IN VITRO PROPAGATION OF ALTERNANTHERA VERSICOLOR (LINN.) - A MEDICINAL HERB

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ABSTRACT
A complete protocol for micropropagation of Alternanthera versicolor using leaf and internodal explants was developed. An explants from A. versicolor established on Murashige and Skoog medium were treated with various concentrations and combinations of auxins and cytokinins to determine the best method for callus formation, shoot regeneration and root formation. MS medium supplemented with 1 mg/l BAP and 1 mg/l IAA was determined to be the most suitable for callus induction. After 2 weeks, the callus was then transferred to shooting media which consisted of half strength MS basal medium, 1 mg/l IAA and 1 mg/l BAP. After 4 weeks, structures were observed with their basal ends embedded on the callus. These structures turned into purple colored shoot buds. MS medium supplemented with 1 mg/l IAA and 1 mg/l BAP showed maximum shoot multiplication. For rooting, the optimal medium was half strength MS medium supplemented with 1 mg/l IAA. Rooted plantlets were then transferred to sand bag vessels which provided the high humidity environment. The hardened plants were then successfully established in the soil medium and can function in the natural environment.

Key words: Alternanthera versicolor, micropropagation, callus, shooting.

1. INTRODUCTION
Plant tissue culture techniques are being increasingly exploited for clonal multiplication and in vitro conservation of valuable indigenous germplasm threatened with extinction. Greater demand for these plants especially for the purpose of food and medicine is one of the causes of their rapid depletion from primary habitats. Micropropagation offers a great potential for large scale multiplication of such useful species and subsequent exploitation (Boro et al., 1998).
Micropropagation is the practice of rapidly multiplying stock plant material to produce a large number of progeny plants, using modern plant tissue culture methods. Micropropagation is used to multiply novel plants, such as those that have been genetically modified or bred through conventional plant breeding methods. It is also used to provide a sufficient number of plantlets for planting from a stock plant which does not produce seeds, or does not respond well to vegetative reproduction. Frederick Campion Steward was the person who discovered and pioneered micropropagation and plant tissue culture in the late 1950s and early 1960s. He was one of Cornell's finest.

Plants are the main source of many modern medicines. It is estimated that approximately one quarter of prescribed drugs contain plant extracts or active ingredients obtained from or modeled on plant substances. Recent estimates suggest that over 9,000 plant species have known medicinal applications in various cultures and countries and this is without comprehensive research amongst several indigenous and other communities. In India, approximately 1700 plants species are used in Ayurveda, 500 for Siddha, 400 for Unani, 300 for Amchi systems of medicine with substantial overlaps of common plants among the system. Green leafy vegetables (greens) play a major role in the Sri Lankan diet, probably due to the influence of traditional herbal medicine, easy accessibility and low cost. Furthermore, green leaves are considered as a main source of vitamins, minerals and fibre for the local consumers. Due to their dietary importance, many scientific studies have been carried out on the nutritive values of green leaves (Gayathri et al., 2006).

The in vitro propagation is an alternative tool for large scale multiplication and may increase the number of propagules for cultivation as well as aid the replacement of natural populations (Kumaraswamy and Anuradha, 2010; Jaimsha et al., 2010; Gokhale and Bansal, 2009; Bashir et al., 2008; Roy, 2008; Johnson et al., 2007; Johnson et al., 2005; Johnson, 2004; Johnson and Manickam, 2003; Boro et al., 1998). Callus production and shoot regeneration from primary callus or hypocotyl explants have been reported by Flores et al. (1982), Flores and Teutonico (1986) in A. hypochondriacus, A. tricolor and by Bagga et al. (1987) in A. paniculatus. Bennici et al.(1992) studied 4 different species of Amaranthus (A. audatus, A. hypochondriacus, A. cruentus and A. hybridus) for callus induction, growth and organogenesis. Bennici et al. (1997) studied in vitro behavior of A. cruentus, A. hybridus and A. hypochondriacus. Preliminary work on in vitro callus production was reported by Kayani, (2008). A few published reports are available on micropropagation of A. sessilis (Singh et al., 2009; Boro et al., 1998; Bennici and Schiff, 1997).

*Alternanthera versicolor*, Amaranthaceae family is a weed and occurs in both wetlands and uplands and can grow on a variety of soil types. The plant spreads by seeds, which are wind and water-dispersed and by rooting at stem nodes (Scher, 2004). It is a weed of rice throughout tropical regions and of other cereal crops, sugarcane and bananas. Although it is a weed, it has many utilities. Young shoots and leaves are eaten as a vegetable in southeast Asia (Scher, 2004). The young tips are eaten as a vegetable. The leaf is very rich in iron, vitamin A and dietary fiber. The plant contains protein and soups made with the leaf are given to anemic patients in rural areas. It contains abundant carotene, therefore it is used for curing night blindness. The plant enhances the secretion of milk in new mothers (Naples, 2005) and it is used as a remedy against intestinal cramps, diarrhoea and dysentery (intestinal disorder), and externally for curing night blindness. The plan t enhances the secretion of milk in new mothers (Naples, 2005) and it is used as a hair tonic.

In previous research, Bennici et al. (1992) studied 4 different species of Amaranthus (A. caudatus, A. hypochondriacus, A. cruentus and A. hybridus) for callus induction, growth and organogenic processes from hypocotyl and epicotyl cultures and found that 5.4 µM of naphthalene acetic Acid (NAA) plus 4.4 µM of benzyl amino purine (BAP) or 2.3 µM of 2,4-dichlorophenoxyacetic acid (2,4-D) plus 2.3 µM of kinetin were the best combinations for callus induction and complete plantlets were obtained from rooted adventitious shoots. Callus production and shoot regeneration from primary callus or hypocotyl explants have been reported by Flores et al. (1982), Flores and Teutonico (1986) in A. cruentus, A. hypochondria, A. tricolor and by Bagga et al. (1987) in A. paniculatus. Strong cytokinin such as BAP or isopentenyl adenine (2-iP) seems to be effective agents for shoot regeneration. The endogenous auxin/cytokinin balance and age dependant competence of explants tissues may play an important role in regeneration (Bennici and Schiff, 1997). A rapid in vitro propagation system has been established from mature shoot tip and nodal segments of a highly valuable medicinal plant *Alternanthera sessilis* Linn., (Gnanaraj et al., 2011). This study describes the results of the study taken up for developing effective reproducible and simple protocol for the large scale multiplication of the economically and medicinally important plant *A. versicolor*.
2. MATERIALS AND METHODS

Plants were collected from SRM University campus, Kattankulathur, Kancheepuram, Tamilnadu, India. Young shoots were harvested and washed with running tap water and surface sterilized in 0.05 and 0.1% mercuric chloride for 2, 3 and 5 min. After rinsing 3-4 times with sterile distilled water, shoot tip, leaves, stem nodes and internodes were cut into smaller segments (0.5 to 1.0 cm) used as the explants. The explants were placed horizontally (leaves and internodal segments) as well as vertically (shoot tip and nodal segments) on solid basal Murashige and Skoog (1962) medium supplemented with 3% sucrose, 0.7% (w/v) agar (Hi-Media) and different concentration (0.5-2.0 mg/l) and combination of BAP and Kin for in vitro shootlets regeneration. Regenerated shoots were separated from the cultures individually and used for root induction. The pH of the medium was adjusted to 5.8 before autoclaving at 121°C for 15 min. The cultures were incubated at 25 ± 2°C under cool fluorescent light (3000 lux 16 hr/d photoperiod). For rooting, the in vitro raised shootlets were transferred to the ½ MS medium augmented with different concentrations of auxins (IAA & IBA). Each and every experiment was performed with 20 replicates and repeated twice. For hardening, the in vitro raised plantlets were removed from culture, washed thoroughly with tap water planted in small polycups filled with sterile garden soil (3:1), covered by unperforated polybags, and hardened for 4 weeks in a mist chamber before transfer to field.

3. RESULTS AND DISCUSSION

3.1. Callus induction

Callus initiation was observed on the surface or cut ends of the explants after 14 days of inoculation. The best callus induction response for leaf explants of A. versicolor was observed on MS medium supplemented with 1 mg/l - IAA and 1 mg/l BAP. No callus induction was noted on 1 mg/l IAA or 1 mg/l BAP and the explants withered away. When equal concentrations of auxins and cytokinins were supplemented in the media high frequencies of callus induction were noted. When a high concentration of auxin (IAA) was used in combination with lower concentration of cytokinin (BAP), a low frequency of callus induction was noted. Similar development was observed in Amaranthus by Flores et al. (1982) and Bennici et al. (1992). When a high concentration of cytokinin was used with a low concentration of auxin, a low frequency of callus induction was noted. Hence the callus obtained from MS medium supplemented with 1 mg/l IAA and 1 mg/l BAP were used for further analysis. The callus obtained from internodal segment is 89.9± 0.83% on the MS basal medium supplemented with IAA(table2) The calli were subcultured into fresh medium every 2 weeks.

<table>
<thead>
<tr>
<th>MS medium plus growth hormone</th>
<th>% of leaves from A. versicolor forming callus</th>
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<tbody>
<tr>
<td>1 mg/L IAA + 1 mg/ L BAP</td>
<td>98 ± 2.82</td>
</tr>
<tr>
<td>1 mg/L IAA</td>
<td>0</td>
</tr>
<tr>
<td>1 mg/ L BAP</td>
<td>0</td>
</tr>
<tr>
<td>0.5 mg/L IAA + 1 mg/ L BAP</td>
<td>49.5 ± 6.36</td>
</tr>
<tr>
<td>0.5 mg/L IAA + 0.5 mg/ L BAP</td>
<td>77.5 ± 3.53</td>
</tr>
<tr>
<td>1 mg/L IAA + 0.5 mg/ L BAP</td>
<td>53.5 ± 2.12</td>
</tr>
</tbody>
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Table 2. Effect of IAA on callus production from internodal segment A. versicolor.

<table>
<thead>
<tr>
<th>MS medium plus growth Hormone IAA in mg/l</th>
<th>Mean % of callus induction ± S.E</th>
</tr>
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<tbody>
<tr>
<td>0.0</td>
<td>00.0± 0.0</td>
</tr>
<tr>
<td>0.5</td>
<td>37.6±0.43</td>
</tr>
<tr>
<td>1.0</td>
<td>59.7±0.48</td>
</tr>
<tr>
<td>1.0</td>
<td>70.7±0.67</td>
</tr>
<tr>
<td>2.0</td>
<td>88.9± 0.83</td>
</tr>
<tr>
<td>2.5</td>
<td>63.4 ± 0.47</td>
</tr>
<tr>
<td>3.0</td>
<td>48.7± 0.64</td>
</tr>
</tbody>
</table>

Figure Shows the callus proliferation from the explants(21 days)

3.2. Shoot regeneration from callus

Callus obtained from leaf and internode explants of A. versicolor were transferred into shooting medium which consist of half strength medium, 1mg /l IAA and 1mg /l BAP .After 4 weeks structures were observed with their basal ends embedded on the callus and turned into purple coloured shoot buds. These structure were transferred into tissue cultured tubes consisting of half strength MS basal medium. After 21 days the individual shoots were cut of and transferred into tissue culture tubes containing MS medium supplemented with 1mg /l IAA and 1mg/l BAP. This combination show maximum shoot multiplication of 10 shoots / culture(table 3). Bennici et al (1997) found that high cytokinin:auxin ratio favour shoot regeneration in Amaranthus. In this study lowest number of shoot regeneration was observed on half strength MS medium 1 mg IAA only. No shoot multiplication was observed on half strength MS medium supplemented with 1 mg/ l BAP. Shoot multiplication was noted after the cultures were inoculated into the medium

Table 3.Effect of growth regulators on shoot multiplication.

<table>
<thead>
<tr>
<th>MS medium</th>
<th>Shoot multiplication of A. versicolor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mg/L IAA + 1 mg/ L BAP</td>
<td>10 shoots per culture</td>
</tr>
<tr>
<td>1 mg/L IAA</td>
<td>2 shoots per culture</td>
</tr>
<tr>
<td>1 mg/ L BAP</td>
<td>0</td>
</tr>
</tbody>
</table>
3.4. Rooting of regenerated shoots

For rooting, the shoots developed from callus of *A. versicolor* were cultured on rooting medium consisting of half strength MS medium containing different concentration of either IAA or IBA. Optimal rooting were observed on half strength MS medium supplemented with 1mg/l IBA. Some rooting response was also observed on MS media supplemented with 1 mg/l IAA. Bagga et al (1987) found that hypocotyl segment of *A. paniculatus* forms root on B5 medium supplemented with NAA and Bennici et al(1992) reported that Amaranthus responded well informing in roots with IAA plus kinetin and / IAA plus BAP.
Figure shows the root formation from the shoots (15 days)

Figure shows the root formation from the shoots (21 days)

3.5. Hardening of plantlets

The regenerated plantlets from the explant with healthy root and shoot system were transferred after washing with distilled water to remove the media traces from the roots. They were transferred into bottles containing sterilized soil and covered with sunbag vessels to maintain a high humidity environment for a week. Thereafter the sunbags were removed and the plantlets were transferred into bottles containing normal soil and they were grown under full sunlight.
4. CONCLUSION

Tissue culture was carried out by the supplementation of plant growth regulators such as auxins (IAA, NAA and 2,4-D) and cytokinins (BAP and KINETIN) to the MS medium for the induction of callus. The type of growth regulators and concentrations used varied according to the cell purpose. In auxins, 2,4-D was better when compared to IAA and NAA. In cytokinins, kinetins was better than BAP on callus induction. Both auxins and cytokinins were well suited for shoot initiation (apical bud, nodal explants). Both auxins and cytokinins produced better results on callus induction and shoot initiation. Auxins (IAA) and cytokinins (BAP) were supplemented in the MS medium to induce callus in the leaf and internode explants. The maximum callus induction from the leaf explants was 98% at the concentration of 1 mg/l IAA and 1 mg/l BAP and maximum callus induction from the internode explants was 88% when the medium supplemented with 2 mg/l IAA. The leaf explants gave better response than the internodal explants. The maximum number of shoots obtained was 10 from internodal explant when BAP and IAA amended in the MS medium at 1 mg/l and 1 mg/l BAP. The minimum number of shoots obtained was 2 from nodal explant when IAA was amended in MS medium at 1 mg/l. The optimal rooting was observed on the half strength MS medium supplemented with 1 mg/l IBA and 1 mg/l IAA.

The present study has resulted in the establishment of protocol for micropropagation of *Alternanthera versicolor* (L.) through leaf, shoot tip and nodal segments. This technique could be used as a tool for the large scale multiplication programmes.

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APPENDIX:

IAA – Indole Acetic Acid
IBA – Indole Butyric Acid
NAA – Naphthalene Acetic acid
2,4-D – 2,4 – Dichlorophenoxy Acetic Acid
BAP – Benzyl Amino Purine
MS Medium - Murashige and Skoog (1962) medium
REFERENCES