In vitro micropropagation in *Polygonatum verticillatum* (L.) All. an important threatened medicinal herb of Northern India

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Abstract An ideal micropropagation method of *Polygonatum verticillatum* has been developed using stem disc explants. Multiple shoots were initiated from stem disc explants on Murashige and Skoog (MS) medium fortified with different concentrations ($0.25\text{--}10.0\text{ mg l}^{-1}$) and combinations of cytokinins (BAP, Kn and TDZ) along with ($0.5\text{--}1.0\text{ mg l}^{-1}$) auxins (NAA/IBA/IAA). 1.0 mg l^{-1} BAP with 0.5 mg l^{-1} NAA was found to be the most effective in producing maximum number of shoots. Regular subculturing of these in vitro multiple shoots induced profuse growth of lateral roots in the same medium. Individual shoots were excised and rooted in vitro on half strength MS medium with 1.0 mg l^{-1} NAA. Regenerants were hardened in growth chamber with high humidity and showed a high rate of survival.

Keywords Liliaceae · Meda/Mahameda · Micropropagation

Introduction

Polygonatum verticillatum (L.) All. (Family- Liliaceae) commonly known as whorled solomon's seal (Meda) is a distinguished medicinal plant of temperate Himalaya. It is distributed frequently as undergrowth in forest of temperate regions between 2,000 and 3,000 m elevations. The rhizome of this plant constitutes an important ingredient of Ashtavarga, a group of eight drugs used extensively in

Ayurveda mainly as a tonic and aphrodisiac. It has been reported to pacify pitta dosa, increase cough and shukra and is useful in phthisis, burning sensation in the body, fever and has significant antifungal activity (Chaumont 1979; Varier 1994). In trade, its rhizome is known as Meda/Mahameda and its medicinal attributes are ascribed mainly to the presence of steroidal saponins and polysaccharides in the rhizome. The plant is also useful in the preparation of cosmetics, skin tonic and as a vegetable.

Due to large scale and indiscriminate collection of wild material, *P. verticillatum* is rapidly disappearing. There is a need for conservation and mass multiplication of this plant. To fill the gap between demand and supply of its tuberous roots for commercial gains and to provide cost-effective planting material for conventional propagation methods at a rapid rate in a short span of time, plant tissue culture approach seems to be a profitable alternative.

Materials and methods

Plant material

Plants of *P. verticillatum* were collected from natural habitat (from the forest division in Pauri Garhwal and Mandal in Gopeshwar) and were maintained in earthen pots containing mixture of sand, soil and farmyard manure (1:1:1) v/v. These plants were used as the mother source for obtaining explants for tissue culture experiments.

Establishment of aseptic cultures

The young rhizomes were washed thoroughly with 1 % (v/v) Labolene detergent for 15 min in a gentle continuous flow of tap water followed by 0.1 % w/v bavistin for 1–2 h and later

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surface sterilized with 0.1 % (w/v) mercuric chloride (HgCl_2) with two drops of Tween 20 per 100 ml of solution for 3–6 min. Following surface sterilization, explants were rinsed 4–5 times with sterile distilled water to remove all traces of HgCl_2 and were blotted on sterile filter paper discs. Initially, aseptic culture were established by culturing stem discs/shoot bases bearing 2 or 3 shoot buds on MS (Murashige and Skoog 1962) medium supplemented with different phytohormones benzylaminopurine (BAP), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), kinetin (Kn), 1-naphthaleneacetic acid (NAA), thidiazuron (TDZ) used in the present study. For all the experiments pH of the medium was adjusted to 5.86 ± 0.1 prior to autoclaving at 15 lbs at 121°C for 15 min. The culture conditions (temperature $25 \pm 2^\circ\text{C}$, 16 h photoperiod using cool white fluorescent light, and 3,000–4,000 lux light intensity) were maintained throughout the course of study.

Rooted plantlets growing in rooting medium were removed from the culture media. Plantlets were thoroughly and gently washed with running tap water to remove agar from the roots, and planted in thermocol cups (5 cm dia.) having sterilized mixture of garden soil and organic compost manure in the ratio of 1: 1 (v/v). Plants planted in thermocol cups were kept in environmental chamber for 2 weeks. Potted plantlets were irrigated with water during the plant growth in environmental chamber. Plantlets were covered with polythene bags with small holes for air ventilation to ensure high humidity at initial stages. The polythene bags were removed after 2 weeks and the plants were maintained in the growth chamber under 16 h photoperiod at $28 \pm 2^\circ\text{C}$.

Plantlets were transferred to earthenware pots (25 cm dia.) containing garden soil and organic compost manure. Planted earthenware pots were kept in shade for 2 weeks and watered regularly.

Shoot regeneration from shoot bases

Shoot bases were cultured in MS medium with 3 % (w/v) sucrose and 0.8 % (w/v) agar (Hi-Media) fortified with various levels (0.25 – 10.0 mg l^{-1}) of different cytokinins like BAP, Kn and TDZ. Similarly, different auxins (IAA, IBA and NAA) were also added in combinations with different cytokinins for selecting optimal medium for

direct multiple shoot regeneration from shoot bases. Growth response of cultured shoot bases was examined after 90 days of culture.

In vitro rhizogenesis in micro- shoots

In vitro regenerated shoots were excised from the cluster and transferred on half strength MS medium supplemented with different auxins viz. IAA, IBA and NAA (0.5 – 1.0 mg l^{-1}) and was kept in dark. Frequency of root induction was examined after every 15 days of culture. BAP in combination with NAA induced rooting frequency of root growth; number of root/explants was quantified after 90 days of inoculation.

Analysis of data

In all the experiments standard deviation was calculated using following formula:

$$\sqrt{\frac{n \sum \chi^2 - (\sum \chi)^2}{n(n-1)}}$$

Where,

n = number of replicates observed; $\sum$ = summation; χ = observation of the replicate.

Results and discussion

Shoot regeneration from shoot bases

BAP (3.0 – 8.0 mg l^{-1}) alone in MS medium showed better response than TDZ (0.25 – 3.0 mg l^{-1}) in terms of shoot regeneration but shoot length and number of leaves were comparatively more in TDZ supplemented medium (Table 1, Fig. 1a). This is due to the promoter effect of TDZ regarding growth due to its own biological activities similar to N-substituted cytokinin or it may induce the synthesis and accumulation of an endogenous cytokinin. Highest number of shoots (7.86 ± 1.30) with an average length of $2.10 \pm 0.36 \text{ cm}$ was recorded on BAP (5.0 mg l^{-1}) supplemented medium (Table 2, Fig. 1b). A similar response of maximum shoot regeneration in high concentration of BAP was also observed

Table 1 Effect of TDZ supplementations in MS medium on the growth response of cultured shoot bases of *P. verticillatum* after 90 days of culture

Growth parameters	TDZ (mg l^{-1})				
	0.25	0.50	1.00	2.00	3.00
Total no. of shoots/explant	2.00 ± 0.18	2.33 ± 0.32	3.70 ± 0.33	4.00 ± 0.32	3.00 ± 0.57
Shoot length (cm)	3.60 ± 0.40	4.26 ± 0.49	8.03 ± 1.10	8.60 ± 0.92	6.23 ± 0.82

± Standard deviation

Fig. 1 Multiple shoot regeneration in *P. verticillatum* in MS medium supplemented with : 2.0 mg l⁻¹ TDZ (a); 5.0 mg l⁻¹ BAP (b); 1.0 mg l⁻¹ BAP and 0.5 mg l⁻¹ NAA (c); Rooting in half strength MS medium supplemented with 0.5 mg l⁻¹ NAA (d); Rooted plantlets successfully transferred to thermocol cup (e); One month old plants (f)

in *Chlorophytum* genus of a related family (Liliaceae) which required a very high concentration of this hormone for shoot regeneration (Purohit et al. 1994).

The best response in terms of multiple shoot bud initiation and shoot regeneration was recorded after 90 days of culture in 1.0 mg l⁻¹ BAP and 0.5 mg l⁻¹ NAA. The positive effect of cytokinin and auxin combination is explained by the enhanced RNA synthesis. The applied combination provokes the peak of RNA synthesis. This peak is associated with the appearance of first bud primordial, thus conditioning bud formation. Synergistic

effect of a range of growth regulator combinations on promotion of shoot multiplication is well documented for medicinal plants. Various successful combinations have been reported in various studies such as BA + IAA for *Adhatoda beddomei* (Sudha and Seenii 1994), *Valeriana wallichii* (Mathur et al. 1987), BA + IBA for *Rheum emodi* (Lal and Ahuja 1993) and BA + NAA for *Gompherena officinalis* (Mercier et al. 1992) but the increase in the concentration of auxin suppresses the bud differentiation. This may be explained by assuming that the amount of auxin synthesized endogenously was more than sufficient

Table 2 Growth response of cultured shoot bases of *P. verticillatum* in MS medium after 90 days of culture under the influence of BAP and NAA (0.5 mg l⁻¹)

MS + BAP (mg l ⁻¹)	NAA (mg l ⁻¹)	Total no. of shoots/explant	Shoot length (cm)
1.00	–	3.10±0.20	0.98±0.06
2.00	–	4.00±0.57	1.16±0.08
3.00	–	4.66±0.33	1.55±0.16
4.00	–	5.00±0.57	2.00±0.34
5.00	–	7.86±1.30	2.10±0.36
6.00	–	6.80±0.57	1.96±0.21
7.00	–	6.42±1.25	1.75±0.14
8.00	–	6.12±1.00	1.56±0.10
9.00	–	4.60±0.89	1.00±0.06
10.00	–	2.40±0.54	0.74±0.02
1.00	0.5	8.60±0.58	4.66±1.07
2.00	0.5	7.00±1.00	4.15±0.70
3.00	0.5	6.20±1.00	4.00±1.00
4.00	0.5	6.60±0.56	3.78±1.07
5.00	0.5	5.60±0.58	3.46±0.50
6.00	0.5	4.30±1.07	3.00±0.32
7.00	0.5	4.00±0.0	2.86±0.98
8.00	0.5	3.60±0.58	2.50±0.57
9.00	0.5	2.60±1.15	1.86±0.15
10.00	0.5	2.30±1.07	1.24±0.08

± Standard deviation

to interact with added cytokinin to achieve the balance, necessary for bud induction.

Supplementation of lower levels of NAA (0.5 mg l⁻¹) in the medium synergized the morphogenic response. Highest number of shoots (8.60±0.58) with an average length of 4.66±1.07 cm was obtained in BAP and NAA (1.0 mg l⁻¹: 0.5 mg l⁻¹) supplemented medium (Table 2, Fig. 1c). These

results furthermore coincide with the earlier reports (Yamaner and Erdag 2008; Murthy et al. 2010; Islam et al. 2008).

In vitro rhizogenesis in microshoots

Data recorded periodically after every 15 days of inoculation of in vitro regenerated shoots revealed that, amongst all the auxins i.e. IAA, IBA or NAA tested only NAA and IBA could induce root on half strength MS basal media whereas IAA did not respond. 0.5 mg l⁻¹ NAA in half strength MS medium reflected better rooting and longer roots than half strength MS medium and IBA (0.5–1.0 mg l⁻¹). Highest number of roots (4.79±0.69) was recorded on NAA (0.5 mg l⁻¹) at 60 day stage (Table 3, Fig. 1d). Similar result was also obtained by Murthy et al. (2010) in *Ceropegia spiralis* L. Rooting induced by IBA treatments occurs by direct origin for active division of the cambial cells, resulting in the development of a functional root system (Pasqua et al. 1993). In contrast to this use of half strength of MS media for rooting has been reported by several workers. However, supplementing NAA (1.0 mg l⁻¹) in MS medium significantly enhanced rooting frequency in *Mentha arvensis* (Kukreja et al. 1991). IBA in 1.0 mg l⁻¹ was most effective in *Ocimum americanum* while in *O. sanctum* 1.0 mg l⁻¹ NAA was best for rooting (Pattnaik and Chand 1996).

The rooted plantlets were successfully transferred to soil (Fig. 1e and f). The present study indicate that forest soil with organic compost (1:2 v/v) kept in controlled environment for a longer duration and regular irrigation by keeping the plants in shade are desirable conditions to achieve high survival percentage of *P. verticillatum*.

Tissue culture and biotechnological studies in *P. verticillatum* (Meda) has not to our knowledge, been previously reported. The culture work distinctively elaborates the use of stem disc part. Moreover, the present investigation describes a low auxin and cytokinin single step system for production of multiple

Table 3 Periodic variation in root number (RN) and root length (RL) in half strength MS medium supplemented with varying levels of NAA and IBA

MS ½ + NAA (mg l ⁻¹)		Number of days			
		15	30	45	60
0.5	RN	2.06±1.08	3.96±1.73	4.18±0.52	4.79±0.69
	RL (cm)	0.72±0.26	0.88±0.31	0.97±0.29	1.00±0.30
1.0	RN	1.63±0.61	2.19±0.32	3.06±0.49	4.18±0.52
	RL (cm)	0.61±0.22	0.71±0.25	0.84±0.29	0.97±0.29
MS ½ + IBA (mg l ⁻¹)					
0.5	RN	1.00±0.06	1.21±0.54	2.00±1.10	2.76±0.18
	RL (cm)	0.43±0.12	0.57±0.21	0.74±0.32	0.86±0.14
1.0	RN	0.76±0.12	1.12±0.64	1.76±0.58	2.08±0.57
	RL (cm)	0.34±0.06	0.42±0.08	0.68±0.16	0.74±0.12

± Standard deviation

shoots through de novo shoot regeneration, which could probably be triggered by the endogenous cytokinin level.

Therefore present study was undertaken to devise methods for possible recovery of this endangered and over-harvested medicinal plant species.

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