An efficient *in vitro* regeneration protocol for a natural dye yielding plant, *Strobilanthes flaccidifolious* Nees., from nodal explants

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Adventitious shoot buds formation from axillary buds of nodal segments of *S. flaccidifolious* was achieved on MS medium containing sucrose (3%, w/v), and α-naphthalene acetic acid (NAA; 3 µM) and benzyl adenine (3 µM) in combination. The nodal segments were primed on ‘Growtak Sieve’ for 48 h on MS medium containing sucrose (2%), polyvinyl pyrollidone (200 mg L⁻¹) as antioxidant. About 80% of primed nodal segments responded positively and formed ~12 adventitious shoot buds per explants from explants collected during October-November months of every year. The shoot buds converted into plantlets on MS medium containing sucrose (3%) and kinetin (3 µM) where ~7 micro shoots developed per subculture after 8 weeks of culture. The regenerated micro shoots induced average 14 roots/plant on medium containing NAA (3 µM). The regenerates were hardened for 6-7 weeks on medium with ½MS salt solution and sucrose (2%) under normal laboratory condition before transferring to potting mix. About 70% transplants survived after two months of transfer.

**Keywords**: Micropropagation, Natural dye yielding plant, Nodal explants, *Strobilanthes flaccidifolious*, Tissue culture

Micropropagation of economically important forest trees through organogenesis has the potential for rapid capture of benefits from breeding programme and to improve the quality and uniformity of nursery stocks. In forest plant species seed propagation does not assure genetic stability and a particular characteristic can be lost. Assays of vegetative propagation can be made by conventional vegetative propagation methods such as rooting of cuttings and by micropropagation methods. However, the frequencies of rooting are quite low especially when mature cuttings are used. Micropropagation may be a valuable alternative when: (i) it is difficult to achieve through conventional propagation methods, (ii) problems of rejuvenation persist and (iii) pressure to increase multiplication rates occurs.

Various techniques have been employed for *in vitro* propagation of forest plants and economically important plants such as somatic embryogenesis, organogenesis, axillary shoot proliferation. Amongst the different methodologies used for *in vitro* propagation, the use of lateral buds and or stem segments with axillary buds has been an efficient plant propagation method, besides allowing for the conservation of genetic stability of the propagated plants.

*Strobilanthes flaccidifolious* Nees. (Acanthaceae) is one of the very important dyes yielding plant and plays a key role in indigenous handloom industry. Generally dye is released from the young leaves and extracted by pounded the leaves and boiled with water. The processed leaves produce a light to deep blue colour dye, used for dying fibers/cloths. The population of the species is down sized because of over collection of young twigs and leaves for extracting the dye. The seed propagation of this species is very limited as seed germination rate is very poor. A part from this, seed propagated plants exhibits variability due to cross pollination. Besides seed propagation, the species may also be propagated through stem cutting. But due to poor rooting ability, stem cutting is not an attractive approach. Hence, it is necessary to develop suitable tools for improving the genetic quality and uniformity of planting stock. The present communiqué describes an efficient regeneration protocol of *S. flaccidifolious* from nodal segments of mature plants.

**Materials and Methods**

*Explants source*—The plants were maintained in the Botanical garden of Nagaland University after...
authentic identification of the species by the taxonomist from our University. The nodal segments were collected from these plants in different seasons. Explants were collected in different seasons throughout the year at monthly interval and used for the experimental purpose.

Sterilization, priming of explants and initiation of culture—The nodal explants were freed from leaves and scale before scrubbing by a soft brush with ‘Labolene’ (a commercial laboratory detergent, diluted with water at 1:100 ratio, v/v) followed by washing under water for 10 min. Subsequently, explants were sterilized with aqueous solution of HgCl₂ (0.3%, w/v) for 5 min and then rinsed 4-5 times with sterilized distilled water and kept soaked in sterilized distilled water till cultured on medium.

A part of the pre-soaked sterilized nodal explants were maintained on a ‘Growtak Sieve’ (Hi-Media) containing MS medium having sucrose (2%; w/v) and polyvinyl pyrollidone (PVP) (200 mgL⁻¹) as antioxidant for 48 h to remove the dye from the explants. The pre-soaked and primed nodal segments were used for initiation of culture. About 1.0 cm long sterilized nodal segments was cultured on initiation medium. In the present study full strength MS medium was used for initiation of culture from nodal explants. The medium was fortified with sucrose (0-4%, w/v, as organic carbon source), different concentrations of plant growth regulators (PGRs) like α-naphthalene acetic acid (NAA) and benzyl adenine (BA) (0-15 µM) either singly or in combination and agar (0.8%, w/v) as gelling agent. The pH of the medium was adjusted to 5.6 with NaOH and HCl (0.1 N). About 15 mL medium was dispensed in each test tube (size: 25×150 mm) and cotton plugged before autoclaving at 1.05 kg cm⁻² pressure and at 121°C for 20 min.

Plant regeneration, culture proliferation and rooting of regenerates—The shoot buds developed from cultured nodal segments were maintained on optimum initiation medium for another two passages. The young plantlets and shoot buds were then transferred on MS medium containing sucrose (3%) and different PGRs such as BA and kinetin (Kn; 0-9 µM) either singly or in combination for plant regeneration and culture proliferation. The micro shoots were separated at every subculture and transferred on fresh regeneration medium. The cultures were maintained for 2-3 passages for culture proliferation. About 4 cm long plantlets/shoots with well expanded leaves from the regeneration medium were selected for inducing rooting. The selected shoots were treated differently for inducing roots. One set of the shoots were pulse treated with NAA (0-20 µM) for 3 h followed by culturing on MS plain medium containing sucrose (3%). While, another set of shoot buds were cultured on MS medium containing sucrose (3%) and NAA (0-6 µM) and maintained in normal laboratory condition as stated above.

Hardening and transplantation in potting mix—The well rooted plantlets were taken out from the rooting medium and transferred on ½MS strength semi-gelled medium containing sucrose (2%) but no PGRs and maintained in normal laboratory condition for 6-7 weeks. The hardened plants were then transplanted onto plastic pots containing a mixture of soil, sand, decayed wood powder at 1:1 ratio with a moss topping. The pots were covered with transparent plastics for two weeks followed by without plastic cover and maintained in the shad-house. The plants were fed with 1/10th MS salt solution once in a week for 3-4 weeks. The potted plants were maintained in the poly house for two months or till the formation of new leaves before transferring them in the natural habitat.

Experimental design—A completely randomized experimental design was performed. In all experiments, each treatment had at least three replicates and there were 25 explants per replicates. The cultures were maintained at 25±2 °C under cool white fluorescent light at 40 µmol m⁻² s⁻¹ and 12:12 h (light/dark) photoperiod. All the cultures were sub-cultured at 4-5 weeks interval unless mentioned otherwise. Regeneration capacity of nodal segments was evaluated based on the percentage of explants forming shoot buds and number of buds formed per segments after 5 weeks of culture and data were expressed as the mean of replicates ± SE.

Results and Discussion

Seasonal effect of explants collection and priming of explants—The first objective towards the establishment of in vitro regeneration protocol for S. flaccidifolius was to optimize the time of the year for nodal explants collection from the field grown plants. After sterilization the nodal explants were soaked in sterilized distilled water till culturing on nutrient medium. Soaking of nodal explants in water improved the morphogenetic response over non-soaked
segments. It was observed that pre-soaked nodal segments leached lesser dye in the medium in comparison to non-soaked segments. Alternatively a part of the pre-soaked nodal segments were also maintained on a ‘Growtak Sieve’ containing MS medium containing sucrose (2%) and PVP (200 mgL⁻¹) as antioxidant for 48 h before transferring on initiation medium (Fig. 1a). During this period the blue dye released in the liquid medium of the ‘Growtak Sieve’ and better morphogenetic response was achieved as compared to control and pre-soaked explants.

The nodal segments were collected round the year at one month interval starting from January till December. The explants were collected for three repeated years. It was observed that seasonal changes greatly influenced the explants establishment. Generally the actively growing season is known to be more responsive for bud break in Bauhinia vahlii which is contrary to the present study, where maximum establishment was achieved just before the onset of winter season. It was observed that amongst the different collection seasons, the nodal explants collected during May-July were least responsive and tissues turned necrotic. While explants collected during October-November responded optimally where as much as 80% nodal explants responded by sprouting the axillary buds (Table 1). It was observed that explants collected during May to July released dye in the culture and tissue became necrotic (Fig. 1b). This is probably due to the fact that during this period due to favorable rainfall and temperature dye production is maximal. With the decrease in rainfall during October and November the leaching of dye is also decreased considerably and explants yielded morphogenetic response. Mangal et al. in Guava nodal explants culture reported the effect of time of year on culture initiation and reported that explants collected during February responded optimally under culture condition.

Culture initiation—Under the condition employed, the primed nodal segments from mature plants were free from phenolic exudates and dye; the resident axillary buds were induced to proliferate into multiple shoot buds. Swelling of the axillary buds was observed within a week followed by differentiation.

Fig. 1—Different stages involved in breaking of axillary buds of nodal segments and regeneration of micro shoots of S. flaccidifolius. (a) Sterilized nodal segments are primed on ‘Growtak Sieve’, (b) Cultured nodal segment releasing ‘blue dye’ in the medium, (c) Breaking of axillary bud and sprouting of shoot buds, (d) Multiple shoot buds formation from the nodal segment, (e) Multiple micro shoots formation on regeneration medium showing formation of few roots, (f) Plantlets on rooting medium, (g) Plantlets under hardening condition, (h) A potted plant ready to be transferred to the wild
into multiple shoot buds/micro shoots formation in 3-4 weeks (Fig. 1c). Incorporation of sucrose in the medium was pre-requisite for induction of morphogenetic response. On medium free of sucrose, nodal explants failed to respond and degenerated subsequently. Of the different concentrations of sucrose tested, better morphogenetic response was achieved on medium enriched with sucrose (3%, w/v) (unpublished). Medium containing higher sucrose concentration, explants turned brown while, at lower concentration fewer shoot buds formed. Of the two PGRs incorporated at differential concentrations, NAA singly did not impact impressive morphogenetic response, while BA singly at a concentration of 3 µM supported maximum number of shoot bud formation. But when NAA and BA used in combination, nodal explants registered optimum response. About 80% nodal explants responded positively on medium containing sucrose (3%) and NAA + BA (3 µM each in combination) where as many as 12 shoot buds/micro shoots formed without callus formation (Table 2, Fig. 1 d).

Table 1—Seasonal effect of explants collection on in vitro morphogenetic response of nodal explants of *S. flaccidifolious*

<table>
<thead>
<tr>
<th>Time of explants collection</th>
<th>% response (±SE)*</th>
<th>Type of response**</th>
</tr>
</thead>
<tbody>
<tr>
<td>January</td>
<td>25 (1.50)</td>
<td>Sprouting of axillary buds, leaves crowded at the top, fewer shoot buds formation</td>
</tr>
<tr>
<td>February</td>
<td>25 (1.00)</td>
<td>As above</td>
</tr>
<tr>
<td>March</td>
<td>30 (0.50)</td>
<td>Sprouting of axillary buds from both side of the node, leaves light green, fewer shoot buds formation</td>
</tr>
<tr>
<td>April</td>
<td>30 (0.75)</td>
<td>Greening of nodal segments, sprouting of axillary buds in both side of the node, leaves light green, plantlets healthy.</td>
</tr>
<tr>
<td>May</td>
<td>15 (1.50)</td>
<td>Release of phenolics and dye in media, Callusing of explants and in most cases tissue become necrotic.</td>
</tr>
<tr>
<td>June</td>
<td>14 (1.50)</td>
<td>As above</td>
</tr>
<tr>
<td>July</td>
<td>15 (1.00)</td>
<td>Release of phenolics and dye in media, tissue become necrotic, swelling of node but no shoot buds formation.</td>
</tr>
<tr>
<td>August</td>
<td>25 (0.75)</td>
<td>As above but few shoot buds sprouted.</td>
</tr>
<tr>
<td>September</td>
<td>40 (2.00)</td>
<td>As above</td>
</tr>
<tr>
<td>October</td>
<td>75 (2.00)</td>
<td>Release of dye and phenolics reduced, in most cases explants callused and fewer shoot buds formed.</td>
</tr>
<tr>
<td>November</td>
<td>80 (1.00)</td>
<td>Axillary shoot buds sprouted, multiple shoot buds formation, no release of dye, plantlets were healthy, leaves were dark green.</td>
</tr>
<tr>
<td>December</td>
<td>57 (1.00)</td>
<td>As above but fewer shoot buds formed.</td>
</tr>
</tbody>
</table>

* Standard error; ** On MS medium containing sucrose (3%), NAA and BA (3µM each in combination). Data represents the mean of three replicates.

Table 2—Effects of PGRs on in vitro morphogenetic response of nodal segments of *S. flaccidifolious*

<table>
<thead>
<tr>
<th>PGRs Conc. (µM)*</th>
<th>% response (±SE)**</th>
<th>No. of shoot buds formed/explants**</th>
<th>Type of response</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAA 0</td>
<td>0</td>
<td>0</td>
<td>Greening of explants but degenerated</td>
</tr>
<tr>
<td>-</td>
<td>3</td>
<td>43 ±2.0*</td>
<td>Greening of explants followed, sprouting of axillary buds, multiple shoot buds formation, leaves green.</td>
</tr>
<tr>
<td>0</td>
<td>9</td>
<td>43 ±2.5*</td>
<td>Multiple shoot buds sprouted but callusing at the base.</td>
</tr>
<tr>
<td>0</td>
<td>12</td>
<td>20 ±1.5*</td>
<td>Shoot buds formed but degenerated.</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>67 ±0.5*</td>
<td>Swelling of explants at node and shoot buds formed.</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>40 ±1.0*</td>
<td>As above.</td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>0</td>
<td>Swelling of nodal explants but degenerated subsequently</td>
</tr>
<tr>
<td>12</td>
<td>0</td>
<td>25 ±2.0*</td>
<td>As above.</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>80 ± 1.0*</td>
<td>Greening of nodal segments followed by sprouting of shoot buds at both side of the node, leaves are well expanded and green, partial swelling at the base.</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>57 ±2.5*</td>
<td>Greening of nodal explants and shoot formation, swelling of leaves.</td>
</tr>
<tr>
<td>3</td>
<td>9</td>
<td>33 ±2.0*</td>
<td>Greening of nodal explants followed by callusing of explants.</td>
</tr>
</tbody>
</table>

* Only the significant treatments are computed; ** Standard error; *** On MS medium containing sucrose (3%) (w/v); Data represents the mean of three replicates; Data scored after 5 wk of culture initiation. In the same column, figures followed by the same letter are statistically identical to the threshold of 5% (Newman-Keuls, ± standard error).
Earlier Britto et al.\textsuperscript{16} and Karuppusamy et al.\textsuperscript{17} also reported the synergistic effect of NAA and BA on nodal explants culture of \textit{Ceropogia bulbosa} and \textit{Hydrocotyle conferta} respectively. Dhavala and Rathore\textsuperscript{18} reported that cytokinin alone could not promote axillary bud breaking in \textit{Embelia ribes} unless one of the auxin especially IAA is incorporated in the medium in conjunction with cytokinin. While, in Guava nodal segment culture, incorporation of GA\textsubscript{3} along with BA was pre-requisite for axillary bud breaking\textsuperscript{15}. In case of sugarcane leaf culture, NAA and BA in combination promoted shoot bud formation but NAA alone induced somatic embryogenesis\textsuperscript{2}. 

**Plant regeneration and culture proliferation**—The shoot buds/micro shoots developed on initiation medium were maintained for another two passages. The micro shoots are than maintained on MS medium containing sucrose (3\%) and different PGRs. For regeneration of plantlets, incorporation of one of the PGRs was obligatory. In absence of PGRs all the cultures degenerated. Amongst the two PGRs tested, BA in the entire range did not support optimum culture proliferation. When BA tested singly, at concentration of 3 \(\mu\)M supported 4 shoot bud formation after 8 weeks of culture where plant height was stunted (\(~3.1\) cm) with only two roots. But incorporation of Kn singly proved to be superior for shoot proliferation, root formation and plant height. About 7 shoot buds with an average of 8 roots were formed on medium containing Kn (3 \(\mu\)M) where average plant height was \(~4.5\) cm (Table 3, Fig. 1 e). While BA and Kn in combination exhibited a more or less a similar response in the entire range studied and did not support optimum plant regeneration and culture proliferation. The effectiveness of cytokinin on plant regeneration and culture proliferation is reported\textsuperscript{19,20}. In \textit{Acacia confusa}, BA, NAA and Kn in combination (0.05 and 0.05 mgL\textsuperscript{-1} respectively) produced maximum shoot buds where as many as 25 shoot buds developed in culture\textsuperscript{3}.

**Rooting, hardening and transplantation to potting mix**—Though there were some roots formations in some regenerated shoots on regeneration medium, but roots were not fully developed and shorter in length. Regenerated shoots (\(~4\) cm in length, sources directly from regeneration medium) induced roots when transferred on rooting medium. The shoots were treated differentially for inducing roots. In general, pulse treatment with NAA was found to be inferior over incorporation of NAA in the medium. Of the different concentration of NAA used for inducing roots, a concentration of 3 \(\mu\)M supported maximum root growth where as many as 14 roots per plant developed after 4 weeks of culture (Table 4, Fig. 1 f). At lower concentrations roots were shorter and plantlets were etiolated while, at higher concentrations, roots formation was impaired accompanied by swelling of plants as well as roots.

In comparison to above, the pulse treatment of micro shoots with NAA, roots formation as well as shoot growth was poor (Fig. 2). Under optimum condition only 8 roots were formed after ~4 weeks of culture against 14 roots when NAA incorporated in the medium. Amongst the different pulse treatments, pulse treatment with NAA concentrations of 10 and 15 \(\mu\)M were equally effective for inducing roots and vertical increase in plant height. Auxins have been shown to act as a local morphogenetic trigger on the formation of lateral roots in \textit{Arabidopsis}, leading to the specification of founder cells of the new organ from previous differentiated cells\textsuperscript{31}. The promatory effect of NAA on rooting is also described in rice\textsuperscript{22}, in \textit{Populus euphratica}\textsuperscript{23}. In \textit{Acacia confusa} NAA was better option followed by IAA and IBA\textsuperscript{3} while, in some other reports IBA was found to be superior over other PGRs for rooting\textsuperscript{3,20}.

\begin{table}[h]
\centering
\begin{tabular}{llllll}
\hline
PGRs & Conc. (\(\mu\)M) & Average height of plantlet (cm) & No. of shoot buds formed/explant & No. of roots formed per plantlet \\
\hline
BA & Kn & & & & \\
0 & 0 & - & - & - & - \\
3 & 0 & 3.1 \(\pm\)0.1\textsuperscript{c} & 4\textsuperscript{b} & - & - \\
6 & 0 & 4.1 \(\pm\)0.1\textsuperscript{b} & 3\textsuperscript{c} & - & - \\
9 & 0 & 3.6 \(\pm\)0.2\textsuperscript{b} & 2\textsuperscript{d} & - & - \\
0 & 3 & 4.5 \(\pm\)0.2\textsuperscript{a} & 7\textsuperscript{c} & 8\textsuperscript{b} & 8\textsuperscript{b} \\
0 & 6 & 3.0 \(\pm\)0.3\textsuperscript{c} & 3\textsuperscript{c} & 3\textsuperscript{b} & 3\textsuperscript{b} \\
3 & 0 & 2.9 \(\pm\)0.2\textsuperscript{c} & 2\textsuperscript{d} & 3\textsuperscript{b} & 3\textsuperscript{b} \\
0 & 3 & 4.3 \(\pm\)0.1\textsuperscript{a} & 5\textsuperscript{b} & 4\textsuperscript{b} & 4\textsuperscript{b} \\
3 & 6 & 3.5 \(\pm\)0.3\textsuperscript{b} & 3\textsuperscript{c} & 3\textsuperscript{b} & 3\textsuperscript{b} \\
3 & 9 & 3.5 \(\pm\)0.2\textsuperscript{b} & 3\textsuperscript{c} & 3\textsuperscript{b} & 3\textsuperscript{b} \\
6 & 3 & 3.0 \(\pm\)0.2\textsuperscript{a} & 3\textsuperscript{c} & 2\textsuperscript{d} & 2\textsuperscript{d} \\
6 & 6 & 2.5 \(\pm\)0.2\textsuperscript{c} & 2\textsuperscript{d} & 1\textsuperscript{c} & 1\textsuperscript{c} \\
6 & 9 & 3.3 \(\pm\)0.5\textsuperscript{b} & 3\textsuperscript{c} & 3\textsuperscript{b} & 3\textsuperscript{b} \\
9 & 3 & 2.8 \(\pm\)0.1\textsuperscript{d} & 2\textsuperscript{d} & 2\textsuperscript{d} & 2\textsuperscript{d} \\
9 & 6 & 2.5 \(\pm\)0.2\textsuperscript{d} & 2\textsuperscript{d} & 3\textsuperscript{c} & 3\textsuperscript{c} \\
9 & 9 & 2.8 \(\pm\)0.2\textsuperscript{d} & 2\textsuperscript{d} & 2\textsuperscript{d} & 2\textsuperscript{d} \\
\hline
\end{tabular}
\caption{Effects of different PGRs on plant regeneration and mass multiplication of \textit{S. flaccidifolius}\textsuperscript{*}.}
\textsuperscript{*}On MS medium containing sucrose (3\%) (w/v).
\textsuperscript{**}Only the significant treatments are computed.}
\end{table}
The rooted plants were hardened on medium with ½MS salt solution containing sucrose (2%) and maintained for 6-7 weeks under normal laboratory condition (Fig. 1 g). The hardened plants were transferred to plastic pots as mentioned in the materials and methods (Fig. 1 h). The plants were successfully transferred to a shade-house and then to field. About 200 plants were tested for survival and about 70% survival was registered after two months of transfer.

### Table 4—Role of NAA on induction of rooting in plantlets regenerated from nodal segments of S. flaccidifolious

<table>
<thead>
<tr>
<th>NAA Conc. (µM)</th>
<th>No. of roots formed/plantlet</th>
<th>Type of response*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2c</td>
<td>Roots were very small and degenerated.</td>
</tr>
<tr>
<td>1</td>
<td>5c</td>
<td>Plantlets etiolated, leaves light green, roots short</td>
</tr>
<tr>
<td>2</td>
<td>8b</td>
<td>Plantlets slightly etiolated, leaves dark green and healthy roots but shorter</td>
</tr>
<tr>
<td>3</td>
<td>14a</td>
<td>Healthy plantlets, profuse rooting, healthy roots with distinct root hairs.</td>
</tr>
<tr>
<td>4</td>
<td>9b</td>
<td>As above but root hairs not distinct</td>
</tr>
<tr>
<td>5</td>
<td>6c</td>
<td>Plantlets swelled and swelling of roots.</td>
</tr>
<tr>
<td>6</td>
<td>6c</td>
<td>As above</td>
</tr>
</tbody>
</table>

* On MS medium containing sucrose (2%).

Data represents the mean of three replicates.

Data scored 4 wk of culture on the above media.

In the same column, figures followed by the same letter are statistically identical to the threshold of 5% (Newman-Keuls, ± standard error).

**Fig. 2—Effect of plus treatment with NAA on root induction of S. flaccidifolious micro shoots**

**Conclusion**

In the present study, an effective *in vitro* regeneration protocol via axillary bud breaking of nodal explants for rapid propagation of *S. flaccidifolious* was developed. The procedure reported in this communication suggests that multiplication via nodal explants culture could be commercially feasible methods for *S. flaccidifolious*. Micropropagation is more rapid, continuous and efficient than propagation via conventional cutting because it can supply uniform and consistent plant materials for investigations of important secondary metabolites (dye) produced by this species.

**References**


