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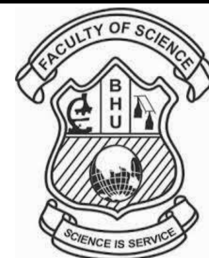

In vitro Propagation of *Polygonatum verticillatum* All. A Threatened Medicinal Herb through Seed Explant

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Abstract: **To conserve and overcome the limitations of traditional propagation, a need was felt to develop an *in vitro* propagation protocol for *Polygonatum verticillatum* (L.) All. by using seeds as explant. The present study was undertaken to evaluate the most suitable concentration of plant growth regulators on callus induction and subsequent rhizome and shoot regeneration. Murashige and Skoog (MS) medium fortified with Kn (8mg/l) in combination with IAA (2mg/l) proved most effective in producing maximum amount of callus from *in vitro* sprouted seed explants which were pre-treated with GA_3 (1 mg/l) and kept at -20°C for 30 days. Subculturing of callus on MS medium fortified with BAP (4mg/l) and NAA (1mg/l) in combination regenerated rhizomes. Shoots regenerated (13.25±2.32) from rhizomes on MS medium fortified with Kn (2mg/l) in combination with BAP (2mg/l). Roots (1.93±0.34 cm) regenerated from shoots inoculated on full strength MS medium fortified with IBA at a concentration of (2.0 mg/l) within 90 days. These *in vitro* raised shoots with well developed roots were successfully acclimatized in vermicompost with 80% survival rate. In addition to *in vitro* shooting, *ex vitro* regeneration of shoots from *in vitro* raised rhizomes was also achieved in vermicompost within 49 days.**

Index Terms: **BAP, Explant, *In vitro*, IAA, Kn, MS medium, *Polygonatum verticillatum*, Rhizome**

I. INTRODUCTION

Biodiversity is necessary for economic well being and human survival (Singh 2002; Vibhuti *et al*. 2018). Over exploitation, habitat destruction and species introduction are recognized as major causes of loss of biodiversity in India (UNEP, 2001; Pande 2016). Due to this most of the species are facing the pressure to survive in nature and *Polygonatum verticillatum* is one of them.

Polygonatum verticillatum, commonly known as 'Whorled Solomon's Seal' (Ballabh and Chaurasia, 2009), is a rhizomatous perennial herb (Fig. 1a) distributed in Afghanistan, North and Central Asia, Europe, Pakistan, Turkey and Tibet at an elevation of 4500m above sea level (asl). In India it is found in temperate Himalayas from Kashmir to Sikkim, Himachal Pradesh and Uttrakhand at an altitude of 1600-3500m asl (Balkrishna *et al*. 2012). In Kashmir it is found in Aharbal, Dara, Goguldara, Gulmarg, Sonmarg, and Tangmarg at an altitude above 2000m asl. *P. verticillatum* is an attractive source of drugs and possess various medicinal properties. The rhizome of this plant species is used in Ayurveda (Bisht *et al*. 2012). It has anticancer activity (Singh and Patra, 2018), aphrodisiac activities (Kazmi *et al.* 2012), antipyretic, anticonvulsant (Khan *et al*. 2013), antispasmodic, antidiarrheal (Khan *et al.* 2016), antimalarial antioxidant (Khan *et al.* 2011a), antinociceptive (Khan *et al*. 2011b), insecticidal, leishmanicidal (Saeed *et al.* 2010), bronchodilator, trachea relaxant, lipoxygenase, urease inhibition antifungal and antibacterial activity (Saboon *et al*. 2016). *P. verticillatum* also possess some ethno medicinal importance. The powder of this plant species is used for treating gastric ailments, wounds (Nautiyal *et al.* 1998; Gaur, 1999), asthma, inflammation (Khan *et al.* 2013), tuberculosis, leucorrhoea (Bhatt *et al.* 2014), jaundices (Sharma *et al*. 1999) and is used as an emollient (Ghayur, 2004). Several active compounds are isolated from this plant species which include Lectins (Antoniuk,

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1993), 5-hydroxymethyl-2-furaldehyde, Diosgenin (Khan *et al.* 2011a), Beta sitosterol, Quinine (Virk *et al*. 2016), Alpha bulnescene, Calarene, Docasane, Eicosadienoic, Lenalyl acetate Pentacosane, Piperitone, Santonin (Saboon *et al*. 2016), Aspartic acid, Digitalis glucoside, Lysine, Serine, Threonine and Saponosides A, B, C and D (Anonymous, 1969).

P. verticillatum is assigned as threatened under vulnerable category by IUCN (Bhat *et al.* 2014). The plant species is rapidly disappearing and is facing threat due to over exploitation, poor seed germination, overgrazing, habitat destruction and unscientific collection of plant material from its natural habitats. There is a need for conservation of this plant species. Thus during present study, tissue culture techniques which is an alternative for conservation of threatened medicinal plants have been extended to this medicinal species to save it from the brink of extinction.

II. MATERIALS AND METHODS

A. Plant Material

Mature fruits of *P. verticillatum* were collected from KUBG (Kashmir University Botanical Garden) in the month of July. Seeds from mature fruits were excised from the pulp and washed thoroughly under running tap water, dried at room temperature and stored in falcon tubes for *in vitro* studies. To confirm whether the seeds used during present study were viable or not, viability test using the tetrazolium chloride technique (Peters, 2000) was performed (Fig. 1b). For *in vitro* studies, seeds were given pre culture treatment as adopted by Prakash *et al*. 2011 with some modifications. For *in vitro* seed germination, seeds were stratified at different temperatures (4ºC, -20ºC, -80 ºC) for different time periods (10, 20 and 30 days) along with 1mg/l gibberellic acid (GA_3) solution, but seeds stratified at -20ºC for 30 days along with GA_3 (1mg/l) solution were used because this was the only pre culture treatment which responded to *in vitro* growth conditions.

B. Establishment of Aseptic Cultures

For standardization of *in vitro* protocols, seeds were thoroughly washed under running tap water for 30 minutes followed by washing with detergent labolene (1 % v/v) treated with few drops of surfactant tween-20. The detergent was removed by washing the seeds first under running tap water and finally washed with double distilled water (DDW). Then the seed explants were given chemical sterilant (0.01% mercuric chloride) for 15 minutes under Laminar Air Flow Hood and then rinsed 2-3 times with autoclaved DDW in order to remove traces of $HgCl_2$. Prior to inoculation, the seeds were scarified by rubbing with sand paper and were given a cut at pointed end and then inoculated on MS (Murashige and Skoog's, 1962) medium augmented with different concentrations and combinations of Plant Growth Regulators (PGRs). Among auxins, Indole-3-aetic acid (IAA), Indole-3-butyric acid (IBA) and 1-Napthaleneacetic acid (NAA) were used and among cytokinins 6-Benzylaminopurine (BAP) and Kinetin (Kn) were used in concentration range of 0.5-10 mg/l. The pH of the media was adjusted to 5.8 by adding few drops of 1N NaOH/HCl and finally autoclaved at 121 °C and 15 lb pressure for 20 minutes. The cultures were maintained in culture room, incubated at 22±4°C and illuminated to a regular photoperiod of 18 hours with fluorescent light providing 3000-4000 lux light intensity.

C. Acclimatization

For acclimatization the *in vitro* regenerated plantlets were taken out of culture tubes and thoroughly washed with DDW in order to remove the adhering medium and then transferred to plastic pots containing Vermicompost (100 g) and PGRs.

D. Data analysis

The experiments were repeated thrice and the data was analysed through Microsoft Excel by calculating Standard Error (SE) of various treatments.

Fig. 1. (a) Taxonomical characters of plant

Fig. 1. (b) Seed viability test showing stained embryos

III. RESULTS AND DISCUSSION

A. In Vitro Seed Germination

During the present study, different treatments were given to seeds in order to see their effect on germination. Among different treatments seeds stratified at -20°C for 30 days along with GA_3 (1mg/ml) solution inoculated on MS basal medium gave best response with 90% seed germination within 36±0.57 days of inoculation (Fig. 2a). Seed germination was also observed on MS medium fortified with different concentrations of BAP (1.0, 1.5 and 2.0 mg/l) in combination with NAA (1.0 mg/l). In addition, successful seed germination was also obtained on MS medium fortified with different concentrations of Kn

(0.5, 1.0, 1.5 and 2.0mg/l; Table I). A similar result of seed germination was obtained by Nasri *et al.* (2013) in *Alstroemeria ligtu*. They achieved 76.76% seed germination on MS basal medium when seeds were stratified at 5±1°C for 21 days and 100 ppm GA_3 treatment for 24 hours. Prakash *et al.* (2011) observed enhancement in seed germination of *P. verticillatum* by giving chilling treatment (at -20°C for 15 to 60 days and exogenous application of 100 ppm GA_3) before sowing in soil. Toma and Rasheed (2012) achieved highest *in vitro* seed germination of *Asparagus Densiflorus* L. by giving the seeds stratification prior to culture. The present study is in accordance with Valizadeh and Tabar (2009) who achieved *in vitro* seed germination in *Bunium persicum* on MS basal medium after stratification at 4°C in dark for 80 days.

Table I: Effect of different treatments on *in vitro* seed germination in *P. verticillatum*

MS+PGR's (mg/l)			Mean no. of days taken for seed germination± SE	% Culture Response
BAP	KN	NAA		
-	**-**	**-**	**36±0.57**	**90**
1	-	1	44.33±0.66	80
1.5	-	1	53.33±0.88	70
2	-	1	57.66±0.33	60
-	0.5	-	70.33±0.33	20
-	1	-	50.66±0.66	50
-	1.5	-	73.33±0.33	20
-	2	-	77.00±0.57	20

20 replicates per treatment

B. Multiple Shoot Regeneration from Seed Explant

During present study, multiple shoots were regenerated from seeds on MS medium augmented with different PGR's i.e., BAP (4.0 mg/l) + NAA (1.0 mg/l); BAP (4.0 mg/l) + IAA (1.0 mg/l); BAP (2.0 mg/l) + IAA (1.0 mg/l); Kn (2.0 mg/l) + IAA (1.0 mg/l) (Table II). Among these PGR's, MS medium fortified with BAP (4mg/l) in combination with NAA (1 mg/l) proved best in terms of mean number of shoots (4.5±0.5), mean length of shoots (2.58±0.6cm), minimum number of days (31.66±0.88) taken for direct shoot regeneration and percent (50%) culture response (Fig. 2b). Our results are in accordance with that of Aasim *et al.* (2011) who achieved direct shoot regeneration from seed explants of *Vicia villosa* Roth on MS medium supplemented with Thidiazuron and IBA in combination. Ghosh and Sen (1994) also obtained maximum shoot regeneration from shoot tip explants of *Asparagus cooperi* Baker on MS medium supplemented with BAP in combination with NAA.

Table 11: Effect of PGRs on multiple shoot regeneration from seed explants on MS medium in *P. verticillatum*

MS+ PGRs (mg/l)				Mean number of shoots ± SE	Mean length of shoots (cm) ±SE	Days taken for regeneration±SE	(%) Culture response
BAP	KN	IAA	NAA				
-	2	1	-	2.0±1.0	0.97±0.22	60.66±0.66	30
2	-	1	-	2.0±1.00	1.46±0.28	51.66±0.88	40
4	**-**	**-**	**1**	**4.5±0.50**	**2.58±0.63**	**31.66±0.88**	**50**
4	-	1	-	4.0±1.00	1.9±0.42	46.00±0.57	40

20 replicates per treatment

C. Callus Production from Sprouted Seeds

The *in vitro* sprouted seeds produced callus when subcultured from MS basal medium to MS medium augmented with different concentrations of Kn (5.0, 6.0, 7.0, 8.0, 9.0 mg/l) in combination with IAA (2.0 mg/l) (Table III). Among all these combinations, Kn (8.0 mg/l) + IAA (2.0 mg/l) proved best in terms of number of days (33±0.57) taken for callus production and percent culture response (60%) (Fig. 2c). In case of *Hybanthus enneaspermus* L. Muell, similar results were achieved by Prakash *et al.* (1999) by subculturing the sprouted seeds on MS medium containing auxin and cytokinin in combination. Shah *et al.* (2003) while working on the cereal monocots was successful in achieving similar results in case of *Triticum aestivum* L. by using the above mentioned hormone combination. Bano *et al.* (2005) also achieved callus induction on MS medium fortified with Kn in combination with 2, 4-D from seeds of *Oryza sativa* cv. Swat-II

Table III: Effect of PGR's on callus production from sprouted seeds on MS medium in *P. verticillatum.*

MS + PGRs (mg/l)		No. of days taken for callus induction	%Culture response
KN	IAA		
5	2	72.66±0.66	30
6	2	60.33±0.88	50
7	2	52.66±0.33	50
8	**2**	**33±0.57**	**60**
9	2	40.66±0.66	40

20 replicates per treatment

D. Indirect Rhizome Regeneration

During present study we were successful in regenerating rhizomes *in vitro* and to the best of our knowledge this is the first report of *in vitro* rhizome regeneration from *P. verticillatum*. Callus obtained from sprouted seed explants regenerated rhizomes when sub cultured on MS medium fortified with cytokinins i.e., BAP (1.0-5.0 mg/l) and MS medium augmented with different concentrations of BAP (2.0, 3.0, 4.0, 5.0 mg/l) in combination with NAA (1.0 mg/l) (Table IV). Maximum culture

response (90%) and regeneration rate within 37±0.67 mean no. of days was observed when MS medium was fortified with BAP (4.0 mg/l) in combination with NAA (1.0 mg/l) (Fig. 2d). Our results are in accordance with that of Shimasaki and Uemoto (1991) who also obtained rhizomes from flower buds in *Cymbidium goeringii* on MS medium augmented with BAP and NAA. Chen *et al*. (2005) used immature seeds of *Cymbidium faberi* as explant and obtained rhizomes on MS medium fortified with BAP (5.0 mg/l) + NAA (1.0mg/l). Rout *et al.* (2001) also achieved *in vitro* rhizome formation in *Zingiber officinale* cv. on MS medium augmented with cytokinin (BA) in combination with auxin (IAA) from *in vitro* raised shoots. Similar results of *in vitro* rhizome regeneration in *Curcuma Longa* Linn was achieved by Devi *et al*. (2001) from shoot explants on MS medium fortified with cytokinin (Kn) auxin (NAA) combination.

Table IV: Effect of PGR's on rhizome regeneration from seed callus on MS medium in *P. verticillatum*

MS+PGR's mg/l		Mean no. of days ± SE	% culture response
BAP	NAA		
1	-	72±0.29	20
2	-	68±0.54	40
3	-	52±0.25	50
4	-	60±0.38	60
5	-	55±0.45	60
2	1	50±0.54	60
3	1	48±0.67	80
4	**1**	**37±0.67**	**90**
5	1	42±0.33	70

20 replicates per treatment

E. Shoot Regeneration From In Vitro Raised Rhizomes

In vitro regenerated rhizomes produced shoots when subcultured on MS medium fortified with cytokinin-cytokinin (BAP + Kn) and cytokinin-auxin (Kn + IAA) combination (Table V). MS medium supplemented with Kn (2mg/l) in combination with BAP (2mg/l) produced 15.25±0.92 shoots with mean shoot length of 6.12±0.34cm within 18±0.64 days with 100% culture response (Fig. 2e) and proved best in terms of shoot length, shoot number, percent culture response and minimum no. of days taken for shoot regeneration. The results obtained were better when compared to those obtained during an earlier study carried out by Bisht *et al.* 2012 in which direct shoot regeneration was achieved from stem explants on MS medium fortified with BAP and NAA (1mg/l + 0.5mg/l) with 8.60±0.58 highest numbers of shoots with an average length of 4.66±1.07. In our study, shoot regeneration also occurred on MS medium supplemented with Kn (1.0-5mg/l) in combination with BAP (2mg/l). In addition to these PGR's, shoot regeneration was also effectively induced from rhizomes on MS medium augmented with Kn (4mg/l) + IAA (1mg/l) and Kn (5mg/l) + IAA (1mg/l) with 8.0±0.24 and 3.23± 0.54 mean number of shoots, 3.33±0.52 and 2.66± 0.66cm mean shoot length with culture response of 50 and 40% within 44±0.34 and 48±0.34 days respectively. Chen *et al.* (2005) also initiated shoots from *in vitro* raised rhizomes of *Cymbidium faberi* on MS medium augmented with BAP (5.0mg/l) + NAA (1.0mg/l) in 90% cultures within 60 days.

Table V: Effect of different PGRs on shoot regeneration from *in vitro* raised rhizomes on MS medium in *P. verticillatum*

MS+PGR's mg/l			No. of shoots ± SE	Length of shoots (cm)± SE	Mean no. of days± SE	% Culture Response
BAP	KN	IAA				
2	1	-	7±0.88	3.80±0.63	24±0.29	60
2	**2**	**-**	**15.25±0.92**	**6.12±0.34**	**18±0.64**	**100**
2	3	-	13.25±0.45	5.90±0.73	25±0.83	90
2	4	-	11±0.85	5.20±0.23	28±0.66	80
2	5	-	9.0±0.66	4.00±0.73	36±0.58	70
-	4	1	8.0±0.24	3.36±0.52	44±0.34	50
-	5	1	3.23 ± 0.54	2.66± 0.66	48±0.34	40

20 replicates per treatment.

F. Root regeneration of in vitro raised shoots

For root induction, MS medium fortified with auxins like IAA, IBA and NAA individually and in combination with cytokinins like BAP and Kn were used. Roots with 1.93±0.34 cm mean length regenerated from shoots when inoculated MS medium augmented with IBA (2.0 mg/l) within 26±0.55 days (Fig. 2f) which is in accordance with the results obtained by Karpov (2004) in case of *Yucca aloifolia* L. The results are in agreement with Shekhawat and Ravindran (2015) who also achieved rooting on MS medium supplemented with IBA (2.5mg/l) in *Passiflora foetida.* Similar results of *in vitro* root regeneration were achieved by Shekhawat and Manokari (2015) in *Artemisia absinthium* L. on MS medium fortified with IBA (2.0 mg/l)

G. Acclimatization

In vitro raised plantlets were transferred to plastic pots containing 100 g vermicompost supplemented with PGR's. The *in vitro* raised plantlets were successfully acclimatized under green house conditions and successful hardening was obtained within 3 weeks in vermicompost containing PGRs (IBA (2.0 mg/l), NAA (4.0 mg/l) + BAP (1.0 mg/l), IBA (0.8 mg/l 80% survival was obtained in vermicompost containing BAP (4mg/l) + NAA (1mg/l) (Fig. 2g).

H. Ex Vitro Shoot Regeneration from In Vitro Raised Rhizomes.

The *in vitro* raised rhizomes were fragmented into pieces containing buds and were transferred to disposable cups containing vermicompost (70 g). In order to maintain the humidity, the disposable cups were covered with polythene bags and watered at regular intervals. The rhizomes sprouted successfully within 49 days (Fig. 2h).

Fig. 2: *In vitro* propagation of *P. verticillatum* All. through seed explant

(a) Seed germination on MS basal medium
(b) Multiple shoot regeneration from seed explant on MS medium fortified with BAP (4 mg/l) + NAA (1 mg/l)
(c) Callus induction on MS medium supplemented with Kn (8 mg/l) + IAA (2mg/l)
(d) Rhizome regeneration from seed callus on MS medium fortified with BAP (4 mg/l) + NAA (1 mg/l)
(e) Shoot regeneration from *in vitro* raised rhizomes on MS medium fortified with Kn (2 mg/l) + BAP (2 mg/l)
(f) Root regeneration of shoots on MS medium augmented with IBA (2mg/l)
(g) Acclimatization
(h) *Ex vitro* shoot regeneration from *in vitro* raised rhizomes

CONCLUSION

The purpose of the present study was to develop different conservation strategies for the dwindling population of *Polygonatum verticillatum*. Poor seed germination which further jeopardises the already threatened status of *P. verticillatum* was dealt by setting forth a protocol for the stratification and *in vitro* seed germination where in 90% of seeds geminated within 36±0.57 days. A full developed protocol for the *in vitro* propagation of this medicinal plant was also developed using seed as explant which proved to be a better explant as compared to the stem that was used in an earlier study. The generation of the plantlets directly from the *in vitro* raised rhizomes in just 49 days was reported in this plant for the first time. This holds the promise of a time saving and cost effective conservation strategy as it bypasses the usual method of obtaining plantlets from callus through organogenesis. Last but not the least the *in vitro* regeneration of rhizomes from the callus and the subsequent use of these for the isolation of the bioactive compounds will help to buffer the pressure which the wild population of this plant is experiencing.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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