
4.0	1.0	85	3.4±0.93 <sup>cd</sup>	4.17±0.57 <sup>b</sup>
5.0	1.0	75	2.4±0.81 <sup>f</sup>	3.51±0.35 <sup>d</sup>

Values represent means ± SD of three independent experiments. Values followed by different letters are significantly different at 5 % level as determined by Duncan’s test (DMRT)

0.75 mg l<sup>-1</sup> BA and 0.25 mg l<sup>-1</sup> Kn after 3rd passage of culture (Fig. 1d, Table 2). The repeated transferring has been recommended as a method for rejuvenation of mature explants for micro-clonal propagation of several woody plants (Pierik 1990; Shekhawat et al. 1993; Phulwaria et al. 2011). The increase in shoot number after repeated transfer of mother explants for different passages may be due to

suppression of apical dominance during subculture that induced basal dormant meristematic cells to form new shoots (Tripathi and Kumari 2010). Shoot regeneration was further increased when multiple shoots were excised into clumps (3–4 shoots) and subcultured on medium containing different combinations of plant growth regulators. About 25.1±1.19 shoots were obtained on MS medium supple-

**Table 5** Effect of various types of auxins (IBA, NOA) on ex vitro root induction from shoots of *Lawsonia inermis*

IBA conc. (mg l <sup>-1</sup> )	NOA conc. (mg l <sup>-1</sup> )	Response (%)	Mean Root No ± SD	Mean Root length (cm) ± SD
0.0	–	10	0.3±0.65 <sup>g</sup>	0.53±1.08 <sup>g</sup>
100	–	25	1.6±0.67 <sup>f</sup>	3.87±0.50 <sup>f</sup>
200	–	40	2.4±1.04 <sup>e</sup>	4.52±0.13 <sup>d</sup>
300	–	70	3.2±1.09 <sup>d</sup>	5.25±0.55 <sup>c</sup>
500	–	90	5.7±0.65 <sup>b</sup>	6.08±0.48 <sup>ab</sup>
700	–	80	3.4±1.04 <sup>d</sup>	5.15±0.36 <sup>c</sup>
–	100	20	2.0±0.79 <sup>ef</sup>	3.79±0.40 <sup>f</sup>
–	200	35	2.5±0.68 <sup>e</sup>	4.05±0.32 <sup>ef</sup>
–	300	60	3.3±0.65 <sup>d</sup>	4.28±0.50 <sup>de</sup>
–	500	80	4.4±0.93 <sup>c</sup>	5.22±0.43 <sup>c</sup>
–	700	75	3.3±1.29 <sup>d</sup>	3.94±0.64 <sup>f</sup>
100	100	50	2.5±0.82 <sup>e</sup>	5.01±0.88 <sup>c</sup>
200	100	80	3.4±0.93 <sup>d</sup>	5.75±0.93 <sup>b</sup>
300	100	95	6.3±0.92 <sup>a</sup>	6.12±0.56 <sup>a</sup>
500	100	90	5.3±0.92 <sup>b</sup>	6.05±0.69 <sup>ab</sup>
700	100	80	4.8±1.86 <sup>c</sup>	5.24±0.75

Values represent means ± SD of three independent experiments. Values followed by different letters are significantly different at 5 % level as determined by Duncan’s test (DMRT)

mented with BA ( $0.25 \text{ mg l}^{-1}$ ), Kn ( $0.25 \text{ mg l}^{-1}$ ), IAA ( $0.1 \text{ mg l}^{-1}$ ) and additives ( $50 \text{ mg l}^{-1}$  ascorbic acid,  $25 \text{ mg l}^{-1}$  of each of adenine sulphate, arginine and citric acid) after 4 weeks (Table 3). This number appears to be the higher as compared to previous report on the same plant species (Rout et al. 2001).

A significant improvement in shoot multiplication ( $28.5 \pm 0.97$ ) and average shoot length ( $6.6 \pm 1.17$ ) also observed when ammonium sulphate ( $150 \text{ mg l}^{-1}$ ) was incorporated in medium (Figs. 1e, 4). The shoots cultures of *L. inermis* were maintained on above mentioned culture condition from last 3 years by regular subculturing. In the present study, incorporation of ammonium sulphate in medium reduced hyperhydricity; shoots tip necrosis and promoted growth of shoots. Similar observations have also been made in a number of plants like *Pterocarpus marsupium* (Husain et al. 2006), *Melia azedarach* (Husain and Anis 2009) and *Leptadenia reticulata* (Rathore et al. 2010). Several lines of evidences also document the sulphate ions promote absorption of nitrate and buffered the culture medium (Ivanova and Staden 2008; Kopriva et al. 2009).

#### In vitro and ex vitro rooting of microshoots and acclimatization of plantlets

Out of two auxins (IBA and NOA) tested, IBA was found to be the most suitable for the root induction. Maximum percent (90 %) response for root induction and highest number ( $5.8 \pm 0.91$ ) of roots per shoot was recorded on 1/4th strength of MS Medium+200  $\text{mg l}^{-1}$  activated charcoal supplemented with  $5.0 \text{ mg l}^{-1}$  of IBA (Fig. 1f, Table 4). Among all the treatments tested, a combination of IBA ( $300 \text{ mg l}^{-1}$ ) and NOA ( $100 \text{ mg l}^{-1}$ ) for 5 min was best for the ex vitro rooting of shoots. After treatment with this combination, about 95 % shoots were rooted ( $6.3 \pm 0.94$  roots per shoot) within 18–20 d (Fig. 1g, Table 5). The role of auxins such as IBA or NOA or NAA on in vitro and ex vitro rooting of shoots have also been well documented (Rathore and Shekhawat 2009; Rathore et al. 2010; Phulwaria et al. 2011). Ex vitro rooting is not only helpful in the reducing resources, time and labour costs but also simplify the protocol by eliminating of rooting step under sterile conditions, an additional step of micro-propagation (Preece and Shutter 1991; Dinkel-Meier et al. 1993; Pospilovia et al. 1999; Yan et al. 2010). Also the ex vitro rooted plantlets are hardened with ease as compared to in vitro rooted plants which require additional steps both in vitro and ex vitro first for root induction and then for hardening. Generally ex vitro developed roots have numerous root hairs while in vitro developed roots possessed few or no root hair and were readily damaged during post-transplantation. Excessive development of root hair may be beneficial in adaptation during acclima-

tization (Sharma et al. 2007). Furthermore, it has also been reported that ex vitro rooted plants are better suited to tolerate environmental stresses (Rathore et al. 1992; Tiwari et al. 2002; Phulwaria et al. 2011). The in vitro and ex vitro rooted plantlets were successfully hardened off inside the green house in soilrite planting substrate for 4 weeks and eventually established in mixture of organic compost, garden soil and sand (Fig. 1h). In comparison to in vitro rooted plantlets (70 %), transplant survival rate of plantlets those rooted ex vitro was higher (80 %). Plantlets developed through ex vitro rooting usually have lateral roots just like natural root system and higher root length resulting higher survival rate than plantlets those developed from in vitro rooting (Yan et al. 2010).

#### Conclusion

In conclusion, the present paper reports an efficient regeneration system for *L. inermis*, a medicinally important and dye yielding plant. The regeneration system is an improved method as compared to earlier published report on same plant. Use of ex vitro rooting technique for plant production makes it an easy and more economical in term of labour cost and save time. Therefore, present method can be used for large scale propagation of this important plant at commercial scale.

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