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# Direct and indirect organogenesis of *Gymnostachyum latifolium* var. decurrens: An endangered plant

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**Abstract**
The study aimed to evaluate the efficiency of direct and indirect organogenesis for large-scale multiplication, propagation and conservation of *Gymnostachyum latifolium* var. *decurrens*, an endemic and endangered plant. Shoot tips and nodal explants were used for direct organogenesis. The highest frequency of shoot initiation from node and shoot tip was observed on MS medium with 2.22 M BAP. Node is used as a source of explant for callogenesis. High frequency of green and friable organogenic callus induction (95%) was observed from the node on MS medium supplied with 13.56 M 2,4-D. The regeneration frequency of shoot induction from the nodal derived calli was high (86%) when MS medium supplemented with 11.10 M BAP. Microshoots regenerated via direct as well as indirect organogenesis were rooted best in ½ MS medium containing 10.74 M NAA. The well rooted plantlets were successfully established in the soil.

**Keywords:** *Gymnostachyum latifolium* var. *decurrens*, endangered, organogenesis

## Introduction
*Gymnostachyum latifolium* (Dalzell) T. Anders. var. *decurrens* Gamble (Family – Acanthaceae) is an endangered plant distributed in the Western Ghats of Kerala, Goa and Karnataka States. This plant grows among laterite rocky slopes near streams in a moist deciduous forests. The plant is having antioxidantal, antimicrobial and antipyretic and traditionally used for curing many disorders [1]. The key feature of this species of *Gymnostachyum* is that the leaves of the plant decurrent on petiole, lamina broadly elliptic, decurrent at base; lower lip shallowly 3-lobed, filament bent above at the connective, stamens and style held within corolla lobe. Poor seed setting, seed germination and seed viability are the certain constrains in its survival in nature. In the present study, efficient protocols were developed for direct and indirect organogenesis, callogenesis, *in vitro* rhizogenesis, large-scale multiplication and subsequent plantlet regeneration for conservation of *Gymnostachyum latifolium* var. *decurrens.*

## Materials and Methods
### Explants used
The Shoot tips and node collected from healthy plants were used as explants for direct organogenesis. The node, leaf lamina and petiole explants were used as explants for callogenesis.

### Sterilization of glassware and materials
The glassware and other materials were sterilized by soaking in solution containing water and 1.0 N HCl and kept overnight. They were washed in tap water to remove the traces of acid and then soaking in detergent solution (Teepol 0.1%) for overnight. The traces of soap solution were removed by running tap water and then rinsed twice in double distilled water. The glasswareswere dried in hot air oven at 100°C for 24 hours. The forceps, scalpels, needles, scissors and spatula were sterilized in an autoclave at 120°C and 15 psi pressure for 20 minutes and flame sterilization was also done at the time of inoculation.

### Preparation of Culture media
MS (Murashige & Skoog, 1962) medium with 3% sucrose at full or half strength was used as the basal medium for all the *in vitro* studies. MS Basal medium was modified by the addition of different growth regulators *viz*., 2,4-Dichlorophenoxy acetic acid (2,4-D), Naphthalene acetic acid (NAA), Indole 3-acetic acid (IAA), Indole-3-butyric acid (IBA), Benzylaminopurine (BAP) and Kinetin (Kn) at different concentrations either singly or in combinations.

### Sterilization of explants
The explants were washed thoroughly with running tap water to remove the external dust particles and then rinsed with water containing small amount of soap (Teepol) for 10 min. and then again rinsed with double distilled water. Thereafter, sterilization was done in laminar air flow chamber; the explants were disinfected with 70% (v/v) ethanol for 60 seconds, 5% (v/v) sodium hypochlorite for 10 minutes and 0.1% (w/v) mercuric chloride for 5 min. Each treatment was followed by 3 rinses of sterile distilled water and then the explants were blotted with sterile filter paper to remove the excess of water.

### Inoculation of explants
The inoculation of explants into culture vessels was carried out in laminar air flow chamber with full aseptic condition. The instruments were dipped into 70% ethanol and flamed over a sprit lamp and after cooling they were used for inoculation. The hands were also swabbed with 70% ethanol before carrying out the work to ensure the aseptic condition.

### Culture Incubation
The culture vessels containing media and inoculated explants were maintained in a culture room having

controlled environmental conditions *i.e.*, where 25 ± 2°C temperature and 60% relative humidity were maintained. The light and dark cycle (16 hours and 8 hours respectively) was also maintained with the use of florescent lamps having 3000 – 4000 lux intensity.

**Hardening and Transplanting**

The plantlets with well-developed roots were carefully removed from the culture tubes and washed gently with sterile distilled water to eliminate the adherent medium from roots and transferred to plastic cups containing the mixture of sterilized sand, red soil and vermicompost (1:1:1) and covered with transparent polythene bags to maintain a humidity. The potted plants were grown under greenhouse environment were transferred to open field condition.

**Observation and Statistical Analysis**

Visual observations were made every day and data on explants response, number of shoot buds/ shoots per explants and numbers of roots per shoot were recorded upto the end of 8$^{th}$ week. Each growth regulator treatment consists of five replicates and was repeated thrice. The Data on shoot bud regeneration, multiple shoot production and rooting were statistically represented by Mean and Standard Error.

## Results and Discussion

**Direct organogenesis**

The shoot tip and nodal explants were inoculated on MS medium supplemented with different concentrations of cytokinins or cytokinins alone (2.22 – 13.2 M BAP), (2.32 – 13.95 M Kn) and in combinations with cytokinins or auxins (2.22 – 13.2 M BAP & 0.53 – 3.22 M NAA; 2.22 – 13.2 M BAP & 2.32 – 13.95 M Kn) for shoot induction. The results obtained are shown in Table 1 & Figure 1. Based on the observation it was found that the highest frequency of shoot initiation from node and shoot tip was observed on MS medium with 2.22 M BAP followed by 4.44 M BAP after 20-22 days of inoculation. MS media supplemented with Kn alone or the combinations of BAP with NAA showed comparatively poor results. The similar type of media was found to be most effective for producing axillary buds in *Blepharis maderaspatensis* [2]. Higher number of shoots per explants observed on MS media fortified with 2.22 M BAP followed by 2.22 M BAP + 2.32 M Kn. Higher concentrations of cytokinins alone, *i.e.*, BAP (13.32 M) or Kn (13.95 M) or in combinations with auxins reduced the number of shoots. The lowest percentage of shoot initiation was observed on MS medium supplemented with 13.32 M BAP + 13.95 M Kn followed by 11.10 M BAP + 11.62 M Kn. The significant role of BAP in bud proliferation and shoot multiplication was also reported in many members of the family Acanthaceae *viz. Adhatoda beddomei* [3], *Barleria prionites* var. *dicantha* [4], and *Strobilanthes tonkinensis*[5].

**Table 1:** Effect of Plant Growth Regulators on shoot induction from shoot tip and nodal explants of *Gymnostachyum latifolium* var. *decurrens* when cultures on MS Basal media

| Plant Growth Regulators (M) | | | % of response | No. of shoots per explant | Shoot length (cm) |
|---|---|---|---|---|---|
| BAP | Kn | NAA | | | |
| 2.22 | - | - | 90 | 3.4 ± 0.24 | 1.84 ± 0.04 |
| 4.44 | - | - | 84 | 3.0 ± 0 | 1.76 ± 0.06 |
| 6.66 | - | - | 76 | 2.6 ± 0.24 | 1.64 ± 0.05 |
| 8.88 | - | - | 76 | 2.4 ± 0.24 | 1.40 ± 0.20 |
| 11.10 | - | - | 64 | 1.6 ± 0.24 | 1.33 ± 0.06 |
| 13.32 | - | - | 72 | 0.70 ± 0.32 | 0.88 ± 0.22 |
| - | 2.32 | - | 69 | 2.6 ± 0.24 | 1.4 ± 0.20 |
| - | 4.65 | - | 57 | 2.4 ± 0.24 | 1.33 ± 0.06 |
| - | 6.97 | - | 65 | 1.8 ± 0.19 | 1.2 ± 0.08 |
| - | 9.30 | - | 75 | 1.4 ± 0.24 | 1.12 ± 0.06 |
| - | 11.62 | - | 72 | 1.4 ± 0.24 | 1.1 ± 0.06 |
| - | 13.95 | - | 75 | 0.70 ± 0.32 | 1.02 ± 0.02 |
| 2.22 | - | 0.53 | 64 | 2.6 ± 0.24 | 1.46 ± 0.05 |
| 4.44 | - | 1.07 | 61 | 2.8 ± 0.19 | 1.64 ± 0.05 |
| 6.66 | - | 1.61 | 68 | 2.4 ± 0.24 | 1.28 ± 0.09 |
| 8.88 | - | 2.15 | 57 | 1.8 ± 0.19 | 1.16 ± 0.06 |
| 11.10 | - | 2.68 | 72 | 1.4 ± 0.24 | 1.12 ± 0.05 |
| 13.32 | - | 3.22 | 69 | 1.2 ± 0.37 | 0.80 ± 0.19 |
| 2.22 | 2.32 | - | 68 | 3.2 ± 0.19 | 1.76 ± 0.05 |
| 4.44 | 4.65 | - | 75 | 3.0 ± 0 | 1.36 ± 0.09 |
| 6.66 | 6.97 | - | 76 | 2.2 ± 0.19 | 1.12 ± 0.06 |
| 8.88 | 9.30 | - | 61 | 1.8 ± 0.19 | 1.02 ± 0.02 |
| 11.10 | 11.62 | - | 54 | 1.4 ± 0.24 | 1.02 ± 0.02 |
| 13.32 | 13.95 | - | 53 | 0.70 ± 0.32 | 0.80 ± 0.19 |

**Callogenesis**

The node, leaf lamina and petiole explants were inoculated were inoculated on MS media supplemented with different concentrations of auxins (4.52 – 13.56 M 2,4-D) and combinations with cytokinins (4.52 – 13.56 M 2,4-D & 0.93 – 4.65 M Kn; 4.52 – 13.56 M 2,4-D & 0.88 – 4.44 M BAP; 5.37 – 16.11 M NAA & 0.88 – 4.44 M BAP). The results are shown in Table 2 & Figure 1. High frequency of green and friable organogenic callus induction (95%) was observed from the node on MS medium supplied with 13.56 M 2,4-D + 4.44 M BAP at an interval of 20-24 days. A similar result of callogenesis from nodal explant was also recorded in *Justicia adhatoda* [6].MS medium supplied with 12.31 M 2, 4-D + 3.55 M BAP also induced semi-friable and green coloured callus from the node at a callusing frequency of 85%. The degree of callus induction from the

leaf and petiole explants was moderate (50-60%) when MS medium supplied with 2,4-D alone or in combination with Kn, BAP or NAA. Even though compact and brown coloured callus initiated from petiole after 15 days of inoculation on different concentrations of auxins and cytokinins turned black after a few days. When the concentrations of 2, 4-D and BAP decreased there was a decreasing tendency of callusing efficiency from the node.

**Table 2:** Effect of Plant Growth Regulators on callus induction from various explants of *Gymnostachyum latifolium* var. *decurrens* on MS media

| Plant Growth Regulators (M) | | | | Explant | % of response | Texture of the Calli | Colour of the Calli |
|---|---|---|---|---|---|---|---|
| 2,4-D | Kn | BAP | NAA | | | | |
| 4.52 | - | - | - | Petiole | 60 | Compact | Brown |
| 6.78 | - | - | - | Petiole | 50 | Compact | Brown |
| 9.05 | - | - | - | Petiole | 50 | Compact | Brown |
| 12.31 | - | - | - | Petiole | 60 | Compact | Brown |
| 13.56 | - | - | - | Petiole | 60 | Compact | Brown |
| 4.52 | 0.93 | - | - | Node | 50 | Compact | Brown |
| 6.78 | 1.86 | - | - | Node | 60 | Compact | Brown |
| 9.05 | 2.79 | - | - | Node | 50 | Compact | Brown |
| 12.31 | 3.72 | - | - | Node | 60 | Compact | Brown |
| 13.56 | 4.65 | - | - | Node | 60 | Semi friable | green |
| 4.52 | - | 0.88 | - | Node | 60 | Compact | Brown |
| 6.78 | - | 1.77 | - | Node | 55 | Compact | Brown |
| 9.05 | - | 2.66 | - | Node | 70 | Compact | Greenish white |
| 12.31 | - | 3.55 | - | Node | 85 | Semi-friable | green |
| 13.56 | - | 4.44 | - | Node | 95 | Friable | Green |
| - | - | 0.88 | 5.37 | Petiole | 60 | Compact | Brown |
| - | - | 1.77 | 8.05 | Petiole | 50 | Compact | Brown |
| - | - | 2.66 | 10.74 | Petiole | 60 | Compact | Brown |
| - | - | 3.55 | 13.20 | Petiole | 60 | Compact | Brown |
| - | - | 4.44 | 16.11 | Petiole | 50 | Compact | Brown |
| 4.52 | - | - | - | Leaf | 40 | Compact | Brown |
| 6.78 | - | - | - | Leaf | 50 | Compact | Brown |
| 9.05 | - | - | - | Leaf | 55 | Compact | Brown |
| 12.31 | - | - | - | Leaf | 50 | Compact | Brown |
| 13.56 | - | - | - | Leaf | 40 | Compact | Brown |

**Indirect organogenesis**

The semi-friable callus produced from the node were cut into small pieces and sub cultured on MS medium with different concentrations of cytokinin (4.44 – 13.32 M BAP) and in combinations with auxins (4.44 – 13.32 M BAP + 0.45 – 2.26 2,4-D; 4.44 – 13.32 M BAP + 0.57 – 2.85 M IAA) for regeneration of shoots. The results are shown in Table 3 & Figure 1. The regeneration frequency of shoot induction from the calli was high (86%) when MS medium supplemented with 11.10 M BAP + 2.28 M IAA followed by 13.32 M BAP + 2.85 M IAA (77%) after 30 days of sub-culture. The maximum number of multiple shoots was produced on MS media with 13.32 M BAP + 2.85 M IAA followed by 11.10 M BAP + 2.28 M IAA. Maximum shoot length was observed on the MS media supplemented with 11.10 M BAP + 2.28 M IAA followed by 8.88 M BAP + 1.71 M IAA. The regeneration frequency and the number of shoots produced were comparatively less when MS media supplemented with different concentrations of BAP alone.

The similar type of media was found to be superior for the *in vitro* regeneration from calli in *Eranthemum nervosum* [7] and *Feronia limonia* Linn. [8]. this showed that the requirement of cytokinin appears to be vital for shoot regeneration and the effect of BA was more significant than Kn.

**Table 3:** Effect of Plant Growth Regulators on shoot induction from callus derived from nodal explants of *Gymnostachyum latifolium* var. *decurrens* on MS Media

| Plant Growth Regulators (M) | | | % of response | No. of shoots per explant | Shoot length (cm) |
|---|---|---|---|---|---|
| BAP | 2,4-D | IAA | | | |
| 4.44 | - | - | 43 | 2.8 ± 0.19 | 1.06 ± 0.03 |
| 6.66 | - | - | 51 | 2.4 ± 0.24 | 1.04 ± 0.04 |
| 8.88 | - | - | 57 | 3.6 ± 0.24 | 0.82 ± 0.09 |
| 11.10 | - | - | 52 | 2.6 ± 0.24 | 0.60 ± 0 |
| 13.32 | - | - | 53 | 1.8 ± 0.19 | 0.54 ± 0.02 |
| 4.44 | 0.45 | - | 64 | 3.8 ± 0.19 | 1.46 ± 0.05 |
| 6.66 | 0.90 | - | 54 | 5.8 ± 0.19 | 1.64 ± 0.05 |
| 8.88 | 1.36 | - | 53 | 6.4 ± 0.24 | 1.74 ± 0.08 |
| 11.10 | 1.81 | - | 52 | 4.4 ± 0.39 | 1.12 ± 0.05 |
| 13.32 | 2.26 | - | 61 | 4.0 ± 0.31 | 1.04 ± 0.04 |
| 4.44 | - | 0.57 | 67 | 4.4 ± 0.39 | 1.98 ± 0.04 |
| 6.66 | - | 1.14 | 69 | 6.6 ± 0.24 | 2.24 ± 0.02 |

| | | | | | |
|---|---|---|---|---|---|
| 8.88 | - | 1.71 | 73 | 7.0 ± 0.31 | 2.50 ± 0.03 |
| 11.10 | - | 2.28 | 86 | 9.8 ± 0.37 | 3.04 ± 0.07 |
| 13.32 | - | 2.85 | 77 | 11.0 ± 0.44 | 2.20 ± 0.05 |

***In vitro* rhizogenesis**

The *in vitro* derived shoots from the node as well as the nodal callus were transferred to ½ MS medium fortified with different concentrations of auxins (2.46 –12.30 M IBA, 2.68 – 13.20 M NAA, 2.85 – 14.27 M IAA) for root induction. The results are shown in Table 4 & Figure 1. The high percentage of rooting of *in vitro* derived shoots was observed on ½ MS medium supplemented with 10.74 M NAA after 15 days. The number of roots was high on ½ MS medium supplemented with 10.74 M NAA followed by 13.20 M NAA. Compared to other auxins used, NAA showed better performance in initiation of roots and its elongation. Higher concentration of IAA (14.24 M) reduced the rooting frequency and the number of roots. The lowest percentage of rooting was noticed on ½ MS medium with 11.42 M IAA followed by 7.38 M IBA.

**Table 4:** Effect of Plant Growth Regulators on Rooting from *in vitro* Derived Shoots of *Gymnostachyum latifolium* var. *decurrens* in Half-MS Media

| Plant Growth Regulators (M) | | | % of response | No. of roots | Root length (cm) |
|---|---|---|---|---|---|
| IBA | NAA | IAA | | | |
| 2.46 | - | - | 67 | 3.8 ± 0.19 | 1.36 ± 0.09 |
| 4.92 | - | - | 69 | 4.0 ± 0.31 | 1.20 ± 0.08 |
| 7.38 | - | - | 59 | 5.8 ± 0.19 | 1.12 ± 0.06 |
| 9.84 | - | - | 73 | 5.4 ± 0.24 | 1.10 ± 0.06 |
| 12.30 | - | - | 65 | 4.2 ± 0.37 | 1.02 ± 0.02 |
| - | 2.68 | - | 67 | 6.4 ± 0.24 | 1.64 ± 0.06 |
| - | 5.37 | - | 65 | 7.0 ± 0.32 | 1.68 ± 0.04 |
| - | 8.05 | - | 67 | 7.6 ± 0.24 | 1.86 ± 0.04 |
| - | 10.74 | - | 83 | 8.8 ± 0.19 | 2.00 ± 0.03 |
| - | 13.20 | - | 77 | 8.6 ± 0.24 | 1.84 ± 0.04 |
| - | - | 2.85 | 84 | 4.4 ± 0.39 | 1.64 ± 0.06 |
| - | - | 5.71 | 76 | 5.8 ± 0.19 | 1.74 ± 0.08 |
| - | - | 8.56 | 66 | 4.4 ± 0.39 | 1.36 ± 0.09 |
| - | - | 11.42 | 57 | 3.8 ± 0.19 | 1.12 ± 0.06 |
| - | - | 14.27 | 61 | 2.6 ± 0.24 | 1.10 ± 0.06 |

The requirement of half-strength MS medium for root induction has also been reported in many plant species including *Melia azedarach* [9], *Pterocarpus marsupium* [10], *Acacia sinuata* [11] and *Adhatoda vasica* [12].

## Conclusions

An efficient direct and indirect shoot organogenesis protocols were developed for large-scale multiplication, propagation and conservation of *Gymnostachyum latifolium* var. *decurrens.* This is the first report on *in vitro* propagation of this endangered plant. This protocol will be helpful for conservation of other *Gymnostachyum spp*. as well as medicinally important species of the family Acanthaceae.

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