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Role of polyamines on *in vitro* regeneration and podophyllotoxin production in *Podophyllum hexandrum* Royle

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Podophyllum hexandrum is a popular medicinal plant endemic to Himalayas. In the present investigation, we report an efficient mass propagation protocol and Podophyllotoxin (PTOX) production in *in vitro*-derived plants of *P. hexandrum*, cultured on MS medium containing various plant growth regulators and polyamines. A combination of BA (1.5 mg/L), IAA (0.2 mg/L) and spermidine (20 mg/L) produced the highest number of multiple shoots per explant (23 shoots) after 6 weeks of culture; the regenerated shoots were elongated on the same medium. The elongated-shoots were rooted on root induction medium (half strength MS medium) supplemented with IBA (1.5 mg/L) and putrescine (15 mg/L). The rooted plantlets were successfully hardened and acclimatized with a survival rate of 68% in the greenhouse. The highest content of PTOX (4.23 mg/g DW) was recorded in *in vitro* derived roots followed by leaves when compared to field-grown parent plants. The present system offers the possibility to use *in vitro* culture techniques for mass propagation and PTOX production for commercial utilization.

Keywords: Indian Mayapple**,** Mass culture**,** PTOX**,** Putrescine, Shoot production, Spermidine**,** Zygotic embryo

Podophyllum hexandrum Royle (Indian May apple) belonging to *Berberidaceae*, is an endangered medicinal plant which grows in the Northern Himalayan region at an altitude of 3500-4000 meters[1]. Podophyllotoxin (PTOX; Fig. 1) is a plant-based pharmacologically active lignan, which is synthesized in this plant[1]. The drug obtained from this plant has remarkable activities in lung cell cancer, testicular cancer, neuroblastoma, hepatoma and other tumour diseases. The plant drug has a cytotoxic action due to inhibition of topoisomerase II, and it acts as an inhibitor of the microtubule assembly[1]. The Indian *P. hexandrum* is superior to its American counterpart, *P. peltatum* in terms of its higher PTOX content (4%) as compared to only 0.25% of *P. Peltatum*[2]. Thus, the plant becomes an important resource for the synthesis of two chemotherapeutic agents, teniposide and etoposide[3]. Due to the presence of high valued medicinal properties as well as high market demand of PTOX production, the plant has been uprooted and utilized for commercial drug preparation. Hence, many pharmaceutical chemical companies have devoted their efforts to develop synthetic PTOX production in Laboratory, but failed due to complicated steps involved in it[4].

Owing to the steady decline in its natural populations, largely due to reckless harvesting for pharmaceutical purposes, the species has been declared in the list of "critically endangered" species as per IUCN criteria[4]. Moreover, the plants flower only after attaining the age 5–6 years (following seed germination) representing a relatively extended juvenile phase[5]. This species is propagated both by

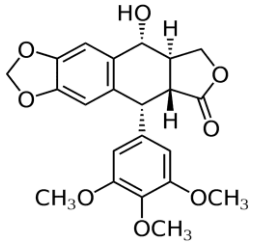

Fig. 1 — Structure of Podophyllotoxin

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vegetative means as well as seed. Natural population is, however, restricted by poor, erratic seed setting and limited germination; seed may remain dormant for 1-2 years and a period of up to 2 months is required for harvest ripening[6]. Under these ruthless situations, it is, therefore, vital to open steps for large-scale multiplication of this plant for sustainable production of PTOX and its conservation.

During the last few decades some attempts have been made to produce/improve PTOX content in cell/organ cultures[7], but the product yield has not been ample from industrial point of view. Available couple of reports are restricted to *in vitro* regeneration of *P. Hexandrum*[5,8] but failed to reveal any application due to lower frequency of *in vitro* response. Recent citations revealed that polyamines have improved the multiple shoot regeneration frequency[9,10]. Polyamines interact with phytohormones, act as plant growth regulator/hormonal secondary messenger, and as a reserve of carbon and nitrogen in culturing tissues[11]. Martin-Tanguy[12] postulated that exogenous polyamines treatment would trigger proliferation and growth of plant cells, and would lead to adventitious shoot formation. None of the available reports documented PTOX production in *in vitro* regenerated *P. hexandrum*. Therefore, an efficient mass propagation protocol from zygotic embryo of *P. hexandrum* through the addition of polyamines in the medium and, to scale up PTOX synthesis in *in vitro* regenerated plants were attempted in the present study. The role of polyamines on the efficiency of multiplication and rooting in the *in vitro* culture of *P. hexandrum* has been studied for the first time.

Materials and Methods

Plant material

Mature seeds of *P. hexandrum* were collected from Parkachi (3200 m), Kargil District, Jammu and Kashmir, India and these collections were designated as Parkachi variety. Surface sterilization was performed as per our earlier method[13]. In brief, mature seeds were separated from the ripened fruits, dried under shade and stored at 4ºC for further use. The mature seeds were washed in running tap water and followed by rinsing in Teepol® (Reckitt Benckiser Ltd. India) solution for 10 min. The seeds were surface sterilized with 70% (v/v) alcohol for one min, and followed by 10 min with 0.1% (w/v) mercuric chloride. Finally, the seeds were rinsed several times with sterile double distilled water to remove the complete sterilants. They were imbibed for 24 h in sterile distilled water and placed on orbital shaker (Orbitek, India) at 120 rpm. The zygotic embryos were carefully excised from the imbibed seeds without any damage and used to raise plants from them.

Influence of cytokinins on multiple shoot production

Excised zygotic embryos were cultured in 150 mL Erleynmayer flask containing 30 mL MS medium and five cytokinins (BA, TDZ, KIN, 2-iP and zeatin) individually in the range of 0.1–2.0 mg/L were added to MS medium in a completely randomized design. The pH of the medium was adjusted to 5.8 prior to autoclaving (20 min at 121°C; 1.4×104 kgm^{-2}) and solidified using phytagel (0.2% w/v). All the cultures were incubated at 25±2°C with a 16-h photoperiod (50 µmol m^{-2} s^{-1}) provided by cool-white fluorescent lamp. The control was maintained without any PGRs. The cultures were subcultured once at the end of the third week up to 6 weeks. After 6 weeks of culture, the shoot number, their length, and frequency of cultured zygotic embryos producing shoots were recorded and tabulated.

Influence of auxins and cytokinin on multiple shoot production

On the basis of the results obtained from the preceding experiment, different concentrations (0.1–0.5 mg/L) of auxins (IBA, IAA and NAA) were combined with an optimum concentration of BA (1.5 mg/L) in a completely randomized design. MS medium devoid of any auxin served as a control. The cultures were incubated and subcultured as described earlier. After 6 weeks of culture, the shoot number, their length, and frequency of cultured explants producing shoots were recorded and tabulated.

Influence of polyamines on multiple shoot production

Polyamines such as spermidine, spermine and putrescine at the concentration of 5, 10, 15, 20 and 25 mg/L were added individually to the optimal concentration of BA (1.5 mg/L) and IAA (0.2 mg/L) to further enhance the shoot proliferation from excised zygotic embryos. The control was served as the explant cultured in the MS medium without polyamines. The cultures were incubated and subcultured as described above. After 6 weeks of culture, the shoot number, their length, and frequency of cultured explants producing shoots were recorded and tabulated.

Influence of auxins and polyamines on rooting

After 6 weeks, shoots longer than 5 cm were selected and transferred to half strength MS medium

supplemented with IBA, IAA and NAA (0.5–2.0 mg/L). After 5 weeks of culture, optimized concentration of auxin (IBA at 1.5 mg/L) was combined with different concentrations (5–25 mg/L) of spermidine, spermine and putrescine and was incubated for 5 weeks to improve root induction. Appropriate control was maintained for each experiment. The cultures were incubated and subcultured as described above. After 5 weeks of culture, the root number, length and response of cultured explants producing roots were recorded and tabulated. After 5 weeks, the rooted plants were transferred to plastic cups containing mixture of sterilized perlite, peatmoss and vermiculate (1:1:1v/v/v) and covered with polythene bags to maintain high humidity and further hardening was carried out in the plant growth chamber (Sanyo, Japan) under controlled conditions.

PTOX extraction and HPLC analysis

The plant materials (shoots and roots separately) were dried and ground into fine powder (1 g DW). Extraction was performed as described by Rajesh *et al*.[14] with some modifications. PTOX quantification was determined using a Waters system (Waters HPLC, Vienna, Austria) equipped with a PDA detector and a reverse-phase Luna® column C18 (5 µm; 30×4.6 mm). Twenty microliters of a syringe-filtered (0.22 µm) samples were injected into the column and eluted isocratically with HPLC-grade methanol and water (65:35 v/v) [Himedia, Mumbai, India] at a flow rate of 1.0 mL/min. PTOX was detected with a PDA detector at a wavelength of 250 nm. The relative amounts of PTOX were calculated by comparing their peak areas with standard curve generated using different amounts of external standards. PTOX data were expressed as milligram per gram dry weight. Each sample was run in triplicate manner to check the consistency over the previous results. Standard sample of PTOX (RT 7.0 min) was obtained from Sigma-Aldrich (Laguna Hills, CA, USA).

Statistical analysis

A completely randomized design was used for all treatments. All the experiments were repeated thrice with three replicates for each treatment. Data were statistically analyzed using analysis of variance (ANOVA). Data were presented as the mean ± standard error (SE). The mean separations were carried out using Duncan's multiple range test and significance was determined at the 5% level (SPSS 11.5).

Results and Discussion

Plant material

In the present investigation, zygotic embryos excised from imbibed seeds were used as a starting material for multiple shoot induction. Earlier researchers used rhizome, leaf, and zygotic embryos for *in vitro* shoot regeneration from *P. Hexandrum*[5,8]. In high altitude alpine plants, seed germination and its conservation are difficult. Utilization of excised-zygotic embryo is one of the promising approaches for mass propagation and its conservation through various biotechnological tools. According to Rajesh *et al*.[13], the utilization of immature zygotic embryo as an initial explant is reasonable in the sense that they have diverse physiological prominence, which could aid to different results in the culture when compared to other explants. Kapildev *et al*.[15] reported that the selection of appropriate explant is considered more important than the plant genotype. In the period of zygotic embryo development, cells differentiate early and rapidly, which is accompanied by a loss of mitotic and morphogenetic ability. Moreover, levels of endogenous hormones which differ among organs, tissues and cells, may also influence regeneration responses[16]. Hence, we have employed zygotic embryo as starting material for exploitation of plants under *in vitro* culture conditions.

Influence of cytokinins on multiple shoot production

In the present investigation, zygotic embryos kept on MS medium supplemented with various cytokinins (BA, TDZ, Zea, KN and 2-ip) showed protuberant bulges after 3-4 days. Initiation of multiple shoot primordia was observed on all over the surface of the zygotic embryo after 6–7 days. After 10–20 days, these shoot primordia differentiated into multiple shoots. Table 1 shows the influence of different types and concentrations of cytokinins on multiple shoot production. Shoot numbers and their percentage of response were significantly improved at all cytokinin concentrations when compared to control which favoured germination of zygotic embryos only. Analysis of variance inferred that percentage of response, number of shoots and their length were affected by the concentrations and types of cytokinins tested (Table 1 and Fig. 2). Of different concentrations of cytokinins tested, 1.5 mg/L BA was most effective for multiple shoot production with a mean number of 6.61 shoots/zygotic embryo and with a response of 74.6% after 6 weeks of culture. Exogenously applied cytokinins alter the development

Table 1 — Effect of cytokinins on multiple shoot induction from zygotic embryos of *P. hexandrum* after 6 weeks of culture

Cytokinins (mg/L)	Percentage of responding explants (%)	Mean number of shoots per responsive explant	Mean shoot length (cm)
*Control	54.3c	1.00±0.25f	2.28±0.14c
BA			
0.5	58.6c	4.03±0.26c	4.31±0.11a
1.0	65.3b	5.56±0.25b	4.36±0.13a
1.5	74.6a	6.61±0.21a	4.48±0.16a
2.0	68.0b	5.34±0.20b	3.03±0.17b
TDZ			
0.1	64.1b	5.30±0.26b	3.87±0.19b
0.2	59.3c	4.93±0.23c	3.52±0.13b
0.3	45.9d	3.56±0.27d	3.07±0.17b
0.4	38.6e	3.37±0.26d	2.93±0.10c
0.5	33.5e	2.95±0.22e	2.43±0.12c
Zea			
0.2	60.3b	4.84±0.26c	3.24±0.18b
0.4	52.5c	3.83±0.27d	3.05±0.13b
0.6	44.7d	3.14±0.21d	2.77±0.16c
0.8	34.6e	2.46±0.26e	2.13±0.18c
1.0	30.5e	2.15±0.22e	1.43±0.12d
2-iP			
0.5	40.3d	2.03±0.26e	1.06±0.13d
1.0	51.5c	2.73±0.23e	2.15±0.18c
1.5	40.7d	2.06±0.24e	1.83±0.12d
2.0	28.6f	1.37±0.26f	1.24±0.14d
Kin			
0.5	44.3d	2.30±0.21e	2.45±0.16c
1.0	57.5c	3.53±0.23d	3.07±0.11b
1.5	38.7e	3.12±0.20d	2.54±0.10c
2.0	31.6e	2.34±0.29e	1.63±0.15d

[Control: MS basal medium. *Zygotic embryo germination was observed in control cultures. Values represent the mean±standard error of three experiments. Mean values followed by same letters within a column are not significantly different according to Duncan's multiple range test at 5% level]

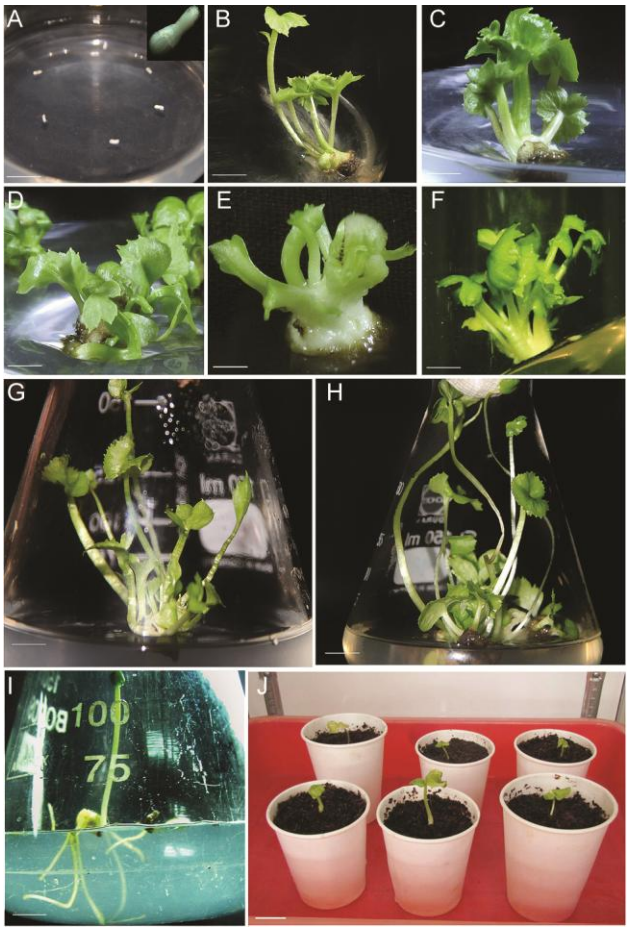

Fig. 2 — The effect of plant growth regulators and polyamines on multiple shoots production and rooting efficiency in *P. hexandrum*. (A) Zygotic embryos (size 2 mm) (*bar* 0.5 cm) [Inner image showing magnified embryo under microscope (*bar* 2 mm)] (B) Shoot development after six weeks on MS Medium supplemented with BA 1.5 mg/L (*bar* 0.5 cm) (C) Shoot development after six weeks on MS Medium supplemented with TDZ 0.1mg/L (*bar* 0.5 cm) (D) Multiple shoots on MS medium supplemented with KN 1.0 mg/L (*bar* 0.5 cm) (E) Multiple shoots on MS medium fortified with Zea (0.2 mg/L) (*bar* 0.5 cm) (F) Multiple shoots on MS medium fortified with BA (1.5 mg) and IAA (0.2 mg/L) after six weeks of culture (*bar* 0.5 cm) (G) Multiple shoots on MS medium with BA, IAA and spermine (15 mg/L) (*bar* 1 cm) (H) Multiple shoots on MS medium with BA, IAA and spermidine (20 mg/L) (*bar* 1 cm) (I) Shoot with profuse rooting on ½ strength MS medium supplemented with IBA (1.5mg/L) along with putrescine (15 mg/L) (*bar* 1 cm) (J) Hardened plants (*bar* 1 cm).

of meristems, promote the proliferation of meristematic cells from explants[17,10].

Amoo *et al.*[18] stated that BA is reported to be among the most effective and affordable cytokinin used in micropropagation techniques. Moreover, the efficiency of BA over other cytokinins on development of shoots possibly ascribes to the ability of plant tissues to metabolize natural hormones more freely than the synthetic growth regulator[19]. Further, BA could persuade the production of natural hormones such as zeatin within tissues. Buah *et al.*[20] stated that the amount of BA conjugated to medium was less when compared to other regulators. As a result, a large amount of BA exists as free or severable forms, which are readily available to plant tissues from the medium. According to Dal Cin *et al.*[21], although BAs mechanisms of action and intentions for differential effects are undefined; the pre-eminence of BA predicted might be due to its easy penetrability and increased affinity for active cell uptake. By enhancing the concentrations of cytokinins, the response of cultured explants producing shoots and the number of shoots increased. However, intensifying the concentration of cytokinins beyond the ideal level in the medium reduced morphogenetic response (data not shown). In the present investigation, next to BA, TDZ (0.1 mg/L)

supplemented medium also was effective for multiple shoot production from zygotic embryo. However, the number of shoots and response of cultured explants producing shoots were comparatively lesser than those recorded on a medium containing BA (1.5 mg/L).

A maximum of 5.3 shoots/zygotic embryo were produced with 64% of response from cultured explants on MS medium containing TDZ (0.1 mg/L). The higher concentration (above 0.2 mg/L) of TDZ exhibited hyperhydricity or morphological abnormalities in the regenerated shoots (data not presented). It may possibly due to over activity of TDZ or due to the activity of phenyl groups in TDZ[22]. Previous reports on the use of TDZ in tissue culture of *W. somnifera* have shown that TDZ in multiple shoot induction medium resulted in the production of altered morphology of multiple shoots, i.e., internode shortening or lengthening, differentiation of smaller or bigger leaves, suppression of shoots and reduced proliferation rate[9,10]. Cytokinins, KN, 2-iP and zeatin amended media showed poor shoot regeneration response (Table 1). In the present experiment, we achieved a higher number of multiple shoots from excised zygotic embryos. Sivanandhan *et al*.[10] reported that the nutrient components in the medium and exogenously added cytokinins stimulated and favoured the development of growth of multiple axillary buds and two to three fold enhancement in shoot production within a shorter period under optimized culture conditions. The present study indicates that among the cytokinins tested, BA can induce *in vitro* development of meristems from zygotic embryos of *P. hexandrum*.

Influence of auxins and cytokinin on multiple shoot production

The effect of auxin and optimized-concentration of BA on frequency of explants producing shoots and mean number of shoots per explant are shown in Table 2 and Fig. 2. The highest number of shoots and their percentage of response were recorded as 10.7 shoots/explant and 83.6%, respectively in the treatment with BA 1.5 mg/L in combination with IAA 0.2 mg/L. Even though, the mean number of shoots and their response in most treatments were almost higher than that of the respective controls (Table 2). In most cases, inclusion of IAA with BA in the medium significantly improved shoot number when compared to other auxins, suggesting a synergistic/additive effect of IAA with BA on shoot induction and proliferation in *W. somnifera* as suggested by Sivanandhan *et al*.[10]. Auxins have known to reveal synergistic, antagonistic and additive interactions with cytokinins at multiple levels (depending on the plant species and tissue type) in regulating physiological response[23]. Coenen and Lomax[23] and Kamínek *et al*.[24] hypothesized that the exogenous application of PGRs may modify the synthesis, breakdown, activation, sequestration or translocation of, as well as sensitivity to endogenous PGRs of the same or different type. Nordstrom *et al*.[25] described that while the active cytokinin level in plant can be regulated by auxin and vice versa. Furthermore, an increase or decrease in any auxin concentration except optimized concentration in combination with BA has led to the reduction in shoot number and their percentage of response. This observation proved that higher concentrations have shown inhibitory effect on the regenerability of explant and lower concentrations have shown a minimal effect on explant's regeneration. The same trend was documented by Sivanandhan *et al*.[9] in multiple shoot culture of *Withania somnifera*.

Nadeem *et al*.[5] in their study stated that highest shoot multiplication (5 shoot/explant) was observed from zygotic embryos on medium containing both BA and IAA (each at 1.0 µM) after 4-5 weeks of culture in *P. hexandrum.* Recently, Chakraborty *et al*.[8] reported

Table 2 — Effect of auxins in combination with BA (1.5 mg/l) on multiple shoot induction from zygotic embryos of *Podophylum hexandrum* after 6 weeks of culture

Plant growth regulators (mg/L)				Percentage of responding explants (%)	Mean number of shoots per responsive explant	Mean shoot length (cm)
BA	IAA	IBA	2,4-D			
Control	-	-	-	74.6^{b}	6.61±0.21^{e}	4.48±0.16^{b}
1.5	0.1	-	-	80.3^{a}	9.45±0.26^{b}	5.02±0.11^{a}
1.5	0.2	-	-	83.6^{a}	10.73±0.25^{a}	5.27±0.13^{a}
1.5	0.3	-	-	76.2^{b}	8.41±0.21^{c}	4.89±0.15^{b}
1.5	0.4	-	-	71.6^{b}	7.42±0.23^{d}	4.52±0.17^{b}
1.5	0.5	-	-	69.0^{c}	5.04±0.28^{f}	4.45±0.10^{b}
1.5	-	0.1	-	56.4^{d}	4.54±0.21^{g}	4.21±0.13^{b}
1.5	-	0.2	-	60.3^{c}	6.67±0.24^{e}	4.86±0.17^{b}
1.5	-	0.3	-	53.6^{d}	5.75±0.29^{f}	4.15±0.12^{b}
1.5	-	0.4	-	46.5^{e}	4.12±0.26^{f}	4.05±0.16^{b}
1.5	-	0.5	-	40.2^{e}	3.32±0.22^{h}	3.78±0.10^{c}
1.5	-	-	0.1	49.7^{e}	2.64±0.25^{h}	3.43±0.14^{c}
1.5	-	-	0.2	52.5^{d}	3.95±0.28^{h}	3.94±0.19^{c}
1.5	-	-	0.3	43.3^{e}	2.25±0.26^{i}	3.58±0.15^{c}
1.5	-	-	0.4	35.2^{f}	1.43±0.28^{j}	2.86±0.18^{d}
1.5	-	-	0.5	31.1^{f}	1.57±0.20^{j}	2.11±0.13^{d}

[Control: BA (1.5 mg/L) supplemented medium. Values represent the mean±standard error of three experiments. Mean values followed by same letters within a column are not significantly different according to Duncan's multiple range test at 5% level]

that combination of BA and NAA at higher concentrations was favourable for shoot regeneration from rhizome explant of *P. hexandrum*, whereas callus induction response was observed from leaf explant at same PGR concentration. The type and concentration of PGRs have confirmed differential response depending upon culture conditions, genotype, explant and their age[26,9]. In addition to that, Chakraborty *et al*.[8] noted efficiency of activated charcoal and IAA supplemented MS medium in shoot regeneration (7 shoots/explant) and 20% of response from rhizome explants. They also reported that plant growth was very slow and acquired one month for shoot emergence and three months for maximum shoot proliferation in *P. hexandrum*. In the present investigation, maximum number of shoots with higher efficiency was achieved in MS medium amended with BA+IAA combination without activated charcoal after 6 weeks of culture. Activated charcoal may suppress the uptake of PGRs from the culture medium and thereby hindering the regeneration capacity of the explant[27].

In other related species, an optimum combination of cytokinin and auxin combinations have shown higher shoot multiplication in *in vitro* propagation[9,28]. Kapildev *et al.*[15] suggested that the proper ratio of cytokinin and auxin is essential as their interaction can elevate the development of shoots. From the present study, it was observed that BA (1.5 mg/L) and IAA (0.2 mg/L) provided a 1.6-fold increase in the mean number of regenerated shoots when compared to control. These results are in support of the hypothesis that cytokinin and auxin combinations induced rapid divisions of cells in meristematic tissues and hence, resulting in better growth[28]. Other researchers also have noted the synergistic/additive effect of auxins with cytokinins on shoot production in *W. Somnifera*[9,10]. In the present study, it has been observed that the type and concentration of exogenously added auxins with cytokinin favoured shoot growth response in *P. hexandrum* and reduced the time taken for multiplication.

Influence of polyamines on multiple shoot production

The morphogenic responses of zygotic embryos of *P. hexandrum* reared on MS medium supplemented with various concentrations of polyamines are presented in Table 3 and Fig. 2. Different concentrations of polyamines along with optimized concentrations of BA and IAA were used to improve the efficiency of shoot multiplication. Among the different polyamines concentrations tested, MS medium supplemented with BA (1.5 mg/L) and IAA (0.2 mg/L) in combination with 20 mg/L spermidine was found most effective for shoot induction and proliferation. Higher number of shoots (23.7/explant) with higher response (95.4%) was recorded in the treatment with the same concentration of BA, IAA and spermidine. Spermine (15 mg/L) also showed significant (but lesser than spermidine) improvement in mean shoot number (16.3 shoots/explant) and response (87.6%) on the medium containing the optimal concentration of BA and IAA after 6 weeks of culture followed by putrescine (15 mg/L) [Table 3; Fig. 2]**.** Frequency of explants showing shoot formation and number of shoots increased with an increase in the concentration of polyamines and these two factors have drastically decreased shoot production beyond the ideal concentration of all polyamines tested. Statistically, the percentage of response and number of shoots were significant at different levels of polyamines analysed. Shoot elongation also occurred with shoot multiplication in *P. hexandrum*, when the medium was amended with polyamines in combination with BA and IAA.

Table 3 — Effect of polyamines in combination with BA (1.5 mg/l) and IAA (0.2 mg/L) on multiple shoot induction from zygotic embryos of *P. hexandrum* after 6 weeks of culture

PAs (mg/L)	Percentage of responding explants (%)	Mean number of shoots per responsive explant	Mean shoot length (cm)
Control	83.6^{b}	10.73±0.55^{i}	5.27±0.13^{b}
Spermidine			
5	79.1^{c}	13.42±0.29^{f}	5.84±0.12^{b}
10	80.9^{b}	16.33±0.26^{c}	6.17±0.18^{a}
15	84.5^{b}	18.75±0.24^{b}	6.35±0.16^{a}
20	95.4^{a}	23.78±0.21^{a}	6.78±0.17^{a}
25	70.3^{c}	14.40±0.27^{e}	5.20±0.19^{b}
Spermine			
5	71.8^{c}	11.24±0.26^{h}	5.11±0.17^{b}
10	75.7^{c}	13.87±0.29^{f}	5.54±0.15^{b}
15	87.6^{b}	16.32±0.24^{c}	5.36±0.13^{b}
20	83.4	14.53±0.27^{e}	5.17±0.18^{b}
25	66.3	10.20±0.28^{i}	4.83±0.12^{c}
Putrescine			
5	65.2^{d}	12.51±0.26^{g}	4.60±0.12^{c}
10	74.3^{c}	13.25±0.28^{f}	5.18±0.19^{b}
15	85.3	15.34±0.21^{d}	5.76±0.15^{b}
20	76.6^{c}	14.28±0.27^{e}	4.39±0.18^{c}
25	68.9^{d}	10.39±0.24^{i}	4.10±0.14^{c}

[Control: BA (1.5 mg/L) and IAA (0.2 mg/L) supplemented medium. Values represent the mean±standard error of three experiments. Mean values followed by same letters within a column are not significantly different according to Duncan's multiple range test at 5% level]

Moreover, in this study, it was noted that polyamines at higher concentrations have impeded multiple shoot forming capacity; however, their exact concentration was probably essential for the induction of shoots from zygotic embryo.

In the present report, maximum multiple shoot production was achieved due to direct contact of growing tissue with polyamines and PGRs and thereby facilitated the formation of *de novo* and pre-existing meristem in *W. somnifera* as suggested by Sivanandhan *et al*.[9,10]. The number of shoots produced in spermidine and spermine treatment was significantly higher (2.22 and 1.52 times, respectively) when compared to shoot production in BA or TDZ in combination with IAA (Table 3). The beneficial effect of spermidine along with BA and IAA in multiple shoot formation has also been established in *W. somnifera* nodal explants[9, 10]. Vasudevan *et al*.[29] suggested that besides the key roles of cytokinin and auxin on shoot induction, very important function in multiple shoot induction and differentiation process can be accelerated by polyamines in the cultured medium. Furthermore, they suggested that polyamines have a broad spectrum of action with some resemblances both with auxin and cytokinin and in co-operation with plant phytohormones modulated morphogenic process.

Regeneration and organ differentiation in plant species could be improved by application of polyamines; however, polyamines could not compensate cytokinin and auxin activity in micropropagation[30]. Polyamines interact with phytohormones; act as plant growth regulator or hormonal secondary messenger, and as a reserve of carbon and nitrogen in culturing tissues[11]. Martin-Tanguy[12] postulated that exogenous polyamines treatment would trigger proliferation and growth of plant cells, and would lead to adventitious shoot formation. As suggested by Sivanandhan *et al*.[9,10], in the present study, it was found that multiple shoot induction capacity was synergistically increased by manipulating the balance of PGRs and polyamine.

Effect of auxins and polyamines on rooting

The shoots that attained a height of 4-5 cm were axenically excised from explants and then transferred onto half strength MS medium containing different concentrations of auxins and then subsequently onto polyamines amended medium. Root induction capacity of elongated shoots cultured in auxins supplemented medium varied significantly with the type and concentration (Table 4). Rooting was achieved at all the tested concentrations of IBA, IAA and NAA within 5 weeks of culture. Among the different auxins tested, IBA at 1.5 mg/L in the rooting medium exhibited maximum root induction response, root number and their length. The control culture failed to show rooting response from the elongated shoots. IBA induced the maximum response of rooting (41%) and root numbers (3.6 roots/explant) at 1.5 mg/L followed by IAA at 1.5 mg/L (33.4% of response and 2.7 roots/explant) [Table 4]. The highest root length (4.7 cm) was also observed at same ideal concentrations of IBA followed by IAA (3.8 cm root length). NAA in the culture medium responded poorly for root induction when compared to IBA and IAA. In the present study, it was noticed that the roots produced by IBA were long, tuberous, thick and whitish in nature which help in acclimatization process whereas other auxins (IAA and NAA) showed stunted, swollen roots which did not support acclimatization process (Data not shown).

Nadeem *et al*.[5] showed that the rate of rooting in *P. hexandrum* was 25% when the medium was supplemented with IAA 0.5 µM and the use of activated charcoal was found ineffective. It has been reported that IBA promotes better root induction in plants such as *W. Somnifera*[9], *Dysosma versipellis*[31]. In order to achieve a higher root induction in *P. hexandrum*, we have successfully used polyamines

Table 4 — Effect of auxins on root induction from elongated shoots of *Podophylum hexandrum* after 5 weeks of culture

Auxins (mg/L)	Percentage of responding explants (%)	Mean number of roots per responsive shoot	Mean root length (cm)
Control	0.0e	0.0d	0.0e
IBA			
0.5	28.7^{c}	1.82±0.24^{c}	3.9±0.16^{b}
1.0	31.1^{b}	2.54±0.26^{b}	4.3±0.14^{a}
1.5	41.3^{a}	3.66±0.28^{a}	4.7±0.17^{a}
2.0	25.6^{c}	2.18.±0.29^{b}	4.1±0.14^{a}
IAA			
0.5	19.3^{d}	1.64±0.25^{c}	2.2±0.17^{c}
1.0	25.9^{c}	1.76±0.22^{c}	3.1±0.16^{b}
1.5	33.4^{b}	2.72±0.27^{b}	3.8±0.12^{b}
2.0	21.8^{c}	1.50±0.22^{c}	3.4±0.19^{b}
NAA			
0.5	13.2^{d}	1.14±0.25^{c}	1.7±0.17^{d}
1.0	15.1^{d}	1.53±0.22^{c}	2.1±0.16^{c}
1.5	19.5^{d}	2.16±0.27^{b}	2.5±0.12^{c}
2.0	14.3^{d}	1.28±0.22^{c}	1.4±0.19^{d}

[Control: MS basal medium. Values represent the mean±standard error of three experiments. Mean values followed by same letters within a column are not significantly different according to Duncan's multiple range test at 5% level]

in the culture medium. There was a gradual increase in the number of roots as well as length of roots per shoot up to 15 mg/L putrescine in combination with IBA (1.5 mg/L), and above that there was a decline (Table 5). After 5 weeks of culture in the rooting medium containing 15 mg/L putrescine in combination with IBA (1.5 mg/L), each shoot produced an average of 5.7 roots with a length of 5.2 cm. [Table 5; Fig. 2]. In the present study, IBA alone did not evoke root induction significantly when compared to putrescine and IBA combination thereby implying the specificity of specific PGR requirement. Couee *et al*.[11] described that many plant species share common features of polyamine involvement especially putrescine in root development process. Inclusion of polyamine, putrescine enhanced rooting response quickly in *W. somnifera* when compared to IBA treatment[9]. They added that root number and root length of *in vitro* raised shoots would play an important role in the successful hardening and acclimatization process. In the present study, rooted plants were acclimatized in perlite, peat moss and vermiculite mixture in the ratio 1:1:1 in plant growth chamber with 68% survivability.

Quantification of PTOX production

The quantity of PTOX content in the methanolic extract of leaves, stems and roots of *in vitro* plants were analyzed by HPLC and their productions were compared with the field grown parent plant (which was collected from Parkachi, Karkil district, Jammu and Kashmir and stored in −80°C) of *P. hexandrum*. Figures 3 and 4 represent the PTOX content in *in vitro* and field-grown plants. PTOX content in the individual plant parts exhibited a marked increase over the respective organs of field grown parent plants. PTOX content was 4.23 mg/g DW in the roots of *in vitro* derived plants and it was 2 fold higher when compared to field-grown parent plant roots (Figs. 3 and 4). The leaves and stems from *in vitro* grown plants also exhibited significant accumulation of PTOX content (2.58 mg/g DW and 1.18 mg/g DW, respectively) with 1.8- and 2-fold higher levels respectively over parent plant leaves and stems (Figs. 3 and 4). It has been reported earlier that the plants derived from *in vitro* conditions undergo some stress conditions due to the synergistic effects of PGR, physical and chemical conditions of the medium. In addition to that, PGR's played a crucial factor in secondary metabolite production in organ cultures[9,10]. Sakakibara *et al*.[32] postulated that plant growth regulates significantly transporters of some macronutrients such as nitrate, ammonium, sulphate and phosphate on one hand, while nitrate on the other hand regulates the expression of genes involved in secondary metabolite pathway. It might be a possible reason for the enhancement of PTOX level in *in vitro* cultured plants. In addition to that polyamines along with PGRs in culture medium might mimic the biosynthesis of secondary metabolites through activation of specific genes involved in secondary product pathways as in shoot and root

Table 5 — Effect polyamines on root induction from elongated shoots on MS medium containing IBA (1.5 mg/L) after 5 weeks of culture

Pas (mg/L)	Percentage of responding shoots (%)	Mean number of roots per responsive shoot	Mean root length (cm)
Control	41.3c	3.66±0.28c	4.7±0.17b
Spermidine			
5	0.0d	0.0d	0.d
10	0.0d	0.0d	0.0d
15	0.0d	0.0d	0.0d
20	0.0d	0.0d	0.0d
25	0.0d	0.0d	0.0d
Spermine			
5	0.0d	0.0d	0.0d
10	0.0d	0.0d	0.0d
15	0.0d	0.0d	0.0d
20	0.0d	0.0d	0.0d
25	0.0d	0.0d	0.0d
Putrescine			
5	45.7c	4.3±0.25b	4.1±0.25b
10	51.1b	4.9±0.25b	4.3±0.25b
15	62.3a	5.7±0.25a	5.2±0.25a
20	56.6b	5.3±0.25a	4.8±0.25b
25	41.5c	4.1±0.25b	3.7±0.25c

[Control: IBA (1.5 mg/L) supplemented medium. Values represent the mean±standard error of three experiments. Mean values followed by same letters within a column are not significantly different according to Duncan's multiple range test at 5% level]

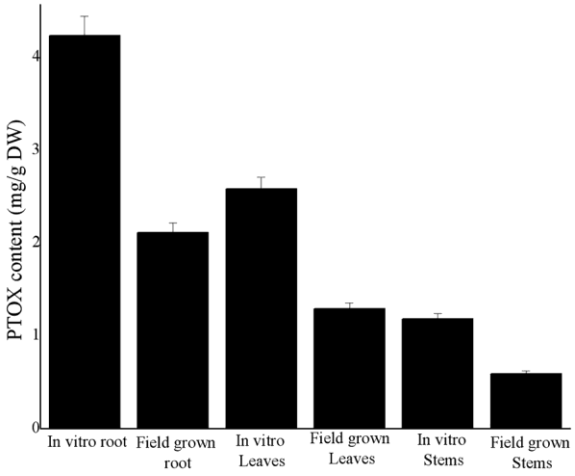

Fig. 3— Quantities of PTOX in *in vitro*-cultured plant parts and its respective field-grown parent plant parts of *P. hexandrum*. Data represents the mean±standard error of three replicates.

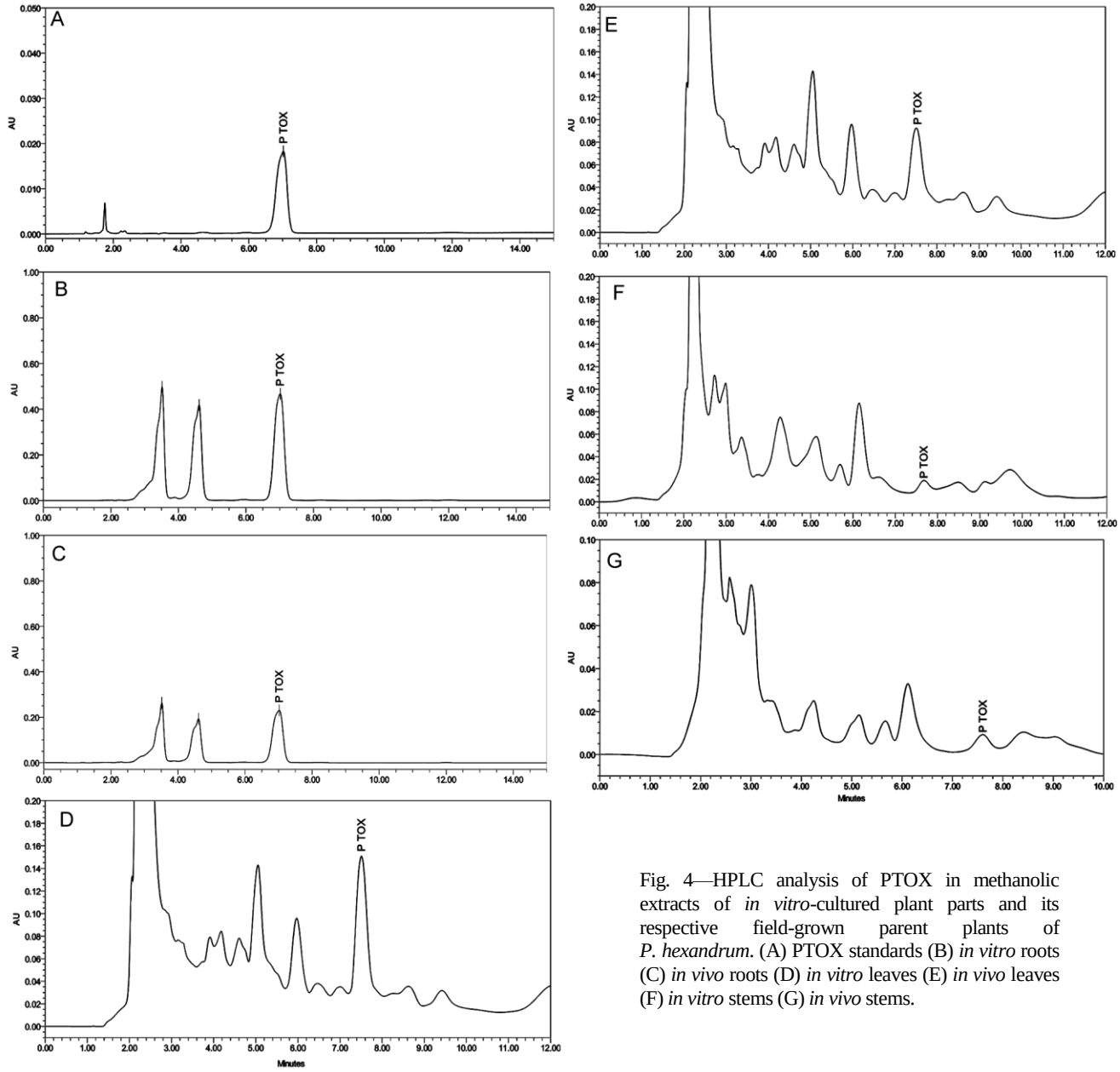

Fig. 4—HPLC analysis of PTOX in methanolic extracts of *in vitro*-cultured plant parts and its respective field-grown parent plants of *P. hexandrum*. (A) PTOX standards (B) *in vitro* roots (C) *in vivo* roots (D) *in vitro* leaves (E) *in vivo* leaves (F) *in vitro* stems (G) *in vivo* stems.

cultures of *Cicorum intypus* and *W. Somnifera*[9,10,31]. The present study provides evidences that *in vitro* plant parts can enhance the biochemical quantity of the plant. The system described here will be useful for conservation of elite germplasm of this important medicinal plant. This protocol can also be used for large-scale propagation of plants and PTOX production utilizing bioreactor design in laboratory and gene transfer studies in the future.

Conclusion

The present experiment demonstrated that zygotic embryo when cultured in MS medium containing combination of 20 mg/L spermidine, 1.5 mg/L BA and 0.2 mg/L IAA showed effective multiple shoot production. The higher efficiency of root induction was achieved on medium amended with 15 mg/L putrescine in combination with IBA (1.5 mg/L). The *in vitro* roots produced maximum PTOX level than field grown plants. This protocol will be useful for mass propagation of *P. hexandrum* for conservation and PTOX production.

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Conflicts of interest

The authors declare no conflict of interests.

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