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Callus induction in *Polygonatum verticillatum* (L.) All.: An Astavarga medicinal herb

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Abstract
Polygonatum verticillatum, a highly valued medicinal herb of temperate Himalaya is extensively used to cure various health troubles as cardiotonic, antiperiodic, demulcent, diuretic, energizer, aphrodisiac, sedative, hypoglycemic, antitumor, pain reliever, general tonic, nervine tonic etc. The present investigation deals with the establishment of callus culture from leaf and hypocotyls segments of *in vitro* raised seedlings of *P. verticillatum*. Hypocotyls showed better callogenic response as compared to leaf explants. Callus induction was achieved in 22.4±0.51 to 30.8±1.59 days and 27.8±1.28 to 37.4±0.81 days after inoculation using hypocotyls and leaf explants respectively. Among all tested treatments, MS+TDZ (1.0mg/L)+2,4-D(1.0mg/L) caused maximum per cent callus induction in both hypocotyl (70±5.00 %) and leaf explant (45±5.00%). Maximum 1.70±0.26 g fresh weight of callus was achieved from hypocotyls inoculated in TDZ (1.0mg/L)+2,4-D(1.0mg/L) fortified MS media. Thus, obtained callus can be used for further biochemical and biotechnological investigation with aim to enhance pharmaceutical potential of this highly valued Ayurvedic medicinal herb.

Keywords: *Polygonatum verticillatum*, medicinal herb, astavarga, callus culture, conservation

Introduction
Polygonatum (Solomon’s seal), a genus comprising of around 57 species has world wide distribution in the warm temperate, subtropical and boreal regions of the northern hemisphere [1]. *P. verticillatum,* commonly known as Meda, is a distinguished medicinal herb of temperate Himalaya. *P. verticillatum* is claimed to possess vast range of pharmacological properties *viz*., antitussive, antipyretics, cardiotonic, antiperiodic, demulcent, diuretic, energizer, aphrodisiac, sedative, pain reliever, hypoglycemic, appetizers, general tonic, nervine tonic and are used for curing pulmonary troubles and tuberculosis [2-3], interrupted menstruation, uterine tumor and leucorrhoea [4]. Together with *P. sibiricum, P. verticillatum* has been found to cure chronic hepatitis B [5]. Rhizome of *P. verticillatum* is used as an effective drug to treat respiratory troubles and inflammatory problems [6]. The plant is also used to prepare beauty products [7]. Being a member of Astavarga group of eight medicinal plants, *P. verticillatum* is extensively used in the Ayurvedic system of medicine. In traditional and folkloric systems of medicine, it is used to increase body temperature, to improve sexual potency and to treat gastric troubles [8]. Due to high medicinal importance, it is facing imprudent harvesting from the wild without having any approach for its cultivation. *P. verticillatum* has been recorded as vulnerable in IUCN Red List [9].

Callus culture is one of the reliable alternatives to conserve the germplasm and enhance the biomass of medicinal plants of threatened status. By manipulating nutrients and hormonal combinations, callus culture can be used for whole plant regeneration and for somatic embryo formations. Callus culture can be used to perform various biochemical assays and to produce various commercially valued secondary metabolites. It also pave the way for isolating economically valuable bioactive compounds [10-11] and reduces the burden of natural stock to meet the need of pharmaceutical ventures. To the best of our knowledge, *P. verticillatum* has not been grown as callus in nutrient medium under *in vitro* conditions. Hence, the present communication is our effort to establish callus culture to conserve the germplasm of *P. verticillatum* and provide a basis for further scientific investigation.

Materials and Methods
Fruits of *P. verticillatum* were collected from wild population of Bhowali region, dist. Nainital of Uttarakhand (India). Seeds were extracted by hands and washed vigorously with running tap water for about 30 min. followed by continuously shaking in distilled water containing 2% detergent solution (Tween 20) for 10 to 15 min. Fungicidal treatment of Bavistin [0.5% (w/v)] was given for 15 min. After 5 to 6 times washing with autoclaved distilled water,

seeds were disinfected with aq. mercuric chloride [0.1 % (w/v)] for 5 min. and finally rinsed with six changes of double distilled water inside laminar air flow. Surface sterilized seeds were kept aseptically on glass Petri plates containing two layers of filter paper moistened with dist. water on alternate days. Seeds were kept at 16/8 light/ dark condition using cool-white fluorescent light (40 µmol $m^{-2}s^{-1}$) in tissue culture lab at 25±1^{0}C. Hypocotyl segments and leaf from *in vitro* raised seedlings were used as explants for inducing callus. For all treatments and control, MS (Murashige and Skoog medium) medium fortified with 100 mg/l myo-inositol, 0.05% (w/v) Polyvinylpyrrolidone (PVP), 0.7% (w/v) Agar was used. MS (full strength) was supplemented with 3% (w/v) sucrose whereas ½ MS medium (MS salts diluted to the half) was augmented with 1.5% (w/v) sucrose. Full strength MS medium devoid of any plant growth regulators (PGRs) was served as control whereas full and half strength MS medium fortified with different conc. and combinations of plant growth regulators namely 2,4-D, IAA, IBA, NAA, BA, KN and TDZ were used as different treatments. The culture conditions *viz*., temperature 25±1°C, 16/8 hours photoperiodic condition using cool-white fluorescent tube (intensity 40 µmol $m^{-2}s^{-1}$) were maintained throughout the course of study. Whenever required, sub culturing was done to avoid the browning of media. Growth responses were recorded at regular basis. Days to callus induction, % response and fresh weight of callus were assessed. Callus induction (%) was calculated by using the following formula=

(No. of explants producing callus/ Total no. of explants on callus induction media) × 100

Data analysis

All treatments had five replicates having four explants each (5 replications x 4 explants = 20 explants). Presented data is mean values ± SE. Mean values of different treatments were subjected to statistical analysis using one way ANOVA. The differences among the mean values were compared using significant differences at $p < 0.05$.

Results and Discussion

Hypocotyl and leaf segments (1.5-2.0 cm) of *in vitro* raised seedlings of *P. verticillatum* were inoculated in full strength (MS+3% sucrose) and half strength (½ MS+1.5% sucrose) medium supplemented with various conc. and combinations of plant growth regulators to initiate callus induction (Table 1). Hypocotyls responded better for callogenic response as compared to leaf explants (Figure 1). Hypocotyls showed callus induction response in 22.4±0.51 to 30.8±1.59 days after inoculation. Leaf explants took 27.8±1.28 to 37.4±0.81 days for callus induction. Maximum per cent callus induction was achieved using MS+TDZ (1.0mg/L)+2,4-D(1.0mg/L) in both hypocotyl and leaf explants showing 70±5.00 % and 45±5.00 % callus induction frequency respectively. The induced callus was granular, pale to yellow in color in both the explants. Callus fresh weight ranged from 0.30±0.02 to 1.70±0.26 g in hypocotyl derived callus and 0.35±0.06 to 0.91±0.04 g in leaf derived callus. The differential response of explants in callusing may be due to varying conc. of endogenous levels of phytohormones in different explants [12-13] or due to intrinsic quality of phytohormones which relatively effects the

Table 1: Effects of different conc. and combinations of plant growth regulators on *in vitro* callus induction and further proliferation from hypocotyls and leaf explants of *in vitro* raised seedlings of *P. verticillatum*

Medium	Plant growth regulators (mg/L)	Days to callus induction		Callus induction (%)		Fresh weight (g)	
		Hypocotyls explant	Leaf explants	Hypocotyls explant	Leaf explant	Hypocotyl explant	Leaf explant
MS+ 3% sucrose	MS basal media	-	-	-	-	-	-
MS+ 3% sucrose	IAA (1.0)	-	-	-	-	-	-
MS+ 3% sucrose	IBA (1.0)	-	-	-	-	-	-
MS+ 3% sucrose	NAA (1.0)	-	-	-	-	-	-
MS+ 3% sucrose	2, 4-D (1.0)	24.6±1.81 ab	37.4±0.81 c	40±6.12 ab	25±7.91 a	0.66±0.08 b	0.47±0.08 b
MS+ 3% sucrose	2,4-D (2.0)	24.2±0.66 ab	36.6±1.21 c	50±11.18 b	35±6.12 ab	0.70±0.06 b	0.51±0.06 bc
MS+ 3% sucrose	TDZ (0.5)+2,4-D(2.0)	24.8±1.53 ab	-	40±6.12 ab	-	0.51±0.19 ab	-
MS+ 3% sucrose	TDZ (1.0)+2,4-D(1.0)	22.4±0.51 a	27.8±1.28 a	70±5.00 c	45±5.00 b	1.70±0.26 d	0.91±0.04 d
MS+ 3% sucrose	KN (1.0)+IAA(1.0)	-	-	-	-	-	-
MS+ 3% sucrose	KN (1.0)+IBA(1.0)	-	-	-	-	-	-
MS+ 3% sucrose	KN (1.0)+2, 4-D(1.0)	30.8±1.59 c	-	30±9.35 a	-	0.30±0.02 a	-
MS+ 3% sucrose	BA (1.0)+IAA(0.5)	-	-	-	-	-	-
MS+ 3% sucrose	BA (1.0)+NAA(0.5)	26.0±1.30 b	31.4±1.91 b	55±9.35 bc	35±6.12 ab	0.46±0.05 ab	0.38±0.08 ab
MS+ 3% sucrose	BA(1.0)+ 2,4-D(1.0)	26.0±1.48 b	35.2±1.62 c	45±9.35 ab	30±5.00 ab	0.68±0.10 b	0.39±0.04 ab
MS+ 3% sucrose	BA (0.5)+2, 4-D(1.0)	27.2±1.39 b	31.2±2.44 b	55±9.35 bc	30±9.35 ab	0.55±0.09 ab	0.35±0.06 a
MS+ 3% sucrose	BA(1.0)+2,4-D(0.5)	25.6±1.33 b	28.4±1.91 ab	60±6.12 bc	40±6.12 b	1.17±0.19 c	0.45±0.06 ab
1/2 MS+ 1.5 % sucrose	2,4-D(1.0)	23.6±1.63 ab	37.2±0.58 c	45±5.00 ab	25±7.91 a	0.54±0.09 ab	0.41±0.02 ab
1/2 MS+ 1.5 % sucrose	2, 4-D(2.0)	24.6±1.75 ab	35.4±1.60 c	45±9.35 ab	25±7.91 a	0.72±0.06 b	0.58±0.04 c
1/2 MS+ 1.5 % sucrose	TDZ (1.0)+ 2,4-D(1.0)	26.4±1.44 b	30.8±0.86 ab	60±6.12 bc	45±5.00 b	1.19±0.18 c	0.65±0.06 c
1/2 MS+ 1.5 % sucrose	BA (1.0)+ NAA (0.5)	25.4±0.24 ab	-	45±9.35 ab	-	0.67±0.10 b	-
1/2 MS+ 1.5 % sucrose	BA (1.0) + 2,4-D (0.5)	22.8±1.07 ab	35.2±1.43 c	50±7.91 b	30±5.00 ab	0.87±0.04 b	0.46±0.03 b

Different letters following mean±SE indicate significant difference among treatments (P<0.05)

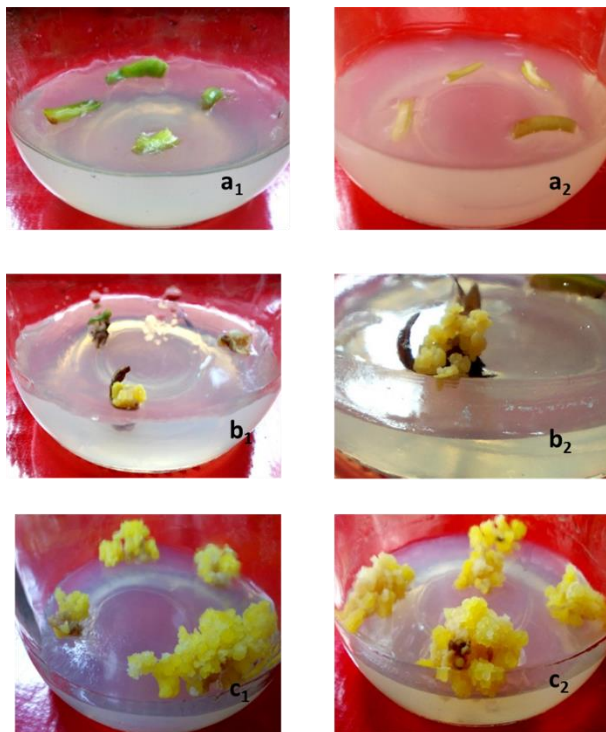

Fig 1: Callus induction and further proliferation from leaf (a_1-c_1) and hypocotyl explants (a_2-c_2) of *in vitro* raised seedlings of *P. verticillatum*

Genes for triggering differentiation of cells. In the present study, TDZ(1.0mg/L)+2,4-D(1.0mg/L) proved better for callogenic responses when used in combination with full strength MS+3% sucrose as compared to ½ MS+3% sucrose.
Among all tested auxins, only 2,4-D given individually was found effective for inducing callus. Effectiveness of 2,4-D was increased when used in combination with most of the cytokinins and their tested conc. The efficacy of 2,4-D alone and in combination with different cytokinins in initiating callogenic response may be due to their role in mitosis and DNA synthesis [14].
TDZ (1.0 mg/L) in combination with 2,4-D (1.0 mg/L) was proved optimum for callogenic response in *P. verticillatum*. TDZ was found most effective cytokinin for callus induction in *Beta vulgaris* [15]. TDZ is chemically different from usually used auxins and cytokinins, but has been shown to exert both auxin and cytokinin like responses due to its capability to regulate endogenous level of phytohormones [16-17]. Combination of TDZ (1.0 mg/L) and 2,4-D (0.1 mg/L) was found best for callus induction in *Beta vulgaris* L. regardless of explants used [15]. TDZ + 2,4-D at different conc. in Driver and Kuniyuki (DKW) medium gave 88.93 to 100 % callogenic response in *Hibiscus sabdariffa* L. [18]. 100 % callus induction response was observed in leaf explants of *Primula vulgaris* cultured in MS+TDZ (2.0 mg/L)+2,4-D (4.0 mg/L) [19]. TDZ and 2,4-D at 1.0 and 0.5 mg/L conc. caused highest (65%) per cent callus induction from cotyledon segments of *Vigna subterranean* L. [20].

Conclusion

During past few decades, medicinal plants have drawn much commercial and scientific consideration due to escalating faith in herbal medicine vis-à–vis allopathic medicines. Medicinal plants are elite premise of life saving medication because of their ability to produce diverse group of bioactive compounds through the progression of metabolism. Majority of plants of therapeutic significance are confronting threat of extinction just for their overexploitation for commercial purposes. There is an earnest need to guide exhaustive *in-situ* and *ex-situ* conservation measures for the management of high-risk medicinal plant species and to meet the industrial demand. Considering the pharmacological importance of plant secondary metabolites, pharmaceutical ventures are deeply interested in utilizing plant tissue culture techniques for the production and further enhancement of their bioactive compounds. Callus culture system is being utilized for biological investigations and for enhancement of secondary metabolites. Using optimized treatment of this protocol, it is possible to produce callus of *P. verticilltum* within 1 month that can be used for further study of different aspects, biochemical assays, biotechnological applications and conservation of *P. verticillatum.*

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