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Establishment of Plantlet Regeneration System from Nodal, Internodal and Leaf Explants of *Sauropus androgynus* (Sweet Shoot)

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ABSTRACT

Propagation of *Sauropus androgynus* (SA) by tissue culture technology provides a better alternative than conventional propagation, as it ensures uniformity in production of sustainable plantlets with desirable traits. The objective was to establish an efficient plant regeneration protocol using leaf, nodal and internodal explants of SA, starting from the establishment of aseptic cultures to the acclimatization stage of plantlets. The effects of plant growth regulators on the growth performance of SA were studied. Results showed *in vitro* mass multiplication of SA was achieved via indirect organogenesis and shoot regeneration pathway. After 30 days of culture inoculation, high percentage of aseptic cultures were established from nodal (85%), internodal (85%) and leaf (91%) explants sterilized with 70% (v/v) ethanol for 1 min followed by 20% (v/v) Clorox for 20 min. MS medium supplemented with 2.0 mg L^{-1} BAP (6-benzylaminopurine) and 0.5 mg L^{-1} IAA (indole-3-acetic acid) proved to be effective in inducing adventitious shoots from internodal (95%), nodal (96%) and leaf (90%) explants, after 60 days of culture. High frequency of callus formation (38%) was obtained from leaf explants cultured on MS medium containing 2.0 mg L^{-1} IAA and 0.5 mg L^{-1} BAP. Rooting response of 90% was achieved from cultured shoots using half-strength MS medium enriched with 1.0 mg L^{-1} IAA and from these, 85% survived acclimatization and grew vigorously after 4 weeks. This optimized protocol established a complete *in vitro* plant regeneration of SA and has never been reported previously. This may form the basis for future research in germplasm conservation and genetic transformation for sustainable production of high quality SA.

Key words: *Sauropus androgynus*, surface sterilization treatments, adventitious shoot and callus regeneration, rooting, acclimatization

INTRODUCTION

Sauropus androgynus L. Merr., commonly known as 'Cekur manis' and 'Sweet shoot' is a green leafy perennial underutilized shrub belonging to the Phyllanthaceae family (Benjapak *et al*., 2008). It is cultivated commercially in tropical regions of Southeast Asia, notably for its high yields and palatability (Benjapak *et al*., 2008). The genus *Sauropus* consists of 40 species of plants, but only a few are economically and medicinally important (Wiart, 2006). For instance, *S. brevipes* and *S. spatulifolius* are used to treat diarrhea, sore throat and cough, while the trunks of *S. villosus* are used as a construction material in Philippines (Wiart, 2006). For *S. androgynus*, it has been used for many traditional purposes such as fever reduction, ulcer healing, prevention of urinary

disorders, alleviation of food poisoning, stimulation of breast milk production and for post-partum uterine contractions (Kanchanapoom *et al*., 2003; Lee *et al*., 2011). Besides that, secondary metabolites, such as quercetin, kaempferol, lignin, papaverine, sauroposide, ferulic acid, chlorogenic acid, megastigmane and corchoionoside can be extracted from the leaf extract of sweet shoot (Lee *et al*., 2011; Selvi and Basker, 2012). It has been proven in animal studies that these secondary metabolites, namely quercetin and kaempferol have both antimicrobial and antioxidant properties, via it's inhibitory effects on lipid oxidation and propagation of oxidizing chain reactions (Lee *et al*., 2011; Selvi and Basker, 2012; Khoo *et al*., 2015). United States Department of Agriculture (USDA) has accorded sweet shoot as a highly nutritive source of crude fiber, protein, vitamins A, B and C mineral salts and antioxidants (Lee *et al*., 2011; Selvi and Basker, 2012; Khoo *et al*., 2015). Thus, it is imperative to exploit this vast potential of sweet shoot using plant tissue culture technology for the benefit of the masses especially in treating and preventing diseases related to these deficiencies.

Sweet shoot has attracted worldwide attention as a multipurpose shrub. Recent literature claimed that sweet shoot has positive effects in reducing body weight in Wistar rats with a sweet shoot isolated compound, 3-O-b-D-glucosyl-(1→6)-b-D-glucosyl-kaempferol (GGK) and such it has been proposed as a safe method in fighting obesity especially with the current global obesity pandemic (Yu *et al*., 2006). Moreover, there was also a study using sweet shoot in poultry egg farming and it showed increased egg production with the additional benefits of reduced cholesterol in each egg (Santoso *et al*., 2005). Furthermore, it has also been shown to aid in reducing the glucose levels in patients with type 2 diabetes mellitus (Sai and Srividya, 2002). Despite its importance, sweet shoot still appears to be underutilized with restricted usage (Wiart, 2006). In order to unlock the true potential of sweet shoot, researchers need to harness these many positive attributes of sweet shoot by formulating a method to mass produce sweet shoot in a sustainable manner for future research and also secondary metabolite extraction (Wiart, 2006).

Sweet shoot is traditionally propagated via woody shoot cuttings and seeds. Propagation of sweet shoot via these methods appear to be restricted due to its high susceptibility to fungal and bacterial diseases, labour intensiveness and time consuming with low survivability and possibility of growth and yield retardation (Kregor, 1999; Kartha *et al*., 1974). Furthermore, the recalcitrant nature of the seeds also causes difficulties in generating the plantlets from seed and the seed raised plants exhibit genetic variations (Davidonis and Knorr, 1991; Li and He, 2006). Hence, *in vitro* propagation of sweet shoot serves as an alternative method in increasing productivity and developing a sustainable agricultural system. The pharmaceutical production could also be upregulated using plant tissue cultures under optimized conditions. These automated controls of tissue growth would definitely contribute to cost reduction and productivity improvement of plantlets with desirable traits. The objective of this study was to develop a protocol for plant regeneration and to evaluate the effects of plant growth regulators on the growth performance of sweet shoot. This experiment helped to reveal the importance of plant growth regulators in bringing about complete morphogenesis in sweet shoot under the present experimental conditions.

MATERIALS AND METHODS

Plant materials and growth conditions: The healthy plants of high antioxidant yielding strain (about 32.4 mg/100 g kaempferol and 46.1 mg/100 g quercetin) of *S. androgynus* (sweet shoot) obtained from the University Putra Malaysia (UPM), Selangor, Malaysia were grown in pots containing a mixture of perlite and compost (1:1) and maintained in ambient conditions in a

Table 1: Surface sterilization treatments tested for the elimination of contaminants in the establishment of aseptic cultures of sweet shoot

	Surface sterilization treatment			
Treatment	Ethanol (%)	Duration (min)	Clorox (%)	Duration (min)
T1	-	-	10	10
	-	-	10	20
	-	-	10	30
T2	-	-	20	10
	-	-	20	20
	-	-	20	30
T3	70	1	10	10
	70	1	10	20
	70	1	10	30
T4	70	1	20	10
	70	1	20	20
	70	1	20	30

shade house at the University of Nottingham Malaysia Campus (Selvi and Basker, 2012). Sweet shoot grown in pots was identified by post-harvest horticulturist, Dr. Mahmud bin Tengku Muda Mohamed, Faculty of Agriculture, UPM, Selangor, Malaysia, prior to usage.

Surface sterilization of nodal, internodal and leaf explants: Juvenile leaf, nodal and internodal segments of sweet shoot were used as explants for the establishment of aseptic cultures. Juvenile explants (1.5 cm) were washed thoroughly under running tap water for 5 min and surface sterilized using a range of treatments (Table 1). Treatment 1 and 2 consisted of a single step surface sterilization with Clorox plus a drop of Tween 20, whereas treatment 3 and 4 consisted of two steps, where the explants were prewashed with 70% (v/v) ethanol for 1 min followed by Clorox plus a drop of Tween 20. Disinfected explants were rinsed 3 times with sterile purified water to remove all traces of sterilants.

After surface sterilization of explants, all the explants were inoculated aseptically onto Murashige and Skoog (1962) medium supplemented with 3% (w/v) sucrose (Duchefa, Netherlands) and 0.25% (w/v) Phytagel™ (Sigma-Aldrich, USA) (Murashige and Skoog, 1962). The pH of media was adjusted to pH 5.8±0.1 and autoclaved at 121°C with 15 psi for 15 min. All culture jars were incubated at 26±2°C under 16-h photoperiod, provided by cool white fluorescent light at a photosynthetic flux of 40-50 μmol m^{-2} sec^{-1}.

The axenic status of cultured tissues was confirmed by using nutrient broth culture assay. Samples of tissues were cultured in nutrient broth for 5-7 days to detect the presence of microbial contaminants. Data on contamination, mortality and survival percentage were recorded after 30 days from culture initiation.

Optimization of adventitious shoot and callus production: Juvenile leaf, nodal and internodal explants were cultured on semi-solid MS medium supplemented with IAA (0, 0.10, 0.25, 0.50, 1.00 and 2.00 mg L^{-1}) (Sigma-Aldrich, USA) in combination with BAP (0, 0.10, 0.50, 1.00, 2.00 and 3.00 mg L^{-1}) (Sigma-Aldrich, USA). The culture medium was solidified with 0.25% (w/v) Phytagel™ and supplemented with 0.3% (w/v) sucrose. Adventitious shoots and calluses formed were subcultured onto fresh MS media at intervals for mass multiplication of sweet shoot. The percentage of responded explants, total number of shoots formed per explant, shoot length, percentage of callus formation and estimated callus fresh weight were recorded after eight weeks of culture initiation.

Morphological characteristics: Observations were made every 3 weeks and the morphogenesis of shoot and callus regeneration in cultures were observed using a Nikon® ECLIPSE 80i stereomicroscope (Nikon, USA) with 100x total magnification. Cultures with any potential physiological abnormalities were also recorded every 4 weeks.

***In vitro* root induction:** Proliferated shoots of 3-5 cm in length were harvested from *in vitro* cultures and implanted on half-strength semi-solid MS medium supplemented with different concentrations of IAA (0, 0.5, 1.0 and 1.5 mg L^{-1}) for root induction. All media were enriched with 3% (w/v) sucrose and 0.25% (w/v) Phytagel™. Cultures were maintained in the dark and the data on rooting were evaluated after 10 days of rooting period.

Hardening and acclimatization of plantlets: Plantlets with well developed roots were washed thoroughly under running tap water to remove any residual agar and soaked in 0.2% (v/v) carbendazim for 5 min. For hardening-off, the rooted plantlets were placed into culture jars containing purified water and maintained at ambient conditions in tissue culture laboratory for 1 month. The plantlets were then transplanted to pots containing equal parts (1:1) of perlite and compost (peat moss, topsoil and potting soil) for establishment *ex vitro* in shade house. High humidity was maintained in the shade house with 65% shading and intermittent misting during the first 4 weeks of acclimatization. The survival percentage of plantlets was recorded after 2 months of acclimatization.

Statistical analysis: The experiments were conducted in a completely randomized block design with 3 replicates of 20 cultures for each treatment. Each experiment was repeated thrice. Data in percentages were transformed using logistic regression analysis. All data were analyzed statistically by analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) with significant difference level at $p<0.05$ using Statistical Package for Social Sciences (SPSS) software version 16.

RESULTS

Surface sterilization of nodal, internodal and leaf explants: In the present study, the analysis of variance showed significant effects of surface sterilization treatments in reducing the incidence of microbial contamination in leaf, nodal and internodal explants of sweet shoot ($p<0.05$). The two step surface sterilization approaches using ethanol and Clorox (treatment 3 and 4) was proven statistically to be effective in reducing the incidence of microbial contamination, ranging from 6.67-25.00% in leaf explants, from 15.00-35.00% in nodal explants and from 11.67-31.67% in internodal explants ($p<0.05$) (Table 2). For treatment 3 and 4, the survival percentage of cultured explants significantly increased as the duration of surface sterilization treatment increased. However, the detrimental effects of prolonged exposure to sterilization treatment also increased as there were higher numbers of necrotic cultures observed, especially in leaf, nodal and internodal explants treated with treatment four at 30 min. Therefore, surface sterilization with 70% (v/v) ethanol for 1 min followed by 20% (v/v) Clorox for 20 min was the preferred method for sweet shoot, as it managed to have a low contamination (6.67%-15.00%) with low mortality (1.67%) and high survivability (85.00-91.67%) percentages of cultured explants after one month of surface sterilization.

Table 2: Effects of surface sterilization method and duration on the incidence of contamination, mortality and survival of leaf, nodal and internodal explants, after 30 days of culture

Explant type and treatment	Clorox duration (10 min)			Clorox duration (20 min)			Clorox duration (30 min)		
	CON (%)	MOR (%)	SUR (%)	CON (%)	MOR (%)	SUR (%)	CON (%)	MOR (%)	SUR (%)
Leaf segment									
Control (T0)	100.00^{Aa}	0.00	0.00	100.00^{Aa}	0.00	0.00	100.00^{Aa}	0.00	0.00
10% clorox (T1)	25.00^{Abc}	6.67	68.33	18.33^{Ab}	5.00	76.67	15.00^{Ab}	5.00	80.00
20% clorox (T2)	38.33^{Ab}	5.00	56.67	33.33^{ABb}	5.00	61.67	18.33^{Bb}	5.00	76.67
EtOH+10% clorox (T3)	25.00^{Abc}	1.67	73.33	10.00^{Bb}	3.33	86.67	8.33^{Bb}	3.33	88.33
EtOH+20% clorox (T4)	11.67^{Ac}	3.33	85.00	6.67^{Bc}	1.67	91.67	8.33^{Bb}	1.67	90.00
Internodal segment									
Control (T0)	100.00^{Aa}	0.00	0.00	100.00^{Aa}	0.00	0.00	100.00^{Aa}	0.00	0.00
10% clorox (T1)	46.67^{Ab}	0.00	53.33	33.33^{Ab}	0.00	66.67	30.00^{Ab}	0.00	70.00
20% clorox (T2)	36.67^{Ab}	0.00	63.33	31.67^{Ab}	0.00	68.33	26.67^{Ab}	0.00	73.33
EtOH+10% clorox (T3)	31.67^{Abc}	0.00	68.33	23.33^{ABbc}	1.67	75.00	13.33^{Bc}	3.33	86.67
EtOH+20% clorox (T4)	21.67^{Ac}	0.00	78.33	15.00^{Ac}	0.00	85.00	11.67^{Ac}	1.67	86.67
Nodal segment									
Control (T0)	100.00^{Aa}	0.00	0.00	100.00^{Aa}	0.00	0.00	100.00^{Aa}	0.00	0.00
10% clorox (T1)	50.00^{Ab}	0.00	50.00	43.33^{Ab}	0.00	56.67	38.33^{Ab}	0.00	61.67
20% clorox (T2)	43.33^{Ab}	0.00	56.67	33.33^{Ab}	0.00	66.67	28.33^{Abc}	0.00	71.67
EtOH+10% clorox (T3)	35.00^{Abc}	0.00	65.00	20.00^{Abc}	1.67	78.33	18.33^{Bcd}	1.67	80.00
EtOH+20% clorox (T4)	21.67^{Ac}	0.00	78.33	15.00^{Ac}	0.00	85.00	13.33^{Ad}	1.67	85.00

CON: Percentage contaminated, MOR: Mortality percentage, SUR: Percentage survived, EtOH: Ethanol. Values expressed as the mean of three replicated experiments, each replicate consisted of 20 cultures. Different letters within column (lowercase) indicate a significant difference ($p<0.05$) according to ANOVA and DMRT

Based on the results exhibited in Table 2, the two bleach concentrations tested (treatment 1 and 2) failed to reduce the incidence of microbial contamination in leaf (15.00-38.33%), nodal (28.33-50.00%) and internodal (26.67-46.67%) explants of sweet shoot ($p>0.05$). Likewise, increasing the duration of these treatments also did not reduce the risk of contamination.

For treatment 4 at 20 min, the contamination percentage of nodal and internodal explants (15.00%) was greater than leaf explants (6.67%). Qualitative nutrient broth assay was carried out to detect the presence of microbial contamination in tissue cultures of sweet shoot. Microbial contamination was observed in the nutrient broth assay and on culture media between 3 and 21 days after surface sterilization of explants. It was noted that both bacterial and fungal pathogens grew rapidly in this study and colonized the culture media, which resulted in increased tissue mortality. In some cases, some bacteria were not even detected visually at the initial or later stages of cultures, but it may have been contaminated after several subcultures.

Optimization of adventitious shoot and callus production: The initiation and proliferation of adventitious shoots and calluses were strongly influenced by both the types of explants and the hormonal treatments ($p<0.05$) (Table 3). All explants were found to be responsive towards the tested hormonal treatments with the exception of the control treatment, where there was no shooting response recorded in both the leaf and internodal explants (Table 3). Of the different hormonal treatments tested, semi-solid MS medium supplemented with 2.00 mg L^{-1} BAP and 0.50 mg L^{-1} IAA was noted to be effective for shoot induction and proliferation, where 96.67% of nodal explants were able to produce adventitious shoots, followed by internodal and leaf explants producing 95 and 90% of shooting responses respectively. This medium also produced the highest number of longest shoots (2.86-7.65 shoots) from leaf, nodal and internodal explants with a mean greater than 4.10 cm (Table 3).

In this study, the effect of hormonal treatments on the number and length of adventitious shoots was found to be dependent on both the cytokinin concentration and the types of explants

Table 3: Effects of plant growth regulator treatment on the number of shoots, shoot length, incidence (%) of callus formation and callus fresh weight on leaf, nodal and internodal explants in vitro of sweet shoot, 60 days after culture initiation

Growth regulators (mg L^{-1})		Adventitious shoot production			Callus formation	
IAA	BAP	Responding explants (%)	No. of shoots per responding explant	Longest shoot length per responding explant (%)	Cultures with callus (%)	Estimated callus fresh weight per responding explant (mg)
Leaf segment						
0.00	0.00	0.00^{c}	0.00^{e}	0.00^{d}	0.00^{d}	0.00^{e}
0.10	3.00	68.33^{b}	10.09^{a}	0.46^{e}	5.00^{d}	1.08^{e}
0.25	3.00	86.67^{a}	9.16^{ab}	0.72^{e}	11.67^{cd}	3.47^{e}
0.50	2.00	90.00^{a}	7.65^{b}	4.10^{a}	21.67^{bc}	17.23^{d}
1.00	1.00	90.00^{a}	3.44^{d}	3.20^{b}	28.33^{ab}	26.24^{c}
2.00	0.50	85.00^{a}	7.65^{b}	2.93^{b}	38.33^{a}	32.08^{a}
2.00	0.10	81.67^{a}	5.85^{c}	2.31^{b}	33.33^{ab}	29.55^{b}
Internodal segment						
0.00	0.00	78.33^{b}	0.00^{e}	0.00^{f}	0.00^{d}	0.00^{e}
0.10	3.00	93.33^{a}	5.51^{a}	0.54^{e}	0.00^{d}	0.00^{e}
0.25	3.00	93.33^{a}	4.39^{b}	0.82^{e}	6.67^{cd}	2.36^{e}
0.50	2.00	95.00^{a}	2.86^{c}	4.97^{a}	16.67^{bc}	11.39^{d}
1.00	1.00	93.33^{a}	1.97^{d}	4.23^{b}	23.33^{ab}	17.04^{c}
2.00	0.50	96.67^{a}	4.35^{a}	3.43^{c}	33.33^{a}	28.72^{a}
2.00	0.10	95.00^{a}	3.30^{c}	2.80^{d}	30.00^{a}	22.07^{b}
Nodal segment						
0.00	0.00	83.33^{b}	3.13^{de}	1.76^{e}	0.00^{c}	0.00^{e}
0.10	3.00	96.67^{a}	7.76^{a}	0.88^{f}	0.00^{c}	0.00^{e}
0.25	3.00	96.67^{a}	7.17^{ab}	1.87^{e}	3.33^{c}	1.60^{e}
0.50	2.00	96.67^{a}	6.37^{b}	5.10^{a}	8.33^{bc}	6.39^{d}
1.00	1.00	96.67^{a}	3.03^{e}	4.24^{b}	16.67^{ab}	10.80^{c}
2.00	0.50	96.67^{a}	5.21^{c}	3.17^{c}	25.00^{a}	23.79^{a}
2.00	0.10	96.67^{a}	3.99^{d}	2.64^{d}	20.00^{a}	16.60^{b}

Values expressed as the mean of three replicated experiments, each replicate consisted of 20 cultures. Different letters within column (lowercase) indicate a significant difference ($p<0.05$) according to ANOVA and DMRT, IAA: Indole acetic acid, BAP: Benzylaminopurine

($p<0.05$) (Table 3). Higher concentrations of BAP (2.00 and 3.00 mg L^{-1}) significantly enhanced the number of adventitious shoots, whereas the average shoot length decreased as the concentration of BAP increased. At lower concentrations of BAP (0.10, 0.25, 0.50 and 1.00 mg L^{-1}), the leaf, nodal and internodal explants produced fewer number, but longer adventitious shoots.

The interaction of IAA with BAP in the concentrations tested was also found to be effective in obtaining compact calluses from all the tested explants ($p<0.05$) (Table 3). As shown in Table 3, the percentage of cultured explants forming callus was generally low. The highest callus formation was observed in leaf explants (38.33%) followed by internodal (33.33%) and nodal (25.00%) explants, when cultured in semi-solid MS medium containing 2.0 mg L^{-1} IAA and 0.50 mg L^{-1} BAP ($p<0.05$) (Table 3). The types of callus grown were hard green globular calluses, which showed good growth, from this optimum hormonal composition. The estimated callus fresh weight on leaf, nodal and internodal explants was also found to be significantly higher ($p<0.05$) on media containing 2.00 mg L^{-1} IAA and 0.50 mg L^{-1} BAP, when compared to the other concentrations of IAA and BAP. The control explants did not promote callus formation due to the absence of growth regulators. Furthermore, nodal and internodal explants cultured on medium containing 0.1 mg L^{-1} IAA and 3.0 mg L^{-1} BAP did not promote callus initiation, as it was too weak to facilitate the callus initiation, after 60 days of cultivation. Likewise, brownish green calluses with low callus fresh weight were also observed from all the tested explants on the MS medium supplemented with 3.0 mg L^{-1} BAP and 0.25 mg L^{-1} IAA. The callus growth only improved on MS medium enriched with decreasing concentrations of BAP and increasing concentrations of IAA, producing green-coloured calluses. Thus, these findings suggest that the frequency of callus production increased with respect to different concentrations of IAA in the MS medium and this increment was significant ($p<0.05$).

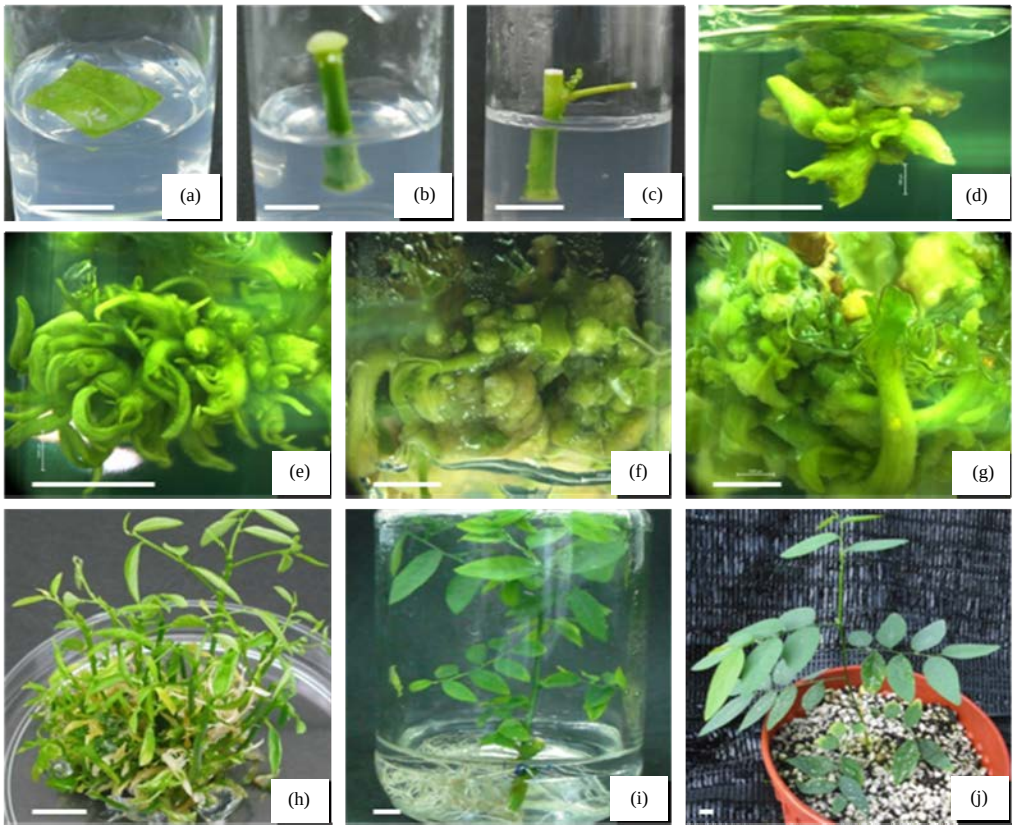

Fig. 1(a-j): *In vitro* shoot regeneration of sweet shoot from leaf, nodal and internodal explants, (a) Responding leaf explants after culture for seven days, (b) Basal end of internodal explants enlarged after three days of culture, (c) Shoot growth from axillary bud after 3 days of initiation, (d) Induction of shoot buds from internodal explants, (e-g) Callus and early stage of adventitious shoots generated from internodal explants on MS medium supplemented with 2.0 mg L^{-1} BAP and 0.5 mg L^{-1} IAA, (h) Development of adventitious shoots after 60 days of culture, (i) Root development at the base of *in vitro* shoot cultured on MS medium containing 1.0 mg L^{-1} IAA and (j) Establishment of *in vitro* sweet shoot plant after one month of transfer. Bar represents 1.0 cm (a-h)

Morphological characteristics: Leaf, nodal and internodal explants of sweet shoot responded well in the cultures and produced calluses and adventitious shoots within 60 days of initiation (Fig. 1). The formation of adventitious shoots and calluses from leaf, nodal and internodal explants occurred via indirect organogenesis and shoot regenerative pathway. After three weeks of culture initiation, the explants had enlarged and developed the globular structures on the margins of leaf explants and at the basal end of nodal and internodal explants (Fig. 1a-c). After 30 days, the cut surface of leaf and the basal end of nodal and internodal explants were covered with hard globular callus and subsequently proliferated into shoot buds (Fig. 1d-f). Leaf primordia were also noted (Fig. 1g). The formation of shoot buds on callus which developed into adventitious shoots was visible after 60 days from culture initiation (Fig. 1h). Subculture of these shoots resulted in the development of more shoots without any apparent

Table 4: Effect of IAA on rooting percentage, number of roots and root length on proliferated shoots of sweet shoot, after 10 days of rooting period

IAA concentration (mg L^{-1})	Rooting (%)	No. of roots	Longest root length (cm)
0.0	30^{b}	1.33^{b}	9.13^{a}
0.5	40^{b}	2.25^{b}	8.85^{a}
1.0	90^{a}	6.22^{a}	6.72^{b}
1.5	90^{a}	6.56^{a}	6.10^{b}

Values expressed as the mean of three replicated experiments, each replicate consisted of 20 cultures. Different letters within column (lowercase) indicate a significant difference ($p<0.05$) according to ANOVA and DMRT, IAA: Indole acetic acid

decline in vigour or reduction in proliferation over a period of 3 months. A two-fold ratio of adventitious shoot and callus proliferation was achieved at every subculture.

***In vitro* root induction:** In the present study, a significant interaction between the hormonal treatments was observed for root induction of sweet shoot ($p<0.05$) (Table 4). A relatively low percentage (30%) of microshoots rooted in the hormone-free control medium. The number of rooted microshoots significantly increased from 30.00-90.00%, as the concentration of IAA increased from 0.0-1.5 mg L^{-1}. After 10 days of culture, primary roots were formed in 90% of shoots on half-strength semi-solid MS medium supplemented with 1.0 mg L^{-1} of IAA with an average of 6.22 roots per shoot and an average root length of 6.72 cm. Well developed primary and lateral root system were recorded in 10 days on this IAA supplemented medium (Fig. 1i).

Acclimatization of plantlets: Out of 20 rooted shoots transplanted *ex vitro*, survival of 85% was achieved after 1 month of transfer to the shade house (Fig. 1j). All these rooted plantlets were maintained in the shade house in temperatures ranging between 25.5 and 33.5°C, with the relative humidity of 62.3-90.4% and photosynthetically active radiation levels from 40.85-94.61 μmol m^{-2} sec^{-1}. All these acclimatized plantlets grew vigorously in compost and showed high homogeneity with no visual evidence of somaclonal variation. A development period of 4 months was required for the micropropagation of sweet shoot starting from surface sterilization stage to acclimatization stage.

DISCUSSION

The establishment of aseptic cultures of a plant requires a thorough understanding of the physiological status of the plant together with its susceptibilities to pathological contaminants in order to eradicate microbial infection in tissue cultures. In this study, it was observed that the highest microbial contamination occurred in the control cultures (T0 with sterile purified water only) when the various sterilants and their respective concentrations were evaluated. This attest to the publications by many authors including Cassels (1996), on the importance of surface sterilization of explants in micropropagation (Cassels, 1996).

Till date, there are no articles reporting on the percentage of aseptic cultures obtained in the study of sweet shoot. The most commonly adopted procedure involved surface sterilization of nodal explants of sweet shoot with 0.1% (w/v) mercuric chloride for 10 min followed by three rinses with sterile purified water (Li and He, 2006; Eganathan and Parida, 2012). Shortly after Li and He's research (Sai and Srividya, 2002; Tejavathi *et al*., 2010) also treated nodal, shoot tip and leaf explants using Tween 20 for 15 min followed by 0.1% (w/v) Bavistin for 10 min (Tejavathi *et al*., 2010). However, the use of mercuric chloride and fungicides in explant surface sterilization is not recommended due to potential toxicity to both the researcher and the environment and furthermore, there is also an increased risk of plant growth retardation (Matthew and Duncan,

1993). High concentrations and extended exposure of mercuric chloride has been shown to be also phytotoxic, which resulted in high plant tissue mortality and tissue necrosis. Similarly, increasing the exposure duration to 10% (T3) and 20% (T4) Clorox from 20-30 min had successfully reduced the rate of contamination in all tested explants, but slightly reduced number of explants survived to grow and develop. This phenomenon was attributed to the high risk of tissue death associated with the extended exposure to sterilants, as previously reported by Hammond *et al*. (2014). For these reasons, surface sterilization with 70% ethanol for one minute followed by 20% Clorox for 20 min was the preferred method in reducing microbial contamination of sweet shoot. This method of sterilization has been reported in tissue cultures of Jamaican yams, sugarcane and *Carissa carandas* (Mitchell *et al*., 1995; Moutia and Dookun, 1999; Rai and Misra, 2005).

According to Razdan (1993), the type of sterilant used, concentration and exposure duration depends on the nature of explant and species (Razdan, 1993). In this study, the contamination percentage of nodal explants was greater than leaf explants. This could be due to the poor contact of nodal explants with the disinfecting agents, whereby contaminants entrapped in between the nodes and petioles could not be adequately expelled. Furthermore, trimming nodal and internodal explants prior to surface sterilization exposed the intercellular spaces and vessel cavities of the cut ends to contaminants (Moutia and Dookun, 1999). These contaminants may have remained within the tissues and escaped from the surface sterilization treatments, which resulted in the occurrence of contamination in *in vitro* cultures. In this experiment, juvenile leaf explants showed a higher percentage of phytotoxicity when compared to nodal and internodal explants. There is a possibility that the thin cuticle layer of leaf explant may not be able to withstand the strong concentration of the disinfectant as there was presence of necrosis at the trimmed edges of the leaf explant. These trimmed edges served as a site for deeper penetration of the disinfectant into the left explants tissues, leading to its toxic effects and resultant reduction in growth responses (Thakur and Sood, 2006). Similar observations were seen in numerous plant species such as in potato and oil palm (Villafranca *et al*., 1998; Omamor *et al*., 2007).

To date, scientific studies assessing the potentially important benefit of sweet shoot for use as medicinal plants are still limited with only four scientific studies reporting on the micropropagation of sweet shoot (Li and He, 2006; Tejavathi *et al*., 2010; Eganathan and Parida, 2012; Philomena, 1993). Indirect shoot development is the common regenerative pathway observed in nodal, internodal and leaf explants of sweet shoot, where the explants are cultured on MS medium supplemented with 2.0 mg L^{-1} BAP and 0.5 mg L^{-1} IAA to promote adventitious shoot proliferation. Preliminary studies showed that more numbers of adventitious shoots were obtained via indirect shoot development than direct shoot development (data not shown). BAP has been considered to be one of the most effective cytokinins for shoot induction in plant tissue culture (Baskaran and Jayabalan, 2005). Malik *et al*. (2005), Naik and Chand (2011) suggested the advantage of BAP for shoot induction may be attributed to the ability of plant tissues to metabolize BAP more readily and also the ability of BAP to induce the production of natural hormones, such as zeatin within the tissue (Malik *et al*., 2005; Naik and Chand, 2011). Moreover, BAP is not easily metabolized and thus, persists in the medium in either free or ionised forms (Buah *et al*., 2010). The results obtained in this study were in agreement with previous observations of pistachio cultivar, *Plumbago* species and *Spilanthes acmella*, where the introduction of high BAP concentrations enhanced the number of shoot production (Tilkat *et al*., 2008; Das and Rout, 2002; Saritha and Naidu, 2008).

Several attempts have been made to enhance shoot formation using different types and combinations of auxins and cytokinins (Li and He, 2006; Tejavathi *et al*., 2010; Eganathan and Parida, 2012; Philomena, 1993). In this study, the combination of BAP and IAA was proven to be effective in producing sweet shoot plantlets from leaf, nodal and internodal explants, which supports the findings from Li and He (2006). In Philomena (1993) studied the effects of coconut water on induction and growth of shoots from nodal explants of sweet shoot and the plantlets showed increased shoot formation and growth with NAA, whereas kinetin had no effect (Philomena, 1993). The addition of adenine sulphate (10.86 μM) and gibberellic acid (1.44-2.88 μM) into L2 medium also facilitated direct regeneration of sweet shoots with roots, however, the published data was not analyzed in detail (Tejavathi *et al*., 2010). Furthermore, Eganathan and Parida (2012) also reported that maximum number of shoots (10.13 shoots) and shoot length (2.07 cm) was observed on MS medium containing 1.0 mg L^{-1} BAP and 0.1 mg L^{-1} kinetin (Eganathan and Parida, 2012). Although the number of shoots produced from the study done by Eganathan and Parida (2012) is high (10.13 shoots), the shoot length obtained in this study is about 2 folds longer than their experiment (Eganathan and Parida, 2012). All the results obtained in this study showed slight variation when compared to previous studies done by other scientists, such as the presence of coconut water, gibberellic acid, NAA and kinetin in the culture medium for shoot regeneration.

Organogenesis is an indispensable tool for plant regeneration using tissue culture technologies for plant transformation and for the extraction of bioactive metabolites. In this study, plant regeneration of sweet shoot via indirect organogenesis has been accomplished from leaf, nodal and internodal segments on semi-solid MS medium containing 2.00 mg L^{-1} IAA and 0.5 mg L^{-1} BAP. Earlier studies reported poor callusing from both nodal and leaf explants of sweet shoot. This might be due to the slow growth and the tendency towards the incidence of necrosis in sweet shoot calluses (Li and He, 2006; Tejavathi *et al*., 2010; Eganathan and Parida, 2012; Long *et al*., 2010). Based on the above mentioned results, leaf explants had a higher callus production capacity than internodal and nodal explants and the differences were significant. The midrib region of leaf explants exhibited more potential for callus formation, which was in agreement with the results obtained by Kumar *et al*. (1992) and Bejoy *et al*. (2008). The variation of callus induction in different explants may be due to the level of maturity of leaf, nodal and internodal explants in response to different growth regulators (Ahmad *et al*., 2010). This has been highlighted in a study on orchid, whereby 60% of the juvenile leaf explants responded well with callusing, while only 35% of nodal explants formed callus when cultured on MS basal medium containing optimum concentration of 2,4-D and kinetin (Janarthanam and Seshadri, 2008).

In Tejavathi *et al*. (2010) also exhibited high amounts of callus from sweet shoot leaf explant when cultured on L2 medium individually supplemented with IBA and kinetin (Tejavathi *et al*., 2010). Moreover, Eganathan and Parida (2012) also mentioned that nodal explants of sweet shoot growing under high concentrations of BAP and kinetin showed callus formation at the proximal edge of the nodal explants (Eganathan and Parida, 2012). However, these studies failed to mention the actual concentration of plant growth regulators used for callus induction and the percentage of callus formation in their experiment. As such, it is difficult to compare between this present study and other studies on which combination of plant growth regulators is better for callus formation. In other words, this is the only standardized study to evaluate the effects of plant growth regulators in sweet shoot.

For any micropropagation protocol, successful rooting of shoots is a pre-requisite to facilitate their establishment *ex vitro*. Several attempts have been made to enhance the rooting efficacy in sweet shoot using various concentrations of different auxins, such as IAA, indole-3-butyric acid (IBA), α-naphthalene acetic acid (NAA) and 2,4-dichlorophenoxy acetic acid (2,4-D) ranging from 0.1-2.0 mg L^{-1}. Tejavathi *et al*. (2010) reported that sweet shoot can root well in half-strength L2 medium containing optimal concentrations of NAA, Tejavathi *et al*. (2010) but the rooting response might be suppressed with an increase in NAA, as previously found in *Striga hermonthica* and *Vanilla planifolia* (Janarthanam and Seshadri, 2008; Ma *et al*., 1998). Similarly, the root development was poor when IBA (0.5 mg L^{-1}) and NAA (0.25 mg L^{-1}) were added to half-strength MS medium, as reported by Eganathan and Parida (2012). The root length of sweet shoot obtained in this study was about 2.6 folds longer than their optimized culture medium (Eganathan and Parida, 2012). Therefore, IAA was then selected as a preferred auxin to induce roots from shoot. Although IAA has been shown to have weak auxin activity for root formation in some studies, the positive effects of IAA found in this study on sweet shoot may be postulated to be due to its preferential uptake, transport and stability of IAA together with the effects on gene activation. The beneficial effects of IAA on root development *in vitro* have also been noted in studies performed on *Azadirachta indica*, *Vitis thunbergii* and *Hoslundia opposite* (Venkateswarlu *et al*., 1998; Lu, 2002; Prakash and van Staden, 2007).

Successful acclimatization of rooted plantlets and their subsequent transplantation to the field is a critical step for commercial exploitation of *in vitro* technology. The acclimatization of rooted sweet shoot was relatively easy compared to other woody plant species, in which the survival rate of 85% was recorded after one month of acclimatization in the shade house. Low mortality percentage (15%) obtained in sweet shoot upon transfer of rooted plantlets to *ex vitro* conditions could possibly be due to the higher exposure of radiation and the development of nonfunctional stomata, cuticle and root system, as previously reported by Mathur *et al*. (2008). Therefore, the physiological and anatomical characteristics of rooted plantlets necessitate gradual acclimatization to the shade house environment.

CONCLUSION

A novel regeneration protocol for sweet shoot has been successfully developed using leaf, nodal and internodal explants via indirect organogenesis and shoot regenerative pathway. The protocol described above has the potential for rapid and mass propagation of sweet shoot for commercialization, conservation purposes, genetic improvements and the production of bioactive metabolites. Various types of DNA-based molecular markers (RAPD, RFLP, ISSR, AFLP) can be used for future studies in order to identify the true to type plants with no somaclonal variation from the mother plant. A more detailed study such as histological analysis and scanning electron microscopy analysis should be conducted in the future for better characterization of the callus present in this study.

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