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PLANT GENETICS • ORIGINAL PAPER

Genome size and gas chromatography-mass spectrometry (GC–MS) analysis of field-grown and in vitro regenerated *Pluchea lanceolata* plants

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Abstract

Pluchea lanceolata is a threatened pharmacologically important plant from the family Asteraceae. It is a source of immunologically active compounds; large-scale propagation may offer compounds with medicinal benefits. Traditional propagation method is ineffective as the seeds are not viable; and root sprout propagation is a slow process and produces less numbers of plants. Plant tissue culture technique is an alternative, efficient method for increasing mass propagation and it also facilitate genetic improvement. The present study investigated a three-way regeneration system in *P. lanceolata* using indirect shoot regeneration (ISR), direct shoot regeneration (DSR), and somatic embryo mediated regeneration (SER). Aseptic leaf and nodal explants were inoculated on Murashige and Skoog (MS) medium amended with plant growth regulators (PGRs), 2,4-dichlorophenoxy acetic acid (2,4-D), 1-naphthalene acetic acid (NAA), and 6-benzyl amino purine (BAP) either singly or in combinations. Compact, yellowish green callus was obtained from leaf explants in 1.0 mg/l BAP (89.10%) added medium; ISR percentage was high, i.e., 69.33% in 2.0 mg/l BAP + 0.5 mg/l NAA enriched MS with 4.02 mean number of shoots per callus mass. Highest DSR frequency (67.15%) with an average of 5.62 shoot numbers per explant was noted in 0.5 mg/l BAP added MS medium. Somatic embryos were produced in 1.0 mg/l NAA fortified medium with 4.1 mean numbers of somatic embryos per culture. On BAP (1.0 mg/l) + 0.5 mg/l gibberellic acid (GA_3) amended medium, improved somatic embryo germination frequency (68.14%) was noted showing 12.18 mean numbers of shoots per culture. Histological and scanning electron microscopic (SEM) observation revealed different stages of embryos, confirming somatic embryogenesis in *P. lanceolata.* Best rooting frequency (83.95%) of in vitro raised shootlets was obtained in 1.0 mg/l IBA supplemented half MS medium with a maximum of 7.83 roots per shoot. The regenerated plantlets were transferred to the field with 87% survival rate. The 2C genome size of ISR, DSR, and SER plants was measured and noted to be 2.24, 2.25, and 2.22 pg respectively, which are similar to field-grown mother plant (2C = 2.26 pg). Oxidative and physiological events suggested upregulation of enzymatic activities in tissue culture regenerated plants compared to mother plants, so were photosynthetic pigments. Implementation of gas chromatography-mass spectrometry (GC–MS) technique on in vivo and in vitro raised plants revealed the presence of diverse phyto-chemicals. The yields of alpha amyrin and lupeol (medicinally important triterpenoids) were quantified using high-performance thin-layer chromatography (HPTLC) method and enhanced level of alpha amyrin (2.129 µg g^{-1} dry wt) and lupeol (1.232 µg g^{-1} dry wt) was noted in in vitro grown leaf tissues, suggesting in vitro conditions act as a potential trigger for augmenting secondary metabolite synthesis. The present protocol represents a reliable mass propagation technique in producing true-to-type plants of *P. lanceolata*, conserving 2C DNA and ploidy successfully without affecting genetic homogeneity.

Keywords Asteraceae · Organogenesis · Histology · Photosynthetic pigments · Genetic homogeneity · Triterpenoids

Abbreviations

MS	Murashige and Skoog
PGRs	Plant growth regulators
BAP	6-Benzylamino purine
2,4-D	2,4-Dichlorophenoxy acetic acid
NAA	α-Naphthalene acetic acid
IBA	Indole-3-butyric acid

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SEM	Scanning electron microscopy
FCM	Flow cytometry method
GC-MS	Gas chromatography-mass spectrometry
HPTLC	High-performance thin-layer chromatography

Introduction

Pluchea lanceolata (Oliver & Hieren), commonly known as Rasana, is one of the most important perennial medicinal herbs from the family Asteraceae. It is a tropical flora, thrives well on sandy coverings, and found in many parts of India, viz., Uttar Pradesh, Punjab, and Rajasthan as well as other East Asian regions (Pandey 2018). Chemical investigations revealed that the whole plant is a rich source of pharmaceutically important compounds (sesquiterpenoids, monoterpenes, glycosides, triterpenoids, and flavonoids) which act against different diseases (Srivastava and Shanker 2012). Alpha amyrin and lupeol are important triterpenoids with anti-inflammatory and anti-carcinogenic activity (Martelanc et al. 2009). Recently, quercetin was also reported as an effective compound against COVID-19 (Derosa et al. 2020). Due to over exploitation and improper cultivation, the plant is kept under "threatened" category (Arya and Patni 2013). The seeds are not viable and the propagation through root sprouts produces a limited number of clones (Kher and Nataraj 2019). Tissue culture has emerged as an alternative approach for propagation as it conserves threatened, endangered, and rare occurring plant populations (Sommerville et al. 2020). For mass propagation, the effectiveness of somatic embryogenesis has extensively been reported in several plant species using various explants (Lü et al. 2013; Feher 2015; Espinosa et al. 2018; Bączek et al. 2021). In *Pluchea*, the induction of callus and direct shoot formation were reported in the past (Arya and Patni 2013) but the somatic embryogenesis report and the genetic homogeneity status of derived plants have not been conducted yet. Flow cytometry method (FCM) is a technique used for rapid ploidy testing and it has several advantages. It examines a large number of nuclei and cells at a time unlike chromosome counting method (Dhooghe et al. 2011).

In vitro cultures exposed to different PGRs, light, and humidity produce free radicals like superoxide anion (O2• −), hydroxyl radical (•OH), and non-radical molecules like hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) in inducing stress. In vitro stresses and pathogen invasion promote production of reactive oxygen species (ROS) due to cellular homeostasis in triggering apoptotic mechanism (Jahan et al. 2014). As a defense response, various enzymes like catalase, superoxide dismutase, ascorbate peroxidase, and other antioxidant enzymes are produced (Dharmaraja 2017; Malik et al. 2020a, b). The activated enzyme levels ameliorate the effectiveness of produced ROS during adverse in vitro conditions (Kapoor et al. 2019).

Chemo-profiling of plant extracts through GC–MS is an important research approach in areas like pharmacognosy and pharmaceutical biotechnology (Al-Rubaye et al. 2017). Recent studies ensure that the GC coupled with mass spectroscopy (MS) is used in separation, identification, and detection of multi-component mixtures like essential oils, hydrocarbons, and secondary metabolites (Mohammed et al. 2016; Ali et al. 2019). Similarly, the GC–MS and HPTLC technique are utilized in several cultivated tissues but the compounds present in regenerated *Pluchea* plant parts have not been analyzed yet.

The objective of the present investigation was to evaluate the micropropagation prospects of *P. lanceolata* by using different PGRs in direct, indirect, and somatic embryogenic regeneration methods. The genome size of regenerated *Pluchea* plants was measured and compared with field-grown plants through flow cytometry as to ascertain the genetic homogeneity. The photosynthetic pigments, the enzymatic activity, and the chemical profiling (via GC–MS) of in vitro raised cultures were examined for the first time in response to stress. The quantification of triterpenoids (alpha amyrin and lupeol) has also been carried out by HPTLC. This is perhaps the first ever report of genome size analysis of in vitro regenerated *P. lanceolata*. The study would help in conserving this threatened plant by mass production without altering its ploidy.

Material and methods

Explant disinfection and culture establishment

In the present study, fresh young leaves and nodes of 4-year-old field-grown plant of *P. lanceolata* from herbal garden, Jamia Hamdard, New Delhi, were harvested and used as an experimental material. Surface sterilization of fresh plant tissues was done as described by Zafar et al. (2019). The culturing of the disinfected excised plant material was aseptically carried on basal MS (Murashige and Skoog 1962, HiMedia®, Mumbai, India) medium augmented with various concentrations of different plant growth regulators (PGRs). The cultures were placed in a growth room equipped with white fluorescent light (Philips, Gurugram, India) of 100 µmol m^{-2} s^{-1} intensity, 12/8 h photoperiod, and temperature regulated at 23° ± 2 °C with 30–50% relative humidity. One explant per test tube was inoculated with three replicates of each treatment. In vitro grown tissues were regularly sub-cultured within 3–4-week interval.

Callus formation and proliferation

The harvested leaves and nodes were inoculated on MS containing 0.5, 1.0, 1.5, 2.0, and 3.0 mg/l of BAP (Sigma-Aldrich®) and 0.5 mg/l of 2,4-D (Sigma-Aldrich®) for induction of callus. Callus started to form within 10–14 days of inoculation and proliferated well with successive sub-culturing. The callus induction efficacy was noted after 4 weeks of inoculation. The proliferated shoots were sub-cultured every 3–4 weeks.

Indirect shoot regeneration (ISR)

Profuse callus obtained from explants was inoculated on MS medium augmented with various PGR concentrations. The ISR was noted in varying concentrations (1.0, 1.5, 2, and 3 mg/l) of BAP and 0.5 and 1.0 mg/l NAA (Sigma-Aldrich®). The shootlet development percentage was recorded after 4 weeks of treatment. The average number of shoots per explant was also noted to monitor the best PGR treatments used. Twenty-four test tubes were used per treatment and each of the experiment was replicated thrice. The proliferated shoots were sub-cultured every 3–4 weeks.

Direct shoot regeneration (DSR)

In *P. lanceolata*, the direct shoot regeneration was obtained using leaf explant after culturing on MS medium fortified with different concentrations of BAP (0.25, 0.5, 1.0, 1.5, 2.0, 2.5 mg/l). The sub-culturing of shoot was made in every 3–4-week interval onto a fresh medium and the shoot induction incidence and the mean numbers of shoots per explant were noted after 4 weeks of culture.

Somatic embryogenesis-mediated regeneration (SER)

The leaf mediated friable callus was carried to test tubes containing MS medium added with various NAA concentrations (0.1, 0.5, 1.0, and 1.5 mg/l) alone, and in combination with BAP (0.1 mg/l). The somatic embryo induction percentage and the mean numbers of somatic embryos produced per callus mass (250 mg/l) were recorded after 4 weeks of inoculation. Bipolar embryo structures were obtained within 4–5 weeks of culture in the above embryogenic callus. For germination, the mature green somatic embryos were inoculated on MS augmented with 0.25, 0.5, and 1.0 mg/l of GA_3 (Sigma-Aldrich®) singly, and also in combination with BAP (0.5, 1.0, and 2.0 mg/l). The germination frequency and the average numbers of regenerants via SER per culture were calculated after 3–4 weeks of culture.

Histology

The histological study of the callus tissue was carried out by fixing the samples in a FAA mixture consisting formalin, glacial acetic acid, and 70% ethanol in the ratio of 5:5:90 for 24 h. Thereafter, progressive dehydration of fixed material was done in different ethanolic gradients ranging from 10 to 70% and later on embedded in paraffin as per the protocol laid down by Johansen (1940). The thin Sects. (8 µm) were made from the treated samples by using a rotatory microtome (Spencer, USA) equipped with steel knife. Subsequently, the sections were placed on a new and clean glass slide, de-waxed, and stained with 5% hematoxylin and 2% eosin dye. Finally, the mounting of the stained samples was done on Canada balsam which was further subject to micro photography by observing under light microscope (Nikon Optiphot, Japan).

Scanning electron microscopy (SEM)

To understand the surface morphology and ontogenesis of embryo development, the SEM was performed. The embryogenic callus was used for fixation in 2.0% glutaraldehyde, 2.0% formaldehyde, and 0.1 M phosphate buffer (pH adjusted to ~ 6.8) for 24 h at a temperature of 4 °C. Afterwards, the callus was washed off with buffer and then fixed in similar buffer (1.0% osmium tetroxide) for approximately 2 h followed by sequential ethanol-based dehydration. Finally, the gold palladium was used for coating the tissue and processed for photography by a LEO 435 V P (Zeiss, Oberkochen, Germany) setup which is operational at 15–25 kV.

Rooting and acclimatization of shootlets

The microshoots regenerated from ISR, DSR, and SER were transferred onto the root induction medium. The plantlets generated through SER have inconspicuous root ends so it also requires exogenous supply to grow and function well. Half strength MS added with 0.5, 1.0, 1.5, and 2.0 mg/l of NAA and 0.5, 1.0, 1.5, and 2.0 mg/l of IBA (Sigma-Aldrich®) was used as rooting media. The rooting frequency and the mean numbers of roots/shoot were recorded after a culture period of 4 weeks. The plantlets were thoroughly washed, after removing the gelling agent, shifted to pots containing soil rite and soil in the ratio of 1:1 in order to ensure high survival of the plant. Eventually, the regenerated plants were transferred to the field.

Flow cytometry analysis

2C nuclear DNA content of *P. lanceolata* was investigated using flow cytometry technique by procuring leaf tissues from field-grown plants and in vitro regenerated plants. In order to assess the genetic similarity and ploidy level, five leaf samples from in vitro regenerated *Pluchea* plants were randomly selected from each group of different regeneration pathway with the field-grown plant as control, along with a reference standard *Pongamia pinnata* with known 2C DNA content of 2.51 pg (Choudhury et al. 2014). The sample preparation protocol was optimized according to Doležel and Bartoš (2005). Finally, the prepared samples were analyzed using flow cytometer system of CFM BD FACS Calibur (BD Biosciences, San Jose, CA, USA). The 2C DNA content of *Pluchea* was estimated using the value of fluorescence intensity of G1 peaks for both the field and in vitro samples. The relative nuclear DNA content and the nuclear DNA index were calculated using the given formula:

$$2C\ \text{DNA}\ of\ P.\ lanceolata\ (pg) = 2.51\ \text{pg} \times \frac{\text{Mean of G0/G1 peak of } P.\ lanceolata}{\text{Mean of G0/G1 peak of } Pongamiapinnata}$$

DNA index = 2C DNA content of *Pluchea* / 2C DNA content of *Pongamia* and 1C DNA content was determined by using the method deduced by Greilhuber et al. (2005) which ascertains monoploid genome size. The conversion formula used for determination of genome size (Dolezel 2003) is 1 pg DNA: 1 pg = 978 Mbp or 0.978 × 109 bp.

Physiological analysis

Photosynthetic pigment assay

Quantification of pigments was carried out using 100 mg leaves from tissue culture regenerated plantlets (produced via direct, indirect, and embryogenic pathway) and donor (field) plant. Pigment analysis of chlorophyll a, chlorophyll b, total chlorophyll, and total carotenoid was estimated using the method of Arnon (1949). The extraction of pigment was done by pulverizing the leaf sample in 80% ice cold acetone (Merck). The mixture was filtered with Whatman filter paper (Whatman No. 1, Mumbai, India) followed by centrifugation for 15 min at 4 °C, and lastly kept for whole night incubation in dark. Absorbance was measured at the following wavelengths, i.e., 470 nm, 645 nm, and 663 nm, on a UV–Vis spectrophotometer (UV-1601, Shimadzu, Japan).

Enzymatic antioxidant assay

To determine the antioxidant enzyme activity, 0.5 g of fresh leaf was homogenized in 2.0 ml extraction buffer (1.0% (w/v) polyvinylpyrrolidone (PVP), 0.5 M sodium phosphate buffer, 3.0 mM ethylenediaminetetraacetic acid (EDTA), 1.0% (v/v) Triton X-100) using mortar and pestle at 4 °C in the dark conditions. The pH was adjusted to 7.5. The homogenate was filtered and centrifuged (10,000 g) for about 15 min. The activity of enzymes superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT) was analyzed using the procedure mentioned by Dhindsa et al. (1981), Nakano and Asada, (1981), and Aebi (1984) respectively. Enzyme activity was measured in enzyme unit (EU)/mg/min protein.

Chemical profiling

In vivo and in vitro derived plantlets were used for GC–MS analysis. Fresh and young leaves were collected from 4-year-old donor and in vitro derived plant. Fine powder was prepared using mortar and pestle after drying and crushing the leaves into liquid nitrogen and subsequently Soxhlet extraction was carried out using methanol till the material is completely utilized. This process was done twice after maintaining the volume with the solvent for 24 h. Prior to GC–MS analysis, purification of extract was done using syringe filter (0.22 µm, Genetix, India) and 1 µl extract was taken as the injection volume. The standard settings and preparation were carried out according to Yadav et al. (2019). The HPTLC conditions and standard preparation for quantification of alpha amyrin and lupeol (Sigma-Aldrich, USA) were done as described by Yadav et al. (2014).

Statistical analysis

All investigations under the current study were set up in a completely randomized design. Twenty-four test tubes (one explant per tube) were used per treatment and each of the experiment was replicated thrice. Besides, all other experiments were conducted in triplicates unless specified otherwise; data were analyzed by analysis of variance using SPSS software SPSS v. 16 (SPSS Inc., Chicago, USA). The means were separated using Duncan's multiple range test (DMRT) and the significance was determined at $p \leq 0.05$. The deviation to the mean was calculated as standard error (SE).

Results

Callus induction and proliferation

The leaf and node explants were cultured on MS medium supplemented with different concentrations of BAP. Single use of BAP (0.5–3.0 mg/l) produced enough good quality calluses; when tried with 2,4-D (0.5 mg/l), it did not improve callus induction frequency. The addition of 2,4-D alone in MS medium was also tested for callusing, except explant swelling; no callus induction was noted; hence, the data is not shown. Among all the combinations applied, 1.0 mg/l BAP showed maximum callus induction frequency (89.10%) from the leaf while the nodal explants exhibited 82.8% callusing (Suppl. Figure 1). Lowest callus induction intensity was recorded in high concentration and combination of BAP and 2,4-D, i.e., 3.0 mg/l BAP + 0.5 mg/l 2,4-D added medium from leaf (35.30%) and node (30.61%). The combination of auxin and cytokinin was thus noted to be less responsive in inducing vigorous callus. In *P. lanceolata*, BAP triggered a fairly good amount of callus in comparison to auxin. Leaves were observed to be more responsive as compared to nodal explants as the leaf produced intense callus, which started to appear within a couple of weeks of culture (Fig. 1a, b). Initially, the callus was green and friable but later became compact and yellowish, proliferated well after 3–4 weeks of culture in successive sub-culturing in the same medium (Fig. 1c, d).

Indirect shoot regeneration (ISR)

For indirect shoot regeneration, the yellowish green callus obtained from leaf and nodal parts was cultured on various NAA (0.5 and 1.0 mg/l) and BAP (1.0, 1.5, 2.0, and 3.0 mg/l) added MS medium. The nodal callus was noted to have no response in showing organogenesis, hence not included in data analysis. Within 2 weeks of culture, the small buds were emerged from the leaf callus, which developed into shoots in BAP and NAA added medium (Fig. 2a, b). The MS enriched with 2.0 mg/l BAP + 0.5 mg/l NAA showed the highest frequency of shoot regeneration (69.33%) with mean numbers of shoots (4.02) per callus mass (Fig. 3). Significant elongation and development of shoots were observed with regular sub-culturing on the same PGR added medium (Fig. 2c).

Direct shoot regeneration (DSR)

Fully expanded mature leaf explant in 0.25 to 2.5 mg/l BAP supplemented MS medium produced small bud-like structures directly on leaf surfaces without any intervening callus stage (Fig. 2d, e). After 4 weeks of culture, these direct shoot bud responses were noted; the newly formed shoots were separated carefully from the leaf surface, transferred to flask containing new media (Fig. 2f). The low level of BAP proved to be effective in inducing direct shoot formation, while in higher levels, shoot induction percentage gradually decreased. The highest direct shoot induction frequency (67.15%) and an average 5.62 shoots per explant were noted in 0.5 mg/l BAP added medium (Supp. Figure 2). Direct shoot bud formation was entirely absent in nodal explants.

Somatic embryogenesis mediated regeneration (SER)

The leaf-derived callus started to form embryogenic tissues within about 3–4 weeks of culture (Fig. 2g). The MS augmented with 1.0 mg/l NAA alone was efficient in inducing somatic embryos in *P. lanceolata*; the induction

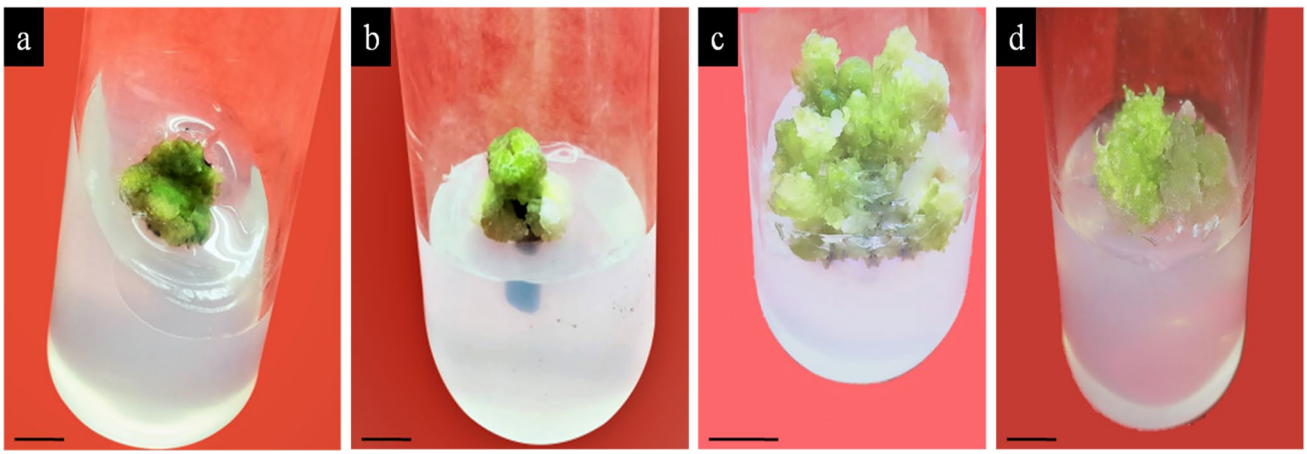

Fig. 1 Callus induction of *Pluchea lanceolata* in MS medium amended with 1.0 mg/l BAP after 4 weeks of inoculation from (**a**) leaf explant, (**b**) nodal explants, and (**c**, **d**) proliferated callus from leaf and node explant after 6 weeks of sub-culturing (bars: **a**, **b**, **c**, 1.5 cm; **d**, 1 cm)

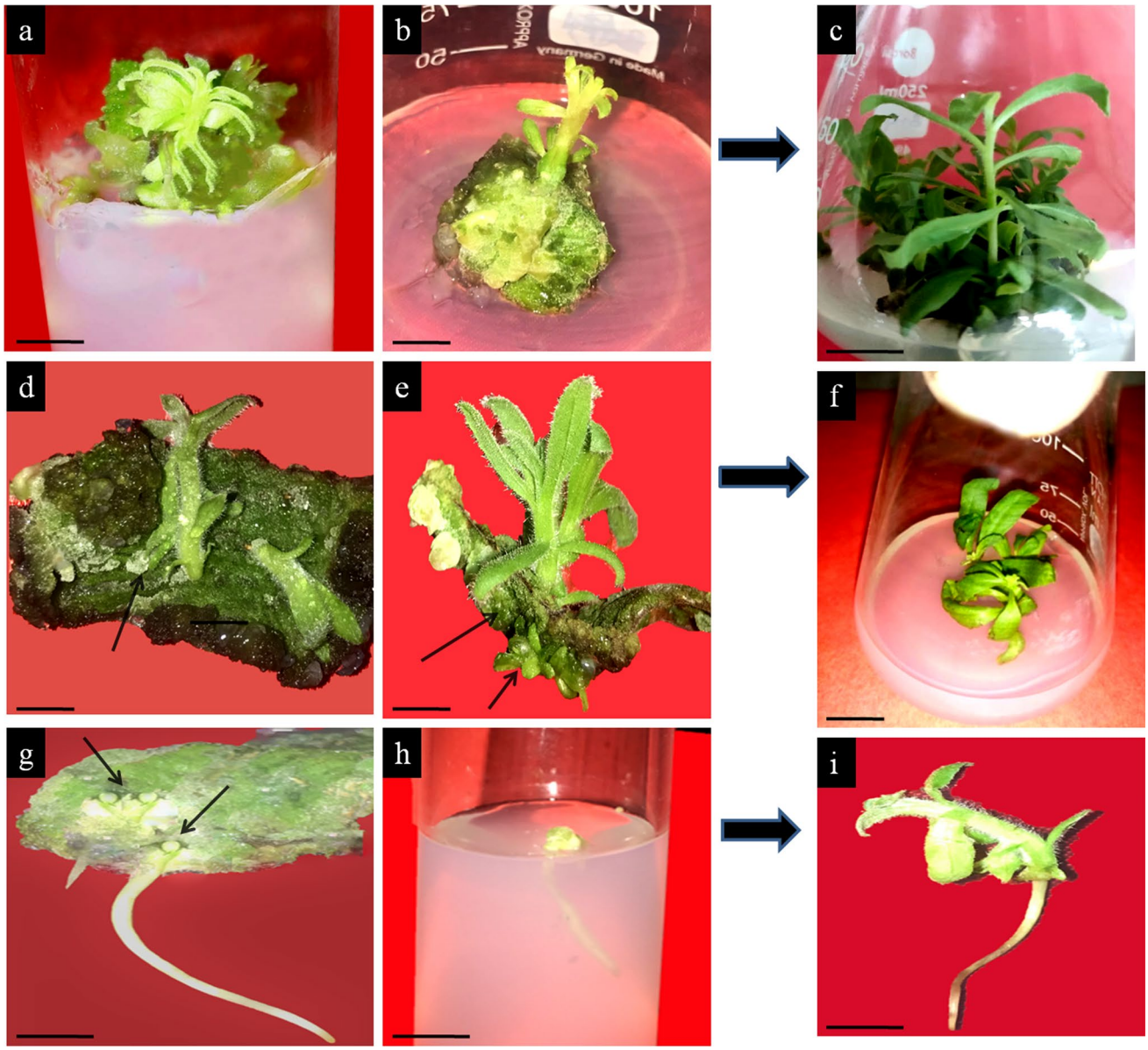

Fig. 2 In vitro regeneration of shootlets in *P. lanceolata* under the influence of various PGRs via somatic embryogenesis, caulogenesis, and direct organogenesis from leaf explants. (**a**) Emergence of shoot buds from callus masses when cultured on MS medium + 2.0 mg/l BAP + 0.5 mg/l of NAA. (**b**) Developing shoot from callus under the influence of 2.0 mg/l of BAP and 0.5 mg/l of NAA. (**d**, **e**) Direct organogenesis from leaf explant in MS medium supplemented with 0.5 mg/l BAP (arrow heads). (**g**) Initiation of embryogenic callus and somatic embryos (SE) from leaf explants on MS medium supplemented with 1.0 mg/l of NAA (arrow heads). (h) Somatic embryo placed in germinating medium enriched with 1 mg/l BAP + 0.5 GA_3 mg/l added MS medium. (**c**, **f**, **i**) Completely regenerated *P. lanceolata* plant from different methods (bars: **a**, **b**, **c**, **d** 1 cm; **e**, **f**, **g**, **i** 1.5 cm; **h**, 2 cm)

percentage was quite moderate, i.e., 39.7%, with production of 4.1 mean numbers of somatic embryos per culture. The least embryogenic potential (14.6%) was noticed in 0.1 mg/l NAA + 0.5 mg/l BAP with 1.2 mean numbers of embryos/culture (Fig. 4). Bipolar embryo-like structures appeared on callus were separated and transferred to GA_3 containing medium for obtaining plantlets (Fig. 2h). Node-derived callus was not responsive in evoking embryogenesis, hence data not shown. MS augmented with various GA_3 concentrations (0.25, 0.5, 1.0 mg/l) alone and in combination with BAP (0.5, 1.0, 2.0 mg/l) was tested for embryo germination. Among those treatments, 0.5 mg/l GA_3 showed good germination frequency of 59.89% with 10.67 mean numbers of shoots/flask (Supp. Figure 3). The addition of BAP (1.0 mg/l) to GA_3 (0.5 mg/l) immensely improved somatic embryo germination frequency (68.14%). The microshoots produced in 4–5 months' time were subjected to rooting before transplantation (Fig. 2i).

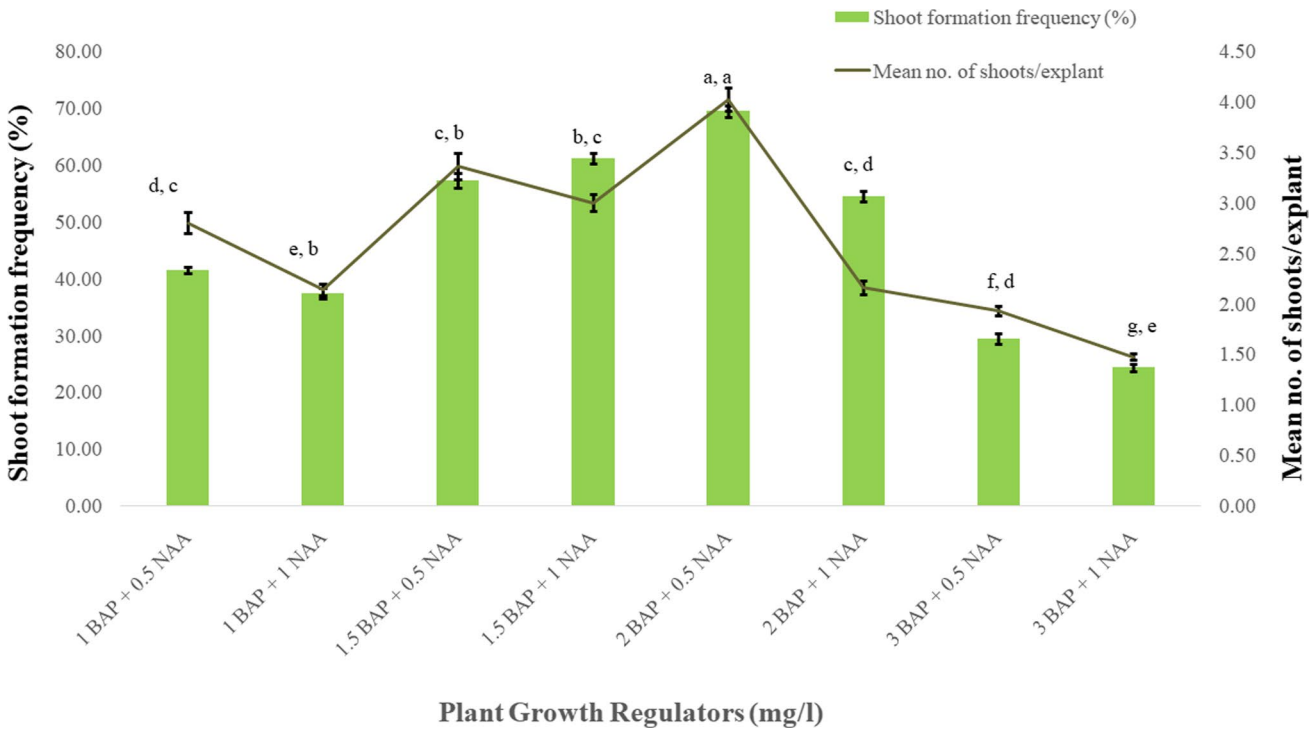

Fig. 3 Effect of plant growth regulators BAP and NAA on in vitro shoot regeneration from leaf-derived callus and mean numbers of shoots per explant. The data was scored after 4 weeks of culture. Mean values followed by different letters are significantly different at $p \leq 0.05$ according to DMRT

Histological and SEM study

Histological study was carried out to get a better understanding of the ontogeny, development, and morphology of embryo/organ through callus. This study helps in monitoring the cellular origin, development of embryos, and plantlet formation (Fig. 5a). Initially, the proembryo stage was observed as a result of division within cells and later cells exhibited rounded morphological structures having dense cytoplasm with abundant starch grain indicating the formation of somatic embryos. Somatic embryo in organogenic callus appeared to be unorganized, with no association to mother tissue leading to the formation of shoot and root meristems (Fig. 5b). The cross-section shows dome-like structure with flanking arms exhibiting shoot apical meristem with leaf primordia (Fig. 5c). SEM investigation also suggested the formation and development of somatic embryo further affirming other developmental characteristics on callus surface prior to proper expansion of shoot and root meristem in embryo. Surface morphology of different developmental stages of embryos, i.e., proembryo (incipient), globular, heart, and cotyledonary embryos, was noted through SEM preparations (Fig. 5d, e, f). These developing embryos following germination were cultured for rooting in obtaining complete plantlets.

Rooting and acclimatization

For successful root regeneration, the regenerated shoots obtained through direct, indirect, and embryogenic pathways were placed in half strength basal medium containing auxins at different concentrations. Microshoots on media containing auxins induced roots within 2 weeks and grew vigorously (Fig. 7a, b). Both IBA and NAA induced roots at variable numbers but the best rooting response was noted in 1.0 mg/l IBA with 83.95% root induction frequency and a maximum of 7.83 roots/shoot after 4 weeks of culture (Fig. 6). Well-developed rooted plantlets (twenty) were hardened inside the culture room in soilrite and soil (1:1) for 4 weeks, followed by transfer to pots containing soil, where the plants were grown under natural light with 87% survival rate (Fig. 7c, d).

Flow cytometry analysis

In vitro grown cultures particularly the callus is affected by stress, PGRs, and chemicals of medium, which trigger genetic instability in cultivated tissues. In order to check the genetic status, the 2C DNA content and the ploidy of in vitro and in vivo procured plants was measured. Very similar peaks for G0/G1 position in all experimental materials of in vitro and in vivo plant samples were noted. Flow cytometry study

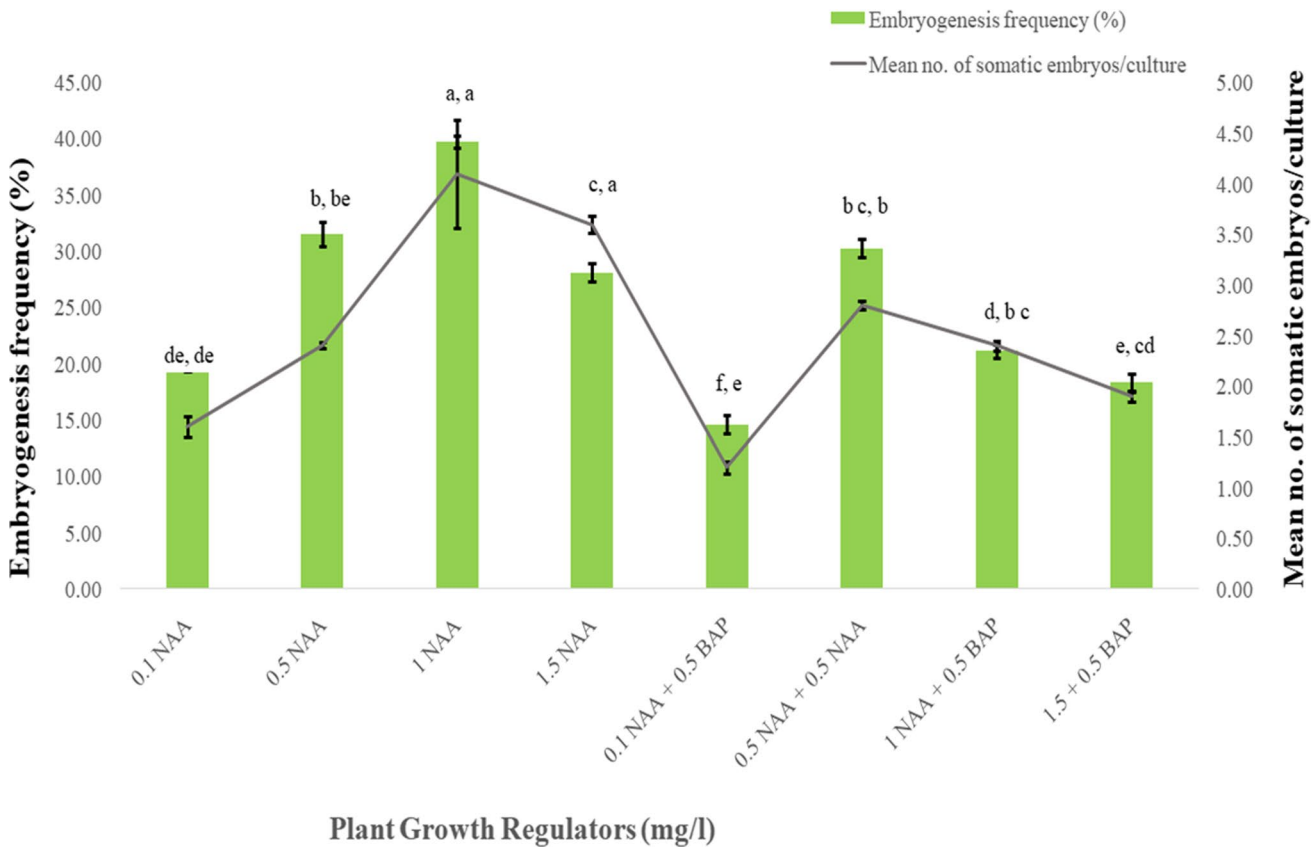

Fig. 4 Effect of different combinations and concentrations of PGRs like BAP and NAA on embryo formation and mean numbers of somatic embryos per culture. The data was scored after 4 weeks of culture. Mean values followed by different letters are significantly different at $p \leq 0.05$ according to DMRT

(Fig. 8a–e) shows that the 2C DNA contents of tissue culture raised, i.e., indirect, direct, and somatic embryo regenerated plants and donor/mother plant of *P. lanceolata*, are equal to 2.24, 2.25, 2.22, and 2.26 pg respectively. The 2C DNA content can also be expressed in Mbp and the in vivo and in vitro regenerated plants are found as 2200.50, 2192.60, 2195.61, and 2175.07 Mbp. Thus, the homogeneity in genome size of both the two groups of plants (in vitro and field grown) was affirmed. The 2C DNA index of mother plant, indirect, direct, and embryo-derived plants is as follows: 0.896, 0.893, 0.894, 0.886. The 1C (monoploid) genome sizes of all the above discussed samples are 1.13 pg, 1.12 pg, 1.12 pg, and 1.11 pg (Table 1). The genetic similarity is maintained in cultured tissues; the regenerants are therefore "true to type" having no real differences. The genetic morphology and ploidy level of in vitro raised plant are the same with the field-grown plant.

Physiological analysis

Photosynthetic pigment and antioxidant enzyme assay

Augmentation of various exogenous PGRs in culture medium triggers stress and overproduction of ROS (reactive oxygen species) which causes cell damage in plants. In order to avert these oxidative injuries, plants adapt themselves by altering various physiological processes including enhanced production of anti-oxidant enzyme activities. In present investigation, the samples from field/donor plant and in vitro regenerated plantlets were examined for photosynthetic pigments and antioxidant enzyme activity. There is significant increase in pigment profiles of in vitro obtained *P. lanceolata*, when compared to parent/field-grown plant. Among all the regenerants, plants derived via caulogenesis showed maximum

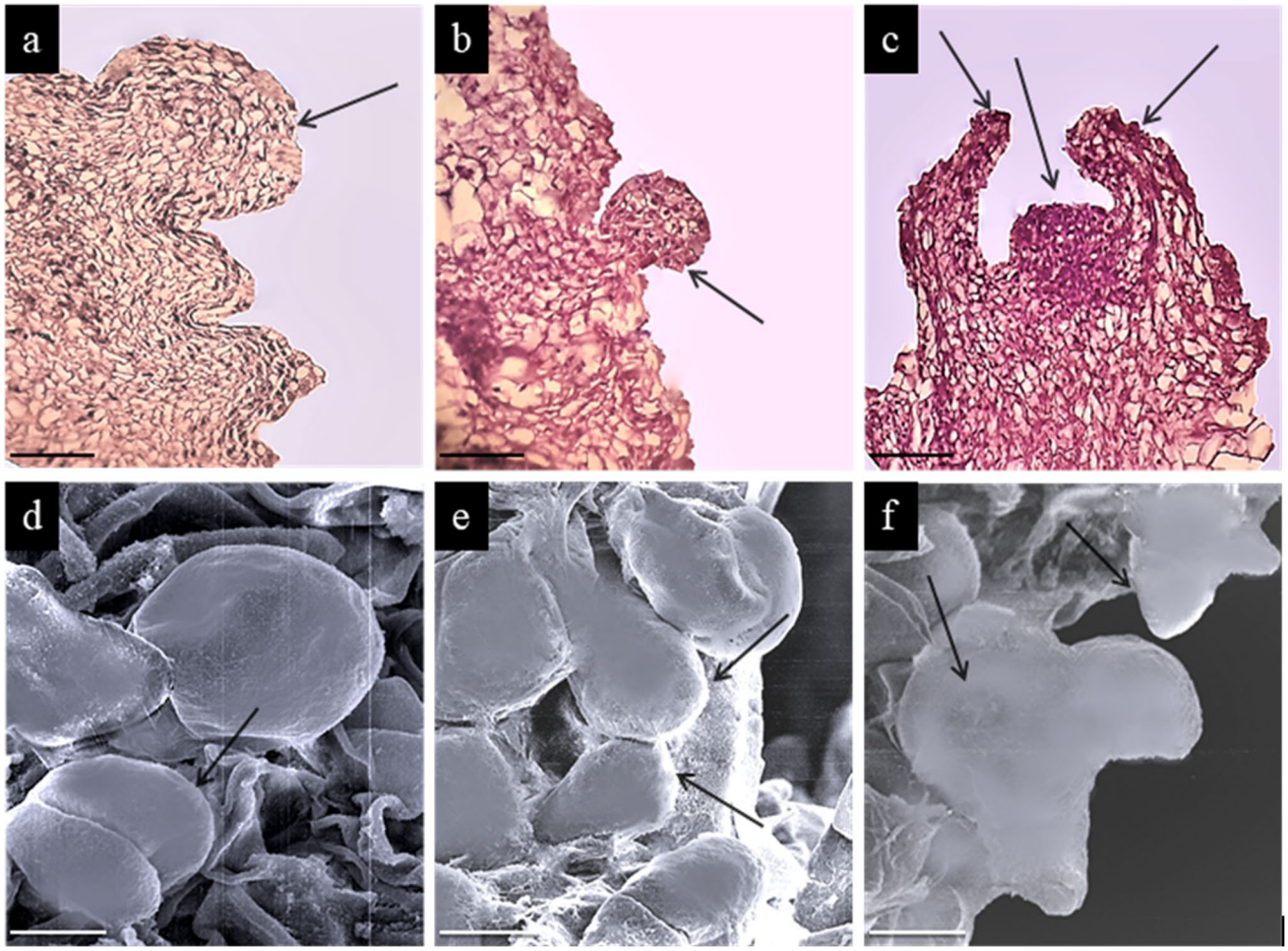

Fig. 5 Histology and SEM investigation confirming SE at different stages in *Pluchea lanceolata*. (**a**) Development of early embryonic mass on the surface of embryogenic callus (arrow head). (**b**) Globular embryo. (**c**) Callus surface with flanking arms showing shoot apical meristem and leaf primordia. (**d**) Proembryo with two incipient structures (arrow head). (**e**, **f**) Globular and heart-shaped stages of somatic embryos (bars: **a**, **b**, **c** 1 mm; **d**, **f** 100 µm; **e**, 10 µm)

pigment content, i.e., total Chl (0.96 ± 0.02 mg/g FW), Chl a (0.91 ± 0.03 mg/g FW), Chl b (0.51 ± 0.04 mg/g FW), and carotenoid (0.79 ± 0.01 mg/g FW) (Table 2). The levels of antioxidant enzymes were also monitored in different in vitro regenerated plants and were compared with donor (mother) plant. The SOD, CAT, and APX were 3.54 ± 0.01 EU/min/mg protein, 2.26 ± 0.01 EU/min/mg protein, and 1.31 ± 0.02 EU/min/mg protein in embryogenic derived regenerants and all were high compared to parent plant (Table 2). Thus, the plants derived from in vitro cultures showed enhanced antioxidant enzyme activities confirming adaptive cellular mechanism against adverse stress situations.

Chemical profiling

GC–MS and HPTLC analysis

Methanolic leaf extract of field and tissue culture regenerated plantlets of *P. lanceolata* was analyzed by GC–MS analysis. The chemical profiling of GC–MS chromatogram reveals the presence of more than 50 phyto-compounds; most of them belong to lignans, flavonoids, phytosteroids, terpenoids, saponins, glycosides, and tannins. It is evident from the GC–MS data that the *P. lanceolata* is an important source of medicines (Fig. 9a, b; Tables 3 and 4). Various

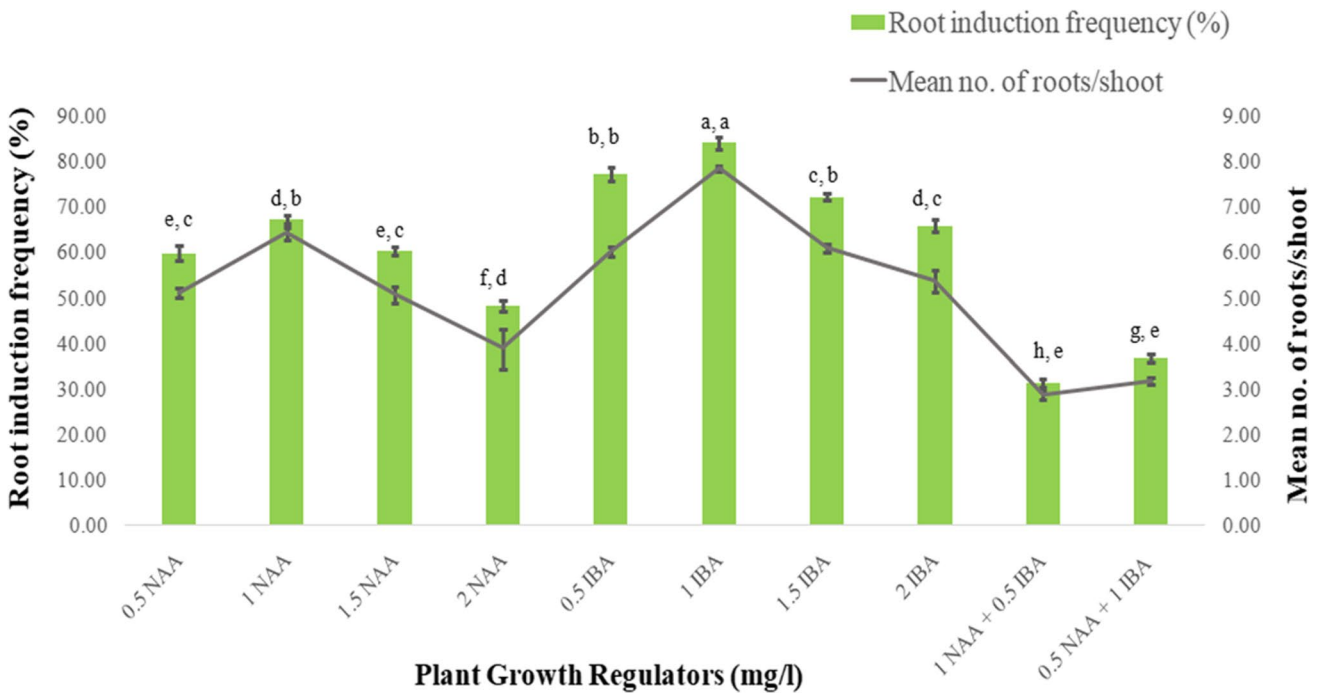

Fig. 6 Effect of PGRs augmented with different concentrations of IBA and NAA on root induction frequency and mean numbers of roots per shoots. The data was scored after 4 weeks of culture. Mean values followed by different letters are significantly different at $p \leq 0.05$ according to DMRT

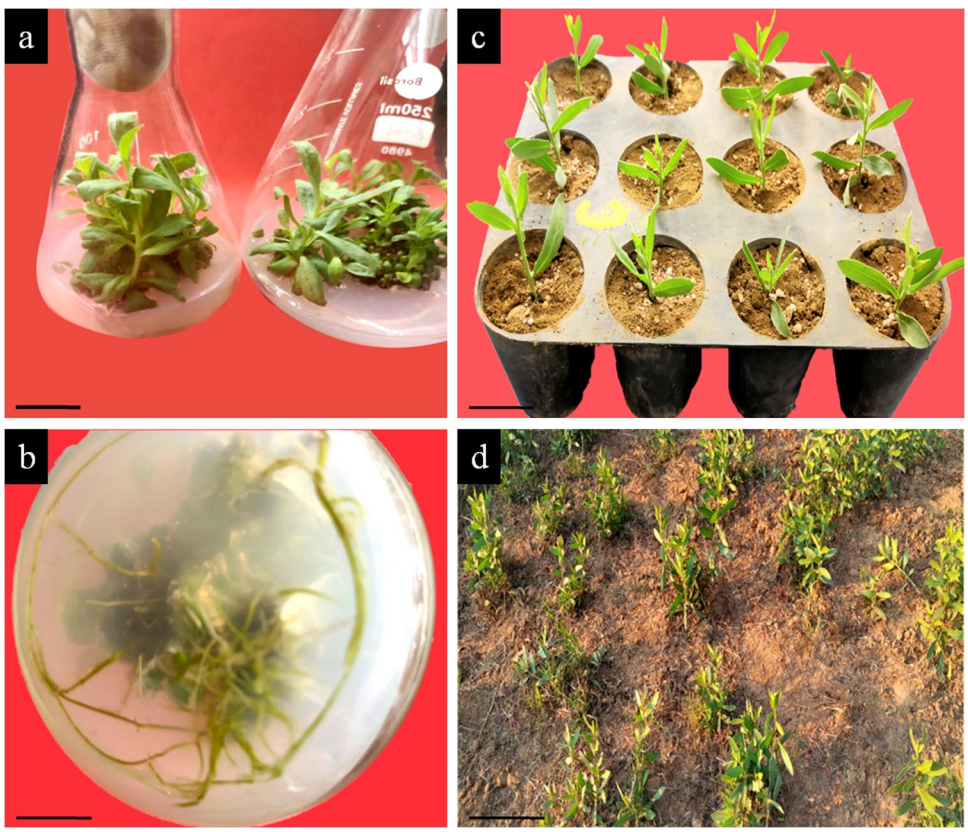

Fig. 7 Rooting and acclimatization of micropropagated *P. lanceolata* plant. (**a**) In vitro grown shoots placed on MS medium fortified with 1.0 mg/l IBA. (**b**) Completely rooted plant of *P. lanceolata*. (**c**, **d**) Acclimatization and field transfer of plant

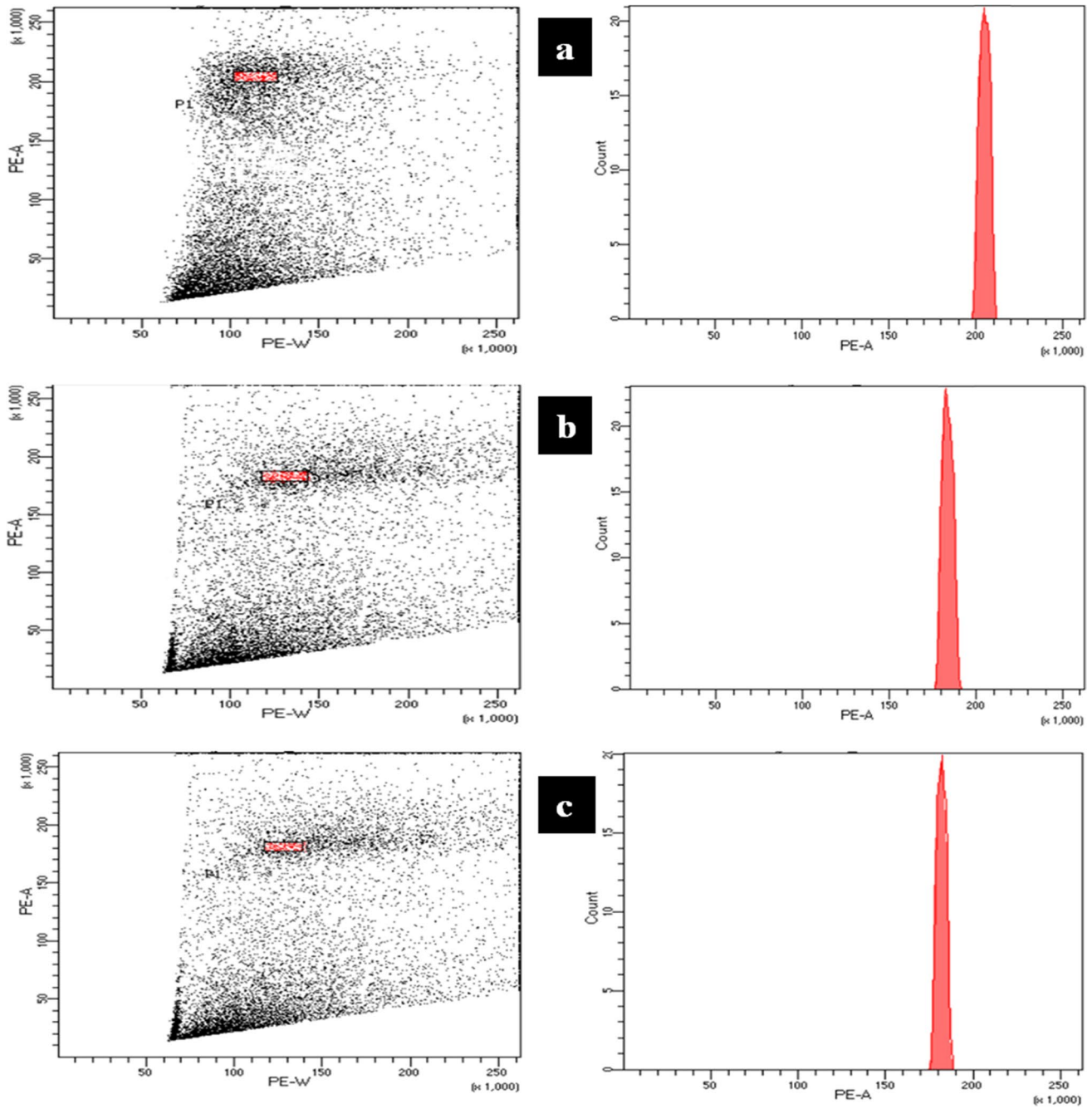

Fig. 8 Flow cytograms exhibiting genome size constancy in *Pluchea lanceolata*. Left panels demonstrates dot plot in which singlet G0/G1 population is gated and observed within the count versus PE-A histogram plot. (**a**) *Pongamia pinnata* (standard), (**b**) indirect, (**c**) in vivo, (**d**) direct induced, and (**e**) somatic embryo regenerated *P. lanceolata*

active compounds identified are 2,6,10-trimethyltetradecane; isopropyl myristate; neophytadiene; phytol acetate; n-hexadecanoic acid; squalene; vitamin E; stigmasta-5,22-dien-3-ol; alpha amyrin; 4,4,6a,6b,8a,11,11,14b-octamethyl-1,4,4a,5,6,6a,6b,7,8,8a; 24-norursa-3,12-diene; lupeol; γ-sitosterol; 9,19-cyclolanost-24-en-3-ol; and lup-20(29)-en-3-yl acetate and were synthesized in considerable quantities (> 1% peak area). Calusterone, eicosanoic acid, furan-2-carboxylic acid, celidoniol, γ-tocopherol, ergost-5-en-3-ol, phytyldecanoate, etc. were produced in low quantities (1% peak area).

In vitro conditions have been proved to be a significant way of enhancing the level of plant metabolites. The quantification of alpha amyrin and lupeol was made using HPTLC; the chromatograms of wild and regenerated tissues showed compact peaks corresponding to the Rf value of alpha amyrin

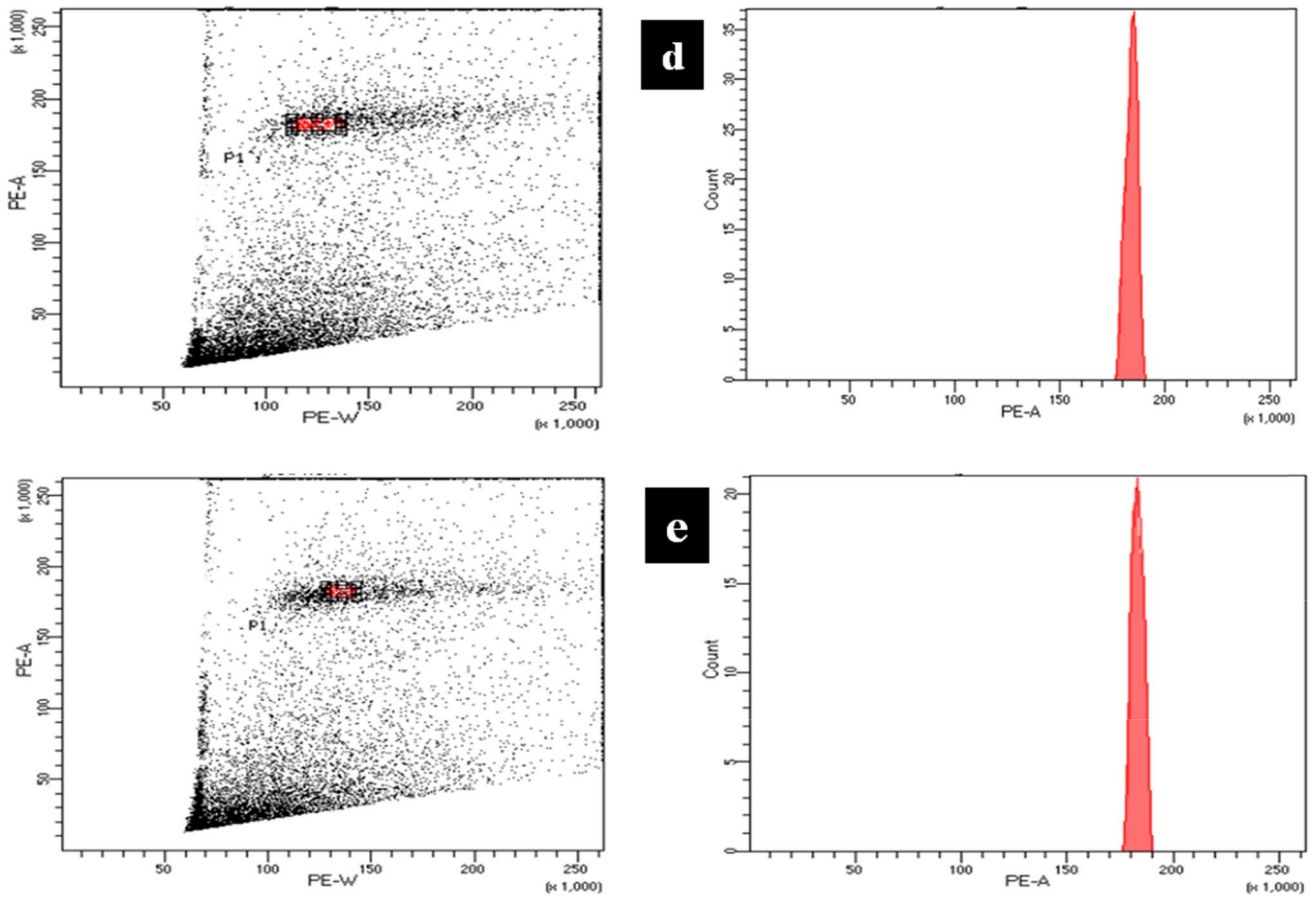

Fig. 8 (continued)

Table 1 Nuclear 2C DNA content of *Pluchea lanceolata* using in vivo and in vitro regenerated plant samples via callus (ISR), direct (DSR), and somatic embryo (SER) for determination of genetic homogeneity

Plant source	*Pongamia pinnata* (reference standard)			
	Nuclear DNA (in pg)		2C DNA genome size (Mbp)	2C DNA index*
	2C	1C		
Field-grown plant	2.26 ± 0.02a	1.13 ± 0.05a	2200.50a	0.896a
Callus regenerated(ISR)	2.24 ± 0.01a	1.12 ± 0.08a	2192.60a	0.893a
Direct regenerated (DSR)	2.25 ± 0.06a	1.12 ± 0.03a	2195.61a	0.894a
Embryo regenerated(SER)	2.22 ± 0.04a	1.11 ± 0.01a	2175.07a	0.886a

Data is represented as mean ± SD ($n = 3$). Mean values in a column followed by different letters are significantly different at $p = 0.05$ as per DMRT

*DNA index = 2C *Pluchea lanceolata* / 2C *Pongamia pinnata*

(0.62) and lupeol (0.38) at 525 nm with their respective standards (Fig. 10). In both the tested leaf tissues (mother and tissue cultured), the increased yield of terpenoids (alpha amyrin and lupeol) was noted in acclimatized leaf tissues (Fig. 11). Enhanced alpha amyrin (2.129 µg g^{-1} dry wt) and lupeol (1.232 µg g^{-1} dry wt) level was noted in in vitro grown tissues with respect to donor/mother tissues (Table 5).

Discussion

This study aims to investigate the possibility of in vitro propagation of *P. lanceolata* by using various PGRs as these signaling elements regulate key roles in growth and development of plants (Méndez-Hernández 2019). The callus was induced from the leaf and node using different

Table 2 Quantification of various photosynthetic pigments and antioxidant enzyme activities in in vitro regenerated plants and parent plant of *P. lanceolata*. *Chl* a (mg/g FW), *Chl* b (mg/g FW), total *Chl* (mg/g FW), total carotenoids (µg/g FW), SOD (EU/min/mg protein), CAT (EU/min/mg protein), and APX (EU/min/mg protein)

Pigment/enzyme	In vitro regenerated plant			Field-grown plant (parent plant)
	DSR plantlets	ISR plantlets	SER plantlets	
Chl a	0.69 ± 0.02b	0.91 ± 0.03d	0.82 ± 0.06c	0.62 ± 0.02a
Chl b	0.32 ± 0.01b	0.51 ± 0.04d	0.47 ± 0.02c	0.21 ± 0.01a
Total *Chl*	0.90 ± 0.07b	0.96 ± 0.02d	0.91 ± 0.05c	0.83 ± 0.03a
Total carotenoids	0.69 ± 0.03b	0.79 ± 0.01d	0.72 ± 0.03c	0.51 ± 0.06a
SOD	2.87 ± 0.02b	3.29 ± 0.08c	3.54 ± 0.01d	2.34 ± 0.08a
CAT	1.71 ± 0.04b	2.03 ± 0.03c	2.26 ± 0.01d	1.23 ± 0.05a
APX	0.93 ± 0.06b	1.12 ± 0.01c	1.31 ± 0.02d	0.46 ± 0.07a

Data is represented as mean ± SD ($n = 3$). Mean values in a row followed by different letters are significantly different at $p = 0.05$ as per DMRT

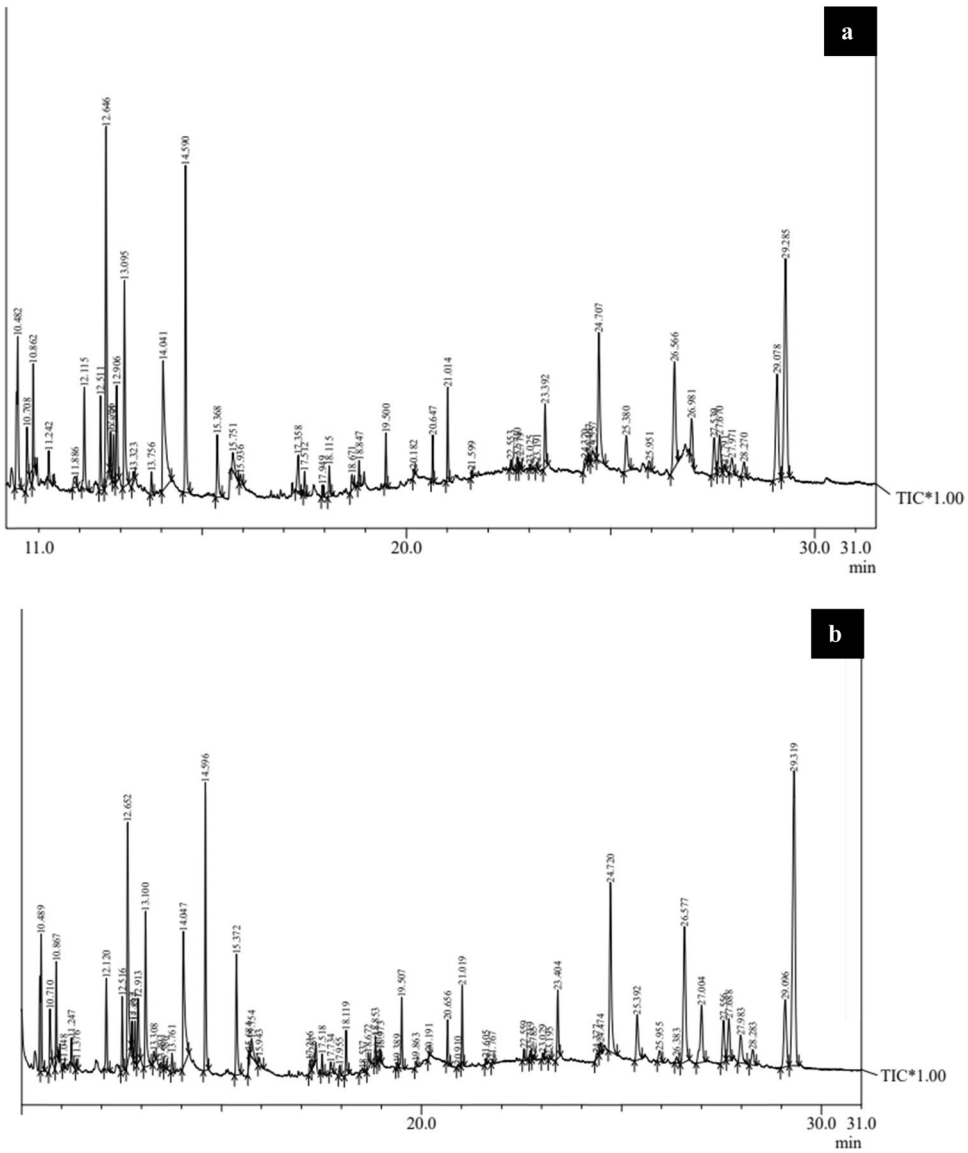

Fig. 9 GC–MS chromatograms of methanolic leaf extract of *P. lanceolata* (**a**) in vivo plant and (**b**) in vitro plant

PGRs and allowed to proliferate. The addition of BAP triggered the initiation and multiplication of cells although the callus growth rate was different in two explant sources, i.e., leaf and node. Here, in this present study, 1.0 mg/l BAP in MS singly and in combination with 2,4-D (0.5 mg/l) were tried but the yield of callus was not that satisfactory.

Table 3 Compound identified in GC–MS analysis of in vivo grown *P. lanceolata*

Peak	R/time	Area	Area %	Name
1	10.482	4,322,344	5.17	2,6,11-Tridecatrien-10-ol, 2,6,10-trimethyl-
2	10.708	1,326,358	1.59	Methyl (3-oxo-2-pentylcyclopentyl)acetate
3	10.862	1,814,357	2.17	1-(4-Isopropylphenyl)-2-methylpropyl acetate
4	11.242	548,344	0.66	3-Methyl-4-(2,6,6-trimethyl-2-cyclohexen-1-y
5	11.886	464,110	0.56	Octanal, 2-(phenylmethylene)-
6	12.115	2,199,881	2.63	Naphtho[2,1-b]furan, dodecahydro-3a,6,6,9a-tetramethyl-
7	12.511	1,758,717	2.10	Isopropyl myristate
8	12.646	7,586,915	9.08	Neophytadiene
9	12.756	632,462	0.76	Oxacyclotetradecan-2-one
10	12.830	933,540	1.12	7-Acetyl-6-ethyl-1,1,4,4-tetramethyltetralin
11	12.906	2,196,959	2.63	Phytol, acetate
12	13.095	4,513,617	5.40	Benzaldehyde 3,7,11,15-tetramethyl-2-hexadecen-1-ol
13	13.323	275,717	0.33	3-Hydroxy-4-benzyloxy-
14	13.756	478,180	0.57	Oxacycloheptadec-8-en-2-one, (8Z)-
15	14.041	5,839,898	6.99	N-Hexadecanoic acid
16	15.368	1,720,287	2.06	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-
17	15.751	696,257	0.83	Cyclopropaneoctanoic acid, 2-[[2-[(2-ethylcyclopropyl)met
18	15.936	292,465	0.35	9-Octadecenoic acid
19	17.358	1,133,441	1.36	1-Phenanthrenecarboxylic acid, 7-ethenyl
20	17.512	447,315	0.54	Methyl abieta-8,11,13-trien-18-oate
21	17.949	204,043	0.24	Methyl abietate
22	18.115	623,234	0.75	Decenyl tiglate, 2E-
23	18.671	443,568	0.53	1-Decanol, 2-octyl-
24	18.847	434,354	0.52	Furan-2-carboxylic acid n′-(2-hydroxy-2-pen
25	19.500	874,241	1.05	5.Alpha.-pregnane-3.beta.,20.beta.-diol, 3-acetate
26	20.182	119,403	0.14	Hexadecane
27	21.014	1,532,257	1.83	Squalene
28	21.599	118,356	0.14	Eicosane
29	22.710	230,805	0.28	Stigmasta-5,22-dien-3-ol, acetate
30	22.779	160,658	0.19	Γ-Tocopherol
31	23.392	1,468,074	1.76	Vitamin E
32	24.370	279,948	0.34	3-Benzofuranmethanol
33	24.467	141,201	0.17	Ergost-5-en-3-ol
34	24.707	4,303,556	5.15	Stigmasta-5,22-dien-3-ol
35	25.951	273,728	0.33	24-Noroleana-3,12-diene
36	26.566	4,152,391	4.98	Alpha.-amyrin
37	26.981	1,338,938	1.60	4,4,6a,6b,8a,11,11,14b-Octamethyl 1,4,4a,5,6,6a,6b,7,8,8a
38	27.539	1,326,106	1.59	9,19-Cyclolanost-24-en-3-ol
39	27.670	1,315,680	1.57	24-Norursa-3,12-diene
40	27.971	677,260	0.81	Lupeol
41	28.270	603,715	0.72	1,1′-Bicyclopentyl, 2-hexadecyl
42	29.285	9,582,638	11.47	Lup-20(29)-en-3-yl acetate

The use of 2,4-D in medium was, however, noted to have significant influence on callusing in many other studied plants (You et al. 2011; Ye et al. 2017).

Callus-mediated embryogenesis involves various stages, i.e., callus induction, acquisition of embryogenic potentiality, embryo formation and development, and plantlet formation (Gulzar et al. 2020). These steps are regulated by complete restructuring of cellular state, involving a complex network of physiological, biochemical, and molecular processes (Sharmin et al. 2014; Feher 2019, Mujib et al. 2022). This study on somatic embryogenesis indicated that the MS added with 1.0 mg/l NAA alone induced maximum number of embryos. The responses also indicated that the organogenesis and somatic embryogenesis were significantly

Table 4 Compound identified in GC–MS analysis of in vitro grown *P. lanceolata*

Peak	R/time	Area	Area %	Name
1	10.489	3,713,976	2.92	2,6-Dimethyl-8-(tetrahydro-2 h-pyran-2-ylox
2	10.710	1,949,693	1.53	Methyl (3-oxo-2-pentylcyclopentyl)acetat
3	10.867	2,506,420	1.97	1-(4-Isopropylphenyl)-2-methylpropyl acetate
4	11.048	159,485	0.13	Methyl (3-oxo-2-pentylcyclopentyl)acetate
5	11.247	644,906	0.51	1-(4-Isopropylphenyl)-2-methylpropyl aceta
6	11.376	277,628	0.22	(3ar,4R,7R)-1,4,9,9-Tetramethyl-3,4,5,6,7,8-hexahydro-2H
7	12.120	2,626,897	2.07	Naphtho[2,1-b]furan, dodecahydro-3a,6,6,9a-tetramethyl
8	12.516	1,925,820	1.52	Isopropyl myristate
9	12.652	8,382,359	6.60	Neophytadiene
10	12.762	690,987	0.54	Oxacyclotetradecane-2,11-dione, 13-methyl-
11	12.837	1,206,176	0.95	7-Acetyl-6-ethyl-1,1,4,4-tetramethyltetralin
12	12.913	2,491,087	1.96	Z-4-Nonadecen-1-ol acetate
13	13.100	5,505,403	4.33	3,7,11,15-Tetramethyl-2-hexadecen-1-ol
14	13.308	432,248	0.34	Benzaldehyde, 3-benzyloxy-2-fluoro-4-methoxy-
15	13.491	183,394	0.14	7-isopropyl-1,9a-dimethyl-4-methylene-2,3,3
16	13.586	119,815	0.09	Eicosanoic acid
17	13.761	611,510	0.48	Oxacycloheptadec-8-en-2-one
18	14.047	6,845,235	5.39	Hexadecanoic acid
19	15.372	4,462,367	3.51	2-Hexadecen-1-ol
20	15.684	346,284	0.27	9,12-Octadecadienoic acid
21	15.754	1,156,655	0.91	(Z)6,(Z)9-Pentadecadien-1-ol
22	17.216	395,150	0.31	Calusterone
23	17.287	56,999	0.04	1-Ethyl-2,3-dimethyl-4-piperidinone
24	17.734	499,207	0.39	9-Octadecenamide
25	17.955	270,347	0.21	Methyl abietate
26	18.672	620,746	0.49	Heneicosyl heptafluorobutyrate
27	18.853	685,593	0.54	Furan-2-carboxylic acid
28	18.973	265,101	0.21	1,2-Benzenedicarboxylic acid
29	19.507	2,068,848	1.63	5.Alpha.-pregnane-3.beta.,20.beta.-diol, 3-acetate
30	20.910	80,869	0.06	Ether, dodecyl isopropyl
31	21.019	2,046,467	1.61	Squalene
32	21.605	147,676	0.12	Celidoniol
33	21.767	104,052	0.08	26,27-Dinorcholesta-5,22-dien-3-ol
34	22.559	378,735	0.30	Stigmasta-4,7,22-trien-3.alpha.-ol
35	22.719	263,901	0.21	Stigmast-5-en-3-ol
36	22.785	135,451	0.11	Gamma.-tocopherol
37	23.404	2,411,229	1.90	Vitamin E
38	24.377	242,577	0.19	3-Benzofuranmethanol, 2,3-dihydro-2-(4-hydroxy-3-methox
39	24.474	470,322	0.37	Ergost-5-en-3-ol
40	24.720	8,240,957	6.49	Stigmasta-5,22-dien-3-ol
41	25.392	2,600,682	2.05	Gamma.-sitosterol
42	26.383	318,555	0.25	9,19-Cyclolanost-24-en-3-ol
43	26.577	8,550,980	6.93	Alpha.-amyrin
44	27.004	3,795,836	2.99	Olean-12-en-3-ol, acetate
45	27.556	2,281,812	1.80	9,19-Cyclolanost-24-en-3-ol
46	27.688	2,257,726	1.78	24-Norursa-3,12-diene
47	27.983	1,929,876	1.62	Lupeol
48	28.283	1,024,493	0.81	Phytyl decanoate
49	29.319	20,578,539	16.20	Lup-20(29)-en-3-yl acetate

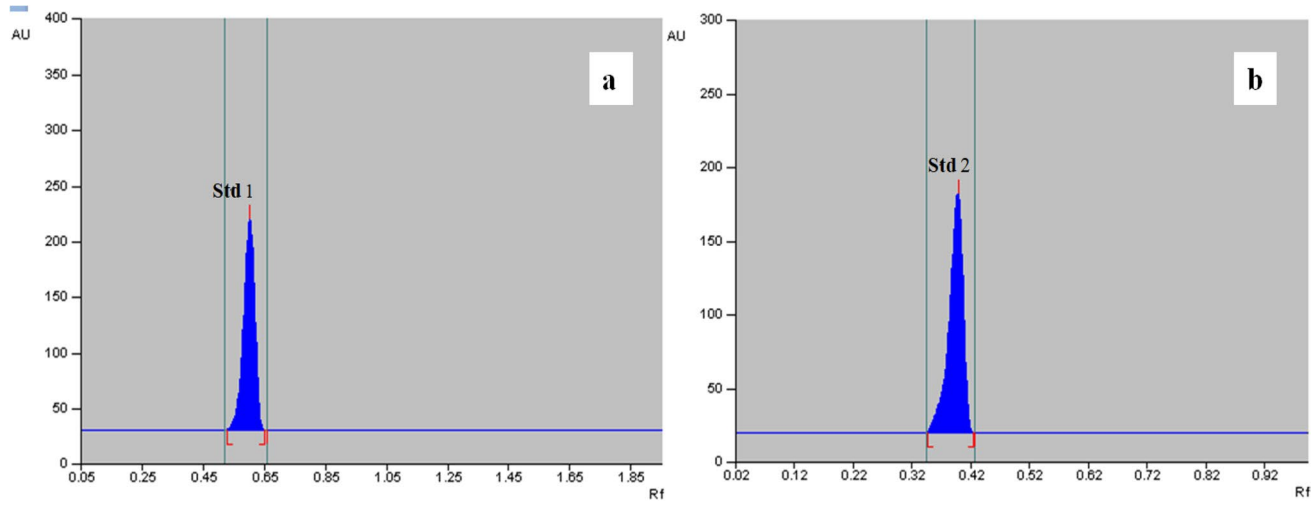

Fig. 10 HPTLC chromatogram of standard compounds. (**a**) Alpha amyrin. (**b**) Lupeol

influenced by the concentration variations of different used PGRs. Beside morphology, the histology and SEM evidences have been presented confirming the incidence of somatic embryogenesis in *Pluchea*. The same histology and SEM technique have been employed widely for understanding the mode of in vitro plantlet formation in many other plant species (Mujib et al. 2005, 2013; Couillerot et al. 2012; Malik et. al. 2020). Kher et al. (2019) recently reported that the MS added with 2,4-D + thidiazuron induced direct shoot regeneration in *P. lanceolata* from leaf and shoot explants. Here, the number of shoots per explant increased with decreased NAA level. The effectiveness of NAA with BAP in inducing shoots was reported in many plant species like *Ledebouria ovatifolia* (Baskaran et al. 2016), *Solanum viarum* (Mahadev et al. 2014), *Solanum nigrum* (Sridhar and Naidu 2011), and *Stevia rebaudiana* (Preethi et al. 2011). In this study, the leaves were noted to be more responsive in inducing shoot and embryo compared to the nodal segments. The leaf was earlier noted to be an effective explant source for shoot formation in other investigated plants like *Hygrophila polysperma* (Karatas et al. 2014) and *Scutellaria bornmuelleri* (Gharari et al. 2019). The varied physiological gradients of endogenous PGR level and the photosynthetic content present in different explants might be responsible for diverse morphogenetic responses; the precise reason of differential response of explants is still not known (Mujib et al. 2017; Ramabulana et al. 2021).

The shoots induced from different pathways need rooting before transplantation. In this study, various PGRs were used in half strength MS medium for roots. Almost all the treatments induced roots at variable numbers but IBA was more efficient in producing good quality healthy roots. Different natural and synthetic auxins have earlier been used in inducing roots and were reported in a variety of plant genera (Aslam 2013; Mujib et al. 2017). Kher et al. (2016) observed a good numbers of roots in *Clerodendrum phlomidis* in 1.5 mg/l NAA added half MS medium.

The medium added with various PGRs especially 2,4-D creates stress and produces genetic instability in older cultures (Chakraborty et al. 2013). These genetic changes are regarded as "unwanted" and need to be detected early in order to avert the loss of genetic stability of somaclones for marketable production (Yang et al. 2011). There are many conventional and modern techniques to recognize these genetic variations (Das et al. 2013; Soares et al. 2016; Vitamvas et al. 2019). Here, the ploidy and the 2C DNA of *P. lanceolata* were checked by flow cytometry. The genome size of in vitro grown plants is the same with that of field-grown donor plant, thus confirming genetic homogeneity in *P. lanceolata*. Similar to our findings, the flow cytometry technique has earlier been used in assessing 2C DNA content of several medicinal and horticultural plants like *Allium*, *Carthamus*, *Zephyranthes*, *Caladium*, and *Tylophora* (Malik et al. 2020a, b; Ejaz et al. 2021; Syeed et al. 2021a, 2021b; Mamgain et al. 2022). Besides, the low cost and high accuracy make the flow cytometry a potent technique to detect variation in ploidy of different studied plants (Ranghoo-Sanmukhiya 2021).

In this study, the alterations in photosynthetic pigments and antioxidant enzyme activity were measured in field and in vitro regenerated *P. lanceolata*. During stress, the photosynthetic pigments get accumulated and the enhanced levels help adapting various stress conditions (Resende et al. 2016). Bose et al. (2016) revealed an elevated level of photosynthetic pigments in in vitro produced *Nardostachys jatamansi* as compared to parent plant. Here, the antioxidant enzyme superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) all showed linear increase in

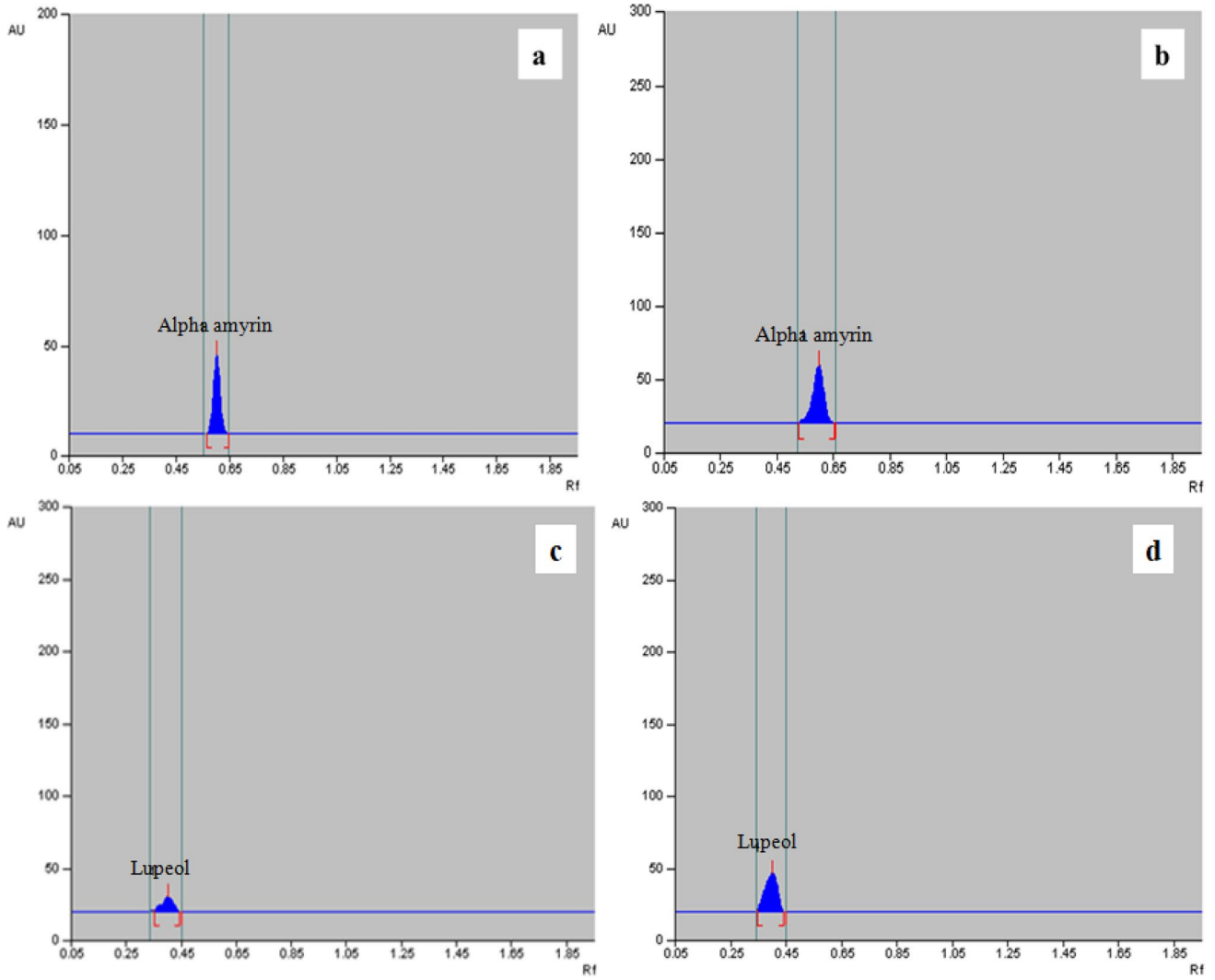

Fig. 11 HPTLC densitograms showing comparative peaks of important terpenoids of *P. lanceolata.* (**a**, **c**) Alpha amyrin and lupeol content in leaves of mother plant. (**b**, **d**) Alpha amyrin and lupeol content of in vitro leaf samples

Table 5 Alpha amyrin and lupeol content (μg g^{-1} dry wt) of in vitro and in field procured samples of *P. lanceolata*

Leaf sample	Solvent system (9.5:0.5)	Alpha amyrin content	Lupeol content
Mother plant	Toluene:ethyl acetate	1.481 ± 0.02a	0.636 ± 0.03a
Acclimatized plant	Toluene:ethyl acetate	2.129 ± 0.03b	1.232 ± 0.02b

Data is represented as mean SD ($n = 3$). Mean values in a column followed by different letters are significantly different at $p = 0.05$ as per DMRT

activity in regenerated plants, maximum being in somatic embryo regenerated shoots. SOD, being potent scavenger of O^{2-}, catalyzes and converts the superoxide radical into hydrogen peroxide (H_2O_2). H_2O_2 is further scavenged by CAT and other classes of peroxidases (Ozyigit et al. 2016). Ascorbate peroxidase also seems to be the enzyme removing excess H_2O_2 (Sofo et al. 2015). In line with our observation, the increased antioxidant enzyme activity was noted in in vitro propagated *Cardiospermum halicacabum* (Jahan et al. 2014). Sudipta et al. (2014) reported higher levels of antioxidants in micropropagated *Leptadenia reticulata* compared to conventionally propagated ones. Beside physiological attributes, chemical profiling was carried out to identify phytoactive compounds through GC–MS. The investigation reveals the presence of different compounds with medicinal potential. The GC–MS study showed little increase of

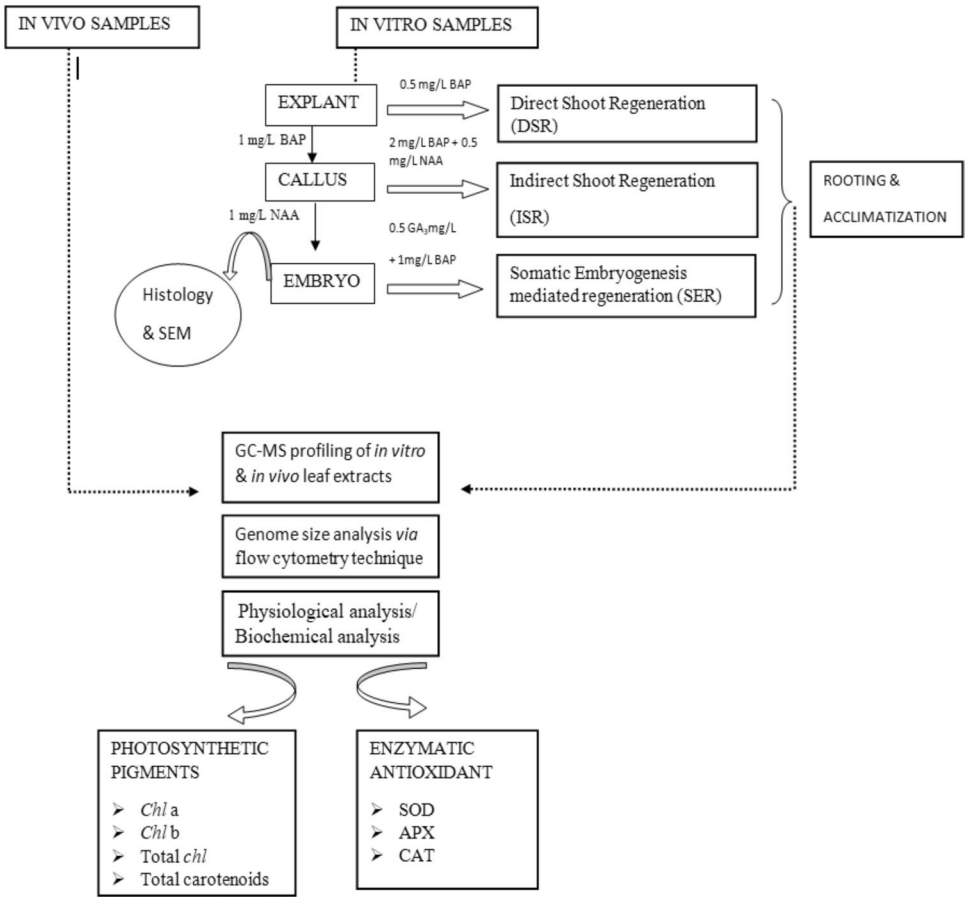

Fig. 12 Summary of suggested protocol depicting flow cytometric and physiological analysis of 3-way regenerated complete plantlets via DSR, ISR, and SER of *P. lanceolata*

phytocompounds in regenerated plantlets (Fig. 12). Similar to our findings in *Cleome rutidosperma*, a noticeable lesser amount of metabolites was observed in wild plants when compared to in vitro propagated plants through GC–MS (Deventhiran et al. 2017). The quantification of triterpenoids was made through HPTLC, which showed in vitro regenerated leaf tissues synthesized enriched level of alpha amyrin (2.129 µg g^{-1} dry wt) and lupeol (1.232 µg g^{-1} dry wt) as compared to field-grown plants. There are reports of GC–MS study on phytocompounds in field-grown plants but the information is very limited in regenerated plant parts (Khodadadi et al. 2013; Ali et al. 2019).

Conclusion

In this study, *P. lanceolata* propagation via ISR, DSR, and SER was standardized. The callus histology and SEM preparation were performed to show the ontogeny and development of regenerated plantlets. The genome size of in vitro produced plants was evaluated by determining the nuclear 2C DNA content and compared with donor/mother *Pluchea* plant. The results of this study showed no alteration of ploidy, which affirms genetic homogeneity in regenerated somaclones of *P. lanceolata*. The enhanced level of photosynthetic and antioxidant enzyme activity represents cellular defense to plants in order to tolerate unfavorable or stressful conditions. GC–MS and HPTLC analysis showed the presence of several compounds of medicinal worth. The regenerated plantlets with roots thrived well on transfer to field conditions. To generate bulk amounts of similar plant materials for isolation of bioactive molecules in pharmaceutical applications, this optimized protocol may be beneficial.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13353-022-00727-7.

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Author contribution Conceptualization, A.M. and J.M; methodology, J.M; data curation, J.M. and A.M; writing. J.M.; editing, A.M; supervision, A.M.; the authors have read and agreed to submit this version of manuscript.

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Data availability The authors declare that the data of this study are available within this manuscript.

Declarations

Competing interests The authors declare no competing interests.

References

Aebi H (1984) Catalase in vitro. Methods Enzymol 105:121–126. https://doi.org/10.1016/S0076-6879(84)05016-3

Ali M, Mujib A, Gulzar B, Zafar N (2019) Essential oil yield estimation by gas chromatography-mass spectrometry (GCMS) after Methyl jasmonate (MeJA) elicitation in in vitro cultivated tissues of Coriandrum sativum L. 3Biotech 9: 41. https://doi.org/10.1007/s13205-019-1936-9

Al-Rubaye AF, Hameed IH, Kadhim MJ (2017) A review: uses of gas chromatography-mass spectrometry (GC-MS) technique for analysis of bioactive natural compounds of some plants. Int J Toxicol Pharmacol Res 9: 81–5 https://doi.org/10.25258/ijtpr.v9i01.9042

Arnon DI (1949) Copper enzymes in isolated chloroplasts: polyphenol oxidase in Beta vulagaris. Plant Physiol 24:1–15. https://doi.org/10.1104/pp.24.1.1

Arya D, Patni V (2013) Micropropagation of Pluchea lanceolata (Oliver & Hiern.) - a potent anti-arthritic medicinal herb. Int J Med Arom Plants. 3:55–60. https://doi.org/10.30495/tpr.2021.680483

Aslam J, Mujib A, Sharma MP (2013) *In vitro* micropropagation of *Dracaena sanderiana* Sander ex Mast: an important indoor ornamental plant. Saudi J Biol Sci 20:63–68. https://doi.org/10.1016/j.sjbs.2012.11.005

Bączek K, Pawełczak A, Przybył JL, Kosakowska O, Węglarz Z (2021) Secondary metabolites of various Eleuthero (*Eleutherococcus senticosus* Rupr. et Maxim./Maxim) organs derived from plants obtained by somatic embryogenesis. Plant Cell and Tissue Differentiation and Secondary Metabolites: Fundamentals and Applications. 433–466

Baskaran P, Kumari A, Naidoo D, Van Staden J (2016) In vitro propagation and ultrastructural studies of somatic embryogenesis of Ledebouria ovatifolia. In Vitro Cell Dev Biol -Plant 52:283–292. https://doi.org/10.1016/j.sjbs.2012.11.005

Bose B, Kumaria SCH, Tandon P (2016) Assessment of genetic homogeneity and analysis of phytomedicinal potential in micropropagated plants of Nardostachys jatamansi, a critically endangered, medicinal plant of alpine Himalayas. Plant Cell Tissue Organ Cult 124:331–349. https://doi.org/10.1007/s11240-015-0897

Chakraborty N, Banerjee D, Ghosh M, Pradhan P, Gupta NS, Acharya K, Banerjee M (2013) Influence of plant growth regulators on callus mediated regeneration and secondary metabolites synthesis in Withania somnifera (L.) Dunal. Physiol Mol Biol Plants 19:117–125. https://doi.org/10.1007/s12298-012-0146-2

Choudhury RR, Basak S, Ramesh AM, Rangan L (2014) Nuclear DNA content of Pongamia pinnata L. and genome size stability of in vitro regenerated plantlets. Protoplasma 251:703–709

Couillerot JP, Windels D, Vazquez F, Michalski JC, Hilbert JL, Blervacq AS (2012) Pretreatments, conditioned medium and co-culture increase the incidence of somatic embryogenesis of different *Cichorium* species. Plant Signal Behav 7:1–11

Das A, Kesari V, Rangan L (2013) Micropropagation and cytogenetic assessment of Zingiber species of Northeast India. Biotechnol 3:471–479

Derosa G, Maffioli P, D'Angelo A, Di Pierro F. (2020) A role for quercetin in coronavirus disease 2019 (COVID-19). Phytotherapy Research 1–7

Deventhiran M, John Wyson W, Sheik Noor M, Jaikumar K, Saravanan P, Anand D (2017) Comparative phytochemical analysis of wild and micropropagated Cleome viscosa L. J Appl Pharm Sci 7:083–088

Dharmaraja AT (2017) Role of reactive oxygen species (ROS) in therapeutics and drug resistance in cancer and bacteria. J Med Chem 60:3221–3240

Dhindsa RS, Plumb-Dhindsa P, Thorpe TA (1981) Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxidation and decreased levels of superoxide dismutase and catalase. J Exp Bot 32:93–110

Dhooghe E, Van Laere K, Eeckhaut T, Leus L, Van Huylenbroeck J (2011) Mitotic chromosome doubling of plant tissues *in vitro*. Plant Cell Tissue Organ Cult 104:359–373

Dolezel J (2003) Nuclear DNA content and genome size of trout and human. Cytom Part a 5:127–128

Dolezel J, Bartos J (2005) Plant DNA flow cytometry and estimation of nuclear genome size. Ann Bot 95:99–110

Ejaz B, Mujib A, Mamgain J, Malik MQ, Syeed R, Gulzar B, Bansal Y(2021) Comprehensive in vitro regeneration study with SCoT marker assisted clonal stability assessment and flow cytometric genome size analysis of Carthamus tinctorius L.: an important medicinal plant. Plant Cell Tissue Organ Cult https://doi.org/10.1007/s11240-021-02197

Espinosa-Leal CA, Puente-Garza CA, García-Lara S (2018) In vitro plant tissue culture: means for production of biological active compounds. Planta 248:1–18

Fehér A (2015) Somatic embryogenesis: stress-induced remodeling of plant cell fate. Biochim Biophys Acta 1849:385–402

Fehér A (2019) Callus, dedifferentiation, totipotency, somatic embryogenesis: what these terms mean in the era of molecular plant biology? Front Plant Sci 10:536. https://doi.org/10.3389/fpls.2019.00536

Gharari Z, Bagheri K, Sharafi A, Danafar H (2019) Thidiazuron induced efficient in vitro organogenesis and regeneration of Scutellaria bornmuelleri: an important medicinal plant. In Vitro Cell Dev Biol -Plant 55:133–138

Greilhuber J, Doležel J, Lysak MA, Bennett MD (2005) The origin, evolution and proposed stabilization of the terms 'genome size' and 'c-value' to describe nuclear DNA contents. Ann Bot 95:255–260

Gulzar B, Mujib A, Malik MQ, Syeed R, Mamgain J, Ejaz B (2020) Genes, proteins and other networks regulating somatic embryogenesis in plants. J Genet Eng Biotechnol 18:1–5

Jahan MS, Nozulaidi MBN, Moneruzzaman MK, Ainun A, Husna N (2014) Control of plant growth and water loss by a lack of light-harvesting complexes in photosystem-II in Arabidopsis thaliana ch1-1 mutant. Acta Physiol Plant 36:1627–1635

Johansen DA (1940) Botanical microtechnique. McCraw-Hill Book, New York

Kapoor D, Singh S, Kumar V, Romero R, Prasad R, Singh J (2019) Antioxidant enzymes regulation in plants in reference to reactive oxygen species (ROS) and reactive nitrogen species (RNS). Plant Gene 19:100182

Karataş M, Aasim M, Çınar A, Dogan M (2014) Adventitious shoot regeneration from leaf explant of dwarf hygro (Hygrophila polysperma (Roxb.) T. Anderson). The Scientific World J 23(9):2190–2194

Kher MM, Soner D, Srivastava N, Nataraj M, Teixeira da Silva JA (2016) Micropropagation of Clerodendrum phlomidis LF. J Hort Res 24(1):21–28

Kher MM, Nataraj M (2019) Direct somatic embryogenesis and shoot regeneration from leaves and internodes of Pluchea lanceolata (DC.) CB Clarke. In Vitro Cell Dev Biol -Plant 55:720–724

Khodadadi E, Aharizad S, Mohammadi SA, Khodadadi E, Kosarinasab M, Sabzi M (2013) Chemical composition of essential oil compounds from the callus of fennel Foeniculum vulgare Miller. Int J Agron Agricul Res 3(11):1–6

Liu W, Liang Z, Shan C, Marsolais F, Tian L (2013) Genetic transformation and full recovery of alfalfa plants via secondary somatic embryogenesis. In Vitro Cell Dev Biol - Plant 49:17–23

Lü J, Chen R, Zhang M, da Silva JA, Ma G (2013) Plant regeneration via somatic embryogenesis and shoot organogenesis from immature cotyledons of Camellia nitidissima. Chi J Plant Physiol 170(13):1202–1211

Mahadev MD, Panathula CS, Naidu CV (2014) Efficient protocol for in vitro callus induction and indirect plant regeneration of Solanum viarum (Dunal)-an important anticancer medicinal plant. Inter J Med Aromatic Plants 4:117–123

Malik MQ, Mujib A, Gulzar B, Zafar N, Syeed R, Mamgain J, Ejaz B (2020) Genome size analysis of field grown and somatic embryo regenerated plants in Allium sativum L. J Appl Genet 61:25–35

Malik MQ, Mujib A, Gulzar B, Zafar N, Syeed R, Mamgain J, Ejaz B, Kanchan (2020) Enrichment of alliin in different in vitro grown tissues of Allium sativum through $CdCl_2$ elicitation as revealed by high performance thin layer chromatography (HPTLC). Ind Crops Products 158:113007

Mamgain J, Mujib A, Ejaz B, Gulzar B, Malik MQ, Syeed R (2022) Flow cytometry and start codon targeted (SCoT) genetic fidelity assessment of regenerated plantlets in Tylophora indica (Burm. f.) Merrill. Plant Cell, Tissue Org Cult 150:129–140

Martelanc M, Vovk I, Simonovska B (2009) Separation and identification of some common isomeric plant triterpenoids by thin-layer chromatography and high-performance liquid chromatography. J Chromatography 1216:6662–6670

Méndez-Hernández HA, Ledezma-Rodríguez M, Avilez-Montalvo RN, Juárez-Gómez YL, Skeete A, Avilez-Montalvo J, De-la-Peña C, Loyola-Vargas VM (2019) Signaling overview of plant somatic embryogenesis. Front Plant Sci 10:77. https://doi.org/10.3389/fpls.2019.00077

Mohammed GJ, Al-Jassani MJ, Hameed IH (2016) Antibacterial, antifungal activity and chemical analysis of Punica grantanum (Pomegranate peel) using GCMS and FTIR spectroscopy. Int J Pharmacog Phytochem Res 8:480–494

Mujib A, Banerjee S, Maqsood M, Ghosh PD (2013) Somatic embryogenesis of some member ornamental genera of Amaryllidaceae and allied families: the similarities and differences. The Open Hort J 6:9–18

Mujib A, Tanu AM, Dipti T, Zafar N, Gulzar B (2017) In vitro propagation of Althaea officinalis: the role of plant growth regulators in morphogenesis. BioTechnol 98:167–173

Mujib A, Bansal Y, Malik MQ, Syeed R, Mamgain J, Ejaz B (2022) Internal and external regulatory elements controlling somatic embryogenesis in Catharanthus: A model medicinal plant. In: Ramírez-Mosqueda MA (eds) Somatic Embryogenesis. Methods in Molecular Biology, Humana, New York, NY, vol 2527, pp 11–27. https://doi.org/10.1007/978-1-0716-2485-2_2

Mujib A, Banerjee S, Ghosh PD (2005) Origin, development, and structure of somatic embryos in selected bulbous ornamentals: BAP as inducer. In: Mujib A, Samaj J (eds) Somatic embryogenesis. Springer, Berlin, pp 15–24

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol Plant 15:473–497

Nakano Y, Asada K (1981) Hydrogen peroxide is scavenged by ascorbate specific peroxidase in spinach chloroplasts. Plant Cell Physiol 22:867–888

Ozyigit II, Filiz E, Vatansever R, Kurtoglu KY, Koc I, Öztürk MX, Anjum NA (2016) Identification and comparative analysis of H_2O_2 scavenging enzymes (ascorbate peroxidase and glutathione peroxidase) in selected plants employing bioinformatics approaches. Front Plant Sci 7:301

Pandey S (2018) In vitro drug release and study of anti-inflammatory effect of RasnaSaptakKwath. Asian J Pharm 11:04

Preethi D, Sridhar TM, Naidu CV (2011) Carbohydrate concentration influences on in vitro plant regeneration in Stevia rebaudiana. J Phytol 3:61–64

Ramabulana AT, Steenkamp PA, Madala NE, Dubery IA (2021) Application of plant growth regulators modulates the profile of chlorogenic acids in cultured *Bidenspilosa* cells. Plants 10:437. https://doi.org/10.3390/plants10030437

Ranghoo-Sanmukhiya VM (2021) Somaclonal variation and methods used for its detection. Vitro Propagation and genetic manipulation of plants. Springer, Singapore, pp 1–18

Resende CF, Braga VF, Pereira PF, Silva CJ, Vale VF, Bianchetti RE, Forzza RC, Ribeiro C, Peixoto PHP (2016) Proline levels, oxidative metabolism and photosynthetic pigments during in vitro growth and acclimatization of Pitcairniaen cholirioides LB Sm. (Bromeliaceae). Braz J Biol 76:218–227

Sharmin SA, Alam MJ, Sheikh MMI, Sarker KK, Khalekuzzaman M, Haque MA, Alam MF, Alam F (2014) Somatic embryogenesis and plant regeneration in Wedelia calendulacea Less. An endangered medicinal plant. Braz Arch Biol Technol 57:394–401

Soares DMM, Sattler MC, da Ferreira MF, Praca S, fontes MM (2016) Assessment of genetic stability in three generations of *in vitro* propagated *Jatropha curcas* L. plantlets using ISSR markers. Tropical Plant Biol 9:229–238. https://doi.org/10.1007/s12042-016-9171-6

Sofo A, Scopa A, Nuzzaci M, Vitti A (2015) Ascorbate peroxidase and catalase activities and their genetic regulation in plants subjected to drought and salinity stresses. Int J Mol Sci 16:13561–13578

Sommerville KD, Cuneo P, Errington G, Makinson RO, Pederson S, Phillips G, Rollason A, Viler V, Offord CA (2020) Conservation in the wake of myrtle rust–a case study on two critically endangered Australian rainforest plants. Pacific Conservation Biol 26:218–229

Sridhar TM, Naidu CV (2011) An efficient callus induction and plant regeneration of Solanum nigrum (L.)-An important antiulcer medicinal plant. J Phytol 3:23–28

Srivastava P, Shanker K (2012) Pluchea lanceolata (Rasana). Chemical and biological potential of Rasayana herb used in traditional system of medicine. Fitoterapia 83:1371–1385

Sudipta KM, Kumara Swamy M, Balasubramanya S, Anuradha M (2014) Assessment of genetic fidelity, antioxidant enzyme activity and proline content of micropropagated and field grown plants of Leptadenia reticulata (Wight & Arn.) an endangered medicinal plant. Plant Cell Biotechnol Mol Biol 15:127–135

Syeed R, Mujib A, Malik MQ, Mamgain J, Ejaz B, Gulzar B, Zafar N (2021) Mass propagation through direct and indirect organogenesis in three species of genus Zephyranthes and ploidy assessment of regenerants through flow cytometry. Mol Biol Reports 48:513–526

Syeed R, Mujib A, Malik MQ, Gulzar B, Zafar N, Mamgain J, Ejaz B (2021) Direct somatic embryogenesis and flow cytometric assessment of ploidy stability in regenerants of Caladium × hortulanum 'Fancy.' J Appl Gen. https://doi.org/10.1007/s13353-021-00663-y

Vitamvas J, Viehmannova I, Cepkova PH, Mrhalova H, Elisova K (2019) Assessment of somaclonal variation in indirect morphogenesis derived plants of Arracacia xanthorrhiza. Pesq Agrop Brasileira 54:0301. https://doi.org/10.1590/S1678-3921.pab2019.v54.00301

Yadav H, Kumar M, Nivsarkar M, Anandjiwala S (2014) Multiple marker-based evaluation of Kalanchoe pinnata, Bombax ceiba, and Morus alba leaves: quantification of α-Amyrin, Lupeol, and β-Sitosterol using High-Performance Thin-Layer Chromatography. Jplanar Chromatography 27:438–443. https://doi.org/10.1556/JPC.27.2014.6.6

Yadav V, Shahzad A, Ahmad Z. Sharma S, Parveen S (2019) Synthesis of nonembryonic synseeds in Hemidesmus indicus R. Br. short term conservation, evaluation of phytochemicals and genetic fidelity of the regenerants. Plant Cell Tissue Organ Cult 138:363–376

Yang JL, Niu YD, Yang CP, Liu GF, Li CH (2011) Induction of somatic embryogenesis from female flower buds of elite Schisandra chinensis. Plant Cell Tissue Organ Cult 106:391–399

Ye S, Cai C, Ren H, Wang W, Xiang M, Tang X, Zhu C, Yin T, Zhang L, Zhu Q (2017) An efficient plant regeneration and transformation system of ma bamboo (Dendrocalamus latiflorus Munro) started from young shoot as explant. Front Plant Sci 8:1298

You CR, Fan TJ, Gong XQ, Bian FH, Liang LK, Qu FN (2011) A high-frequency cyclic secondary somatic embryogenesis system for Cyclamen persicum Mill. Plant Cell Tissue Organ Cult 107:233–242

Zafar N, Mujib A, Ali M, Tonk D, Gulzar B, Malik M, Syeed R, Mamgain J (2019) Genome size analysis of field grown, and tissue culture regenerated Rauvolfia serpentina (L) by flow cytometry: histology and scanning electron microscopic study for in vitro morphogenesis. Ind Crops Prod 128:545–555