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Regeneration of multiple shoots from the callus cultures of *Sauropus androgynus* (L.) Merr.

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ABSTRACT

Morphogenetic potential of shoot tip, leaf and nodal explants of *Sauropus androgynus* was investigated to develop a reliable plant regeneration protocol via indirect organogenesis. Explants were cultured on Murashige and Skoog's (MS) medium and Phillips and Collins (L2) medium supplemented with different concentrations of 6-benzyl aminopurine (BAP) either alone or in combinations with other auxins such as indole-3-acetic acid (IAA), α- naphthalene acetic acid, 4-dichloro phenoxy acetic acid, and indole-3-butyric acid along with gibberllic acid (GA_3). However, L2 medium with hormones was found to be more supportive for induction of profuse callus and subsequent regeneration of shoot buds than the same hormonal composition of MS medium. A maximum number of shoots per culture were differentiated from the organogenic callus raised on L2 + BAP (13.20 μM) + IAA (11.42 μM) + GA_3 (1.44 μM). Thus, regenerated shoots were rooted on ½ L2 + IAA (2.85 μM). The plantlets were acclimatized for 3-4 weeks in pearlite before transferring them into pots containing soil, sand and manure in 1:1:1 ratio. Nearly, 90% of survival was recorded.

KEY WORDS: Callus, indirect organogenesis, *Sauropus androgynus*, somaclonal variations

INTRODUCTION

In vitro techniques for regeneration of plants from various types of cultures have progressed in last few decades to such an extent that a significant contribution has been made in mass propagation of economically important plants. *Sauropus androgynus*, a member of *Euphorbiaceace* is one such important plant. It is commonly known as a multivitamin plant which is consumed as leafy vegetable for its nutritional and medicinal value [1-3]. In recent years, the popularity of this leafy vegetable is deteorating as it is tagged toxic on excessive consumption due to the presence of an alkaloid called "papaverine." This alkaloid is reported to cause severe lung damage manifested by a condition called Bronchiolitis Obliterans on excessive intake [4,5]. Plant tissue culture technology is now well-established as a tool for studying and solving the basic and applied problems in plant propagation and helps in genetic manipulations which are less possible by conventional methods. Further beneficial somaclonal variations which are arising through the callus cultures can be exploited for the genetic improvement of the economically important plants [6,7]. Perusal of literature revealed that this taxon is underexploited except two preliminary reports on *in vitro* studies [8,9]. In this direction, the present work is an attempt to regenerate the plants from the callus cultures which is a prerequisite for any biotechnological approaches to overcome the negative aspects and genetic improvement of the taxon.

MATERIALS AND METHODS

Collection of Plant Material

Healthy plants were procured from the University of Agricultural Sciences, Bangalore. They were maintained in the Botanical garden, Department of Botany, Bangalore University, Bangalore. The third to sixth branches from the tip of the plant were excised and nodal segments of 1-1.5 cm, shoot tip of 0.75-1.0 cm and leaf explants of 1.5-2.0 cm^2 were used as explants.

Surface Sterilization

The explants were washed with Tween-20 for 5-10 min. After thorough washing under running water for 30 min, they were treated with Bevastin, a fungicide (0.1%) for 5 min. Further surface sterilization was carried out by treating the explants with mercuric chloride (0.1%) for

2 min. After each treatment, the explants were washed thoroughly with sterile water.

Culture Medium

The surface sterilized explants were inoculated on Murashige and Skoog's (MS) medium [10] and Phillips and Collins (L2) [11] medium supplemented with various auxins such as indole-3-acetic acid (IAA), α-naphthalene acetic acid (NAA), 2,4-Dichloro phenoxy acetic acid (2,4-D), indole-3-butyric acid (IBA), and cytokinin - 6-benzyl aminopurine (BAP) either alone or in combination along with gibberllic acid (GA_3). Sucrose (3%) and bacteriological grade agar (0.8%) were used as carbon source and gelling agent respectively. pH of the medium was adjusted to 5.6 and autoclaved for 15 min at 108 kpa.

Culture Conditions

The cultures were incubated at 25°C ± 2°C under fluorescent tube lights with 16 h light/8 h dark cycle at a light intensity of 25 μ mol/m^2/s. The cultures were regularly subculture either to the same composition or to other combinations of growth regulators depending on the requirements of the experiment.

Histology

The organogenic callus was fixed in FAA (formalin:acetic acid:alcohol, 90:5:5) for 24 h. Customary paraffin technique was followed. Sections were cut at 20-26 μm thick and stained with Heidenhain's heamatoxylin and counterstained with Orange-G/Eosin/Erythrosin. Photomicrographs were taken with Nikon binocular microscope using Canon camera.

RESULT AND DISCUSSION

In the present work, MS [10] and L2 media [11] were supplemented with various concentrations of different growth regulators to induce multiple shoots from the callus cultures. L2 medium proved to be best over MS in terms of percent response and induction of multiple shoots.

Effect of Auxins

Callus induction was observed from all the explants supplemented with various concentrations of different auxins like IAA, 2,4-D, NAA, and IBA. Callus initiation occurred on sides of the cut ends of the explants and within 2 weeks of culture on NAA and 2,4-D, whereas callus induction was delayed until the 3rd week on IAA supplemented medium and more than a month on IBA supplemented medium. Of all the auxins tried, NAA induced profuse callus from all the explants. The nature of callus varied depending on the auxin supplemented to the medium. Creamish friable callus was obtained on IAA supplemented medium, whereas green hard compact callus was formed in the presence of 2,4-D and NAA in the medium. On IBA supplemented medium, the callus was brown. The brown is mainly due to the presence of phenolics in the cells. Oxidized products of phenolics appear brown [12]. The presence of auxins at lower concentrations in L2 medium was sufficient to promote callus induction unlike MS medium which required a higher concentration of auxins. L2 medium was formulated by L2 to initiate callus and suspension cultures of Red clover and other legumes. Hence, it is known as leguminous medium. It differs from MS medium in not having nicotinic acid which is found to be inhibitory for callus induction and growth in legumes [11]. Leaf explants showed the maximum amount of callus followed by node and shoot tip. Thus, obtained callus remained non-organogenic even after several subcultures onto the media containing only auxins.

Effect of Cytokinin

Multiple shoots were initiated from 6 weeks old green organogenic callus derived from shoot tip and nodal explants on L2 medium supplemented with various concentrations of BAP ranging from 4.44 to 22.20 μM. whereas, the callus derived from leaf explants failed to show any morphogenetic response on BAP alone supplemented medium. BAP is considered as a potential hormone to induce multiple shoots in other taxa *Cucumis melo* [13], *Bauhinia variegata* [14], *Parkinsonia aculeata* [14], *Pistachio* [15], and *Cucurbita maxima* Duch [16]. High frequency of multiple shoots regeneration was obtained on MS + BAP (17.17 and 22.20 μM) and L2 + BAP (8.87 and 13.20 μM) (Table 1). Lesser concentrations of cytokinin gave best results in L2 medium in comparison with MS medium which required high concentrations of cytokinin for best results (Figure 1a and b). Further, when the concentration of BAP was increased beyond this the response or rate of multiple shoot regeneration reduced. This observation is in consonance with observations on *Ruta graveolens* [17] and *Solanum surattense* [18]. The presence of Kinetin alone in both the media failed to induce callus and subsequent shoot regeneration from all the cultures.

Synergistic Effect of Auxin and Cytokinin

Since BAP was found to be more favorable for the induction of multiple shoots from the callus cultures,

Table 1: Effect of cytokinin (BAP) alone or in combination with IAA/2,4-D/NAA and IBA on multiple shoot regeneration from shoot tip, nodal and leaf explants of *Sauropus androgynus* on L2 media

Plant growth regulators (μM)						Mean±SE		
BAP	IAA	2,4-D	NAA	IBA	GA_3	Mean number of shoots per shoot tip explants	Mean number of shoots per nodal explants	Mean number of shoots per leaf explants
4.44	-	-	-	-	-	19±0.894	26±0.615	-
8.87	-	-	-	-	-	29.4±0.725	39.6±0.717	-
13.20	-	-	-	-	-	41.2±0.621	58.4±0.609	-
17.74	-	-	-	-	-	30.4±0.546	41.2±0.622	-
22.20	-	-	-	-	-	29±0.502	34±0.734	-
4.44	2.85	-	-	-	0.72	66.8±0.692	91.4±0.656	55.8±0.773
8.87	5.71	-	-	-	1.44	103.4±0.947	156.4±0.694	89.4±0.546
13.20	8.56	-	-	-	1.44	117.4±0.590	215.6±0.822	102.4±0.757
17.74	11.42	-	-	-	2.88	89±0.632	120.8±0.746	72.2±0.912
4.44	-	2.26	-	-	0.72	83.6±0.886	75.4±0.608	43.4±0.656
8.87	-	4.52	-	-	1.44	53.4±0.695	121.2±0.902	73.4±0.868
13.20	-	6.78	-	-	1.44	92±0.722	136.4±0.695	82.8±0.586
17.74	-	9.05	-	-	2.88	56.2±0.653	92±0.722	51.4±0.695
4.44	-	-	2.85	-	0.72	44.4±0.775	54.6±0.728	38±0.647
8.87	-	-	5.37	-	1.44	59.8±0.715	64.4±0.738	53.2±0.653
13.20	-	-	8.05	-	1.44	71±0.661	82.4±0.592	63.6±0.559
17.74	-	-	10.74	-	2.88	55.2±0.5742	61±0.5318	47.8±0.4271
4.44	-	-	-	2.46	0.72	-	-	-
8.87	-	-	-	4.90	1.44	-	3±0.5785	-
13.20	-	-	-	7.35	1.44	2.8±0.324	7.6±0.5029	-
17.74	-	-	-	9.80	2.88	1±0.3556	6.8±0.3868	-

Values are mean±SE of 5 replicates. 2,4-D: 2,4-dichloro phenoxy acetic acid, BAP: 6-benzyl amino purine, GA_3: Gibberllic acid, IAA: Indole-3-acetic acid, IBA: Indole-3-butyric acid, NAA: α- naphthalene acetic acid, L2: Phillips and Collins, SE: Standard error

synergistic effects of various auxins with BAP was studied. Two sets of experiments were conducted to study the effect of auxins and cytokinins together. In the first set of the experiment, the non-organogenic callus raised on only auxin or cytokinin supplemented media was subcultured to media containing both auxin and cytokinin at various concentrations along with GA_3. In the second set of experiment, the explants directly inoculated onto the combination media.

When the callus raised on 2,4-D, IAA and NAA subcultures to 2,4-D/IAA/NAA + BAP + GA_3 combinations, only a few shoots were observed after 4-5 weeks of culture. However, IBA with BAP + GA_3 promoted only a few shoots. Whereas the organogenic callus raised on BAP responded faster when transferred to combination media. A large number of shoot buds started initiating form the callus within 2-3 weeks. The percent of multiple shoots originated on combination media was much higher than on BAP alone. Maximum number of multiple shoots 215 ± 0.82 was recorded per culture from nodal culture on L2 + BAP (13.20 μM) + IAA (8.56 μM) + GA_3 (1.44 μM).

In the second set of experiments, the explants directly inoculated on auxins + BAP + GA_3 combinations (Figure 1c-f). Plant propagation through callus required the induction of organogenic callus [19,20]. The presence of cytokinin along with auxin is necessary for indirect shoot induction as noted by Skoog and Miller [21]. All the explants started profuse callusing after 2-3 weeks of culture on all the combination. A synergism between L2 + BAP (13.20 μM) + IAA (8.56 μM) + GA_3 (1.44 μM) was found to be best as in the first set of experiments. Such synergistic effect of auxins and cytokinins on multiple shoot regeneration was reported in *Arachis stenosperma* and *Arachis villosa* [22].

A low auxin concentration in combination with a high concentration of cytokinin was observed in the present studies is the most suitable combination for the proliferation of shoots. Similar reports were made, in *Agave vera-cruz* [23] and in *R. graveolens* [24]. The presence of GA_3 along with auxin and cytokinin in the present studies promoted the elongation of shoots. The promoting effect of GA_3 hormone for shoot elongation on BAP containing medium has been reported on several other plants such as *Withania somnifera* [25], *Aegle marmelos* [26], and *Achras sapota* [27].

The overall result of this study reveals that the *in vitro* morphogenetic potential of the explants mainly depends on the type of explants, source of explants, the type of the media and various growth regulators used. This may be due to the differential expression of the genes in cells within the plant during its growth and development that has resulted in distinct pattern of morphogenesis in

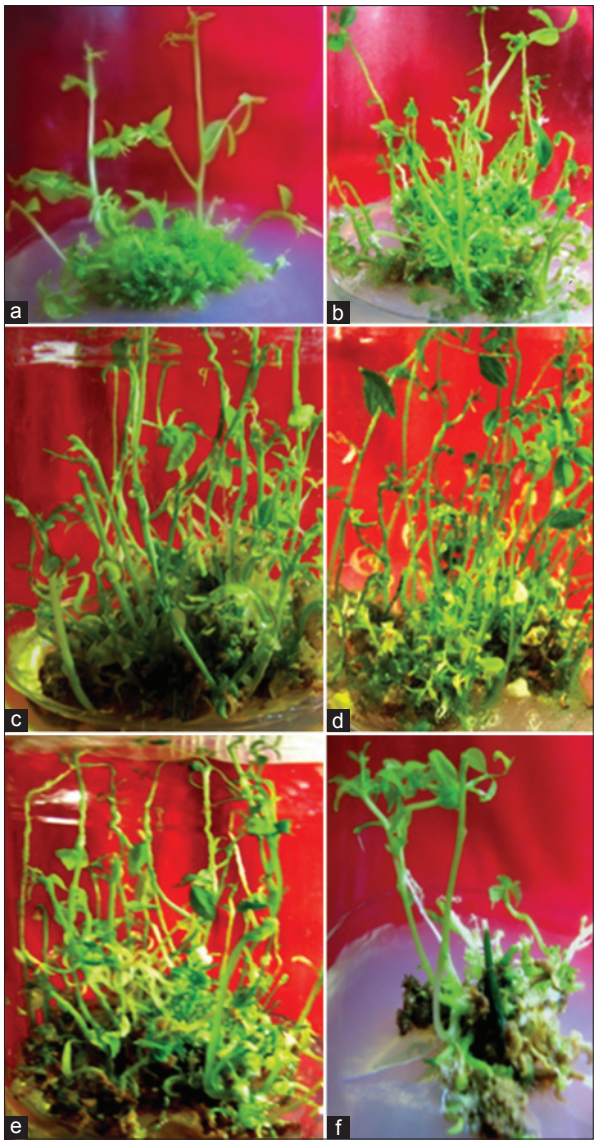

Figure 1: Multiple shoots formation from nodal cultures on various combinations. (a) Multiple shoots formation on MS + BAP (22.20 µM) medium. (b) Multiple shoots formation on L2 + BAP (13.20 µM) medium. (c) Multiple shoots formation on L2 + BAP (13.20 µM) + IAA (8.56 µM) + GA3 (1.44 µM) medium. (d) Multiple shoots formation on L2 + BAP + 2,4-D (6.78 µM) + GA_3 (1.44 µM) medium. (e) Multiple shoots formation on L2 + BAP (13.20 µM) + NAA (8.05 µM) + GA_3 (1.44 µM) medium. (f) Multiple shoots formation on L2 + BAP (13.20 µM) + IBA (7.35 µM) + GA_3 (1.44 µM) medium. MS: Murashige and Skoog's, BAP: 6-benzyl amino purine, L2: Phillips and Collins, IAA: Indole-3-acetic acid, GA_3: Gibberllic acid, 2,4-D: 2,4-dichloro phenoxy acetic acid, NAA: α-naphthalene acetic acid, IBA: Indole-3-butyric acid

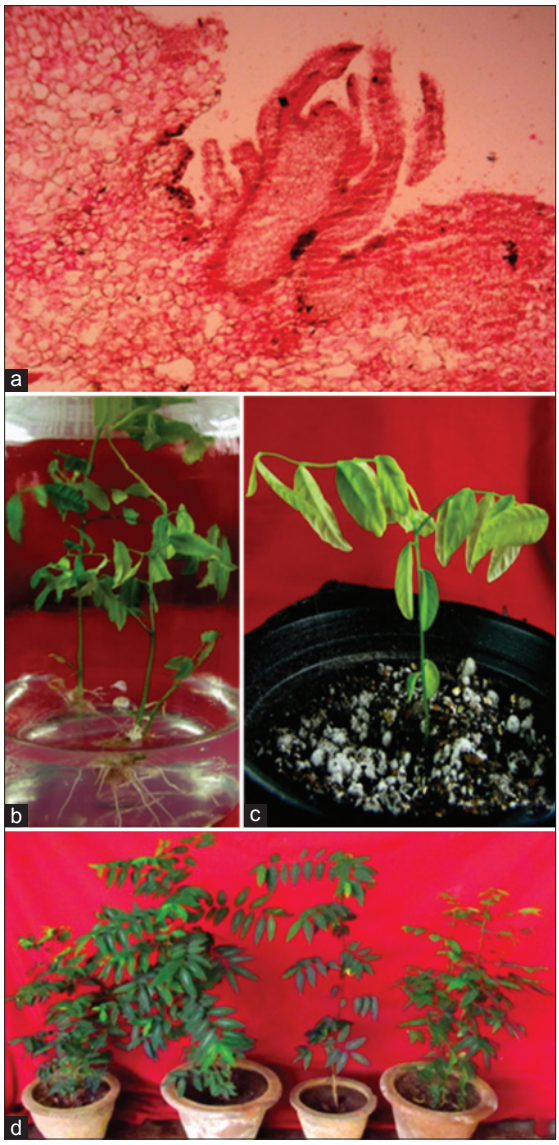

Figure 2: Histology and hardening. (a) Histological details showing indirect regeneration of shoots. (b) Rooting of individual shoots on ½ strength L2 + IAA at 2.85 µM medium. (c) Rooted plantlets hardened in hardened mixture consisting of pearlite. (d) Fully grown plants transferred to earthen pots containing 1:1:1 sand, soil and manure. L2: Phillips and Collins, IAA: Indole-3-acetic acid

various parts of the plant body [6]. Apart from this the morphogenetic potential of the explants also depends on the critical balance between the endogenous and exogenous supplied growth regulators.

Histological Studies

Sections of organogenic callus revealed the meristematic centers at the peripheral regions of the callus. These meristematic centers which are characterized with small cells and dense cytoplasm further differentiated into shoot buds (Figure 2a). Similar type of indirect regeneration was reported in *A. vera-cruz* [23], *Andrographis paniculata* [28], *Macrotyloma uniflorum* [29], and *Piper nigrum* [30]. Critical observation of the callus had shown that the differentiated shoot buds have no connection with the original explant in both the sets of experiments conducted. Hence, the occurrence of genetic variability can be expected in such type of shoots which can be further exploited for the genetic improvement of the taxon through the biotechnological approaches.

Rooting and Acclimatization

Root induction is an important and crucial step in the formation of complete plantlets, usually controlled by the treatment with growth regulators, wherein auxins play a key role in the formation of roots. *In vitro,* elongated shoots were separated and transferred to half strength of L2 medium supplemented with IAA at 2.85 µM and 5.71 µM. IAA at 2.85 µM induced rooting without callusing at the cut ends (Figure 2b). Similar response for rooting was observed in *Solanum dulcamara* [31]. Later, the complete plantlets were taken out from the culture bottles and washed their roots with distilled water and transferred to plastic cups containing pearlite for hardening (Figure 2c). Gradually after 20-30 days, they are transferred to earthen pots containing 1:1:1 sand, soil and manure (Figure 2d) and maintained in polyhouse before transferring to field for better acclimatization. The survival rate was about 90%.

CONCLUSION

The present work is being oriented toward the type and concentration of plant growth regulator used both for induction of callus and shoot initiation. The efficient protocol developed in present studies can be used for large-scale propagation of *S. androgynous* due to high multiplication rate. Since somaclonal variations arising from callus cultures can be harnessed for improved varieties, the high frequency of regeneration from the callus cultures is quiet promising in this direction.

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