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*Full Length Research Paper*

# Adventitious shoot regeneration from leaf explants of the valuable medicinal herb *Plectranthus barbatus* Andrews

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**The objective of this study was to develop an efficient protocol for adventitious shoot regeneration for *Plectranthus barbatus* Andrews using leaf explants. The explants were cultured on MS (Murashige and Skoog, 1962) medium containing various concentration of kinetin (KN), 6-benzylaminopurine (BAP) and thidiazuron (TDZ) (0.5 to 3.0 mg/l) or in combination with α-naphthalene acetic acid (NAA) for shoot development. The highest adventitious shoot regeneration percentage (80%) and average maximum number of 19.7 ± 2.08 shoots per explants was observed on MS medium supplemented with 1.5 mg/l KN followed by 2.0 mg/l BAP with 1.0 mg/l NAA (15.0 ± 2.20 shoots per explants). The shoots were excised and the residual explants were transferred to fresh medium where again they developed shoots. The shoots were elongated when they were cultured on half strength MS medium supplemented with 0.6 mg/l gibberellic acid ($GA_3$) and 0.5 mg/l KN. The *in vitro* developed shoots produced roots when transferred to half strength MS medium augmented with 1.5 mg/l indole-3-butyric acid (IBA). The *in vitro* raised plantlets were successfully transferred to mixture of river sand, garden soil and saw dust (1:1:1) and was hardened in controlled environment. Hardened plantlets were transferred to soil in greenhouse.**

**Key words:** *Plectranthus barbatus,* Lamiaceae, medicinal plant, leaf explants, direct organogenesis, *in vitro*, plant growth regulators.

## INTRODUCTION

*Plectranthus barbatus* (Lamiaceae) is an aromatic, erect herb, from a perennial rootstock distributed in tropical east Africa, West Asia, Himalaya, India and Srilanka (Matthew, 1983). The plant is used to treat a wide range of diseases and accounts for about 68% of all the traditional uses (Lukhoba et al., 2006), which include purgative and nausea (Hamill et al., 2003), abdominal pain, burns, wounds, sores, insect bites, allergies, measles, to relieve colds and coughs (Baerts and Lehmann, 1989) and for general respiratory ailments (Van Puyvelde et al., 1994). The leaves of the plant when burned a little and placed on the skin of the neck relieve stiffness and it is also used in the treatment of bone dislocations (Baerts and Lehmann, 1989). The plant has cytotoxic and anti-tumor promoting activity and can be used in the treatment of cancer and it also has been used against snakebites in India (Schanberg and Ikan, 2003). The whole plant is said to have antibacterial, anti-viral and antifungal activity (Boily and Van Puyvelde, 1986). Natural populations of *P. barbatus* are rapidly disappearing and now it is one of the plant species in India vulnerable to extinction (Reddy et al., 2001). Though the species can be multiplied by seeds and by stem cuttings, there are problem of poor seed viability, low seed germination rate and scanty and delayed rooting of stem cuttings. There are only a few reports on this plant for rapid multiplication, prompting the authors for attempting to propagate plants from leaf explants under *in vitro*

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conditions. Therefore, there is a need to develop a means for rapid regeneration of plantlets.

Micropropagation constitutes a powerful tool for *ex situ* conservation programs of the rich flora, especially for species with reduced populations or low seed production. This technique facilitates the rapid establishment of a large number of stock plants from a minimum impact on the endangered wild population. Therefore, the interest in using these techniques for rapid and large-scale propagation of medicinal and aromatic plants has been significantly increased (Dode et al., 2003). There are a number of reports for the *in vitro* propagation of medicinal plants which includes *Coleus forskohlii* (Reddy et al., 2001), *Ocimum gratissimum* (Gopi et al., 2006) and *Salvia africana-lutea* (Makunga and Staden, 2008).

In recent years there has been an increased interest in *in vitro* culture techniques which offer a viable tool for mass multiplication and germplasm conservation of rare, endangered and threatened medicinal plants (Tiwari et al., 2000). There are reports of adventitious shoot regeneration through direct organogenesis using leaf explants of medicinally important species like *Pothomorphe umbellata* (Pereira et al., 2000), *Tagetes erecta*, *Echinacea purpurea* (Koroch et al., 2002) and *Achras supota* (Purohit et al., 2004). Direct plant regeneration from leaf explant of *Enicostemma axillare* was obtained using different concentrations of BAP with stable concentration of KN (Jeyachandran et al., 2005). Tissue culture techniques are being used globally for the *ex situ* conservation of the plants; reports of the *in vitro* plant regeneration from the tissue of medicinal plants, *Dendrobium candidum* (Zhao et al., 2007), *Ophiorrhiza prostrata* (Martin et al., 2008) and *Phaseolus vulgaris* (Kwapata et al., 2010) are available. Conservation of endangered medicinal plants has also been achieved through cell cultures with significance. Reports of the *in vitro* plant regeneration from tissues of medicinal plants are available (Gopi et al., 2006; Faisal et al., 2007; Divya et al., 2008). The objective of this paper was to describe a simple protocol for the rapid and recurrent propagation of *P. barbatus* through high frequency adventitious shoot regeneration from leaf explants.

## MATERIALS AND METHODS

### Source of explants

*P. barbatus* from natural habitats were collected from Pambar shola in the Palni hills of the Western Ghats. The plants were identified and confirmed at the Rapinat Herbarium (RHT). The plants were established and maintained in the greenhouse of the Rapinat Herbarium and Center for Molecular Systematics, St. Joseph's College, Tiruchirappalli, South India. Non meristematic explants like the first fully expanded and second leaf over 1.0 cm long from apical buds were used for the regeneration in the investigation. The explants were excised with sterile blade and were collected in a beaker. The excised explants were thoroughly washed with running tap water for 10 to 15 min. Thereafter, the explants were washed with detergent (Teepol 5% v/v) solution for 3 min, fungicide (Bavistine 2% w/v) treatment for 2 min then, were soaked in 70% (v/v) ethanol for 30 s, finally, were disinfected with 0.1% (w/v) $HgCl_2$ for 2 min and were rinsed with sterile distilled water five times.

### Inoculation of explants

The leaf explants were inoculated in such a way that either the adaxial or abaxial surface touched the agar stands of the culture tubes on the medium containing the different concentrations with the combination of growth regulators. By means of a long stainless steel forceps, one explant per tube was placed. It was a routine process to flame the mouth of the test tube after uncapping and before recapping the tubes to reduce contamination. All of the experiments were conducted with a minimum of 7 replicates per treatment and each experiment was repeated five times (7×5 = 35 explants were totally cultured for observation). All the cultures were maintained at 25 ± 2°C under 16/8 h light/dark condition of 80 $\mu Ems\text{-}2s^{-1}$ irradiance provided by fluorescent lamps (TL 40W/54 cool-day/light). *In vitro* response of the inoculated explants was assessed every week in the culture by counting the proliferated shoots which were 2.0 cm in length and above. The subsequent subculture was made only on the medium containing the maximum shoot proliferation rate.

### Shoot proliferation and multiplication

The leaf explants were cultured on MS basal medium containing 3% (w/v) sucrose, 0.8% (w/v) agar and various concentrations of BAP, KN, and TDZ (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l) alone or in combination with auxins (0.1 to 2.5 mg/l) and were used for shoot proliferation. After two weeks, the clumps of shoots were sub cultured on MS medium containing 3% (w/v) sucrose and 0.8% agar (w/v) with suitable growth regulators for multiplication and maturation of the shoots. Proliferated multiple shoots were divided into small clusters of 2 to 3 shoots. They were sub cultured on shoot elongation medium containing $GA_3$/KN (0.2 to 1.2 mg/l) alone or in combination with cytokinins like BAP/KN (0.1 to 2.5 mg/l) or NAA (0.1 to 2.5 mg/l). The cultures were incubated at 25 ± 2°C under 16/8 h light/dark photoperiod. After two weeks, shoots longer than 3.0 cm were counted and transferred to rooting medium.

### Root induction and transplantation

The longer shoots (3 cm length) were excised and transferred to MS basal medium containing 3% (w/v) sucrose, 0.8% (w/v) agar and different concentrations of IBA and IAA (0.1 to 2.5 mg/l) alone or in combination with cytokinins such as IBA + KN, IBA + BAP and IAA + KN (1.5 + 0.1 to 2.5 mg/l) for root induction. Rooting was observed from 15 to 20 days. Plantlets with well developed roots were hardened and subsequently transferred to pots. Potted plants were covered with transparent polythene membrane to high humidity and were watered every three days with half strength MS salts solution free of sucrose for two weeks. The survival percentage was observed in all the explants. Samples were photographed at different stages of growth period.

### Statistical analysis

The cultures were examined periodically and the morphological changes were recorded on the basis of visual observation. Whenever possible, the effects of the different treatments were quantified on the basis of percentage of the cultures showing the response per culture. Means and standard errors were used throughout the study and the values were assessed using a

**Table 1.** Effect of cytokinins (KN and BAP) alone or in combination with NAA on direct shoot bud regeneration from leaf explants of *P. barbatus*.

| Plant growth regulator (mg/l) | | | Frequency of culture response | Number of shoots per explant (mean ± SE) |
|---|---|---|---|---|
| KN | BAP | NAA | | |
| 0.5 | - | - | 54.3[c] | 7.21 ± 1.36[c] |
| 1.0 | - | - | 68.6[b] | 13.05 ± 1.75[b] |
| 1.5 | - | - | 80.0[a] | 19.74 ± 2.08[a] |
| 2.0 | - | - | 68.6[b] | 9.79 ± 1.55b[c] |
| 2.5 | - | - | 48.6[cd] | 4.95 ± 1.18c[d] |
| 3.0 | - | - | 40.0[e] | 2.32 ± 0.82[d] |
| - | 0.5 | - | 34.3[d] | 2.63 ± 1.50[e] |
| - | 1.0 | - | 45.7[c] | 4.05 ± 1.31[cd] |
| - | 1.5 | - | 51.4[b] | 7.47 ± 1.35[b] |
| - | 2.0 | - | 60.0[a] | 9.11 ± 1.56[a] |
| - | 2.5 | - | 48.6[bc] | 5.32 ± 1.38[c] |
| | 3.0 | - | 31.4[de] | 2.42 ± 1.30[e] |
| - | 2.0 | 0.1 | 48.6[d] | 7.42 ± 1.35[c] |
| - | 2.0 | 0.5 | 60.0[b] | 12.05 ± 1.78[b] |
| - | 2.0 | 1.0 | 71.4[a] | 15.05 ± 2.20[a] |
| - | 2.0 | 1.5 | 57.1[bc] | 6.00 ± 1.05[cd] |
| - | 2.0 | 2.0 | 45.7[de] | 3.84 ± 1.17[d] |
| - | 2.0 | 2.5 | 34.3[f] | 1.95 ± 1.35[de] |

All of the experiments were conducted with a minimum of 7 replicates per treatment and each experiment was repeated five times (7×5 = 35 explants were cultured for observation).
Mean values within column followed by different letter were significantly different from each other at $P < 0.05$ level comparison by DMRT.

parametric Moods-median test (Snedecor and Cochran, 1989). The data were further analyzed for variance by Duncan's multiple range test (DMRT) and means were compared with $P < 0.05$ as the level of significance.

## RESULTS

The non-meristem like leaf explants were tested on different concentrations and the combinations of the growth regulators were used to define an efficient regeneration medium. Direct bud formation was observed from leaf explants cultured on MS medium supplemented with appropriate plant growth regulators. The earliest visible signs of growth were seen after 2 weeks of inoculation in the form of swelling at one or both the cut ends of the leaf explants on the MS fortified medium. If the treatments failed to induce regeneration within 30 days, it implied that they were unsuitable and their regeneration potential was not further pursued.

### Shoot proliferation and multiplication

Leaf explants grown on the media supplemented with different concentrations of KN, BAP and TDZ alone or in combination with NAA initially responded with the enlargement and swelling of the leaf tissue. They started differentiating the multiple shoot buds within 2 weeks of inoculation, especially at the petiolar ends, where bud initials began to appear, produced through the leaf tissue. Following, was the transfer of the original explants to shoot development as same medium allowed further development and multiplication. Among the various concentrations of cytokinins like KN, BAP and TDZ (0.5 to 3.0 mg/l) that were tested for the leaf segments, the highest adventitious shoot regeneration percentage (80%) was observed on the MS medium at 1.5 mg/l KN and the average maximum number of shoots per explants was 19.7, followed by BAP at 2.0 mg/l which had 60% response with 9.11 shoots per explants. TDZ (1.5 mg/l) showed less frequency (57.1%) and minimum number of 7.89 shoots per explant compared with other cytokinins (Table 1). KN was the most important factor for adventitious shoot regeneration, as no adventitious shoot developed on the explants exposed to the media without KN. The highest percentage of culture response and optimal number of shoots from leaf explants of direct organogenesis is given in Figure 1. Higher concentrations (above 3.0 mg/l) of both BAP and KN alone produced green compact and brown friable callus, respectively. Lower concentrations of cytokinins had no adventitious shoot formation, however, only the optimum concentration showed adventitious shoots from the wound points of leaf explants, after 2 to 3 weeks. The combinations of

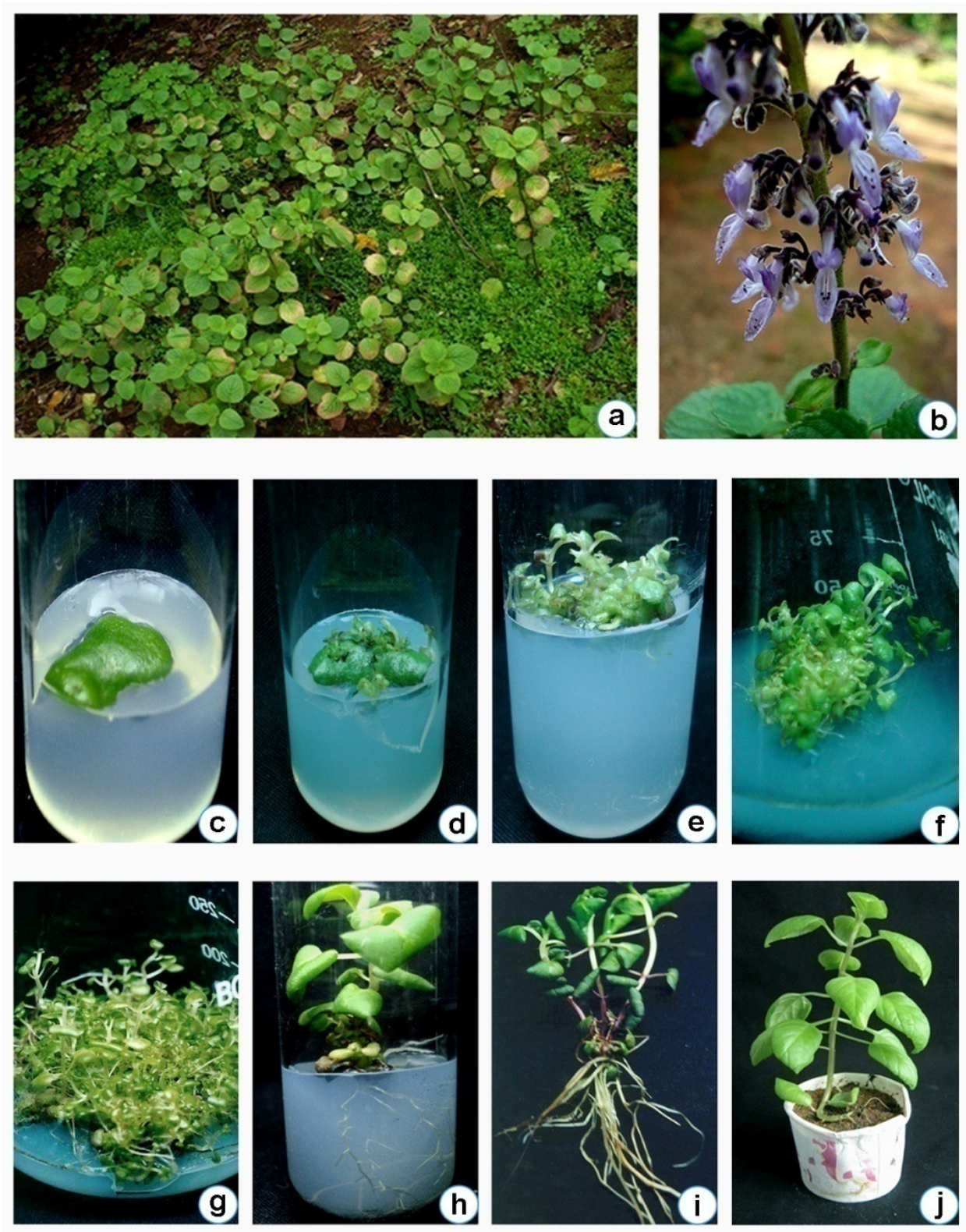


**Figure 1.** Induction of adventitious shoots from leaf explants of *P. barbatus*. A, Habitats of *P. barbatus*; B, inflorescence; C, leaf explants cultured on MS + KN (1.5 mg/l); D, initiation of shoot buds from wounded part of the leaf, after 15 days of culture; E, multiple shoot buds developed from leaf explants on MS + KN (1.5 mg/l); F, shoots elongation on ½ MS + $GA_3$ (0.6 mg/l) + KN (0.5 mg/l); G, clustered shoots rooted in ½ MS + IBA (1.5 mg/l); H, *in vitro* raised single shoot rooted in ½ MS + IBA (1.5 mg/l); I, well developed root system and complete plantlets; J, regenerated plantlet acclimatized to the green house.

**Table 2.** Effect of half strength MS medium with different concentrations of plant growth regulators on shoot elongation from micro shoots of *P. barbatus.*

| Plant growth regulators (mg/l) | | Frequency of culture response | Shoot length (cm) (Mean ± SE) | Number of node (Mean ± SE) | Number of leaf (Mean ± SE) |
|---|---|---|---|---|---|
| $GA_3$ | KN | | | | |
| 0.2 | - | 42.9$^{c}$ | 4.36 ± 1.73$^{d}$ | 3.84 ± 0.90$^{de}$ | 7.68 ± 1.67$^{cd}$ |
| 0.4 | - | 71.4$^{bc}$ | 6.28 ± 1.94$^{b}$ | 5.74 ± 1.73$^{bc}$ | 11.63 ± 2.36$^{b}$ |
| 0.6 | - | 88.6$^{a}$ | 7.54 ± 2.12$^{a}$ | 7.58 ± 1.22$^{a}$ | 15.00 ± 2.79$^{a}$ |
| 0.8 | - | 74.3$^{b}$ | 6.95 ± 3.04$^{ab}$ | 5.84 ± 1.07$^{b}$ | 11.63 ± 2.27$^{b}$ |
| 1.0 | - | 57.1$^{d}$ | 5.60 ± 2.21$^{bc}$ | 4.42 ± 0.84$^{d}$ | 9.05 ± 1.58$^{c}$ |
| 1.2 | - | 40.0$^{ef}$ | 4.08 ± 2.17$^{de}$ | 2.95 ± 0.85$^{e}$ | 6.21 ± 1.93$^{d}$ |
| 0.6 | 0.1 | 74.3$^{b}$ | 6.93 ± 2.19$^{c}$ | 6.79 ± 1.13$^{b}$ | 13.47 ± 2.09$^{b}$ |
| 0.6 | 0.5 | 91.4$^{a}$ | 8.19 ± 3.36$^{a}$ | 7.84 ± 1.07$^{a}$ | 15.26 ± 2.62$^{a}$ |
| 0.6 | 1.0 | 80.0$^{ab}$ | 7.81 ± 4.37$^{ab}$ | 7.05 ± 1.54$^{ab}$ | 13.58 ± 2.46$^{ab}$ |
| 0.6 | 1.5 | 65.7$^{bc}$ | 6.11 ± 3.00$^{d}$ | 6.05 ± 1.39$^{bc}$ | 11.47 ± 2.22$^{c}$ |
| 0.6 | 2.0 | 51.4$^{d}$ | 5.27 ± 1.79$^{e}$ | 5.05 ± 0.97$^{d}$ | 10.63 ± 2.01$^{cd}$ |
| 0.6 | 2.5 | 34.3$^{e}$ | 4.54 ± 1.43$^{cd}$ | 3.32 ± 0.95$^{e}$ | 6.68 ± 1.83$^{e}$ |

All of the experiments were conducted with a minimum of 7 replicates per treatment and each experiment was repeated five times (7 × 5 = 35 explants were cultured for observation).
Mean values within column followed by different letters are significantly different from each other at $P < 0.05$ level comparison by DMRT.

BAP (2.0 mg/l) or KN (1.5 mg/l) with NAA (1.0 mg/l) improved the shoot proliferation rate significantly (15.05 and 11.63 shoots, respectively) compared with BAP and TDZ alone, but shoot height and internodal length was reduced especially at high NAA concentration.

## Shoot elongation

For shoot elongation, 30 days old multiple shoot mass were transferred to half strength MS medium containing $GA_3$/KN (0.2 to 1.2 mg/l) alone or different concentrations of KN, BAP and NAA (0.1 to 2.5 mg/l). Among the various combinations used, $GA_3$ (0.6 mg/l) + KN (0.5 mg/l) combination supported maximum shoot length (8.1 cm length/shoot) and mean number of average node (7.84) with 15.2 leaves/explant within 20 days of the culture (Table 2). When the concentration of $GA_3$ was increased, the shoot length also increased up to the optimum level (0.6 mg/l), afterwards it was decreased with further increase. The optimum concentration of $GA_3$ (0.6 mg/l) + BAP (1.0 mg/l) or NAA (0.5 mg/l) also proved the best shoot elongation and increasing BAP (1.0 mg/l) and NAA (0.5 mg/l) enhanced the number of multiple shoots or basal callusing. Optimum concentration of KN at 0.6 mg/l alone also increased the shoot elongation and further increased the hormone concentration for regeneration of the multiple shoots.

## Rooting of *in vitro* shoots

Well developed shoots (above 3 cm) were excised and grown in half MS medium supplemented with either IBA or IAA (0.1 to 2.5 mg/l) in combination with KN/BAP (0.1 to 2.5 mg/l) individually for rooting. Among the various concentration used, IBA at 1.5 mg/l was found to yield higher percentage of rooting in the *in vitro* derived shoots which had produced, the higher frequency of rooting (85.7%) response with 20.1 roots/shoot and an average of maximum root length of 8.95 cm was observed within 15 to 20 days of culture (Table 3). The maximum (68.6%) frequency of rooting was noticed, when the shoots were cultured in half MS medium fortified with the optimum level of IAA (1.5 mg/l) and produced 13.32 roots with 5.95 cm root length/shoot.

The optimum concentration of IBA/IAA (1.5 mg/l) for rooting selected in the previous experiment was tested in combination with various concentrations of KN/BAP (0.1 to 2.5 mg/l). The combination of IBA (1.5 mg/l) + KN (0.5 mg/l ) showed maximum percentage (80%) response with 17 roots and an average of 8.58 cm root length. The percentage of rooting increased with increasing concentrations of IBA/IAA up to 1.5 (mg/l) and it decreased with further increase of the previously mentioned. The 15 days of culture in rooting medium resulted in maximum percentage of rooting and the 4 to 6 days of dark treatment stimulated maximum rooting in IBA alone or IBA + KN supplemented medium. The hormone concentration with respect to percentage of rooting per shoot and nature of roots were controlled by auxins and their concentrations.

## Acclimatization and hardening

Rooted plantlets of 2 weeks old cultures were transferred to soil under shade for *in vitro* hardening. The plantlets

**Table 3.** Effect of half strength MS medium augmented with different concentrations of IBA alone or in combination with KN on *in vitro* rooting of *P. barbatus*.

| Plant growth regulators (mg/l) | | Frequency of culture response | Number of roots per shoot (Mean ± SE) | Root length (cm) (Mean ± SE) |
|---|---|---|---|---|
| IBA | KN | | | |
| 0.1 | - | 40.0$^{ef}$ | 8.53 ± 1.07$^{ef}$ | 3.32 ± 0.75$^{de}$ |
| 0.5 | - | 62.9$^{cd}$ | 13.11 ± 1.97$^{cd}$ | 5.74 ± 1.28$^{c}$ |
| 1.0 | - | 80.0$^{ab}$ | 15.58 ± 1.57$^{b}$ | 6.89 ± 1.10$^{b}$ |
| 1.5 | - | 85.7$^{a}$ | 20.16 ± 2.24$^{a}$ | 8.95 ± 1.43$^{a}$ |
| 2.0 | - | 62.9$^{c}$ | 13.47 ± 2.06$^{c}$ | 6.11 ± 1.52$^{bc}$ |
| 2.5 | - | 45.7$^{e}$ | 8.95 ± 1.43$^{e}$ | 4.11 ± 0.99$^{d}$ |
| 1.5 | 0.1 | 57.1$^{c}$ | 11.32 ± 1.92$^{bc}$ | 6.05 ± 1.03$^{c}$ |
| 1.5 | 0.5 | 80.0$^{a}$ | 17.00 ± 2.29$^{a}$ | 8.58 ± 1.30$^{a}$ |
| 1.5 | 1.0 | 74.3$^{ab}$ | 13.68 ± 2.21$^{b}$ | 7.26 ± 1.24$^{b}$ |
| 1.5 | 1.5 | 57.1$^{cd}$ | 9.26 ± 1.56$^{c}$ | 5.84 ± 1.71$^{cd}$ |
| 1.5 | 2.0 | 42.9$^{e}$ | 6.26 ± 1.76$^{d}$ | 4.58 ± 1.02$^{e}$ |
| 1.5 | 2.5 | 37.1$^{ef}$ | 3.84 ± 1.17$^{de}$ | 3.37 ± 0.90$^{f}$ |

All of the experiments were conducted with a minimum of 7 replicates per treatment and each experiment was repeated five times (7 × 5 = 35 explants were cultured for observation).
Mean values within column followed by different letters were significantly different from each other at $P < 0.05$ level comparison by DMRT.

were taken out from the flasks, washed with sterile water removed from agar/medium and transferred to paper cups containing river sand, garden soil and saw dust in the ratio of 1:1:1. These plantlets were irrigated with half strength MS medium twice for 20 days in the controlled conditions and were covered with polythene bags. The plants were maintained under controlled temperature (25 ± 2°C) for a week; subsequently, they were transferred in the nursery bags and successfully established to the field. The regenerated plants did not show any detectable variation in morphology or growth characteristics with the respective donor plants. The survival percentage was 90 to 95%.

## DISCUSSION

The objective of the study was to establish an *in vitro* propagation method for *P. barbatus* for conservation purposes as wild natural populations are becoming sparse. The leaf explants were capable of directly regenerating large number of plantlets in the standard (full strength) MS medium containing cytokinins and an auxin with a maximum frequency of response and number of shoots. There was good shoot bud induction and proliferation response only in the presence of cytokinins and no response in the basal medium. Similar results are well documented in several medicinal plants such as *Catalpa ovata* (Lisowska and Wysokinska, 2000) and *P. vulgaris* (Kwapata et al., 2010).

### Shoot proliferation and multiplication

The study demonstrated the suitable method for *in vitro* proliferation of multiple shoots and complete plantlet development for conservation purposes. The observations showed the leaf explants response with respect to hormone and its combinations for shoot buds proliferation, multiplication, shoot elongation, rooting and hardening process. Plant regeneration from leaf explant was reported in *Achras sapota* (Purohit et al., 2004), *E. axillare* (Jeyachandran et al., 2005) and *Prunus serotina* (Liu and Pijut, 2008). By using this method, multiple shoots have been induced directly from leaf explant which produced maximum number of shoots on MS medium containing KN, BAP and TDZ alone or in combination with NAA. Makunga et al. (2005) reported that, KN was more effective for plant regeneration from leaf of *Thapsia garganica*, when compared with BAP during the micropropagation of Fennel. Leaves showed direct multiple shoot formation first from the cut end of the petiole within two weeks of inoculation as was reported by Kelkar and Krishnamurthy (1998). Cytokinins like TDZ proved to have the highest shoot regeneration frequency and comparatively KN indicated poor response of shoot formation in cotyledon as reported by Gua et al. (2005) and Liu and Pijut (2008). This is in contrast, to the results of this study where maximum number of shoot bud proliferation and multiplication was observed in MS medium supplemented with KN (1.5 mg/l). Similar result were obtained in *Enicostemma hyssopifolium* (Seetharam

et al., 2002). NAA had no significant effect on mean number of shoots as reported by Liu et al. (2008). Similar results were also observed in this study.

A combination of NAA at 1.0 mg/l with BAP at 2.0 mg/l induced shoots proliferation. Similar results were observed in leaf explant of *E. purpurea* (Koroch et al., 2002) and *Charybdis numidica* (Kongbengkerd et al., 2005). With increasing or decreasing concentration of NAA from the level of 1.0 mg/l, shoot induction was also dramatically decreased to 20%. So, the range of NAA (1.0 mg/l) combined with BAP (2.0 mg/l) or KN (1.5 mg/l) was the best combination for shoot regeneration from the leaf explant. Similar results were also reported in *Brassica juncea* (Gua et al., 2005), who reported that, the shoot regeneration frequency from leaf segments was also obviously enhanced in the presence of NAA in all the cytokinins used; when 0.5 mg/l NAA was added in the medium combined with other cytokinin 1.0 mg/l BAP and 0.5 mg/l KN gave the highest shoot regeneration potential. Thus, these are in accordance with this investigation. Two major properties of cytokinins useful in culture are stimulation of cell division and release of lateral bud dormancy. The variations in the regeneration potential of leaf explants are attributable to the differences in their physiological and genetic makeup of cells. Auxins affect DNA replication, whereas, cytokinin seems to exert some control over the event of mitosis and cytokinesis. Thus, auxin and cytokinin level in cultures need to be carefully balanced and controlled.

Mangal et al. (2003) reported that, MS medium supplemented with BAP (1.0 mg/l) and $GA_3$ (0.5 mg/l) resulted in desirable shoot elongation in Chrysanthemum plants. MS medium fortified with 0.5 mg/l of BAP and 1.0 mg/l of $GA_3$ promoted good shoot elongation on *P. umbellata* (Pereira et al., 2000). The highest shoot elongation was obtained on MS medium without plant growth regulator (Vanagas et al., 2002). The observation showed that directly regenerated microshoots were subcultured on half strength MS medium supplemented with $GA_3$ at 0.6 mg/l alone or in combination with KN at 0.5 mg/l for maximum shoot elongation; similarly regenerated shoots were further elongated on half strength MS medium without PGR as was reported by Kelkar and Krishnamurthy (1998). This is in accordance with the report of Govindaraju et al. (2003) who reported that shoot bud development was enhanced by the addition of $GA_3$ to the medium.

### Root induction, hardening and acclimatization

Half strength MS medium supplemented with different plant growth regulators were used for root induction; IBA was best suited for inducing roots. Similar results were also reported by Beena et al. (2003). This successful application of IBA root inducing experiments has also been documented in various *in vitro* protocols with *Psoralea corylifolia* (Faisal and Anis, 2006) and cotton (Divya et al., 2008). In contrast, to the previous reports of Pereira et al. (2000) and Ghnaya et al. (2008), the higher frequency of rooting with the highest root length occurred without PGR supplementation medium. This study showed that, the half strength MS medium with IBA at 1.5 mg/l individually produced a greater number of healthy roots than IAA at 1.5 mg/l. The combinations of IBA at 1.5 mg/l with KN or BAP (0.5 mg/l) produced moderate root frequency. A crucial aspect of *in vitro* propagation is to acquire regenerated plants that are capable of surviving outside the sterile and protected *in vitro* environment. The successfully rooted plantlets were transferred to paper cups containing river sand, garden soil and saw dust for hardening. Plantlets were maintained in the culture room (25 + 2°C) conditions initially for 3 to 4 weeks and later transferred to normal environment conditions and were maintained for about 4 weeks. Similar pattern of hardening was observed by Jeyachandran et al. (2005).

In conclusion, the study showed that shoot buds can be successfully regenerated on *P. barbatus* using leaf explants through direct organogenesis. We were able to produced complete plantlets with optimum multiplication rate on MS medium supplemented with KN at 1.5 mg/l followed by BAP and NAA (2.0 + 1.0 mg/l) through leaf explants. The entire procedure could be completed without callus formation, an advantage, since callus may cause genetic variability. By using this protocol, a number of plants of *P. barbatus* can be effectively multiplied. This protocol will enable the conservation of natural populations of this valuable aromatic and medicinal herb.

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