INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
12–628/2013/15–4–755–760
http://www.fspublishers.org

*Full Length Article*

# High Frequency Multiple Shoot Formation of Pygmy Groundcherry (*Physalis minima*): An Endangered Medicinal Plant

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## Abstract

Nodal explants from field grown plants were cultured on Murashige and Skoog (MS) medium + 0.1, 0.5, 1.0, 2.0, 3.0, 4.0 or 8.0 µM of $N^6$-benzyladenine (BA) or Kinetin (Kin) alone or in combination with indole-3-butyric acid (IBA: 0.5, 1.0 or 2.0 µM) for micropropagation of *Physalis minima*. The highest (78.3%) number (6.0) and long axillary shoots (3.3 cm) were produced at 0.5 µM BA after 10 days. Nodal explants from these *in vitro* shoots were further cultured that resulted in 1.6 mean shoots with BA. In subsequent experiment, shoot tips from formerly sub-cultured shoots on BA (4.0 µM) + IBA (2.0 µM) formed 40 shoots with 6.1 cm length on MS liquid basal medium, after 20 days. Shoots were rooted on ½ MS + IBA or α-naphthalene acetic acid (NAA) at 0.1, 0.2, 0.5, 1.0 or 2.0 µM alone or in combinations. The highest rooting (100%) with more than 50 roots per shoot were obtained at 1.0 µM IBA after 15 days. Rooted shoots were acclimatized for 4 weeks in greenhouse with 90% survival. Efficient and simple method using liquid basal medium has been established for high frequency multiple shoot formation with fair survival rate in the field. 

**Keywords:** 6-Benzyladenine; Endangered species; Liquid medium; Multiple shoots; *Physalis minima*

## Introduction

*Physalis minima* is an important medicinal plant of family Solanaceae (Sheeba *et al*., 2010). It is a small herbaceous annual herb grown as weed in crop fields (Nayeemulla *et al*., 2006). This species is used medicinally as tonic, diuretic, laxative, anti-inflammatory, Splenomegaly, and as a helpful remedy in ulceration of the bladder (Mungole *et al*., 2011). The leaves are crushed and applied over snakebite (Karthikeyani and Janardhanan, 2003). Plant extract has anti-cancer and anti-microbial activity (Duke and Ayensu, 1985; Nayeemulla *et al*., 2006). Mixture of mustard oil and water along with leaf extracts of *P. minima* has been used as a remedy for earache (Chopra *et al*., 1986). Pietro *et al*. (2000) reported that plant extract containing physalins display anti-mycobacterial activity against *Mycobacterium tuberculosis*, *M. avium*, *M. kansii*, *M. malmoense* and *M. intracellulare*.

Although the plant has immense medicinal value, it is becoming endangered due to over-exploitation and continuous increase in environmental pollution. Therefore, rapid multiplication of this important drug yielding plant is becoming imperative. The capability to regenerate and propagate plants from cultured cells and tissues is one of the most exciting and useful aspects of *in vitro* cell and tissue culture. Micropropagation offers a great potential for conservation and large scale multiplication of such useful species (Usman *et al*., 2008; Afroz *et al*., 2009).

Very few reports regarding the micropropagation studies of *P. minima* have been found in the contemporary literature. Thus, the main objective of the present study was to utilize this technology for mass propagation of this medicinally endangered plant by developing a reliable protocol for large scale propagation and *in vitro* conservation of *P. minima*.

## Materials and Methods

### Source Material and Culture Conditions

Field grown plants of *P. minima* were collected from Botanical Garden, University of the Punjab, Lahore, Pakistan, in the month of October. Nodal explants (3-4 cm length) were excised and washed under running tap water in glass jars for 10 min to remove dust particles followed by washing with 1% (w/v) detergent for 5-10 min. Explants were then treated with 10% solution of sodium hypochlorite (NaOCl) with one drop of Tween-20 for 7-8 min. Finally explants were rinsed 3-5 times with pre-autoclaved distilled water and inoculated in culture tubes (25 x 150 mm) each containing 10 mL MS (Murashige and Skoog, 1962) agar-solidified (MERCK; 0.8%) medium supplemented with 3% sucrose. The pH of the medium was adjusted to 5.8 before autoclaving at 121°C (103.42 KPa) for 20 min. Cultures were incubated in 16 h photoperiod (35 µmol $m^{-2}$ $s^{-1}$) using cool, white fluorescent tube lights at 25±2°C.

**To cite this paper:** Intzaar, S., M. Akram and H. Afrasiab, 2013. High frequency multiple shoot formation of pygmy groundcherry (*Physalis minima*): An endangered medicinal plant. *Int. J. Agric. Biol*., 15: 755–760

**Axillary Shoot Proliferation**

Dead tissues on both sides of nodal explants were trimmed and 10-20 mm long explants were inoculated in culture tubes (25 x 150 mm) on MS basal medium supplemented with $N^6$-benzyladenine (BA) or Kinetin (Kin: 0.1, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 µM) alone or in combination with Indole-3-butyric acid (IBA; 0.5, 1.0, 2.0 µM) for axillary shoot induction. Culture tubes were capped and incubated under the above mentioned culture conditions. Cultured explants were observed regularly and data were recorded for shoot induction percentage, number and length of shoots per explant (one explant per culture tube) after 10 days.

**Sub-culturing of *In Vitro* Shoots**

*In vitro* grown axillary shoots were sub-cultured and 2.0 cm long nodal explants were prepared for further shoot initiation and proliferation on the same medium composition as mentioned previously. The data were recorded for number of explants forming shoot, number of shoots and shoot length per explant (one explant per culture tube) for 10 days.

**Multiple Shoot Formation and Long Term Maintenance**

Shoot tips ~3.0 cm long from *in vitro* sub-cultured shoots were inoculated on MS basal liquid medium (a disc of cotton swab was immersed to support the explants) without plant growth regulators (PGRs) in glass jars (55 × 125 mm) for multiple shoot formation. Initially the data for number of multiple shoots and shoot length were recorded after 20 days, and then shoot cultures were further maintained up to 80 days by regular sub-culturing after every 30 days on MS basal liquid medium.

***In Vitro* Root Induction and Acclimatization**

*In vitro* maintained shoots (2.0 cm length) were rooted on half strength MS medium supplemented with different concentrations of either IBA, NAA or IAA (0.1, 0.2, 0.5, 1.0, 2.0 µM). After 15 days, data were recorded for percent root induction, number and length of roots per shoot (one explant was inoculated per culture tube). Rooted shoots were taken out from the culture vessels and washed with distilled water to remove agar and transferred to plastic pots filled with mixture of peat moss and sand (1:1) and then placed in greenhouse at 25 ± 5°C. The potted plants were covered with polythene bags to maintain relative humidity and irrigated regularly with ¼ strength MS salts without organics. After 4 weeks, the data for plant survival (%) were recorded and then plants were shifted to soil under natural conditions.

**Data Analysis**

Each treatment contained 10 explants (replicates) and experiment was repeated thrice. Experimental units were arranged using completely randomized design. Data were subjected to analysis of variance (ANOVA). Tukey's HSD was used following ANOVA ($p \leq 0.05$) for statistically significant main effects. Regression analysis ($R^2$) was performed to test significant correlation between factors. The statistical analyses for this study were performed using PASW Statistics 18.

## Results

**Shoot Induction and Proliferation**

*In vitro* shoot induction from nodal explants of *P. minima* on MS medium supplemented with various concentrations of BA or Kin alone or in combination with different concentrations of IBA is shown in Table 1. Generally, the growth of shoots was comparatively good and quite significant results were found with BA and/or IBA. Highest shoot induction (78.3%) with 6.0 mean number and 3.3 cm shoot length was obtained at 0.5 µM BA after 10 days of initial culture (Fig. 1A). Regression analysis indicates moderate correlation between shoot induction ($R^2$= *0.267*; Fig. 2A) and shoot length ($R^2$ = *0.2802*; Fig. 2B) with plant growth regulators (PGRs) whereas least correlation was found with less number of shoots ($R^2$ = *0.0856*; Fig. 2C) induced from field grown plants of *P. minima*. Nodal explants from *in vitro* proliferating shoots were sub-cultured to form multiple shoots on the same treatments. The shoots were quite vigorous (Fig. 1B) with 40% shoot induction (Fig. 3A) after 10 days of initial culture. The number of shoots was highest (1.6) with BA compared with Kin (0.3) alone or in combination with IBA. Similarly, shoot length was also observed highest on the same treatment. There was significant interaction between PGRs and shoot induction ($R^2$ = *0.4769*; Fig. 3A) and number of shoots ($R^2$ = *0.5097*; Fig. 3B), whereas, strong correlation was observed with shoot length ($R^2$ = *0.6671*; Fig. 3C).

**Multiple Shoot Formation and Long Term Maintenance**

A vigorous growth was observed in the beginning at 2.0 µM BA with multiple shoots formation (Fig. 1C, D). However, with the passage of time, growth of these cultures stopped and subsequently glassy appearance was observed in the cultures. To overcome this problem, shoot tips were cultured on MS liquid basal medium in glass jars (Fig. 1E) and maintained under the same culture conditions. The development of shoots was quite promising formerly grown at 4.0 µM BA and 2.0 µM IBA (Table 2). Generally, the number of shoots increased with the increase in pre-cultured BA levels and surprisingly 40 shoots with 6.1 cm length were obtained at 4.0 µM BA in combination with 2.0 µM IBA (Fig. 1E). These cultures were maintained for more than 8 months without any abnormality. We have been able to achieve good results on subsequent liquid medium forming an average of 40 shoots following *ex vitro* establishment in the field.


**Fig. 1:** *In vitro* shoot induction, proliferation and acclimatization from nodal explants of field grown plants of *P. minima* on MS basal medium supplemented with various plant growth regulators (PGRs). **A**) Development of axillary shoots after 10 days. **B**) Shoot proliferation from nodal explants of *in vitro* shoots on MS medium supplemented with BA (2.0 µM) after 10 days. **C, D**) Multiple shoot formation from *in vitro* derived nodal explants at 2.0 µM BA. **E**) Developmental stages of multiple shoot formation from sub-cultured *in vitro* shoots on MS basal liquid medium pre-cultured at BA (4 µM) + IBA (2 µM) after 20 days. **F).** Root induction on ½ MS + IBA (2.0 µM) after 10 days of culture. **G**) Rooted plantlet was initially covered with polythene. **H**) Vigorously growing potted plant in open environment after 10 days of transplantation. **I)** Flowers (dotted red circles) and fruits (complete circle) formation on 45 day-old plants in the pots in green house

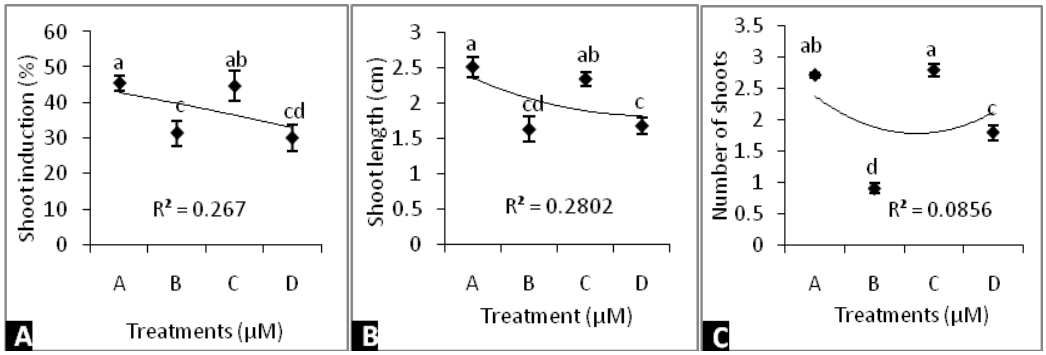


**Fig. 2:** Individual and combined effect of different plant growth regulators (PGRs) on *in vitro* shoot formation from nodal explants of field grown plants of *P. minima*. Treatments: A = BA, B = Kin, C = BA + IBA, D = Kin + IBA. The data were recorded after 10 days of initial culture. Vertical bars are ±SE, and over different letters indicate significantly different results according to Tukey's multiple comparison test ($p<0.05$). Smooth line indicates trend line of polynomial regression analysis

**Table 1:** Effect of different plant growth regulators on *in vitro* shoot formation from nodal explants derived from field grown plants of *P. minima*

| PGRs (µM) | | | Shoot induction (%) | Number of shoots | Shoot length (cm) |
|---|---|---|---|---|---|
| BA | Kin | IBA | | | |
| 0.1 | | | 25.0±2.80$^{defgh}$ | 0.9±0.20$^{ghi}$ | 1.9±0.08$^{abc}$ |
| 0.5 | | | 78.3±4.40$^{a}$ | 6.0±0.26$^{efghi}$ | 3.3±0.31$^{abc}$ |
| 1 | | | 55.0±2.80$^{abcde}$ | 3.0±0.27$^{bcde}$ | 3.4±0.63$^{ab}$ |
| 2 | | | 40.0±5.70$^{bcdefg}$ | 1.5±0.26$^{efghi}$ | 1.1±0.60$^{abc}$ |
| 3 | | | 65.0±2.80$^{abc}$ | 4.0±0.08$^{abc}$ | 4.2±1.40$^{a}$ |
| 4 | | | 19.3±4.71$^{efgh}$ | 4.6±0.33$^{ab}$ | 2.2±0.30$^{abc}$ |
| 8 | | | 36.0±6.31$^{cdefgh}$ | 3.3±0.66$^{bcde}$ | 1.3±0.33$^{abc}$ |
| | 0.1 | | 0$^{h}$ | 00$^{i}$ | 0$^{c}$ |
| | 0.5 | | 0$^{h}$ | 00$^{i}$ | 0$^{c}$ |
| | 1 | | 35.0±2.82$^{cdefgh}$ | 1.2±0.08$^{ghi}$ | 2.8±0.41$^{abc}$ |
| | 2 | | 55.0±2.91$^{abcde}$ | 1.9±0.23$^{defgh}$ | 2.3±0.14$^{abc}$ |
| | 3 | | 74.0±2.90$^{ab}$ | 1.9±0.26$^{defgh}$ | 3.5±0.28$^{ab}$ |
| | 4 | | 24.3±6.90$^{defgh}$ | 0.6±0.17$^{hi}$ | 1.5±0.28$^{abc}$ |
| | 8 | | 30.6±7.20$^{cdefgh}$ | 0.7±0.14$^{hi}$ | 1.4±0.23$^{abc}$ |
| 0.1 | | 0.5 | 75.0±2.81$^{ab}$ | 5.3±0.33$^{a}$ | 2.3±0.60$^{abc}$ |
| 0.5 | | 0.5 | 60.0±11.51$^{abcd}$ | 4.0±0.57$^{abc}$ | 2.9±0.68$^{abc}$ |
| 1 | | 1 | 50.0±11.51$^{abcdef}$ | 2.9±0.37$^{cdef}$ | 3.5±0.08$^{ab}$ |
| 2 | | 1 | 25.0±2.82$^{defgh}$ | 3.0±0.03$^{bcde}$ | 2.6±0.34$^{abc}$ |
| 3 | | 2 | 55.0±8.62$^{abcde}$ | 3.0±0.03$^{bcde}$ | 1.5±0.29$^{abc}$ |
| 4 | | 2 | 31.3±5.71$^{cdefgh}$ | 0.9±0.20$^{ghi}$ | 1.0±0.14$^{abc}$ |
| 8 | | 2 | 16.3±3.21$^{fgh}$ | 0.6±0.17$^{hi}$ | 2.7±1.60$^{abc}$ |
| | 0.1 | 0.5 | 40.0±5.72$^{bcdefg}$ | 2.0±0.05$^{defgh}$ | 2.1±0.10$^{abc}$ |
| | 0.5 | 0.5 | 15.0±2.81$^{fgh}$ | 2.5±0.28$^{cdefg}$ | 1.8±0.18$^{abc}$ |
| | 1 | 1 | 25.0±2.80$^{defgh}$ | 1.2±0.15ghi | 1.0±0.31$^{abc}$ |
| | 2 | 1 | 66.6±10.51$^{abc}$ | 2.1±0.44$^{defgh}$ | 2.7±0.44$^{abc}$ |
| | 3 | 2 | 12.6±3.33$^{gh}$ | 2.9±0.48$^{cdef}$ | 2.4±0.53$^{abc}$ |
| | 4 | 2 | 14.0±1.55$^{fgh}$ | 1.0±0.08$^{ghi}$ | 1.1±0.49$^{abc}$ |
| | 8 | 2 | 15.0±5.55$^{fgh}$ | 1.0±0.57$^{ghi}$ | 0.6±0.66$^{bc}$ |

Data were recorded after 10 days of initial culture
Each value is the mean of 10 replicates and experiment was repeated thrice
Mean values (±SE) followed by different letters are significantly different in columns according to Tukey's multiple comparison test ($p<0.05$)

**Table 2:** Multiple shoot formation on MS basal liquid medium in glass jars from *in vitro* pre-cultured shoot tip explants of *P. minima*

| Pre-culture media (µM) | | Number of shoots | Length of shoots (cm) |
|---|---|---|---|
| BAP | IBA | | |
| 0.1 | | 12.0 ± 1.52$^{b}$ | 10.3 ± 4.9$^{a}$ |
| 0.5 | | 21.6 ± 0.88$^{b}$ | 12.4 ± 4.2$^{a}$ |
| 1 | | 12.6 ± 0.88$^{b}$ | 04.8 ± 1.8$^{a}$ |
| 2 | | 13.6 ± 1.20$^{b}$ | 06.0 ± 2.2$^{a}$ |
| 3 | 1 | 14.0 ± 2.00$^{b}$ | 07.3 ± 2.6$^{a}$ |
| 4 | 2 | 40.0 ± 5.13$^{a}$ | 06.1 ± 2.5$^{a}$ |
| Polynomial Contrast ($p\leq 0.05$) | | | |
| Linear | | <0.0001 | 0.216 |
| Quadratic | | <0.0001 | 0.531 |

Data was recorded after 20 days of initial culture
Mean values followed by same letters are not significantly different according to Tukey's multiple comparison test ($p<0.05$)
Each value is the mean of 10 replicates and experiment was repeated thrice

### *In Vitro* Rooting

For root induction, shoot tips of 2.0 cm length from such maintained cultures were inoculated on MS medium supplemented with IBA, NAA or IAA. Highest rooting (95%) was obtained with 80 mean number of roots and 80 mm length at 0.2 µM IBA after 15 days of inoculation (Fig. 1F, Fig. 4). Similarly, number (80) as well as length (80 mm) of roots was also highest at 0.5 or 1.0 µM of either IBA or NAA, respectively. After 4 weeks, rooted shoots were transferred to plastic pots filled with sand and peat moss (1:1), covered with polythene bags and then placed in greenhouse (Fig. 1G). After 10 days of covering with polythene, plants adjusted themselves at low humidity level in the open environment (Fig. 1H). About 90% of the transplanted plants of *P. minima* survived and produced flowers and pods (fruits) after six weeks (Fig. 1I). During the hardening period length and rigidity of shoots, diameter of leaves increased and regenerated plants showed no morphological variations as compared to normal plants (Fig. 1I).

## Discussion

Medicinal plants play a vital role in pharmaceutical industries. A significant number of compounds used in medicines have been reported from wildly growing herbaceous plant species. *P. minima* is a wild species becoming rare due to its wide spread medicinal usage and over exploitation. It is possible to conserve the local flora from being endangered by using some proper measures (Ashraf and Akram, 2009; Gilani *et al*., 2009). Plant tissue culture technology offers excellent opportunity for conservation of economically important plant species. Shoot induction, proliferation and maintenance for longer period of time is governed by different PGRs and culture conditions (Valizadeh and Valizadeh, 2011).

In the present study, nodal explants cultured in MS medium supplemented with 0.5 µM BA produced 6.0 mean numbers of shoots with 3.3 cm length per explant. In the subsequent experiment, shoot tips from these *in vitro* produced shoots were further sub-cultured in MS liquid medium supplemented with different concentrations of BA and IBA. Maximum number of shoots (40 shoots/explant) with mean shoot length of 6.1 cm were produced in MS liquid medium supplemented with BA 4.0 µM with 2.0 µM IBA. Micropropagation is a technique being used for rapid multiplication of many endangered species. In this technique efforts are directed for obtaining maximum number of shoots from minimum number of shoot explants. In the reported literature for shoot multiplication in *P. minima*, Afroz *et al.* (2009) reported shoot regeneration using BA and coconut water and obtained 95% highest shoot regeneration with 32 mean numbers of shoots on MS medium from nodal explants. Sheeba *et al*. (2010) obtained 84% direct shoot regeneration with 19 mean number of shoots on MS medium supplemented with 2.0 µM BA. In comparison with the results obtained by these workers, we demonstrated an efficient micropropagation procedure with high frequency of shoot formation (40) with excellent proliferation and normal growth using a combination of auxin and cytokinin.

A combination of auxins with cytokinins has been

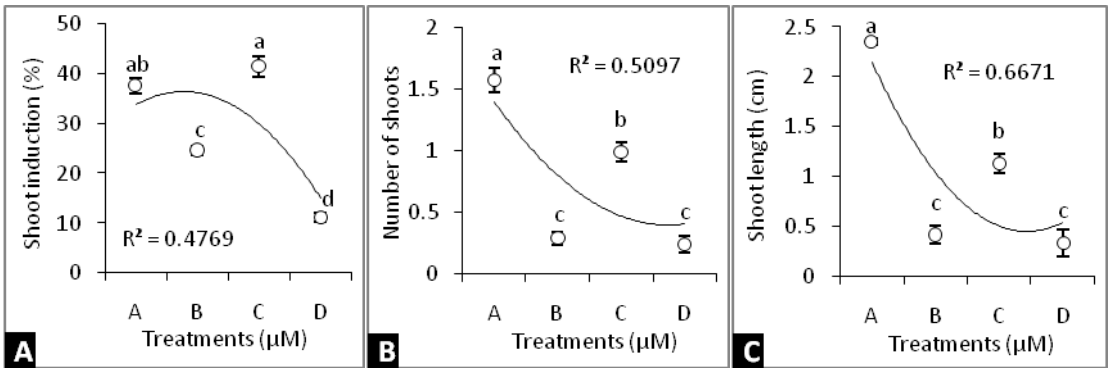


**Fig. 3:** Individual and combined effect of different plant growth regulators (PGRs) on shoot proliferation from nodal explants of *in vitro* grown shoots of *P. minima*. Treatments: A = BA, B = Kin, C = BA + IBA, D=Kin + IBA. The data were recorded after 10 days of initial culture. Vertical bars are ±SE, and over different letters indicate significantly different results according to Tukey's multiple comparison test ($p<0.05$). Smooth line indicates trend line of polynomial regression analysis

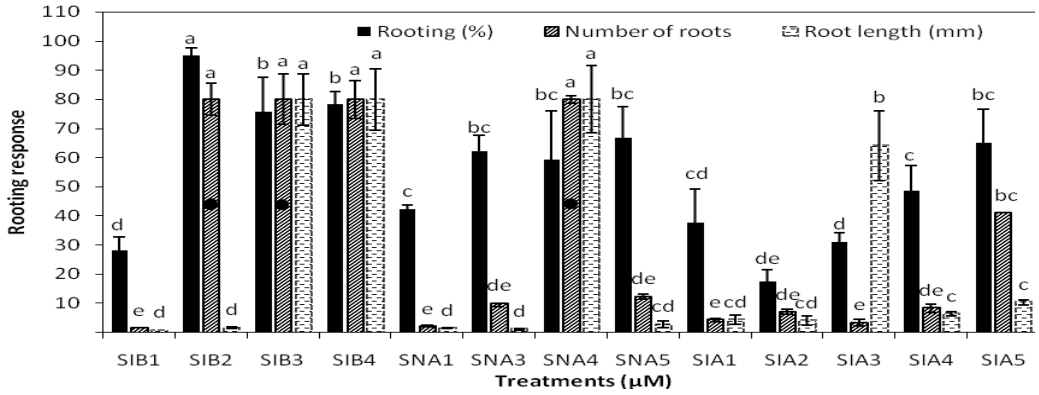


**Fig. 4:** Effect of auxin treatments on *in vitro* rooting of *P. minima*. Treatments are the media codes of five different concentrations (0.1, 0.2, 0.5, 1 or 2 µM) each of IBA (SIB1-4), NAA (SNA1-5) or IAA (SIA1-5). Non-responding experiments were deleted before analysis. Vertical bars over the columns are ±SE and different letters indicate non-significant results according to Tukey's multiple comparison test ($p<0.05$). Each value is the mean of 10 replicates and experiment was repeated thrice. Data were recorded after 15 days of initial culture. Filled circles in the column indicate similar results

reported by many workers for shoot multiplication in different species. Sivansean and Murugesan (2008) obtained multiple shoot production in *Withania somnifera* on MS medium supplemented with 1.5 mg $L^{-1}$ each of BAP and IAA. Similarly, Saritha and Naidu (2007) reported BA (2.0 mg $L^{-1}$) with 0.1 mg $L^{-1}$ NAA to be optimum for multiple shoot formation from nodal explants of *W. somnifera*. In contrast, our results demonstrated more number of shoots on subsequent liquid medium followed by *ex vitro* establishment under the field conditions.

In the present study, few *in vitro* shoot cultures at multiplication stage produced numerous hairy roots like structures in glass jars on liquid medium. However, such shoots did not survive on subsequent transfer to glasshouse conditions. Hairy roots have been reported as a potential source of physalins production (Azlan *et al.*, 2002). In our study, inclusion of auxins (IBA, NAA or IAA) in half strength MS agar medium improved the growth and vigor of roots. Auxin IBA at 0.2 µM improved rooting (95%) with 80 mean number of roots. Similarly, NAA also gave significant *in vitro* rooting percentage of *P. minima*. Afroz *et al*. (2009) reported 37.50 mean number of roots in half strength MS medium supplemented with 0.3 mg/l (16.11 µM) NAA within 15 days of culture of *P. minima*. The effectiveness of IBA for *in vitro* root induction in *W. coagulans* has been reported by Valezadeh and Valezadeh

(2011). Our results are different from these reports in terms of early root induction, large number of roots, vigorous growth and lowest mortality rate under the hardening conditions. Generally, IBA has widely been used as rooting substance to propagate various plants. It is stable and has been reported to increase endogenous level of IAA in stem cuttings of *Arabidopsis thaliana* for improved growth of roots (Kurepin *et al*., 2011).

In our work, *in vitro* grown plants were shifted to glass house for hardening and acclimatization and then to greenhouse where they produced flowers and fruits. Such results have not been previously reported in this species. So, we demonstrated a complete and efficient *in vitro* propagation system for the first time in *P. minima*.

In conclusion, nodal explants from field grown plants were excellent source material for highest shoot induction (78.3%) on MS agar-solidified medium supplemented with 0.5 µM BA after 10 days of inoculation. Rapid multiplication and long term maintenance of *in vitro* shoots was obtained on MS basal liquid medium from pre-cultured shoot tips at BA (4.0 µM) + IBA (2.0 µM). Rooting was significant with IBA and 100% shoots were acclimatized that were established to reproductive stage in the field. A reliable protocol for medicinally endangered species of *P. minima* has been established for the conservation and mass scale propagation that can be used for isolation of medicinally important metabolites for use in BioPharma.

## Acknowledgements

University of the Punjab, Lahore, Pakistan is gratefully acknowledged for financial assistance for this study.

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**(Received 07 August 2012; Accepted 22 February 2013)**