Micropropagation of *Alternanthera sessilis* (L.) using Shoot tip and Nodal segments

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Abstract
A rapid *in vitro* propagation system has been established from mature shoot tip and nodal segments of a highly valuable medicinal plant *Alternanthera sessilis* (L.). The explants were cultured on Murashige and Skoog's medium augmented with different concentrations and combinations of plant growth regulators for shoot bud initiation and multiplications. For shoot tip, highest frequency of shoot proliferation (94.3 ± 0.43) and maximum number per explants (23.4 ± 0.38) was observed in Murashige and Skoog's medium augmented with 2.0 mg/l of 6-Benzyl Amino Purine. For nodal segments, highest frequency of shoot proliferation (90.4 ± 0.82) and maximum number (15.2 ± 0.63) per node was observed in Murashige and Skoog's medium augmented with 1.5 mg/l of 6-Benzyl Amino Purine. Maximum percentage of callus formation (Leaves-92.4 ± 0.61; Inter-nodal -88.9 ± 0.83) was obtained on Murashige and Skoog's basal medium supplemented with 3% and 2, 4-Dichlorophenoxy acetic acid 2.0 mg/l. Highest efficiency (97.4 ± 1.36) of rooting and maximum number (6.3 ± 0.42) of rootlet per shoot let was achieved on half strength Murashige and Skoog's medium fortified with 3 mg/l of Indole-3-Butyric acid. Regenerated plants were successfully transferred to field (78%).

Keywords**:** *in vitro*; Calli; Clonal; Shoot tip; Nodal segments

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INTRODUCTION

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). *Alternanthera sessilis* (L.) (India, Ponnaganni) grows in the tropical regions of the world, especially tropical America, Africa, and Asia and also in temperate Asia. Its leaves and young shoots are eaten as vegetable (Chandrika *et al*., 2006) or cooked in soup. A decoction is recommended as a herbal remedy to treat wounds, flatulence, nausea, vomiting, cough, bronchitis, diarrhea, dysentery and diabetes. Its roots can relieve inflamed wounds (Hosamani *et al*., 2004). *A. sessilis* is known as Matyakshika in Ayurvedic medicine (Shyamala *et al*., 2005). A decoction of the leaves

is drunk for treating itchy and overheated skin (Gayathri *et al*., 2006). In Ghana, a decoction with some salt is taken to stop blood vomiting. In Nigeria, the pounded plant is used for headaches and vertigo and the leaf sap is sniffed up the nose to treat neuralgia. A paste is used to draw out spines or any other object from the body, and is also used to cure hernia. In Senegal and India, the leafy twigs are grounded to a powder and applied on snakebites. The people of Nepal use its roots for the treatment of stomachache (Sreedevi and Chaturvedi, 1993). The plant is also used in veterinary medicine in Kenya. *A. sessilis* is used in local medicine in Taiwan, often in mixtures with other medicinal plants to treat hepatitis, tight chest, bronchitis, asthma and other lung troubles, to stop bleeding, and as a hair tonic. In India, it is used as a cholagogue, abortifacient and febrifuge. In Thailand and Sri Lanka, it is used as a galactagogue. The leaves and shoots are boiled and drunk as an antihypertensive remedy (Acharya and Pokhrel, 2006). *A. sessilis* can be propagated using seeds and vegetative cuttings. Rooting behaviour of stem cutting and non-availability of seeds due to over exploitation (before flowering the leaves *A. sessilis* are harvested for commercial purpose) is major setback for plant propagation. Besides, such conventional propagation processes are season dependent and can be achieved only during monsoon period. 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 *et al*., 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. cruentus*, *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* behaviour of *A. cruentus*, *A. hybridus* and *A. hypochondriacus*. Preliminary work on *in vitro* callus production of *Achyranthes aspera* 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). This study describes the results of the study taken up for developing an effective reproducible and simple protocol for the large scale multiplication of the economically and medicinally important plant *A. sessilis*.

MATERIALS AND METHODS

Plants of *Alternanthera sessilis* (L.) (Amaranthaceae) collected from Kolli Hills, Salem, Tamil Nadu, India and grown in the Botanical garden of Muthayammal college of Arts and Science, Rasipuram, Tamil Nadu, 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, Mumbai) and different concentration (0.5-2.0 mg/l) and combination of BAP and Kin for *in vitro* shootlets regeneration. 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 (2250 lux 12 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 and NAA). 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.

RESULTS

The surface sterilization of *Alternanthera sessilis* was carried with different concentration of mercuric chloride such as 0.05%. 0.1% and 0.15% for different time duration. Among them, 0.1% mercuric chloride for 3½ min showed low percentage contamination and highest (96%) percentage of microbes/contaminants free explants. The explants treated with 0.05%, 0.1% and 1.5% of mercuric chloride for 3 min showed 50-65% of microbes free explants. The explants treated with 0.1% for 4 min and above and 0.15% for 3½ min and

above obtained hundred percentages of microbes free explants with high percentage of explants mortality, high concentration of mercuric chlorides leads the death of the explants (lethal effect). The medium (MS) augmented with different concentrations and combinations of plant growth regulators (BAP (0.5-2 mg/l), Kin (0.5-2 mg/l) and BAP (1.5 mg/l) + Kin (0.05- 0.1 mg/l)) were used for multiple shoots emergence from the shoot tip and nodal segments. The explants (shoot tip and nodal segments) of *A. sessilis* started growing in MS medium supplemented with BAP within a week. Highest percentage (94.3 ± 0.43%) and maximum number (23.4 ± 0.38) of shoot induction from shoot tip was observed on MS medium supplemented with 2.0 mg/l of BAP (Fig. 1 A-H) (Table 1). In case of nodal segments, highest percentage (90.4 ± 0.82%) and maximum number (15.2 ± 0.63) of shoot initiation was observed on MS medium fortified with 1.5 mg/l BAP (Fig. 1 N-R; Table 1). Callus was initiated from the leaves and inter-nodal segments on the

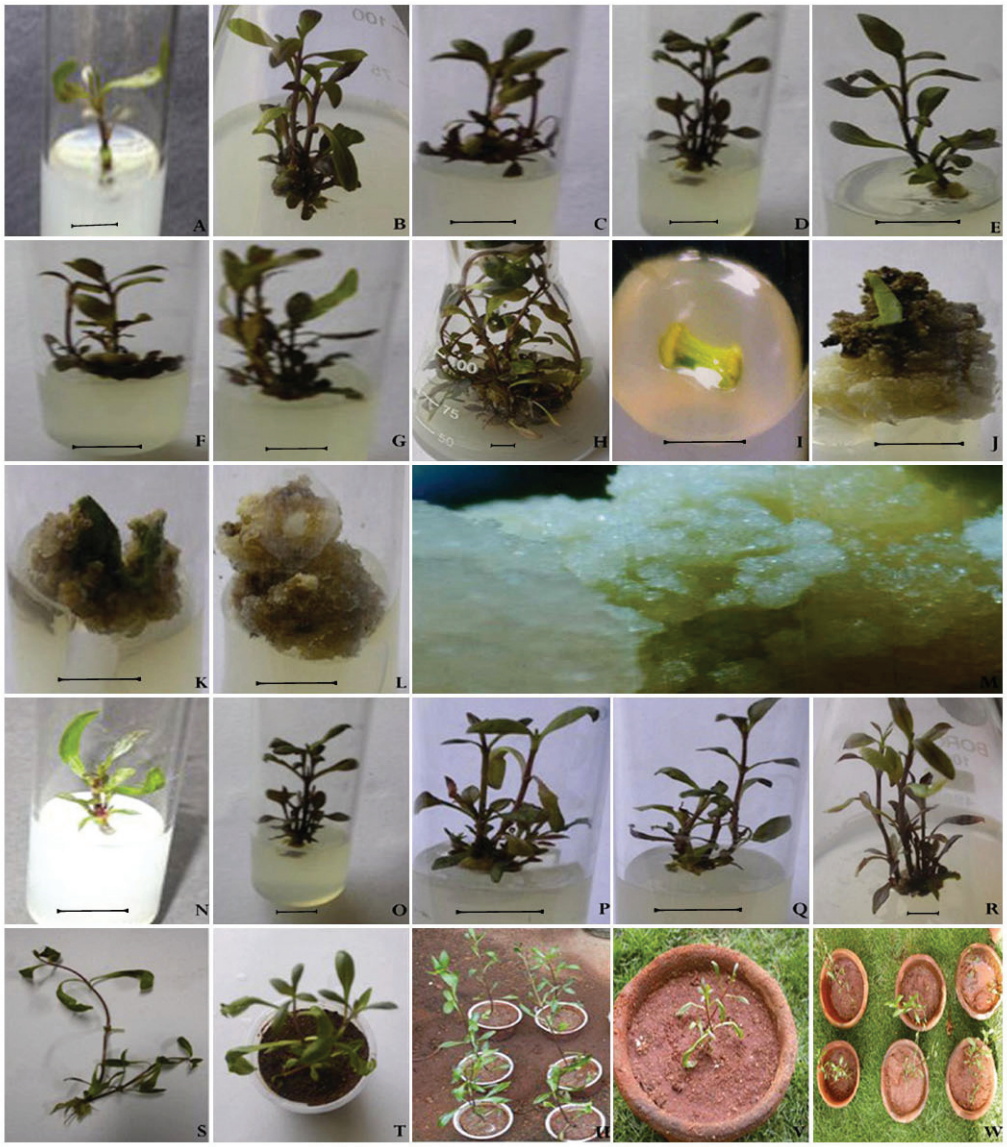

Figure 1. Micropropagation of *Alternanthera sessilis* (L.) using Shoot tip and Nodal segments. A: Multiple shootlets initial stage-Shoot tip B to H-Different stages of multiple shootlets-Shoot tip I- Inter-nodal segment derived calli-initial stage J-M-Leaves segments derived calli-different developmental stages N-Multiple shootlets initial stage-Nodal segments O to R-Different stages of multiple shootlets-Nodal Segments S- *In vitro* derived plantlet with shootlet and rootlets T and U-Different developmental stages of Hardened plants in Poly Cups V and W-Different developmental stages of Hardened plants in Pot Scale Bar 1cm = 1 cm.

Table 1. Effect of cytokinin on multiple shoots formation from shoot tip and nodal segments of *Alternanthera sessilis* L.

MS medium + Cytokinin concentration (mg/l)		Shoot Tip			Nodal Segments		
BAP	KIN	Mean percentage Shoot formation ± S.E.	Mean No. of shoots ± S.E.	Average shoot length (cm)	Mean percentage of Shoot formation ± S.E.	Mean No. of shoots ± S.E.	Average shoot length (cm)
0.0	0.0	46.5 ± 0.36	2.1 ± 0.63	2.0	34.2 ± 0.42	2.7 ± 0.34	3.1
0.5	0.0	62.5 ± 0.42	3.4 ± 0.71	4.0	52.1 ± 0.36	8.4 ± 0.42	4.0
1.0	0.0	80.3 ± 0.63	8.4 ± 0.54	3.5	78.5 ± 0.83	9.3 ± 0.83	5.4
1.5	0.0	86.2 ± 0.84	13.4 ± 0.63	3.7	90.4 ± 0.82	15.2 ± 0.63	5.5
2.0	0.0	94.3 ± 0.43	23.4 ± 0.38	6.3	75.7 ± 0.78	8. 4 ± 0.47	4.1
0.0	0.5	70.4 ± 0.56	3.4 ± 0.49	5.3	64.6 ± 0.53	6 4 ± 0.42	4.6
0.0	1.0	65.3 ± 0.42	4.7 ± 0.34	3.9	55.7 ± 0.72	6.7 ± 0.86	3.9
0.0	1.5	63.2 ± 0.24	5.5 ± 0.47	8.5	63.4 ± 0.57	7.4 ± 0.63	4.5
0.0	2.0	62.3 ± 0.56	5.5 ± 0.35	4.0	65.4 ± 0.34	5.4 ± 0.93	4.0
0.5	0.5	63.2 ± 0.64	7.5 ± 0.25	4.1	56.4 ± 0.56	5.2 ± 0.26	4.1
1.0	0.5	78.6 ± 0.54	9.5 ± 0.65	3.9	75.6 ± 0.94	6.8 ± 0.62	3.9
1.5	0.5	77.9 ± 0.73	10.4 ± 0.37	4.6	78.5 ± 0.73	7.3 ± 1.21	4.6
3.0	0.5	76.5 ± 0.53	15.6 ± 0.75	3.8	69.3 ± 0.86	5.8 ± 0.64	3.8
3.0	1.0	70.5 ± 0.84	17.3 ± 0.44	4.4	76.3 ± 0.78	41.4 ± 0.75	4.4
3.0	1.5	69.5 ± 0.57	81.6 ± 0.96	6.1	68.7 ± 0.76	33.6 ± 0.58	6.1

MS basal medium supplemented with different concentrations and combinations of PGRs (Table 2). Highest percentage of callus on leaves 92.4 ± 0.61% (Fig. 1 J, K, M) and inter-nodal segments 88.9 ± 0.83% (Fig. 1 I and L) were obtained on MS medium supplemented with 2 mg/l of 2, 4-D (Table 2). The calli obtained from leaves and inter-nodal segments were white in colour on MS medium supplemented with 2 mg/l of 2, 4-D. The *in vitro*-raised shootlets were transferred to half-strength MS medium with different concentrations of IBA, IAA and NAA for rooting (Table 3). Highest percentage (97.4 ± 1.36%) and maximum number (6.3 ± 0.42) rootlets were observed on MS medium augmented with 3.0 mg/l of IBA (Fig. 1 S). After 30 days, *in vitro*-raised plantlets were hardened in polycups containing a mixture of sterile garden soil: sand (3:1), covered with polypropylene bags and irrigated with 10x diluted MS liquid medium. The plants were kept in a culture room for 15 days. 88% of plants were successfully established in polycups (Fig. 1 T and U). After 15 days the polycups hardened plants were transferred to pots and kept in green house. Eighty three percent of plants were well established in the green house condition (Fig. 1 V and W). After one month, the plants were transferred to the field. About 78% of plants were established in the field.

DISCUSSION

Singh *et al*. (2009) observed that 5.5 ± 2.12 percentage of contamination when the explants were treated with 0.1% (w/v) mercuric chloride for 5 min and 40% (v/v) Sodium hypochlorite for 20 min. In contrary to Singh *et al*. (2009) observations, the explants treated with 0.1% mercuric chloride for 3½ min showed 4% microbial contamination in our study. In addition, the present study demonstrated that explants treated with 0.1% mercuric chloride for 4 min and above and 0.15% mercuric chloride for 3½ min was lethal to the explants and showed highest percentage of mortality (Above 75%).

The MS medium augmented with cytokinin alone or in combination with auxin induced maximum number of multiple shoots with maximum percentage. The nodal and shoot tip explants cultured on MS medium augmented with cytokinin (BAP) alone induced multiple shoot formation was observed in *Centella asiatica* (Tiwari *et al*., 2000), *Plectranthus ventiveroides* (Siva subramanian *et al*., 2002), *Baliospermum montanum* (Johnson and Manickam, 2003), *Adenia hondala* (Johnson *et al*., 2004), *Vitis thunbergii* (Mei, 2005), *Passiflora mollussima* (Johsnon *et al*., 2007), *Bupleurum distichophyllum* (Karuppusamy and

Table 2. Effect of 2, 4-D on Callus production from the Leaves and Inter-nodal segments of *Alternanthera sessilis* L.

MS medium + Plant Growth Regulator (2, 4-D) in mg/l	Mean percentage of callus induction ± S.E.		Type of Callus	
	Leaves	Inter-nodal	Leaves	Inter-nodal
0.0	00.0 ± 0.0	00.0 ± 0.0	NIL	NIL
0.5	46.4 ± 0.64	37.6 ± 0.43	Friable	Semi-friable
1.0	68.4 ± 0.94	59.7 ± 0.48	Friable	Semi-friable
1.5	73.4 ± 0.86	70.7 ± 0.67	Friable	Semi-friable
2.0	92.4 ± 0.61	88.9 ± 0.83	Friable	Semi-friable
2.5	61.1 ± 0.58	63.4 ± 0.47	Semi-friable	Semi-friable
3.0	52.1 ± 0.67	48.7 ± 0.64	Semi-friable	Semi-friable

Table 3. Effect of Auxins on rooting on *in vitro* derived shootlets of *Alternanthera sessilis* L.

S. No.	Auxins concentration (mg/l)	Mean percentage of Rootlets formation ± S.E	Mean No. of rootlets per shootlets ± S.E	Mean length of rootlets in cm
1	IBA(1.0)	85.8 ± 1.24	5.8 ± 0.46	4.1
2	IBA (2.0)	86.6 ± 1.43	6.1 ± 0.23	3.9
3	IBA (3.0)	97.4 ± 1.36	6.3 ± 0.42	4.2
4	IBA (5.0)	83.5 ± 1.42	4.8 ± 0.67	3.6
5	IAA(1.0)	53.1 ± 1.27	2.4 ± 0.36	2.4
6	IAA (2.0)	60.3 ± 0.89	2.6 ± 0.49	2.4
7	IAA (3.0)	58.4 ± 0.91	3.1 ± 0.44	2.6
8	IAA (5.0)	63.5 ± 1.34	2.5 ± 0.65	2.3
9	NAA(1.0)	41.4 ± 0.67	1.1 ± 0.64	2.4
10	NAA (2.0)	53.4 ± 1.16	1.4 ± 0. 81	2.6
11	NAA (3.0)	61.8 ± 1.43	1.4 ± 1.12	2.8
12	NAA (5.0)	56.4 ± 1.26	1.3 ± 0.92	2.6

Pullaiah, 2007), *Mentha viridis* (David and Arockiasamy, 2008), *Stevia rebaudiana* (Janarthanam *et al*., 2009), *Vitex negundo* (Islam *et al*., 2009) and *Marsdenia brunoniana* (Ugriah *et al*., 2010). The effect of 2, 4-D in the induction of callus was also reported by Manickam *et al*. (2000) in *Withania somnifera*, Johnson *et al*. (2005) in *Rhinacanthus nasutus* and Johnson, (2007) in *Phyllanthus amarus*, the effect of Kin was reported by Rout *et al*., 1999 in *Plumbago zeylanica*. Singh *et al*. (2009) observed the maximum percentage (98 ± 2.82%) of callus induction on leaves segments of *A. sessilis* cultured on MS medium fortified with 1 mg/l 2, 4-D+1 mg/l BAP. In addition to the direct regeneration, we made an attempt to proliferate the callus induction on leaves and inter-nodal segments of *A. sessilis*. In contrary to Singh *et al*. (2009) observation, we observed 92.4 ± 0.61% of callus induction on MS medium augmented with 1 mg/l 2, 4-D alone. Our result was directly consonance with the Manickam *et al*. (2000), Johnson *et al*. (2005) and Johnson, (2007) observations. Bagga *et al*. (1987) found that hypocotyl segments of *A. paniculatus* formed roots on B_5 medium supplemented with NAA. Bennici *et al*. (1992) reported that *Amaranthus* responded well in forming roots with IAA plus kinetin and/or IAA plus BAP. Singh *et al*. (2009) observed the optimal rootlets formation on half strength MS medium supplemented with 1 mg/l IBA. In the present study, we observed the highest percentage and maximum number of rootlets per shootlets on half strength medium supplemented with 3 mg/l IBA. Here the result showed consistency

with other studies where the addition of IBA promoted the induction of roots in several systems including *Citrus reticulata*, *Citrus limon* (Singh *et al*., 1994), *Ocimum basilicum* (Sahoo *et al*., 1997), *Salvia sclarea* (Liu *et al*., 2000), *Artemisia judaica* (Liu *et al*., 2003), *Bixa orellana* (Neto *et al*., 2003), *Dioscorea zingiberensis* (Chen *et al*., 2003), *Woodfordia fruticosa* (Islam *et al*., 2009) and *Ophiorrhiza eriantha* (Jaimsha *et al*., 2010).

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

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