Direct plant regeneration from *in-vitro* derived nodal explants of *Physochlaina praealta* (G. Don) Miers - a vulnerable plant of medicinal importance

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Abstract: *Physochlaina praealta* is a valuable medicinal plant that grows in the high-altitude, cold desert region of Ladakh, Jammu & Kashmir, India. Being a noval and medicinally strategic, this plant is under severe threat and needs germplasm conservation for its progeny to continue. We developed an efficient and reproducible protocol for *in vitro* regeneration from nodal explants cultured on Murashige and Skoog (MS) medium. To determine the optimal conditions for regeneration and multiple shoot induction, we compared callus induction and organogenesis under different hormone combinations and concentrations of 6-benzylaminopurine (BAP), α-naphthalene acetic acid (NAA)/ Indole acetic acid (IAA), thidiazuran (TDZ) and photoperiod regimes (day length of 12, 16, or 24 h). A maximum of 78% shoot regeneration with 9.46 shoots/explants was achieved on a medium containing MS (half strength) and BAP (10μM) +IAA (5μM) + TDZ (5μM). A day length of 12 h was optimal for multiple shoot induction. After sub-culturing on MS (1/2) + IBA (5 μM), 79% of the regenerated shoots developed into plantlets within four weeks. Seventy-three percent of the regenerated plantlets survived and grew well under greenhouse conditions. Our protocol for regeneration and multiple shoot induction has potential use for germplasm conservation of this vulnerable plant and for research on its medicinally-important active constituents.
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1. Introduction

The depletion of wild plant resources has prompted their conservation and propagation, especially for medicinal plants used as therapeutic aid for healing human diseases. The flora of Kashmir Himalaya is extremely rich and houses a diverse variety of medicinal plants. Medicinal practitioners of Kashmir (*Hakims*) and Ladakh (*Amchis*) frequently use local, medicinal plants for treatment of various ailments. However, the medicinal flora of Ladakh has not received much attention. Most of the medicinal plants in Ladakh, such as *Physochlaina praealta*, face extinction due to over-exploitation for medicinal use and other anthropogenic activities. In the Ladakhi system of medicine, there is a great demand for seeds, flowers, roots, rhizomes, leaves and whole plants of *P. praealta*. Although the annual expenditure on global biodiversity has been estimated to be more than ca. $21.5 billion (Waldron *et al*., 2013), insufficient funding levels are a major obstacle to effective global biodiversity conservation and are likely associated with recent failures to meet the United Nations biodiversity targets. In developing countries like India, where insufficient funding is a problem, the conservation of threatened taxa is a far-reaching goal. Therefore tissue culture technique being a viable and a reliable option to be used for the conservation of threatened taxa at minimum costs in countries like India.

Physochlaina praealta is an herbaceous plant of the family Solanaceae, which in local language is known by the vernacular name‘Laltang.’ The herb grows wild in the Lahaulsub-division of Lahaul and Spiti district of Ladakh, and in western Tibet at an altitude range of 3300-4650m.a.s.l. The species conservation status is vulnerable (IUCN, 2000) and the plant is extensively harvested for the herbal industry. The species has great medicinal value due to the presence of tropane group of alkaloids (Gorinova *et al*., 1999). Its roots and leaves contain hyoscyamine, which is converted into atropine and hyoscine and an excellent source of atropine (Kuvaev and Blinova 1960; Gorinova *et al*., 1999). The leaves are narcotic and possess mydriatic properties, causing pupil dilation. The local people use its leaves for treatment of boils, and its seeds as vermifuge to expel roundworms and as an emetic in bilious attack. Its leaves are also used for the treatment of ulcers (Kaul,

1997). In view of its commercial over-exploitation and difficult domesticated cultivation, there is a need to develop efficient propagation methods. A rapid system of plant production is essential to replenish the dwindling populations and support scientific research into the biochemistry and medicinal efficacy of plants (Zhao *et al*., 2013). Micro-propagation is a well-established vegetative propagation technique used for the rapid production of a large number of pathogen-free and genetically uniform plants in a limited space, which can then be used for commercial production of high-quality, plant-based medicines (Xuet *et al*., 2011a,b; Aquil *et al*., 2009; Canter and Thomas, 2005; Zobayed and Saxena, 2003; Santarem and Astarita, 2003; Murchet *et al.,* 2000). This study is first to report on *in vitro* regeneration of *P. praealta*. The main objective was to develop an efficient *in vitro* micro-propagation protocol that could be used for germplasm conservation and mass propagation of this species. Further plants produced with this protocol could be used for phytochemical characterization of active components, and the genetic manipulation of biosynthetic pathways to enhance its medicinal value.

2. Materials and Methods

Plant material and establishment of mother cultures

Authentic and fresh seeds of *Physochlaina praealta* were procured from the Field Research Laboratory of Defense Research Development Organization, Leh, and Amichi Medicine Research unit (CCRAS) Leh, Ladakh (Jammu & Kashmir) in the months of August and September. Whole plants and seeds were also collected from Leh (3000 m a.s.l), Nubra (3048 m a.s.l), Panikher (Kargil 3750 m a.s.l) and Zanaskar (4400 m a.s.l), which are areas of Ladakh between 34°.13′N and 74°.57′E. Voucher specimens were placed in the Herbarium of Department of Botany, University of Kashmir. Both fresh and chilled seeds were used as explants to initiate *in vitro* cultures. Seeds were washed with detergent (Labolene) and surfactant (Tween-20), followed by rinsing with pre-autoclaved double distilled water (DDW). The chemical sterilization was done by treatment with different concentrations and combinations of NaOCl and $HgCl_2$ (0.1%) for different time intervals. Finally, seeds were thoroughly washed three times with sterilized double-distilled water before inoculation. The seeds were pre-treated with GA_3 (1µM) followed by moist chilling treatment at 5±1°C for 15 days. The seeds were inoculated on the hormone-free MS half (Murashige and Skoog, 1962) medium supplemented with 30 g/L sucrose and 8 g/L agar (w/v). After 28 days of inoculation, the shoots (2 to 3 cm long) that germinated from seeds were cultured on MS half medium supplemented with different concentrations of BAP and NAA/IAA. The shoots were sub-cultured on fresh culture medium every 3 to 4weeks, and these were used as mother cultures for supply of nodal segments.

Explant and Culture conditions

The nodal segments obtained from mother cultures were used as explants for multiple shoot-induction experiments. The explants were cultured on MS half medium containing different concentrations and combinations of BAP, NAA/IAA and TDZ. The pH of the media was adjusted to 5.8 prior to autoclaving (120°C for 20 min), and *in vitro* cultures were maintained at 25±2°C with light intensity of 2500–3000 lux, and at a relative humidity of 65-70%. To determine the effect of day length on the morphogenetic response of nodal explants, we cultured the explants under one of three different photoperiods (day/night =24/0,16/8,or 12/12h).

Rooting and acclimatization

For root induction, micro-shoots (2.0-3.5 cm) were isolated and transferred on half strength MS medium containing 30 g/L sucrose under different concentrations and combinations of IAA/NAA (1, 2,3,4,5,7.5 and 10µM). For acclimatization, rooted plantlets were removed from culture vessels and washed carefully with water to remove the adhering medium. The plantlets were planted in plastic cups containing a sterile mixture of sand, soil and peat (1:1:1), and were covered with Pyrex beakers to maintain 90±5% relative humidity. The plantlets were gradually exposed to ambient atmospheric conditions for 2 weeks, and then transferred to a greenhouse.

Data collection and statistical analysis

All the experiments were conducted with 10 replicates (i.e., n = 10 explants) per treatment and repeated three times (i.e., n = 30 explants tested for each treatment). Data for shoot induction, shoot multiplication, and rooting experiments were recorded after 6 weeks of culture. Data were expressed as a mean ± SE from the three repeated experiments for each treatment. The data were analyzed with analysis of variance (ANOVA) using SPSS 17.0. For multiple comparisons of means, Duncan's multiple range test and standard error (SE) were used at $P \leq 0.05$ to establish significant differences among the treatments.

3. Results

Nodal segments from *in vitro* raised seedling were obtained from the sterilized seeds (Fig. 2A) germinated on MS half strength medium after 4 weeks of culture. Germination percentage of seed were very low, to enhance germination percentage seeds were pre-treated with GA_3 (1µM) followed by moist chilling treatment at 5±1°C for 15 days. Maximum seed germination was recorded to be 56%. Sterilization of seeds with maximum efficiency was developed.

Complete seed sterilization was obtained on $HgCl_2$ (0.1%) for 8 minutes followed by sodium hypochloride (5%) for 5 minutes with 92% sterilization rate and 96% explant survival rate. Explants cultured on MS basal (full strength and without growth regulators) showed no regeneration response, while explants cultured on MS (half strength and without growth regulators) showed shoot regeneration. The addition of plant growth regulators enhanced the shoot regeneration rate and the number of shoots per explant. The percentage of response varied with the type of growth regulator and its concentration, besides the photoperiod used. BAP (3 µM) was the most effective, resulting in 1.53 shoots/explants with an average length 2.12 cm after 4 weeks of culturing at 16-h day length (Table 1; Fig. 2B). IAA, when used alone, failed to show any morphogenetic influence on nodal segments. Amongst a range of TDZ concentrations (1-15µM), the most effective was 7.5 µM, which induced axillary multiple shooting in 80% of explants with an average 8.96 shoots/explant and 4.63cm shoot length at 16-h day length (Fig.2D).However, a better response with 86% *de novo* shoot multiplication, 7.67 shoots/explant and 3.25 cm shoot length, was recorded on MS+ BAP (7µM)+NAA (3µM) (Fig 2C). The combination of BAP, IAA/NAA and TDZ was effective for increasing the shoot number, and the maximum mean number (9.46) of shoots per explants was obtained on BAP(10µM)+IAA/NAA(5µM)+TDZ(5µM) (Fig 2E). A 16-h day length was best for multiple shoot induction from nodal explants on all phytohormonal treatments (Fig. 1).

***In vitro* rooting and plantlet acclimatization**

For the development of complete plantlets, *in vitro* raised micro-shoots of appropriate length (2-3cm) were excised and transferred to rooting medium comprised of half strength MS medium with different concentrations and combinations of IAA and IBA. IBA (5µM) was the best (63% success rate) for root induction, with 9.32 average number of roots per inoculated micro-shoot and 7.73cm root length (Table 2, Fig. 2F-G). The micro-propagated plantlets survival rate was 73%, when they were acclimatized in pots containing peat, soil and sand mixture (1:1:1), and maintained under greenhouse conditions with a temperature of 25±5ºC and a relative humidity of 65±5% (Fig. 2H).

4. Discussion

Growth and *in vitro* regeneration is a complex phenomenon that is influenced by a number of genetic and environmental factors. Each species may have specific requirements that help cells to differentiate (Sen *et al*., 2002). We evaluated the effect of various phytohormones and day length on the *in vitro* propagation of *P. praealta*. Our results showed that seeds of *P. praealta* possess endogenous dormancy, which can only be overcome by moist chilling and GA_3 treatment. Various dormancy-breaking and germination-stimulating treatments have been tried with seeds of species such as *Osmorhiza* (Baskin *et al.,* 1991, 1995) *Ptilianium nuttalli* (Baskin *et al.,* 1999) and *Apium graveolens* (Thomas *et al.,* 1985). Gibberellic acid and moist chilling treatment are effective in many species of Apiaceae such as *Ferula ovina* (Amooaghaie, 2006). In present study, the complete seed sterilization of *P.praealta* was obtained on $HgCl_2$(0.1%) for 8 min followed by sodium hypochloride (5%) for 5 minutes with 92 % sterilization rate and 96% survival rate. Earlier, Joshi and Dhar (2003) has also reported sterilization of seeds achieved by $HgCl_2$ (0.1%) for 10 minutes in *Sassurrea obvallata* (Astreaceae) and 0.1% $HgCl_2$ for 02 minutes was found to be effective in seeds of Gerbera (Altaf *et al*., 2009). Time period and rate of sterilization vary perhaps depending upon many ecological factors to which the plants were exposed before taking the material for research purposes. Shoot multiplication is a function of cytokinin activity, and BAP is crucial for stimulating explant growth and development (Barless and Skene, 1980). Similar to our results, the stimulatory effect of BAP on multiple shoot formation occurs in many medicinal plants like *Aegle marmelos* (Yadav and Singh, 2011a) and *Spilanthes acmella* (Yadav and Singh, 2011b). Our observation of the formation of multiple shoots along with considerable callusing at the basal cut ends of nodal segments in *P. praealta* has also been documented in *Azadirachta indica* (Arora *et al*., 2010). This process may be due to the action of accumulated auxin at the basal cut ends, which stimulates cell proliferation, especially in the presence of cytokinins (Marks and Simpson, 1994).

TDZ, a substituted phenyl urea, was first used for the mechanized harvesting of cotton bolls, and then was later incorporated into tissue culture media as a means of inducing regeneration. TDZ acts as a substitute for both the auxin and cytokinin requirements of organogenesis and somatic embryogenesis in several species (Husaini *et al*., 2008; Murthy *et al*., 1998). Husaini and Abdin (2007) demonstrated that long day length, along with high TDZ concentration, provide a trigger that enables cells to undergo changes in the developmental process and allow them to become competent for embryogenic mode of regeneration. Their results support our observation that TDZ (7.5µM) in combination with a 16-h day length produces the maximum number of shoots per explants (8.9); whereas at a day length of 24or 12h, the same TDZ concentration could only result in 2.2 and 5.6 shoots per explant, respectively.

The *in vitro* photoperiod requirements vary among species. Shoot bud regeneration is highest under 24-h illumination in ginger (Rout and Das 1998) and *Brassica* sp. (Jain *et al*., 1988), whereas it is best under 16-h day length in strawberry and lettuce (Husaini and Abdin, 2007; Kadkade and Seibert, 1977). In our study, a 16-h day length was the most effective in increasing shoot number, which demonstrated the importance of photoperiod on shoot multiplication. The effect of photoperiod on growth and multiplication of *in vitro* produced shoots has also been reported by Senapati and Rout (2008). Photoperiod has been implicated in the regulation of cytokinin levels (Forsline and Langille, 1975), as well as in photo-conversion of phytochromes (Torne *et al*., 1996).

Table 1. Effect of BAP, NAA/IAA, TDZ and their combinations on the morphogenesis of *in vitro* raised nodal explants of *Physochlaina praealta* cultured on MS half strength medium*

Treatments	**Morphogenetic Response**		
	Shoots/explants	**Shoot length(cm)**	**Nature & degree of callusing**
Basal	^{a}1.00	^{cd}3.52±0.23	LCC
BAP(1µM)	^{ab}1.35±0.13	^{ab}2.23±0.22	LBLC
BAP(2µM)	^{ab}1.21±0.34	^{ab}2.15±0.27	LBLC
BAP(3µM)	^{abc}1.53±0.23	^{ab}2.12±0.27	LBLC
BAP(4µM) -BAP(15µM)	-	-	-
NAA(1µM)-NAA (15µM)	-	-	-
TDZ(1µM)- TDZ (4µM)	-	-	-
TDZ(5µM)	^{bc}2.91±0.19	^{cd}3.45±0.23	LGLC(+)
TDZ(7.5µM)	^{gh}8.96±0.35	^{d}4.63±0.27	LGLC(++)
TDZ(10µM)	^{c}3.43±0.16	^{c}3.12±0.22	LGLC(+++)
TDZ(15µM)	-	-	-
BAP(5µM)+NAA(2µM)	^{bc}1.72±0.76	^{ab}2.63±0.19	LPC(+++)
BAP(5µM)+NAA(3µM)	^{abc}1.69±0.26	^{ab}2.66±0.11	LPWC(+++)
BAP(5µM)+NAA(5µM)	-	-	-
BAP(6µM)+NAA(5µM)	-	-	-
BAP(6µM)+NAA(3µM)	^{fg}7.23±0.26	^{abc}2.72±0.25	LPC(+++)
BAP(7µM)+NAA(3µM)	^{fg}7.67±0.43	^{c}3.25±0.64	LPC(+++)
BAP(7µM)+NAA(4µM)	^{ef}6.54±0.25	^{c}2.97±0.48	LPC(+++)
BAP(8µM)+NAA(4µM)	^{c}3.92±0.26	2.83±0.44	LPC(+++)
BAP(9µM)+NAA(4µM)	^{c}3.62±0.36	2.84±0.28	LPC(+++)
BAP(9µM)+NAA(5µM)	^{bc}2.13±0.62	^{ab}2.16±0.26	LPC(+++)
BAP(10µM)+NAA(5µM)	^{cd}4.38±0.22	^{bc}2.91±0.22	LGLC(++)
BAP(15µM)+NAA(10µM	-	-	LGLC(++)
BAP(20µM)+NAA(10µM	-	-	LGLC(++)
BAP(5µM) + TDZ(10µM)	-	-	-
BAP(10µM) + TDZ(20µM)	-	-	-
BAP(15µM) + TDZ(20µM)	-	-	-
BAP(5µM)+NAA(5µM)+TDZ(5µM)	-	-	-
BAP(7µM)+NAA(5µM)+ TDZ(5µM)	^{ef}6.46±0.28	^{ab}2.64±0.23	LPC(+++)
BAP(7µM)+NAA(7µM)+TDZ(5µM	-	-	LPC(+++)
BAP(7µM)+NAA(8µM)+TDZ(10µM)	-	-	LGLC(++)
BAP(7µM)+IAA(5µM)+TDZ(5µM)	-	-	LGLC(++)
BAP(10µM)+IAA(5µM)+TDZ(5µM)	^{h}9.46±0.86	^{cd}3.67±0.67	LGNC(++
BAP(12µM)+NAA(5µM)+TDZ(5µM)	-	-	LGLC(++)
BAP(15µM)+NAA(5µM)+TDZ(10µM	-	-	LGLC(++)

Data was scored after 8 weeks of culture. Values are mean ±SE (n=10), Means within a column having the same letters are not statistically significant Duncan's multiple range test ($P \leq 0.05$). Light Green Nodular Callus=LGNC; Light Green Loose Callus=LGLC; Loose Creamy Callus =LCC; Loose Purplish Callus =LPC; Loose Purplish Regenerative Callus=LBLC; ++++= Intense callus; +++= High callus; Moderate callus; + = Low callus. *The *in vitro* raised explants used were 28-42 days old.

Table 2. Effect of MS (1/2) medium augmented with IBA and/or NAA on root induction of *in vitro* raised micro-shoots of *Physochlaina praealta**

Phytohormones	No. of roots /explant.	Root length(cm)	% rooting	Nature of response
Basal	^{ab}5.22±0.34	^{f}8.72±0.38	89	Indirect
NAA(1µM)	^{abc}5.92±0.33	^{ef}8.53±0.39	87	Indirect
NAA(2µM)	^{abc}5.91±0.21	^{def}8.43±0.36	85	Indirect
NAA(3µM)	^{bc}6.15±0.42	^{cde}7.23±0.26	87	Indirect
NAA(4µM)	^{bc}6.32±0.26	^{cd}6.23±0.34	86	Indirect
NAA(5µM)	^{bcd}6.53±0.25	^{bc}5.92±0.28	86	Indirect
IBA(1µM)	^{abc}5.91±0.41	^{cde}7.34±0.25	88	Indirect
IBA(2µM)	^{bcd}6.45±0.27	^{cd}6.54±0.23	88	Indirect
IBA(3µM)	^{d}6.74±0.35	^{cd}6.21±0.37	86	Indirect
IBA(4µM)	^{ef}8.12±0.28	^{cd}6.15±0.31	86	Indirect
IBA(5µM)	^{f}9.32±0.38	^{bc}5.73±0.42	63	Direct
IBA(5µM)+NAA (5µM)	^{ab}5.24±0.43	^{a}4.32±0.43	41	Indirect

Data was recorded after 8 weeks of culture period. Values are Mean±SE (n=10). Means within a column having the same letters are not statistically significant Duncan's multiple range test ($P \leq 0.05$). *The *in vitro* raised plantlets were 4-6 weeks old.

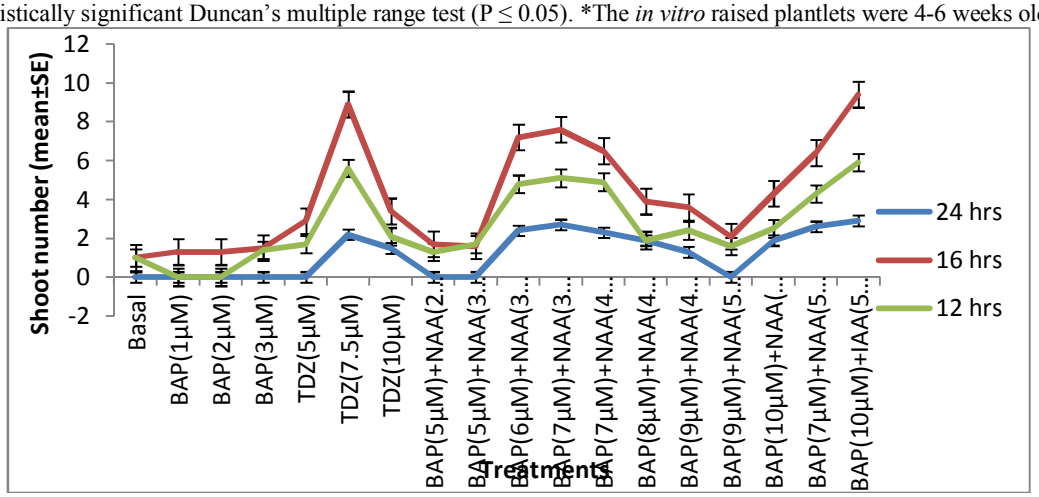

Fig. 1. Interactive effect of growth regulators (BAP, NAA/IAA, TDZ) and photoperiod (24, 16, or 12-h day length) on multiple shoot induction (number of shoots developed per nodal segment) in *Physochlaina praealta.*

Fig 2. Various stages during regeneration and propagation of *Physochlaina praealta.* (A) Seed germinated on MS (1/2) pre-treated with GA_3 (1µM) followed by moist chilling treatment at 5±1°C for 15days;(B)Multiple shooting from callus [1/2 MS+BAP (3 µM)]; (C) Multiple shooting with BAP(7µM)+NAA(3µM);(D)Auxiliary multiple shooting with TDZ (7.5µM); (E) Multiple shooting with BAP(10µM)+IAA(5µM)+TDZ(5µM); (E-F) Rooting with IBA(5µM); (G) Acclimatization of *in vitro* plants.

IBA and NAA induce effective rooting in *Lupinu stexensis* and *Lupinus mutabilis* (Tyub *et al.,* 2005). Similarly, we found that IBA and NAA successfully induced rooting of the *in vitro* raised shoots, while IBA was better for inducing rooting in the isolated shoots. Tyub *et al.,* 2005) also obtained the best rooting in *Lupinus polyphyllus* with IBA.

Conclusions

The present study revealed that TDZ alone is effective for inducing axillary shoot formation, and BAP in combination with NAA is responsible for direct shoot multiplication. MS half strength medium supplemented with BAP (10 µM) + TDZ (5 µM) + IAA (5 µM) is the best combination for shoot multiplication from nodal explants. For root induction of isolated shoots, IBA (5 µM) is the most suitable. With these phytohormonal treatments, a 16-h day length is best for *in vitro* shoot multiplication. This protocol for *in vitro* plant regeneration of *P. praealta* from nodal explants could be used for large scale multiplication and conservation of this medicinally-important, threatened plant.

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