PUBLICATIONS AND PRESENTATIONS

LIST OF PUBLICATIONS

Publications from the thesis:

- **Swati R. Patel**, Aruna G. Joshi, Ashutosh R. Pathak, Neeta Shrivastava and Sonal Sharma (2021) Somatic embryogenesis in *Leptadenia reticulata* (Retz.) Wight and Arn along with assessment of shoot and callus cultures for HPTLC fingerprint and quantification of *p*-coumaric acid. *Plant Cell, Tissue and Organ Culture* 145:173-189. (Impact factor- 2.71)

Other publications:

Book:

- **Swati Patel** and R. Krishnamurthy (2017) Heavy metals effect on Indian mustard: Morphological and physiological aspects. Lambert Academic Publishing, Germany (ISBN: 978-620-2-06154-4).

Book chapter:

- Ashutosh R. Pathak, **Swati R. Patel** Aruna G. Joshi (2021) RNA interference (RNAi): A genetic tool to manipulate plant secondary metabolite pathways. In: RNA-Based Technologies for Functional Genomics in Plants (Eds. Tang G, Teotia S, Tang X and Singh D). Concepts and Strategies in Plant Sciences. Springer, Cham. pp. 169-198.

Research papers:

- Ashutosh Pathak, **Swati Patel**, Aruna Joshi, Neeta Shrivastava and Sonal Sharma (2021) Micropropagation of *Eclipta alba* (L.) Hassk. and chemical profiling of cultures using HPTLC. *Research Journal of Biotechnology* 16(4):31-38. (Scopus indexed)
- **Swati R. Patel**, Ashutosh R. Pathak, Aruna G. Joshi, Neeta Shrivastava and Sonal Sharma (2021) Chemical profiling of *Tylophora indica* (Burm. F.) Merrill. shoot cultures established through leaf explant. *Proceedings of the National Academy of Sciences, India Section B: Biological Sciences* 91:37-43. (Scopus indexed)
- Ashutosh R. Pathak, Aruna G. Joshi, **Swati R. Patel**, Neeta Shrivastava and Sonal Sharma (2021) Evaluation of rutin biosynthesis in shoot cultures of *Hemidesmus indicus* (L.) R. Br. *Journal of Herbs, Spices & Medicinal Plants* 27(1):11-23. (Scopus indexed)
- **Swati R. Patel**, Aruna G. Joshi, Ashutosh R. Pathak and Vinay M. Raole (2020) Micromorphology studies of three important medicinal plants of Asclepiadaceae family. *Notulae Scientia Biologicae* 12(1):22-29. (Scopus indexed)

LIST OF PRESENTATIONS

From thesis:

- **Swati Patel**, Ashutosh Pathak and Aruna Joshi, 'Regeneration of shoots and somatic embryogenesis from callus cultures of *Tylophora indica* (Burm. F.) Merrill.' in National symposium on "Trends in plant biotechnology and agriculture" at Thapar Institute of Engineering and Technology, Patiala, Punjab (6^{th} – 8^{th} February, 2020).-**Oral**

Other presentations:

- Ashutosh R. Pathak, **Swati R. Patel**, Aruna G. Joshi, Neeta Shrivastava and Sonal Sharma, 'Assessment of Rutin Biosynthesis in *Hemidesmus indicus* (L.) R. Br. shoots using HPTLC and gene expression analysis' in National symposium on "Trends in plant biotechnology and agriculture" at Thapar Institute of Engineering and Technology, Patiala, Punjab (6^{th}-8^{th} February, 2020).
- Ashutosh R. Pathak, **Swati R. Patel**, Aruna G. Joshi, Neeta Shrivastava and Sonal Sharma, 'Yeast extract and salicylic acid up-regulates lupeol synthesis in shoot cultures of *Hemidesmus indicus* (L.) R. Br.' in International conference on "Trends in plant sciences and agrobiotechnology-2019" at IIT Guwahati, Assam (14^{th}-16^{th} February, 2019)
- **Swati Patel**, Ashutosh Pathak and Aruna Joshi, 'Micropropagation in *Eclipta alba* (L.) Hassk.' in International symposium on "Conservation of angiosperm diversity: Hidden treasure of today and tomorrow" at The M.S. University of Baroda, Vadodara, Gujarat (29^{th}-31^{st} October, 2018).
- **Swati Patel**, Ashutosh Pathak and Aruna Joshi, '*In vitro* regeneration in *Telosma cordata* (Burm. F.) Merrill' in National seminar on "Impact of climate change on biodiversity-III" at The M.S. University of Baroda, Vadodara, Gujarat (29^{th} November, 2017).
- **Swati Patel**, Ashutosh Pathak and Aruna Joshi, 'Effect of cytokinins, explant age and its orientation on shoot regeneration in *Tylophora indica* (Burm. F.) Merrill. leaf explant', in 4^{th} International congress of the society for ethanopharmacology, India: "Healthcare in 21^{st} century: Perspectives of ethanopharmacology and medicinal plant research" at C.G. Bhakta Institute of Biotechnology, Uka Tarsadia University, Bardoli, Gujarat (23^{rd}-25^{th} February, 2017).

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ORIGINAL ARTICLE

Somatic embryogenesis in *Leptadenia reticulata* (Retz.) Wight and Arn along with assessment of shoot and callus cultures for HPTLC fingerprint and quantification of *p*-coumaric acid

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Abstract

Leptadenia reticulata (Retz.) Wight and Arn is an important medicinal plant of Asclepiadaceae family. In the present study regeneration was attempted using leaf and nodal explants in Murashige and Skoog's (MS) medium fortified with sucrose (3%) and different cytokinins like 6-benzyladenine (BA), kinetin (Kn) and adenine sulphate ($AdSO_4$) in 5–20 µM concentration range and auxins like indole-3-acetic acid (IAA), 1-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) in 0.1–1.0 µM concentration range. Through leaf explants total 21.70 ± 2.06 somatic embryos were recorded in 100% cultures in BA (15 µM) with $AdSO_4$ (15 µM). Further these embryos were transferred to static medium augmented with gibberellic acid (GA_3, 1 µM) facilitated development of plantlets within 8 weeks. Inoculating nodal explants in BA (10 µM) with $AdSO_4$ (10 µM) formed total 5.10 ± 0.33 shoots (100% response). Callus and shoot cultures derived through leaf and nodal explants respectively were further qualitatively and quantitatively analyzed for their biosynthetic potential. High-performance thin layer chromatography (HPTLC) fingerprints of the samples confirmed that the in vitro shoots showed almost similar banding patterns in comparison with in vivo shoots in terms of number of peaks and band on TLC plate, whereas in callus samples the banding pattern was differed. Further quantification of *p*-coumaric acid revealed that in in vivo and in vitro shoots it was in LOD (10 µg) range but out of LOQ (100 µg). Whereas it was quantified 78.08 ± 2.20 mg g^{-1} of dry weight (DW) in 6 weeks old callus cultures obtained from BA (20 µM) + NAA (0.5 µM) fortified medium, which indicate that it can become a chemical marker for *L. reticulata*. This protocol can be utilized for true-to-type plant regeneration as well as conservation of this threatened medicinal plant. Chemical fingerprint can be utilized for authentication of *L. reticulata* and callus cultures as an alternative source to wild plants for production of *p*-coumaric acid.

Key message

SEs were efficiently developed in *L. reticulata*. In vitro cultures were able to synthesize metabolites but callus is prominent source for *p*-coumaric acid. Hence it can be alternative to wild plants.

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Keywords Chemical fingerprint · Leaf and nodal explants · Medicinal plant · Murashige and Skoog's medium · *p*-coumaric acid · Somatic embryos

Introduction

Leptadenia reticulata (Retz.) Wight and Arn is commonly known as Jivanti or Dodi, is a multipurpose medicinal climber of Asclepiadaceae family. According to Atharva-Veda (one of the samhitas), it is an Indian origin plant and known for its medicinal value from 4500 to 1600 BC as a natural life and strength giver (Arya et al. 2003). Plant contains important metabolites like apigenin, diosmetin, luteolin, rutin, *p*-coumaric acid, lupeol, β-sitosterol, quercetin, isoquercetin etc. (Mohanty et al. 2017). The whole plant ameliorates 'tridoshas' (Vatta, Pitta and Kapha), and is mentioned as a Rasayana herb in Ayurveda due to its revitalizing and rejuvenating properties. Patel (1947) documented the use of formulation named 'Leptaden' (equal proportion of *Leptadenia reticulata* and *Breynia patens*) for the first time and it prevents habitual abortion and allied conditions in women as well as useful for its galactogogue properties. The other polyherbal formulation containing *L. reticulata* is Malkanguni which is utilized as an antidepressant (Hakim 1964). Phytochemical profile of the plant revealed the presence of total 46 chemical compounds and pharmacological properties such as antiabortifacient, antianaphylactic, antidepressant, antiepileptic, antiimplantation activity, antimicrobial, antitumor and in vitro cytotoxic activity, antioxidant, antipyretic, analgesic, antiinflammatory, antiulcer, anxiolytic, diuretic, hepatoprotective, immunomodulatory activities and in the treatment of oligospermia (Mohanty et al. 2017).

The natural propagation of *L. reticulata* is through seeds but low seed setting as well as germination rate of seeds reduces its propagation. On the other hand, due to multipurpose medicinal properties annual demand of this plant increased which led to overexploitation due to habitat destruction (Shetty and Singh 1993). The escalating demand for medicinal plants for domestic, national and international market has increased about 15–16% per year between 2000 and 2005. Annual requirement of *L. reticulata* is 200–500 MT and its cost of cultivation in 2016–2017 was US $ 494.88/h and National Medicinal Plants Board (NMPB) has listed it in prioritized plants (Goraya and Ved 2017). The wild resources are unable to meet the current demand because of restricted distribution and seasonal availability. Further, it has also depleted by overexploitation through various anti-social practices, and thus, it became an endangered species (Rathore et al. 2013). Plant regeneration through tissue culture provides an attractive way for conservation of that species as well as utilization of plant genetic resources (and its bioactive compounds) (Bhojwani and Dantu 2013). Somatic embryogenesis is one of the pathways to regenerate true-to-type plants in a short period of time and has been reported in plants like *Phaseolus vulgaris* (Cabrera-Ponce et al. 2015) and *Eclipta alba* (Salma et al. 2019). The development of SEs is influenced by many factors of which plant growth regulators play a central role in determining the morphogenesis (Van Staden et al. 2008). This pathway has several advantages over the other organogenic pathways as somatic embryos are bipolar, the in vitro rooting stage is avoided and facilitates the production of synthetic seeds which aids in germplasm conservation (Cheruvathur et al. 2013). An efficient somatic embryogenesis protocol is also useful in genetic transformation studies as it is reported in *Leptadenia pyrotechnica* (Dutta et al. 2013). There are few reports available on shoot regeneration using leaf (Rathore et al. 2013; Patel et al. 2014) and nodal (Arya et al. 2003; Parabia et al. 2007; Bharat et al. 2011) explants and somatic embryogenesis through node and shoot tip (Martin 2004), stem (Sathyanarayana et al. 2008) and petiole (Rathore et al. 2013) derived callus. As reports are scanty on potency of leaf explant for generation of SEs, therefore, there is need to establish rapid and efficient protocol to enhance number of SEs and improve its development.

L. reticulata possess important phenolic compound such as *p*-coumaric acid, which plays a central role in secondary metabolism because it can be subsequently transformed to other phenolic acids, flavonoids, lignin precursors and other secondary metabolites (El-Seedi et al. 2012). It contains activities like antioxidant, anti-inflammatory, antimutagenic, anti-ulcer, antiplatelet and anti-cancer, in addition to mitigating atherosclerosis, oxidative cardiac damage, UV-induced damage, neuronal injury, anxiety, gout and diabetes (Pragasam et al. 2013). The *p*-coumaric acid content in *L. reticulata* is affected by season as well as geographical location (Mammen et al. 2010) therefore, plant cell and organ cultures offer an attractive alternative for homogeneous and controlled production of metabolites throughout the year (Singh and Chaturvedi 2013). It is synthesized via phenylpropanoid pathway and many studies have documented that the expression of several enzymes like phenylalanine ammonia-lyase (PAL) is affected by cytokinins and auxins (Schmulling and Schafer 1997; Stibon and Perrot-Rechenmann 1997). Geetha et al. (2011) optimized HPLC method for quantification of *p*-coumaric acid from in vivo plants of *L. reticulata*, but the drawback of this technique is that it is costly and only one sample can be analyzed at a time. This can be overcome by high-performance thin layer chromatography (HPTLC), which is comparatively faster, easy, cost effective and several samples can be analyzed simultaneously (Birk et al. 2005). Previously different metabolites of

L. reticulata were used as a marker compound qualitatively (Mammen et al. 2011) and quantitatively for rutin (Prashanth et al. 2003), stigmasterol, 1-α-tocopherol acetate (Hamrapurkar and Karishma 2007), *p*-coumaric acid, rutin and quercetin (Mohanty et al. 2015). Due to less availability of *L. reticulata*, it is routinely adulterated with other commonly available plants like *Holostemma adakodien* Schult., *Flickerengia nodosa* (Dalz.) Seiden f. and *Ichnocarpus frutescens* R. Br., *Dregia volubilis* Linn, *Holostemma annulare* Roxb., *Dendrobium normale* Fale (Mammen et al. 2011; Pal et al. 2012). Moreover, the reports are scanty for chemical fingerprint and *p*-coumaric acid production through in vitro cultures of *L. reticulata*. The qualitative analysis and quantification of chemical markers is the reliable approach for correct identification as well as quality of botanical material (Katakam et al. 2019).

Thus, the aim of present study was divided in two aspects: (1) to evaluate influence of different plant growth regulators on leaves and nodes to obtain in vitro cultures and (2) assessment of nodal derived shoots and callus cultures for biosynthesis of metabolites through HPTLC fingerprint and quantification of a chemical marker i.e. *p*-coumaric acid, which will be reported for the first time.

Materials and methods

Regeneration studies

Plant material

Young healthy leaves and nodes of 1-year-old plant *L. reticulata* were collected from the Botanical Garden of The Maharaja Sayajirao University of Baroda and kept in running water for 1 h followed by washing with labolene (Fisher Scientific, India). These plant materials were treated with chilled PVP (0.01%) solution for 5 min. Antibacterial treatment was given with streptomycin (0.01%) for 4 min under sterile condition. Surface sterilization was carried out using bavistin (0.01%) and $HgCl_2$ (Merck, India) solution (0.1%) for 4 min each. Square pieces (1 cm^2) of leaf containing midrib and 2–3 cm long nodal segments were utilized for establishing in vitro cultures.

Culture media

Leaf explant

Murashige and Skoog's (MS 1962) medium fortified with sucrose (SRL, Mumbai, India) (3%) and plant growth regulators (PGRs) (SRL, Mumbai, India) were used for shoot cultures. Basal MS medium served as a control for the study, different concentrations of cytokinins (5–20 µM) like 6-benzyladenine (BA), kinetin (Kn), adenine sulphate ($AdSO_4$) and auxins (0.1–1.0 µM) like indole-3-acetic acid (IAA), 1-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) were used individually and in combinations (BA + Kn, BA + $AdSO_4$, Kn + $AdSO_4$, BA + IAA, BA + NAA, BA + 2,4-D, Kn + IAA, Kn + NAA and Kn + 2,4-D).

Matured somatic embryos were further developed into plantlets using different combinations of PGRs in static and liquid medium viz. BA (15 µM) + $AdSO_4$ (15 µM), gibberellic acid (GA_3, 0.5–2.0 µM) as well as natural additive like coconut water (4%). The cultures were observed weekly and data were recorded at the end of 8 weeks.

Nodal explants

MS medium fortified with sucrose (3%) and PGRs were used for shoot cultures. Basal MS medium served as a control for the study, different concentrations of cytokinins (5–20 µM) like BA, Kn and $AdSO_4$ were used individually and in combinations (BA + Kn, BA + $AdSO_4$ and Kn + $AdSO_4$) and undefined media supplement like coconut water (2–8%) was also used for elongation of shoots after 8 weeks.

pH of the medium was adjusted at 5.80 using NaOH/HCl (1 N) and 0.8% agar (SRL, Mumbai, India) was used as the solidifying agent. All the media were sterilized at 121 °C (15 psi) for 20 min and the cultures were maintained at 26 ± 2 °C under 16/8 h (dark/light) photoperiod at 40 µmol m^{-2} s^{-1} provided by cool white fluorescent lights (Philips India Ltd., India).

Phytochemical analysis

HPTLC fingerprint

In vivo shoots of *L. reticulata* were collected from the Botanical Garden, washed thoroughly in running water and kept in oven (40°C) for drying. In vitro samples of callus and shoots (given below) were harvested, shoots were washed thoroughly with distilled water and all the samples were dried at 40°C. Dried powdered samples (1 g) were refluxed in water bath (50°C) successively using hexane, ethyl acetate and methanol (AR grade, SRL, Mumbai, India) by following methodology of Pathak et al. (2017). Samples of 15 µl of all the extracts were applied as a band (8 mm) on Silica gel G60 F_{254} precoated TLC plates (0.2 mm thick, E. Merck Ltd., Mumbai, India) and developed in CAMAG twin through glass chamber. Mobile phases were toluene:ethyl acetate:formic acid (7.2:2.5:0.3 v/v), toluene:ethyl acetate:methanol (6.4:2.7:0.9 v/v), toluene:ethyl acetate:formic acid:methanol (6.0:2.7:0.6:0.7 v/v) for hexane, ethyl acetate and methanol extracts respectively. After development, the plates were dried and analyzed

by 254, 366 and 525 nm (after derivatization using anisaldehyde-sulfuric acid reagent). All the densitometric analysis was done in CAMAG TLC scanner-3 linked to winCATS software. Samples taken for phytochemical analysis were as follows:

1. In vivo shoots of *L. reticulata*—L1.
2. In vitro shoots from nodal explant in BA (5 µM) + Kn (5 µM)—L2.
3. In vitro shoots from nodal explant in BA (10 µM) + $AdSO_4$ (10 µM)—L3.
4. In vitro shoots from nodal explant in coconut water (4%)—L4.
5. Callus from leaf explant in BA (15 µM) + 2,4-D (1.0 µM)—L5.
6. Callus from leaf explant in BA (20 µM) + NAA (0.5 µM)—L6.

Preparation of *p*-coumaric acid calibration curve

A stock solution of 1 mg mL^{-1} of *p*-coumaric acid was prepared in methanol. The stock solution was serially diluted to prepare various standard solutions which contain six different concentrations ranging from (100 to 600 µg). A calibration curve was prepared by spotting a fixed volume (10 µl) of different standard solutions in triplicates on TLC plates. Plates were developed in optimized mobile phase for *p*-coumaric acid i.e. toluene:ethyl acetate:methanol (7.2:2.5:0.7 v/v), dried and then scanned at 254 nm and peak area of each concentration was recorded.

HPTLC method development was validated according to International Conference on Harmonisation (ICH) guidelines (ICH 1997) for linearity, repeatability, precision, limit of detection (LOD) and limit of quantification (LOQ). Peak areas were plotted against the pre-determined concentrations of standards and linearity of the method was measured by calculating correlation coefficient (R^2) value. The repeatability was assessed by analyzing the known concentration of a standard spotted multiple times (n = 6) on TLC plate and calculating the relative standard deviation (RSD). The variability was studied by analyzing one sample and six different concentrations of the standard, three times at regular intervals on the same day (intra-day precision) and at a particular time period in 3 different days (inter-day precision). Detection was done at 254 nm and RSD was calculated to determine the precision. Peak area and concentration were subjected to linear regression analysis to calculate the calibration equation and correlation coefficient. LOD and LOQ values were calculated based on signal-to-noise (S/N) ratio which should be 3:1 and 10:1 respectively. For, accuracy three different concentrations of the standard were applied, each in triplicate.

Quantification of *p*-coumaric acid

Quantitative analysis of *p*-coumaric acid was done by densitometry scanning at 254 nm using HPTLC scanner. All the samples were applied (in triplicate) on the TLC plates and developed in aforementioned optimized mobile phase. The peaks in samples corresponding to the standard were validated on the basis of similar Rf values and spectral analysis. For quantification, area under the curve (AUC) was recorded and concentration of compounds was calculated using the regression equation obtained for the standard curve.

Statistical analysis

All the experiments were repeated twice with ten replicates and data was analyzed using one-way analysis of variance (ANOVA, $\alpha = 0.05$), and represented as means and standard errors (SEs). Significant means were further analyzed by Tukey's test using Graph Pad Prism 6.01.

Results and discussion

Regeneration study

Effect of PGRs on leaf explant

Leaf explants when placed on basal MS medium, failed to show any morphogenic response and turned black within 2 weeks. Media fortified with different cytokinins i.e. BA/Kn/$AdSO_4$ (5–20 µM) individually showed only swelling of explants. When different auxins were tried individually, few concentrations of IAA/NAA/2,4-D (0.1–1.0 µM) induced callus but percent response was very less. To enhance the morphogenic response, combinations of cytokinins, and cytokinins with auxins effect on explant was evaluated (Table 1). Combinations of BA with Kn/$AdSO_4$ formed friable as well as embryogenic callus which proliferated till 6 weeks but later turned black along with the explant. Whereas only friable callus was differentiated in presence of Kn with $AdSO_4$ which turned black within 3 weeks. When cytokinins combined with auxins i.e. BA and IAA together induced an embryogenic callus, but the combinations of BA with NAA/2,4-D formed friable callus and maximum proliferation was recorded in BA (20 µM) with NAA (0.5 µM) and BA (15 µM) with 2,4-D (1.0 µM). Replacing BA with another cytokinin (Kn) and combined with auxins, callus differentiation was observed but percent response was recorded less and callus turned black within 4 weeks in all combinations. Embryogenic callus favored formation of somatic embryos which developed into plantlets whereas the proliferative callus from combination of BA + NAA and BA + 2,4-D were utilized for phytochemical analysis.

Table 1 Synergistic effect of different PGRs combinations on leaf explants of *L. reticulata* (6 weeks)

PGRs	Morphogenic response	Proliferation	Response (%)
BA (5–20 µM)—Kn (5–20 µM)	Embryogenic callus	++	90
BA (5–20 µM)—$AdSO_4$ (5–20 µM)	Embryogenic callus	+++	90
Kn (5–20 µM)—$AdSO_4$ (5–20 µM)	Friable callus	+	60
BA (5–20 µM)—IAA (0.1–1.0 µM)	Embryogenic callus	+++	70
BA (5–20 µM)—NAA (0.1–1.0 µM)	Friable callus	+++	70
BA (5–20 µM)—2,4-D (0.1–1.0 µM)	Semi friable callus	+++	50
Kn (5–20 µM)—IAA (0.1–1.0 µM)	Friable callus	+	40
Kn (5–20 µM)—NAA (0.1–1.0 µM)	Friable callus	+	50
Kn (5–20 µM)—2,4-D (0.1–1.0 µM)	Friable callus	+	50

PGRs plant growth regulators, *BA* 6-benzyladenine, *Kn* kinetin, *$AdSO_4$* adenine sulphate, *IAA* indole-3-acetic acid, *NAA*- 1-naphthaleneacetic acid, 2,4-*D* 2,4-dichlorophenoxyacetic acid

In present study it was observed that when cytokinins and auxins used individually they failed to differentiate shoots from leaf explants, which is in line with reports on *Spilanthes acmella* (Singh and Chaturvedi 2010). BA, Kn, IAA, NAA, 2,4-D individually and combinations of BA + NAA and Kn + IAA/NAA induced only callus which was in accordance with observations on leaf explants of *Hemidesmus indicus* (Pathak and Joshi 2017). But in their study the combination of BA-IAA induced indirect organogenesis whereas in the present study this PGR combination induced embryogenesis, which justifies that the PGRs affects in species specific manner. Previous study on leaf explants of *L. reticulata* (Martin 2004; Bharat et al. 2011; Patel et al. 2014) stated that it formed only callus when media supplemented with NAA/2,4-D individually or in combination with BA which is similar with present investigation. It was also observed that in most combinations callus turned brown by 8 weeks which was also reported by Bharat et al. (2011).

Synergistic effect of cytokinins and auxins on somatic embryogenesis

Presence of BA-Kn together, induced somatic embryos (SEs) both directly and indirectly but only few combinations have differentiated direct SEs and percent response was also very less. BA (15 µM) with Kn (5 µM) was one such combination which differentiated direct (Fig. 1a) as well as indirect embryos from the leaf. In lower concentration of BA the number of embryos was less but with increasing concentration formed 20.90 ± 2.86 embryos (90% induction) in presence of BA (15 µM) with Kn (5 µM) (Table 2). Replacing Kn with another cytokinin $AdSO_4$ resulted in development of only indirect SEs. The differentiation of SEs was slightly increased and optimum 21.70 ± 2.06 embryos in 100% cultures was recorded in BA (15 µM) with $AdSO_4$ (15 µM) (Table 2). Observation revealed that callus induction initiated at the end of second week and proliferated in further weeks. SEs were differentiated by the end of 4 weeks (Fig. 1b) and germination passed through heart, torpedo and cotyledonary stages at the end of 6 week (Fig. 1c–e). Different stages were also visualized under microscope (Fig. 1f–h). When cytokinin (BA) and auxin (IAA) were combined, an embryogenic callus was induced and BA (10 µM) with IAA (0.5 µM) formed 12.25 ± 1.20 embryos which was comparatively less than the optimum number which was recorded earlier. Higher concentration of both PGRs gradually decreased the number of embryos and response too (Table 2).

Indirect somatic embryogenesis is a multi-step process and initially the somatic cells dedifferentiate and forms callus, in which embryogenic clumps originate which ultimately develop into SEs (Williams and Maheswaran 1986; Von Arnold et al. 2002). Cytokinins are known to play important role in this transition as well as in stimulating divisions in pro-embryogenic cells (Feher et al. 2003). Results showed that the combinations of BA-Kn, BA-$AdSO_4$ and BA-IAA induced SEs from leaf explant, which justify the fact that BA has pivotal role in inducing SEs (Chang 1991) as well as in conversion (Salma et al. 2019), and is also reported by Martin (2004) in *L. reticulata*. In the present investigation BA alone induced callusing and when combined with Kn or $AdSO_4$, differentiation of somatic embryos was observed. A combination of BA and Kn is rarely reported for somatic embryogenesis (Dhavala et al. 2009; Devendra et al. 2012), but there are many documents on stimulatory effect of $AdSO_4$ when combined with cytokinins, such as in *Acacia farnesiana* and *A. schaffneri* (Ortiz et al. 2000), *Coffea arabica* and *C. canephora* (Samson et al. 2006). It is due to fact that $AdSO_4$ retards the degradation of cytokinins in the medium by feed-back inhibition or by competing for the enzyme systems involved in cytokinin metabolism which in turn facilitates somatic embryogenesis (Van Staden et al. 2008). In the present study optimum SEs were obtained in BA-$AdSO_4$ which is in accordance with the report in *Phaseolus vulgaris* (Cabrera-Ponce et al. 2015). The combination of cytokinins and auxins induces

Fig. 1 Effect of PGRs on development of somatic embryos from leaf explants. **a** Direct SEs on leaf explant in medium fortified BA (15 µM) with Kn (5 µM). **b–e** Indirect SEs development in BA (15 µM) with $AdSO_4$ (15 µM): **b** globular SEs after 4 week, **c** elongated globular SEs at 6 weeks, **d** proliferation of callus and further development of embryos to heart and torpedo stages, **e** germinated embryos. Different stages of SEs under microscope: **f** globular embryos, **g** heart and torpedo stage embryos, **h** separated torpedo stage embryo. **i–m** Development of SEs in GA_3 (1 µM): **i** transferred SE (inset) and development after 2 weeks, **j** formation of direct SSEs at 4 weeks, **k** elongation of SSEs and callus induction at the end of 6 weeks, **l** proliferation of direct SSEs continued till 7 weeks**, m** induction of SEs on callus and development of direct SSEs at the end of 8 weeks. Scale bar = 0.5 mm. *PGRs* plant growth regulators, *SEs* somatic embryos, *SSEs* secondary somatic embryos, *BA* 6-benzyladenine, *Kn* kinetin, *$AdSO_4$* adenine sulphate, *GA_3*- gibberellic acid

Table 2 Effect of different PGRs on induction of somatic embryos from leaf explants of *L. reticulata* (6 weeks)

PGRs (μM)				No. of somatic embryos/explant*	Response (%)
BA	Kn	$AdSO_4$	IAA		
0	0	0	0	0 i	0
5	5	0	0	3.70 ± 0.95 gh	80
5	10	0	0	15.30 ± 2.15 c	90
5	15	0	0	11.10 ± 1.55 cd	90
5	20	0	0	8.70 ± 1.36 de	90
10	5	0	0	6.40 ± 0.92 ef	90
10	10	0	0	7.50 ± 1.50 ef	90
10	15	0	0	9.60 ± 1.50 de	90
10	20	0	0	10.60 ± 1.48 cd	90
15	5	0	0	20.90 ± 2.86 a	90
15	10	0	0	14.00 ± 1.99 c	90
15	15	0	0	9.70 ± 1.73 de	90
15	20	0	0	9.20 ± 1.43 de	90
20	5	0	0	8.90 ± 1.25 de	90
20	10	0	0	7.60 ± 0.95 ef	90
20	15	0	0	4.80 ± 0.97 fg	80
20	20	0	0	2.70 ± 0.81 gh	70
5	0	5	0	3.00 ± 0.69 gh	90
5	0	10	0	3.80 ± 0.75 gh	90
5	0	15	0	8.10 ± 1.39 ef	80
5	0	20	0	10.00 ± 1.61 cde	90
10	0	5	0	12.10 ± 1.66 c	90
10	0	10	0	14.00 ± 2.04 c	90
10	0	15	0	10.40 ± 1.82 cd	80
10	0	20	0	7.50 ± 1.73 ef	80
15	0	5	0	3.30 ± 1.09 gh	70
15	0	10	0	4.50 ± 1.17 fg	80
15	0	15	0	21.70 ± 2.06 a	100
15	0	20	0	14.30 ± 1.39 c	100
20	0	5	0	9.70 ± 1.64 de	90
20	0	10	0	11.50 ± 1.01 cd	100
20	0	15	0	18.60 ± 1.39 ab	100
20	0	20	0	19.30 ± 2.09 ab	100
5	0	0	0.1	3.80 ± 0.52 gh	50
5	0	0	0.5	3.83 ± 0.55 gh	60
5	0	0	1.0	6.86 ± 0.94 ef	70
10	0	0	0.1	9.00 ± 1.21 de	70
10	0	0	0.5	12.25 ± 1.20 c	80
10	0	0	1.0	7.71 ± 1.65 ef	70
15	0	0	0.1	5.43 ± 1.21 fg	70
15	0	0	0.5	4.20 ± 0.72 fg	50
15	0	0	1.0	2.75 ± 0.41 gh	40
20	0	0	0.1	2.33 ± 0.27 gh	30
20	0	0	0.5	1.50 ± 0.35 gh	20
20	0	0	1.0	0 i	0

PGRs plant growth regulators, *BA* 6-benzyladenine, *Kn* kinetin, *$AdSO_4$* adenine sulphate, *IAA* indole-3-acetic acid

*Values represent mean ± SE. Means (n = 10) followed by same letter are not significantly different ($p \leq 0.05$) according to Tukey's test

SEs; these PGRs are involved in the regulation of cell division and differentiation and, therefore, play an important role in inducing somatic embryogenesis (Pinto et al. 2011). The combination of BA and IAA induced SEs in *L. reticulata* which is also reported in *Hybanthus enneaspermus* (Shekhawat and Manokari 2016), but 2,4-D which is a commonly used auxin for somatic embryogenesis (Horstman et al. 2017) have inhibitory activity for embryogenesis in *L. reticulata* (Martin 2004) and is in accordance with current work. Previous report on same plant by Rathore et al. (2013) documented that SEs failed to develop into plantlets, whereas in the present study embryos were successfully developed into plantlets in static medium. The number of embryos developed in the present study is higher as compared to earlier reports on *L. reticulata* (Martin 2004; Sathyanarayana et al. 2008).

Development of somatic embryos

It has been reported that the development of SEs into plantlet formation requires different growth regulators (Pasternak et al. 2002). This is an essential phase, as mature SEs are known to accumulate a sufficient amount of storage proteins and develop into normal plants (Rai et al. 2008). Somatic embryos, which differentiated in various PGRs combinations, were transferred to static and liquid media for further development. MS static media fortified with GA_3 (1 μM) proved to be beneficial for germination of embryos among all the PGRs tried. BA (15 μM) + $AdSO_4$ (15 μM) and coconut water (4%) failed to developed SEs into plantlets and turned black within 2 weeks in both static and liquid media. It has been generally observed that for maturation of SEs, removal of cytokinin and auxin from medium proves to be beneficial (Hazubska-Przybyl et al. 2020), and the same was observed in the present study. In GA_3 medium, the embryos developed into healthy plantlets within 2 weeks and at the same time secondary somatic embryos (SSEs) differentiated from the hypocotyl region and further growth of the plantlet continued till 4 weeks (Fig. 1i, j). These plantlets when transferred to the fresh medium, the SSEs developed into plantlets and simultaneously calli was formed at the end of 6 weeks (Fig. 1k). These direct SSEs were proliferated (Fig. 1l) and by 8 weeks SEs induction was simultaneously initiated from calli (Fig. 1m). The process of generating secondary somatic embryos is easy and quick method and can be kept for extended period of time (Pavlovic et al. 2012). Whereas liquid media fortified with all the PGRs failed to develop embryos, these turned black within 2 weeks. Present results are in accordance with earlier study on same plant

in which presence of GA_3 with BA facilitated maximum conversion of SEs into plantlets (Martin 2004). Similarly beneficial effect of GA_3 for conversion of SEs into plantlets has been documented previously in *H. indicus* (Cheruvathur et al. 2013) and *Haworthia retusa* (Kim et al. 2019). The efficiency of GA_3 could be due to gene activation for the development of embryos (Sharmin et al 2014).

Effect of individual and combinations of cytokinins on nodal explants

Nodes of *L. reticulata* did not give any significant response when placed in basal medium; the elongation of axillary bud into shoots was observed when media were fortified with different cytokinins i.e. BA and Kn in concentration range from 5 to 20 µM. All concentrations of BA induced almost equal number of shoots (more than one) in 100% cultures at the end of 8 weeks. Replacing BA with another cytokinin (Kn) proved more advantageous as it induced healthy shoots with broadened leaves. More than one shoot was recorded in all the concentrations and highest shoots (2.33 ± 0.19) with 3.43 ± 0.36 cm length was obtained in Kn (20 µM, 60% cultures) among all individual cytokinins concentrations tried (Table 3).

When synergistic combination of BA with Kn was tried the shoot number remained similar to that observed in presence of individual cytokinins. Highest 2.40 ± 0.60 shoots in 50% cultures were recorded in BA (10 µM) with Kn (10 µM) among all the concentrations (Table 3). The response was increased when synergistic effect of BA and $AdSO_4$ was used as it resulted in multiple shoot regeneration. The axillary bud break was observed within a week from both sides of node which formed shoots at the end of 2 weeks (Fig. 2a). Within 4 weeks another bud break was observed again from both side (Fig. 2b) and after subculture elongation and proliferation was noticed within 6 weeks (Fig. 2c). Healthy multiple shoots were obtained at the end of 8 weeks (Fig. 2d). Lower concentration of BA (5 µM) formed multiples but hardly two or three shoots were formed and an optimum 5.10 ± 0.33 number of shoots in 100% cultures with 3.74 ± 0.60 cm length was recorded at 10 µM of BA and $AdSO_4$ (Table 3). Further increasing BA concentration failed to induce multiples and resulted in stunted shoots with hyperhydricity. One common observation which was recorded for all PGRs combinations was that the regenerated shoots from nodal explants failed to elongate properly and some remained stunted. Therefore, shoots were transferred to the medium fortified with coconut water at different concentrations (2–8%) (Fig. 2e–h), and 4% proved advantageous because maximum shoot length (6.72 ± 0.38) was achieved in the same (Fig. 3). Hence in vivo nodal explants were also placed in medium supplemented with coconut water (4%) to assess the effect on shoot regeneration. The result showed that healthy elongated shoots were obtained however they failed to form multiples.

Cytokinins are required for cell expansion, its division, regulation of shoot formation and axillary bud multiplication, they also regulate proteins synthesis which is required for formation and functioning of mitotic spindles (Mok and Mok 2001). In the present study optimum shoots were recorded in media fortified with BA and $AdSO_4$; the beneficial effect of $AdSO_4$ was also reported previously in *L. reticulata* (Rathore et al. 2013). In the present study combination of BA-$AdSO_4$ was better as compared to Kn-$AdSO_4$, and this result is in accordance with report of Rout et al. (2008) for *Nyctanthes arbor-tristis*. Similarly, beneficial effect of BAP with $AdSO_4$ for multiple shoot induction is documented in *Solanum tuberosum* (Singh et al. 2017). Whereas Sudipta et al. (2011) documented maximum shoots from nodes of *L. reticulata* in media fortified with BA and Kn, and in the present study similar result for shoot number was achieved. But the shoot number and % response recorded in the present study was higher as compared to other reports for the same plant (Arya et al. 2003; Parabia et al. 2007; Bharat et al. 2011). The findings are at par with report of Rathore et al. (2013) but they further subcultured for enhancing the shoot number which was not the aim of the present study. The observations also revealed that shoots failed to elongate in cytokinin fortified medium and the same has also reported earlier in *L. reticulata* (Arya et al. 2003; Parabia et al. 2007); higher cytokinin concentrations inhibits shoot elongation (Hu and Wang 1983). Coconut water in the medium facilitated elongation of the shoots of *L. reticulata* and similarly observations on nodal explants has been reported in *E. alba* (Baskaran and Jayabalan 2005) and *Capsicum annuum* (Mythili et al. 2017).

Phytochemical analysis

HPTLC fingerprint

Hexane extract of all the samples were subjected to chromatographic separation on TLC plates followed by observation under 254 nm for reveal the variation in fingerprint (Fig. 4a). Further densitometric scanning confirmed the change in peak areas as well as band pattern of different samples (Fig. S1, Table S1a). The change in banding pattern was observed at 366 nm (Fig. 4b, Fig. S1, Table S1b). The plate was then derivatized using anisaldehyde-sulphuric acid reagent (Fig. 4c) and scanned at 525 nm which also showed variation in banding pattern and peak areas (Fig. S1, Table S1c). Similarly in ethyl acetate (Fig. 5a–c) and methanol (Fig. 6a–c) extracts band separation was observed on TLC plate for all the samples. Further densitometry scanning of the plates at different wavelengths (254, 366 and 525 nm) confirmed the change in fingerprint

Table 3 Effect of PGRs on shoot regeneration from nodal explants of *L. reticulata* (8 weeks)

PGRs (µM)			No. of shoots/explant*	Shoot length (cm)*	Response (%)
BA	Kn	$AdSO_4$			
5	0	0	2.20 ± 0.28 bc	2.95 ± 0.25 bc	100
10	0	0	1.78 ± 0.28 de	2.09 ± 0.21 cd	90
15	0	0	2.11 ± 0.29 bcd	2.84 ± 0.20 bc	90
20	0	0	2.00 ± 0.18 cde	2.02 ± 0.08 cd	90
0	5	0	1.67 ± 0.27 ef	1.88 ± 0.19 d	30
0	10	0	1.20 ± 0.18 ef	2.13 ± 0.19 cd	50
0	15	0	1.80 ± 0.33 de	3.13 ± 0.28 ab	50
0	20	0	2.33 ± 0.19 bc	3.43 ± 0.36 ab	60
5	5	0	2.17 ± 0.15 bcd	2.41 ± 0.46 bc	60
5	10	0	1.33 ± 0.27 ef	1.80 ± 0.07 d	30
5	15	0	1.80 ± 0.18 de	1.70 ± 0.16 d	50
5	20	0	1.83 ± 0.18 de	1.89 ± 0.15 d	60
10	5	0	1.33 ± 0.27 ef	2.45 ± 0.18 bc	30
10	10	0	2.40 ± 0.60 bc	2.57 ± 0.3 bc	50
10	15	0	1.40 ± 0.22 ef	1.37 ± 0.09 d	50
10	20	0	1.25 ± 0.22 ef	1.42 ± 0.15 d	40
15	5	0	1.00 ± 0.00 f	1.60 ± 0.00 d	10
15	10	0	1.40 ± 0.22 ef	1.76 ± 0.14 d	50
15	15	0	2.00 ± 0.24 cde	2.42 ± 0.29 bc	60
15	20	0	1.25 ± 0.22 ef	1.34 ± 0.10 d	40
20	5	0	1.33 ± 0.27 ef	1.60 ± 0.29 d	30
20	10	0	1.00 ± 0.00 f	1.50 ± 0.14 d	30
20	15	0	1.00 ± 0.00 f	1.30 ± 0.07 d	20
20	20	0	1.00 ± 0.00 f	0.90 ± 0.07 d	20
5	0	5	2.40 ± 0.25 bc	2.15 ± 0.24 cd	100
5	0	10	3.60 ± 0.57 b	2.80 ± 0.26 bc	100
5	0	15	3.90 ± 0.33 b	2.76 ± 0.27 bc	100
5	0	20	3.90 ± 0.22 b	2.49 ± 0.33 bc	100
10	0	5	3.90 ± 0.26 b	2.59 ± 0.35 bc	100
10	0	10	5.10 ± 0.33 a	3.74 ± 0.60 ab	100
10	0	15	3.50 ± 0.45 b	3.05 ± 0.33 bc	100
10	0	20	2.30 ± 0.25 bc	2.13 ± 0.23 cd	100
15	0	5	2.10 ± 0.22 bcd	2.83 ± 0.37 bc	100
15	0	10	2.10 ± 0.26 bcd	2.16 ± 0.21 bc	100
15	0	15	3.00 ± 0.33 bc	2.14 ± 0.29 cd	60
15	0	20	2.13 ± 0.37 bcd	2.23 ± 0.35 bc	80
20	0	5	1.80 ± 0.18 de	2.16 ± 0.51 cd	50
20	0	10	2.00 ± 0.35 cde	1.91 ± 0.23 d	40
20	0	15	2.67 ± 0.54 bc	1.74 ± 0.23 d	30
20	0	20	1.75 ± 0.41 de	1.69 ± 0.21 d	40
0	5	5	1.00 ± 0.20 f	1.76 ± 0.31 d	100
0	5	10	1.50 ± 0.21 ef	2.67 ± 0.28 bc	100
0	5	15	2.00 ± 0.28 cde	2.80 ± 0.36 bc	100
0	5	20	2.20 ± 0.28 bc	2.99 ± 0.38 bc	100
0	10	5	1.90 ± 0.17 de	4.12 ± 0.68 a	100
0	10	10	3.10 ± 0.33 bc	2.48 ± 0.15 bc	100
0	10	15	2.70 ± 0.28 bc	2.74 ± 0.28 bc	100
0	10	20	1.90 ± 0.28 de	3.82 ± 0.38 ab	100
0	15	5	1.80 ± 0.28 de	2.59 ± 0.29 bc	100

Table 3 (continued)

PGRs (µM)			No. of shoots/explant*	Shoot length (cm)*	Response (%)
BA	Kn	$AdSO_4$			
0	15	10	2.70 ± 0.25 bc	2.52 ± 0.23 bc	100
0	15	15	2.40 ± 0.25 bc	2.16 ± 0.22 cd	100
0	15	20	2.60 ± 0.21 bc	2.50 ± 0.26 bc	100
0	20	5	2.00 ± 0.25 cde	2.32 ± 0.18 bc	80
0	20	10	1.29 ± 0.17 ef	1.88 ± 0.14 d	70
0	20	15	1.20 ± 0.18 ef	2.00 ± 0.22 cd	60
0	20	20	1.20 ± 0.18 ef	1.43 ± 0.17 d	60

PGRs plant growth regulators, *BA* 6-benzyladenine, *Kn* kinetin, *$AdSO_4$* adenine sulphate

*Values represent mean ± SE. Means (n = 10) followed by same letter in each column are not significantly different ($p \leq 0.05$) according to Tukey's test

Fig. 2 Shoot regeneration from nodal explants of *L. reticulata* on medium supplemented with BA (10 µM) with $AdSO_4$ (10 µM). **a** Bud elongation after 2 weeks, **b** shoot formation within 4 weeks, **c** multiples formed after 6 weeks, **d** elongated healthy shoots obtained at the end of 8 weeks. **e**–**h** Elongation of shoots at different concentrations of coconut water after 8 weeks: **e** 2%, **f** 4%, **g** 6%, **h** 8%. BA: 6-benzyladenine, *$AdSO_4$* adenine sulphate

shown by variation in peak areas and numbers of bands in chromatograms of all samples for ethyl acetate (Fig. S2, Table S2a–c) and methanol (Fig. S3, Table S3a–c) extracts.

The profiling of different extract revealed that PGRs of the medium as well as type of culture affected the HPTLC fingerprint of samples. The chemical profile of shoot cultures were at par with in vivo shoots, but in callus samples

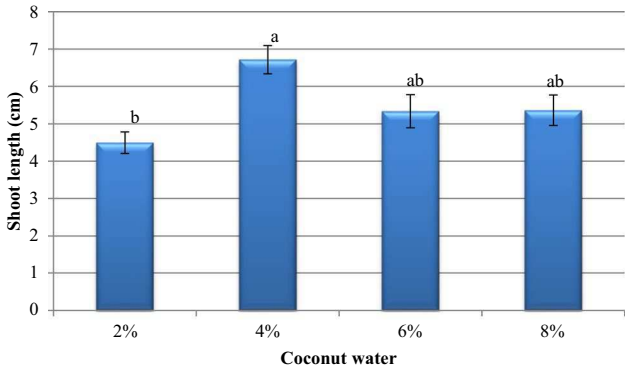


Fig. 3 Elongation of shoots derived through nodal explants after 8 weeks

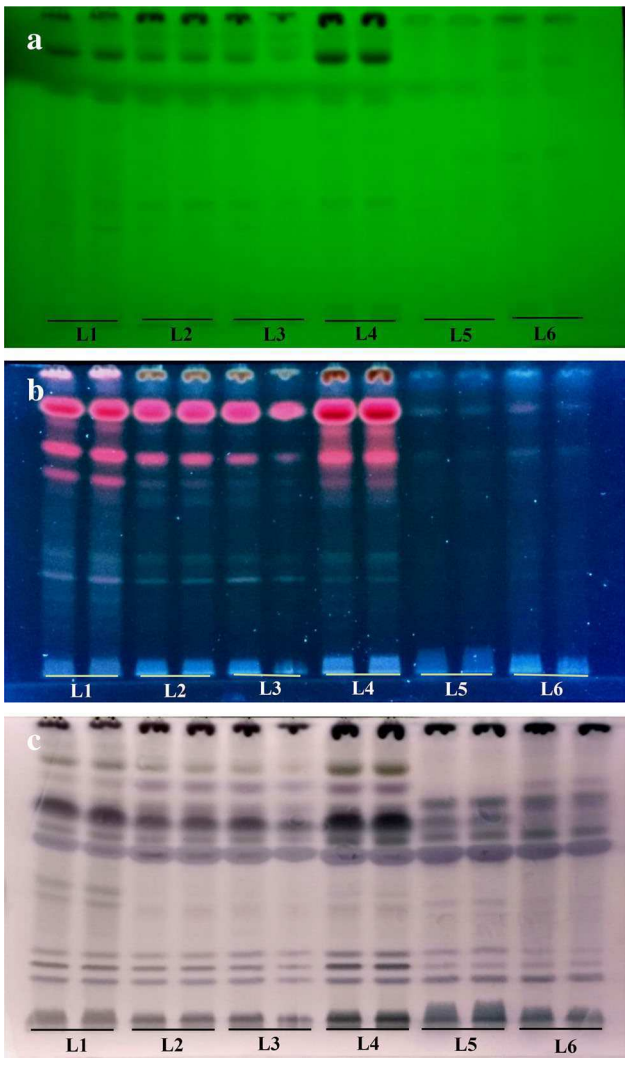


Fig. 4 HPTLC fingerprint of hexane extract for different samples (L1-L6) of *L. reticulata*. **a** UV 254 nm, **b** UV 366 nm, **c** white light after derivatization. *HPTLC* high-performance thin layer chromatography

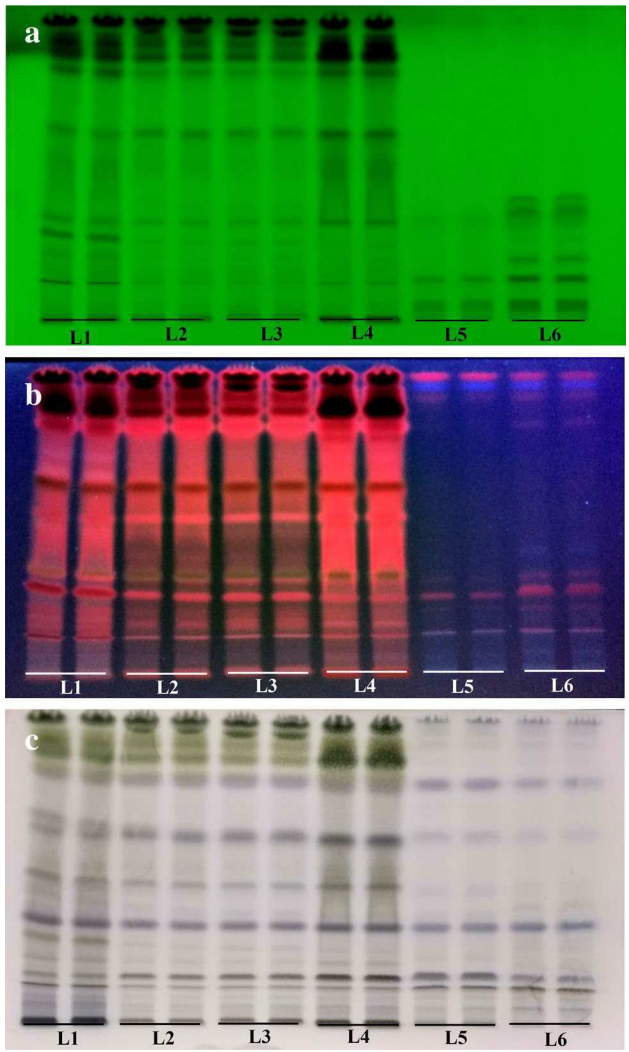


Fig. 5 HPTLC fingerprint of ethyl acetate extract for different samples (L1-L6) of *L. reticulata*. **a** UV 254 nm, **b** UV 366 nm, **c** white light after derivatization. *HPTLC* high-performance thin layer chromatography

the number of bands were less. It may be due to fact that the metabolites synthesis takes place in specific tissues or organs which are lacking in callus tissue (Jain et al. 2011). This has also been documented for picroside production in *Picrorhiza kurroa* where only shoots had the potential to synthesize the secondary metabolite and not callus cultures (Sood and Chauhan 2009). Generally hexane extract contains terpenoids, aglycones, saponins, waxes and fats; ethyl acetate extract contains flavonoids, alkaloids, tannins, phenols, glycosides and chlorophylls; and methanol extract contains polyphenols, phenones, saponins, tannins, flavonoids, anthocyanins and xanthoxyllines (Houghton and Raman 1998), and thus in the present study all these solvents were utilized for extraction to cover all groups of metabolites which is prerequisite for chemical profiling (Harborne 1984). There

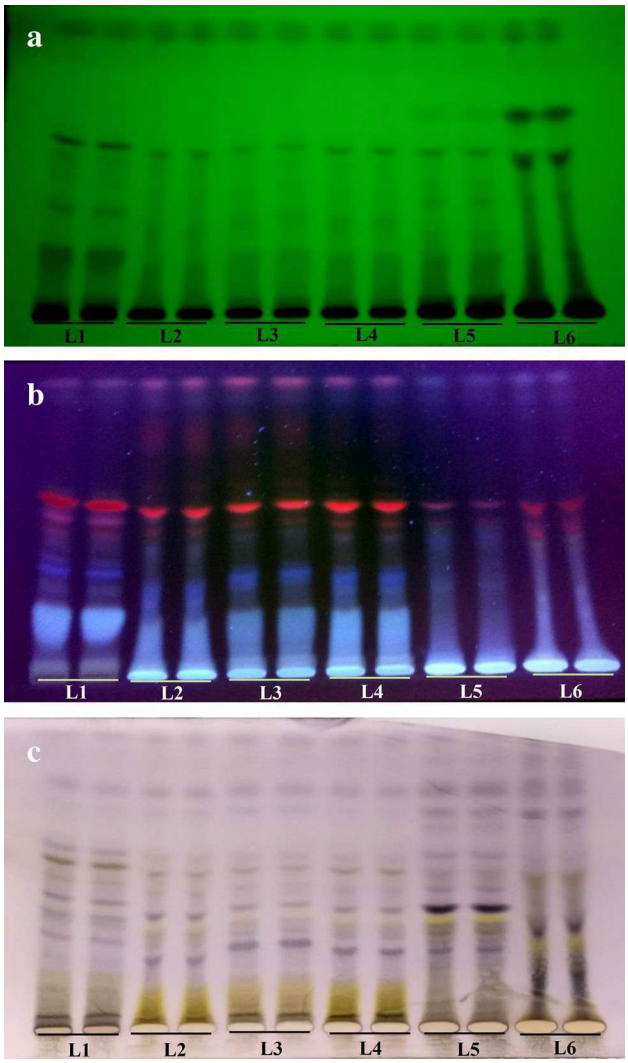


Fig. 6 HPTLC fingerprint of methanol extract for different samples (L1-L6) of *L. reticulata*. **a** UV 254 nm, **b** UV 366 nm, **c** white light after derivatization. *HPTLC* high-performance thin layer chromatography

are reports which suggest that PGRs of the medium especially cytokinins and auxins have influence on biosynthesis of different groups of secondary metabolites (Ekiert et al. 2008; El Tahchy et al. 2011; Grover et al. 2012; Joshaghani et al. 2014; Thiruvengadam and Chung 2015). This technique has also been used to assess the effect of PGRs and biosynthetic potential of in vitro samples of other Asclepiadaceae members like *Ceropegia juncea* (Nikam and Savant 2009), *H. indicus* (Pathak et al. 2017) and *Tylophora indica* (Patel et al. 2020), whereas this study needs to be done in *L. reticulata*. There are number of compounds like diosmentin, luteolin, rutin, quercetin, iso-quercetin, α-amyrin, β-amyrin, lupeol, β-sitosterol, stigmasterol, hentricontanol,

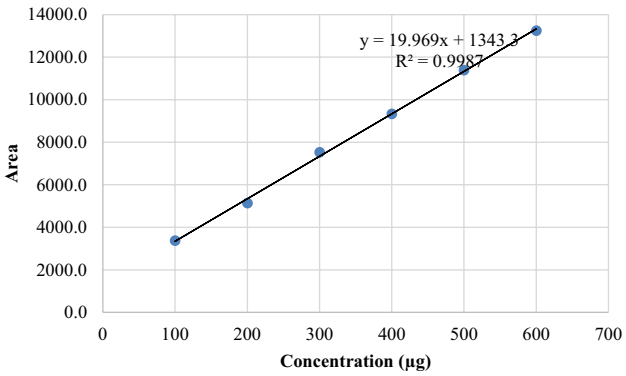


Fig. 7 Standard curve of *p*-coumaric acid

Table 4 Intra-day and inter-day precisions for method validation of *p*-coumaric acid

Sr. No.	Standard concentration (µg)	Intra-day CV (%)	Inter-day CV (%)
1	200	1.9	2.0
2	400	1.7	1.3
3	600	1.5	2.1
Average	–	1.7	1.8

CV coefficient of variation

α-tocopherol, n-triacontane etc. that have been quantified using HPTLC in this plant (Mohanty et al. 2017).

Preparation of *p*-coumaric acid calibration curve and HPTLC method validation

A six point calibration curve over the concentration range (100–600 µg) was prepared with a correlation coefficient of 0.998 (Fig. 7). The slope and intercept of the curve were 1996 and 1343 respectively. The LOD and LOQ were calculated as 10 µg and 100 µg per spot. Specificity was analyzed using standard (*p*-coumaric acid) for all samples and were subjected to TLC analysis. The repeatability of the method was studied by spotting definite amount (10 µl) of standard six times on TLC plate. Intra-day precision values of the HPTLC method for *p*-coumaric acid ranged from 1.5 to 1.9, and similarly inter-day precision values were ranged between 1.3 and 2.1 (Table 4).

Quantification of *p*-coumaric acid

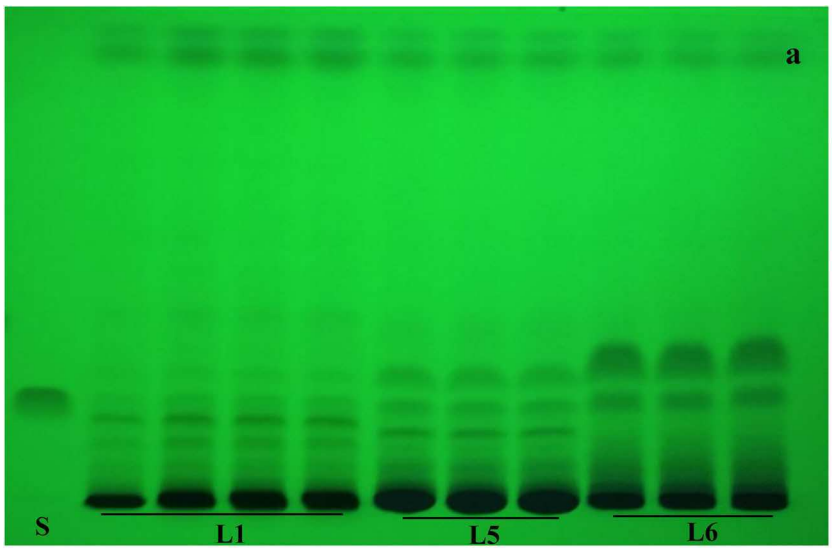
Methanol extract of different samples when separated on TLC plate the presence of *p*-coumaric acid was confirmed in all the samples on plate as band corresponding to standard

(Fig. 8a). Consequential chromatogram showed the peak corresponding to the standard *p*-coumaric acid and in the sample at same Rf as shown in Fig. 8b. Further spectral analysis has been done for confirmation and it revealed that the peaks obtained from both standard and test samples were identical (λ_{max} 254 nm), as they had similar pattern (Fig. 8c). The area under the curve (AUC) obtained after scanning at 254 nm was different for all the samples. It was recorded that in in vivo and in vitro shoot samples (L1-L4) band was observed on TLC plate at similar Rf to standard and similarly corresponding peak also appeared after densitometric scanning, thus it was in range of LOD but it is out of range of LOQ for *p*-coumaric acid through HPTLC. The amount varied in callus samples and it was maximum in sample L6 i.e. 78.08 ± 2.20 mg g^{-1} DW, whereas it was less in sample L5 i.e. 16.22 ± 2.20 mg g^{-1} DW (Table 5).

The effect of PGRs on in vitro cultures is manifested in enhanced growth, differentiation in the cultures as well as secondary metabolite synthesis (Dornenburg and Knorr 1995) and it has been recently documented in *Ipomoea turbinate* for synthesis of *p*-coumaric acid (Ahmad et al. 2019), but it is not yet investigated for in vitro cultures of *L. reticulata*. Thus, shoot and callus cultures derived from different media were analyzed for synthesis of *p*-coumaric acid and it was revealed that amongst all the cultures it was quantifiable only in callus cultures and not in shoots. Our results are in corroboration with results in *Larrea divaricata* (Palacio et al. 2012) and *Aronia melanocarpa* (Szopa et al. 2013) where *p*-coumaric acid synthesized only in undifferentiated cultures and not in shoot cultures. Cytokinins of the medium are known to repress transporters of macronutrients such as nitrate, ammonium, sulphate, phosphate, and regulate the expression of *PAL* gene of phenylpropanoid pathway (Sakakibara et al. 2006). In the present study when auxins were added with cytokinin, the content of *p*-coumaric acid varied as high content was recorded in BA-NAA compared to BA-2,4-D. The type of auxins affects its synthesis as it is well documented that auxin response factors (ARFs) interacts with auxin regulatory genes related to several physiological and biochemical processes, and in turn affects secondary metabolite synthesis (Chapman and Estelle 2009). Study on *Echinacea purpurea* also suggest that phenolics in callus cultures is affected by combinations of cytokinin with either NAA or 2,4-D (Ramezannezhad et al. 2019). Beneficial effect of BA-NAA combination on accumulation of *p*-coumaric acid in *Hypericum perforatum* cvs. Helos (Kwiecień et al. 2015) and phenolic acids in *Ruta graveolens* (Ekiert et al. 2008) is reported. Decreased synthesis of targeted compound in presence of 2,4-D may be due its less efficiency for triggering secondary metabolism (Mantell and Smith 1983; Misawa 1985). In other Asclepiadaceae members, *H. indicus* (Misra et al. 2005) and *C. juncea* (Nikam and Savant 2009), the stimulatory effect of NAA and adverse effect of 2,4-D on production of metabolites through callus cultures is reported. Many-a-time the content of targeted metabolite in callus cultures is high as compared to mother plants, which makes these cultures a promising source for production of valuable secondary metabolites from different important medicinal plants as compared to in vivo plants (Murthy et al. 2014).

Conclusion

In the present study a protocol for efficient regeneration of somatic embryos from leaf explants and axillary bud proliferation using nodal explants was developed. It can be concluded that the leaf explants has potency to develop large number of somatic embryos along with formation of secondary SEs. This can be useful for mass-propagation which ultimately contributes to mitigate the overexploitation of *L. reticulata* from wild. Chemical fingerprint and quantification of *p*-coumaric acid revealed that PGRs of the media and type of culture played a vital role in biosynthesis of metabolites. HPTLC fingerprint confirmed that the synthesis of metabolites in shoot cultures are at par with in vivo shoot for all three extracts, whereas the profiling of callus samples showed variation. The quantity of marker compound was higher in leaf derived callus cultures which indicated that these cultures can become a potential source for production of *p*-coumaric acid as an alternative to wild plants. Future prospects can focus on elicitation of *p*-coumaric acid using callus/cell suspension culture.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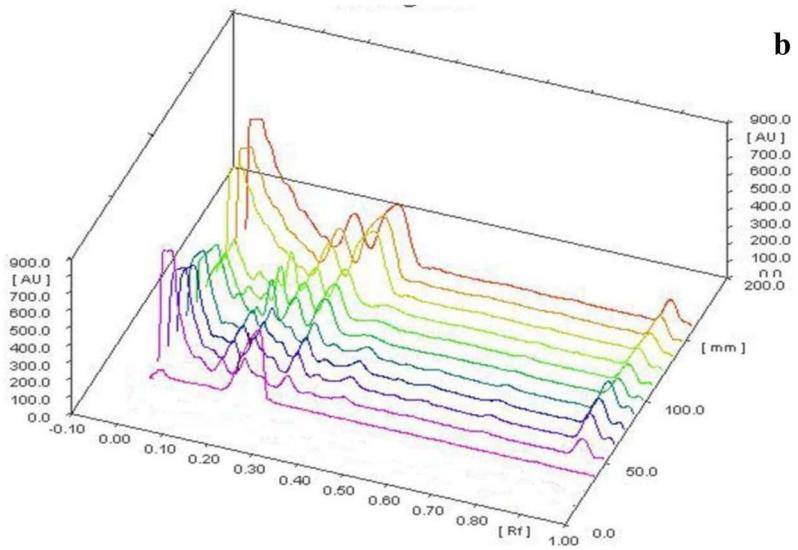
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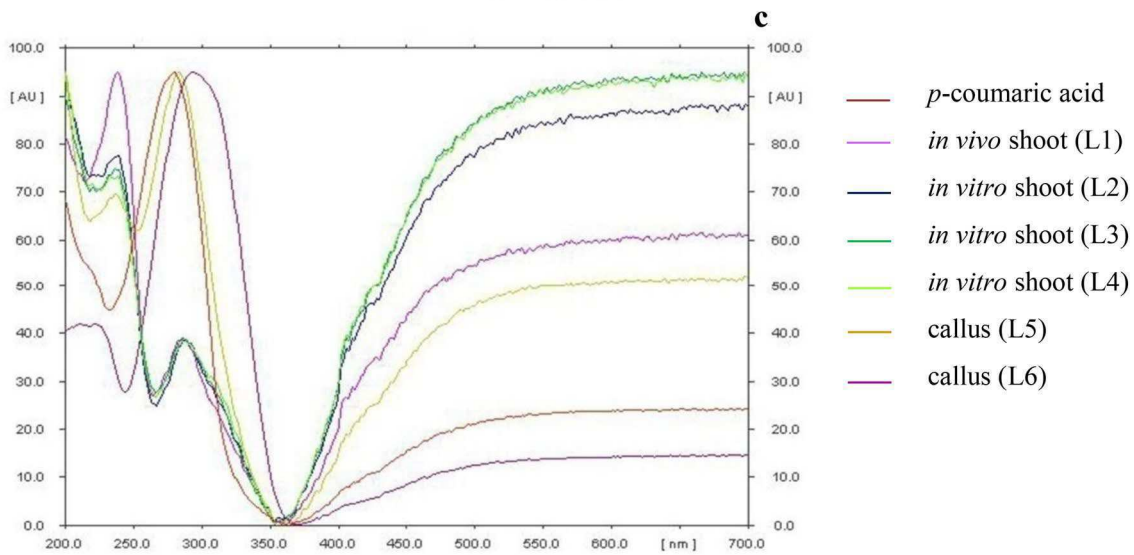
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p-coumaric acid
in vivo shoot (L1)
in vitro shoot (L2)
in vitro shoot (L3)
in vitro shoot (L4)
callus (L5)
callus (L6)

◂**Fig. 8** HPTLC quantification of *p*-coumaric acid in samples of *L. reticulata*. **a** TLC plate under UV 254 nm containing standard (S), in vivo shoots (L1), callus from BA (15 µM) + 2,4-D (1.0 µM) (L5) and callus from BA (20 µM) + NAA (0.5 µM) (L6), **b** densitometric scanning of plate at UV 254 nm, **c** spectra of all samples (L1-L6) with respect to standard *p*-coumaric acid at UV 254 nm. *HPTLC* high-performance thin layer chromatography. *BA* 6-benzyladenine, *NAA*- 1-naphthaleneacetic acid, 2,4-*D* 2,4-dichlorophenoxyacetic acid

Table 5 Quantification of *p*-coumaric acid in in vivo and in vitro samples of *L. reticulata* using HPTLC

Sample	Medium	*p*-coumaric acid quantity (mg g^{-1})*
L1	In vivo shoots	–
L2	BA (5 µM) + Kn (5 µM)	–
L3	BA (10 µM) + $AdSO_4$ (10 µM)	–
L4	Coconut water (4%)	–
L5	BA (15 µM) + 2,4-D (1.0 µM)	16.22 ± 0.09 b
L6	BA (20 µM) + NAA (0.5 µM)	78.08 ± 2.20 a

BA 6-benzyladenine, *Kn* kinetin, *$AdSO_4$* adenine sulphate, *NAA*-1-naphthaleneacetic acid, 2,4-D: 2,4-dichlorophenoxyacetic acid, *HPTLC*: high-performance thin layer chromatography

*Values represent mean ± SE. Means (n = 3) followed by same letter are not significantly different ($p \leq 0.05$) according to Tukey's test

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest to publish this manuscript.

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