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Annals of Biological Research, 2012, 3 (10):4668-4674
(http://scholarsresearchlibrary.com/archive.html)

Annals of Biological Research
Scholars Research Library
ISSN 0976-1233
CODEN (USA): ABRNBW

Effect of growth hormones on callus induction of Sauropus androgynous (Sweet shoot)

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ABSTRACT

Sauropus androgynus (Phyllanthaceae) is an underutilized plant with high nutrient and antioxidant properties. Though traditionally used for food and medicinal purposes, its value is still underutilized for mankind. Developing tissue culture techniques for Sauropus androgynus will permit the application of biotechnology to its culture, and potential for exploitation of phytochemicals that it contains as potential medicine for many remedies. In this study, the effect of type, concentration and ratio of growth regulators on callus regeneration has been investigated. Juvenile leaves will be utilized as explants which were surface sterilized in 70% ethanol for 1 minute followed by 20% Clorox for 20 minutes. The growth regulators dicamba (3, 6- dichloro-2-metaxibenzoic acid) and NAA (α-naphthalene acetic acid) in combination with kinetin (N6-furfuryladenine) were supplemented to MS basal medium at different concentrations. There was a significant difference between the growth regulator concentrations in inducing callus. Callus regeneration was comparatively high in 2mg/l NAA and 1 mg/l kinetin. The callus obtained was friable and greenish white in color. Most of the cultures turned brown when the growth hormones dicamba, NAA and kinetin were used individually and also in a combination of dicamba with kinetin. Browning in culture material indicated the release of phenolic compounds. Further research should be concentrated on reducing the browning effect in culture.

Keywords: *Sauropus androgynus*, dicamba, NAA, kinetin, callus induction, browning.

INTRODUCTION

Sauropus androgynus is one of the underutilized plants in the Phyllanthaceaefamily. It has abundant nutritive and medicinal values. It was originated in India and distributed widely in South East Asia. It is a perennial shrub leaves, flowers and fruits of the plant are edible. Leaves of the plants are rich in vitamin A, B and E, proteins and minerals [1]. It also contains the alkaloid papavarine in substantial amounts, i.e. 580 mg/100g of fresh leaf [2].Co-enzyme Q 10 in *Sauropus androgynus*, which is an antioxidant, is widely employed in preparing nutraceutical products and cosmetics [1,18]. Methanolic extracts of *S. androgynus* have strong activity against pine wood nematode (*Busaphelenchusxylophilus*) [3].It is also known to have anti-bacterial activity against two species of bacteria, *Staphylococcus aureus* and *Klebsiella pneumonia*[21]**.** It is also used in accelerating mother's breast milk [4].

Relatively little is documented about the growth and development of *Sauropus androgynus* in tissue culture. It is desirable to develop this technology to assist future studies on the physiology and biochemistry of plant with potential application to its propagation and genetic improvement for nutritional and medicinal purposes.

The objective of this study is to determine the effect of different types, concentrations and ratios of growth hormones on callus regeneration in *Sauropus androgynus*. This will be achieved through the use of dicamba, NAA and Kinetin in *in vitro* cultures of *Sauropus androgynus***.**

MATERIALS AND METHODS

2.1 Collection of explants

Juvenile leaves of *Sauropus androgynous* were used as explants.Leaves were collected from parent plant grown in the shade house of The University of Nottingham Malaysia Campus, Malaysia. Mother plants were planted in pots with soil compost (3 part soil: 2 part perlite: 1 part peat).

2.2 Surface sterilizationprotocol

Leaf explants were rinsed under running tap water for 5 minutes and trimmed to 2x2 cm. Trimmed explants were treated with 70% ethanol for 1 minute with gentle agitation, then rinsed with sterile water for three times. Rinsed explants were then treated with 20% Clorox for 20 minutes, followed by three rinses in sterile distilled water. Finally, the explants were trimmed to 1x1 cm to remove bleached cut edges, dried lightly on sterile filter paper for 1-2 minutes.

2.3 Callus culture establishment

Sterilized explants were cultured on plain MS medium for 5 days. The inoculated explants were incubated in a culture room at 26±2^{0}C for 16 hours photoperiod under fluorescent light with 2000-3000 lux intensity. Then cultured in 4.42 g/l MS medium (DuchefaBiochemie, Netherland) with 3% (w/v) sucrose (DuchefaBiochemie, Netherland) 0.2% (w/v) Phytagel (Sigma – Aldrich, USA).

After 5 days explants were transferred to growth regulators medium (dicamba with kinetin & NAA with kinetin (Sigma – Aldrich, USA). In total there were 15 media treatments including the control with MS media alone (Table 1). There were six replicates with treatment containing 5 explants.

Table 1: Concentrations and combinations of growth hormones for callus induction

Treatment	Growth regulator	
G1	Control	MS Medium
G2	NAA	1.0 mg/l
G3		2.0 mg/l
G4	Dicamba	1.0 mg/l
G5		2.0 mg/l
G6	Kinetin	0.5 mg/l
G7		1.0 mg/l
G8	NAA + Kn	1.0 + 0.5 mg/l
G9		1.0 + 1.0 mg/l
G10		2.0 + 0.5 mg/l
G11		2.0 + 1.0 mg/l
G12	Dicamba + Kn	1.0 + 0.5 mg/l
G13		1.0 + 1.0 mg/l
G14		2.0 + 0.5 mg/l
G15		2.0 + 1.0 mg/l

2.4 Statistical analysis

Differences between treatments were analyzed by analysis of variance (ANOVA). The numbers of explants that induced callus were transformed into a percentage value and subjected to ANOVA by applying completely randomized design and the level of significance was $P<0.05$. All the treatments were repeated six times and each treatment contained 30 explants.

RESULTS

3.1 Effect of growth hormones on callus

There was considerable variation in callus formation all 15 treatments. In the control (MS medium), no callus induction was observed and 63% of the cultures turned brown in 10-15 days.

Based on size of the callus, each treatment was scored. (Table 2)

Callus scoring Explanation
0 No visible callus
1 < 5mm callus
2 5-10mm callus
3 10-15mm callus

Table 2: Scoring of callus based on size

Treatments	Scoring	Treatments	Scoring	Treatments	Scoring
G1	0	**G6**	0	**G11**	3
G2	0	**G7**	0	**G12**	0
G3	0	**G8**	1	**G13**	0
G4	0	**G9**	2	**G14**	1
G5	0	**G10**	2	**G15**	2

3.2 Effect of auxins and cytokinin
Both auxins, NAA and dicamba when used alone at 1.0 and 2.0 mg/l did not induce callus and explants turned brown ,its browning percentage range from 45 and 55% respectively when the kinetin used alone at 0.5 and 1.0 mg/l also turned the explants brown and the browning percentage ranges from 50-60 %.

3.3 Effect of NAA with kinetin
In treatments G8 to G11, NAA at 1.0 and 2.0 mg/l all was used in combinations with kinetin at 0.5 and 1.0 mg/l. Callus initiation was observed in 30 days, with its incidence ranging from 10 to 20% of the cultures (Table 3). The best response occurred in with 2.0mg/l NAA and 1.0mg/l kinetin. Growth of the callus gradually increased but overall it was very slow (Figure 1).

3.4 Effect of dicamba with kinetin
Combinations of dicamba and kinetin showed poor response in inducing callus. All of the explants in these treatments, including those that induced callus, turned brown in 15 days (Figure 1). The callus produced on the leaf explants was pale green in colour. Examination of the callus under a microscope revealed the callus to be loose, friable and soft in nature (Figure 1). Callus was initiated at the cut surfaces of explants after 27-30 days of incubation.

3.5 Statistical analysis
The probability value generated was below 0.05. This proves that there is a significance difference between the treatments (Table 3).

Table 3: Response of leaf explants to phytohormones in MS medium

Treatment	Growth regulators	Concentration	Browning%	Contamination%	Callus%
G1	Control	MS Medium	63	33	-
G2	NAA	1.0 mg/l	40	20	-
G3		2.0 mg/l	46	20	-
G4	Dicamba	1.0 mg/l	60	30	-
G5		2.0 mg/l	50	26	-
G6	Kinetin	0.5 mg/l	60	33	-
G7		1.0 mg/l	50	20	-
G8	NAA + Kn	1.0 + 0.5 mg/l	43	0	10
G9		1.0 + 1.0 mg/l	53	20	10
G10		2.0 + 0.5 mg/l	33	50	13
G11		2.0 + 1.0 mg/l	13	26	20
G12	Dicamba + Kn	1.0 + 0.5 mg/l	46	30	-
G13		1.0 + 1.0 mg/l	43	40	-
G14		2.0 + 0.5 mg/l	43	36	6
G15		2.0 + 1.0 mg/l	40	36	10

Note: All readings were recorded 35 days after inoculation on growth regulator media.
**Each treatment consisted of 30 explants and the whole experiment was replicated 6 times.*

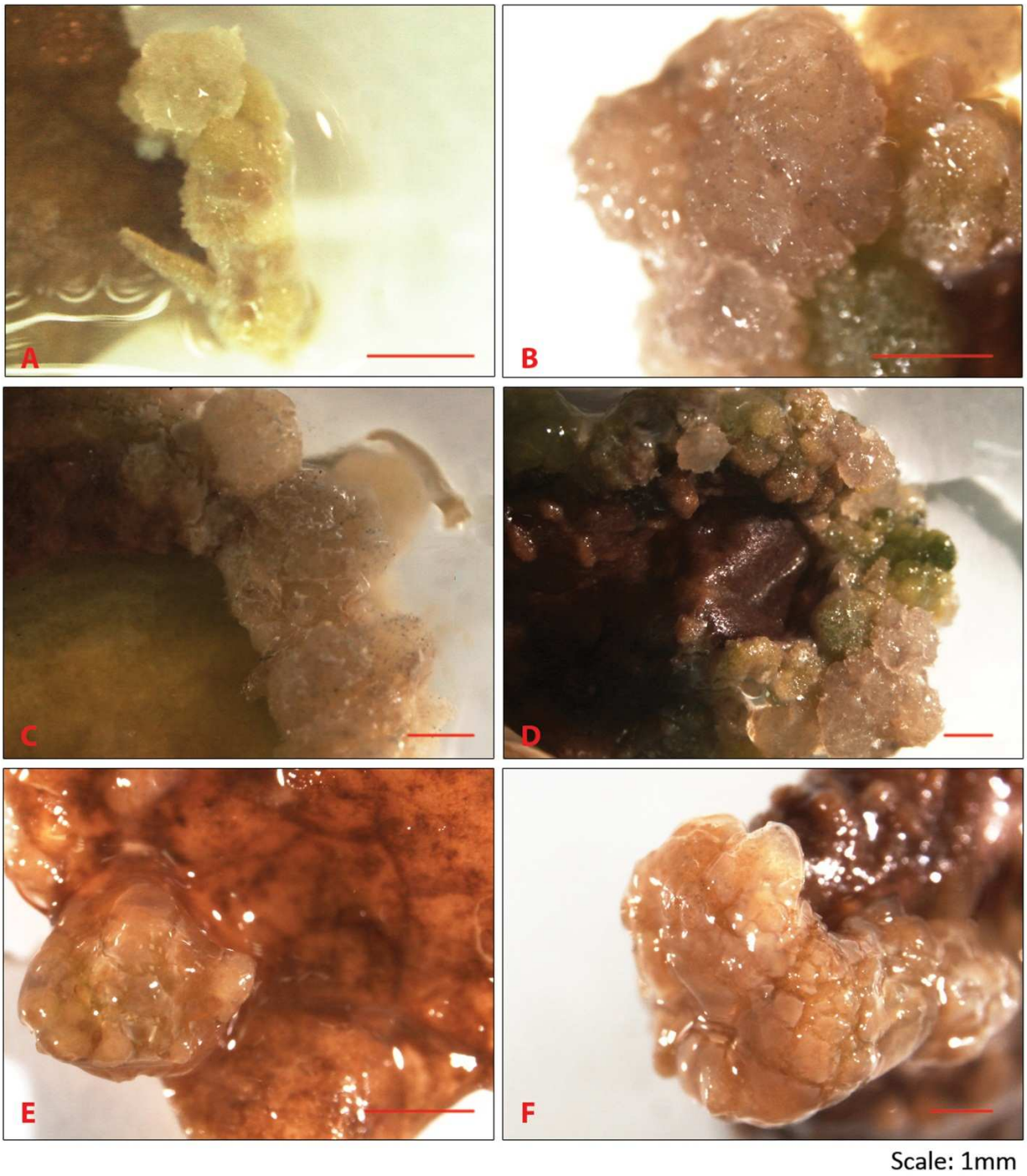

Figure 1: Callus derived from leaf explants of *S. androgynus*

A. Friable callusing at cut ends in MS + 1.0 mg/l NAA with 0.5 mg/l Kn
B. Young friable callus in MS + 1.0 mg/l NAA
C. Moderate greenish white friable callus in MS + 2.0 mg/l NAA with 0.5 mg/l Kn
D. Maximum friable callus regenerated in MS + 2.0 mg/l NAA with 1.0 mg/l Kn
E. Brown callus in MS + 2.0 mg/l Dicamba with 0.5 mg/l Kn undergoing necrosis
F. Brown callus in MS + 2.0 mg/l Dicamba with 1.0 mg/l Kn undergoing necrosis

DISCUSSION

4.1 Callus regeneration in S. androgynus

Gresshoff (1978) documented the following 3 important steps for the efficacious initiation of callus:

a. Initiation of cell division and tissue proliferation
b. Physiological and structural dedifferentiation
c. Using optimal amount of plant growth regulators, mineral salts and organic compounds, including sugar, vitamins and myoinositol.

Gresshoff assured that for callus induction addition of growth regulator medium is necessary. This is similar to the result from the present study in which there was no callus initiation on plain MS medium and the cultures needed growth hormones to initiate callus.

4.2 Effect of auxins

When NAA used alone with MS medium, there was no callus induction and most of the cultures turned brown gradually in 15 days. This agrees with the result achieved by [8]. However, this contradicts with result when *Phyllanthusstipulus* was used for callus induced [7].

When dicamba was used with MS medium, has induced callus in leaves of woody plant species *Eurycoma longifolia* but this contradicts with the present study of *S. androgynus*, dicamba did not initiate any callus[9].

4.3 Effect of cytokinin

Kinetin supplemented alone to MS medium did not initiate any callus. This was in agreement with the result reported by Catapan*et al.,*for *Phyllanthusstipulatus* where low concentrations of kinetin did not provide a positive response for callus induction [7].

In callus induction studies of *Phyllanthustenellus*Roxb. [10], 0.5mg/l of kinetin when used alone in MS medium gave no induction of callus. This result agrees with the present study. Studies reported browning during callus induction in *S. androgynus* cultures on medium containing low concentration of kinetin [8]. This outcome was similar to the current study, where most entire explants turned brown.

4.4 Effect of auxin in combination with cytokinin

NAA when supplemented in combination with kinetin in MS medium favoured induction of callus at the cut edges of the culture material. Better induction of callus was noted while using 2.0mg/l NAA and 1.0mg/l kinetin. Likewise Wee *et al.,*obtained the most callus in *S. androgynus* with similar concentration of NAA and kinetin [8].

When dicamba was used with kinetin, callus was induced at a moderate level. This was also reported for pearl lupin (*Lupinusmutabilis*) with a low concentration of dicamba together with kinetin [11]. Callus induction of *Melastomadedecemfidum*on MS medium containing dicamba and kinetin, in agreement with the present study [12]. George *et al*.,stated that the growth hormone dicamba promotes callus induction when used in appropriate combination with kinetin [13]. Likewise, Wee *et al*., made a general statement that a high concentration of auxin and low concentration of cytokinin in MS medium favour callus induction [8]. The above reports assure that auxin and cytokinin in combination at optimum levels can induce callus, as noted in the present study. The callus induced significantly differs between the treatments at 5% significance.

4.5 Browning of culture material

The browning of culture material in 10-15 days after inoculation on growth regulator medium may have resulted from the release of phenolic compounds or microbial contamination (Figure 2). Since sample of brown and green cultures when assayed by broth test provided no evidence of microbial contamination, it was considered that was due to phenolic compound release.

Phenolic compounds are secondary metabolites released from plants, which are present in high amounts. Browning in plants occurs mainly due to the oxidation of phenolic compounds by phenol oxidase. This phenomenon occurs when the compartmentalized phenolic compounds are released during explant incision and henceforth react with phenolic oxidases and release quinone. Quinone has negative effect a cell growth and can result in death/necrosis of cells (Figure 2).

There is also some evidence that the phytohormone kinetin may cause browning of tissues [15]. In the present study, the presence of kinetin in the medium turns the tissues to brown, the same as report byWee *et al*.,[8].

Observed the browning of the different explants of cotton, including leaf, hypocotyl, cotyledon and root which increased with the increase in age of the explants [16]. In addition, they reported that phenolic oxidation also increased gradually from the 7th to the 28th day of germination on plain MS medium. This is in agreement with the present study in which the browning effect increased with age of explants.

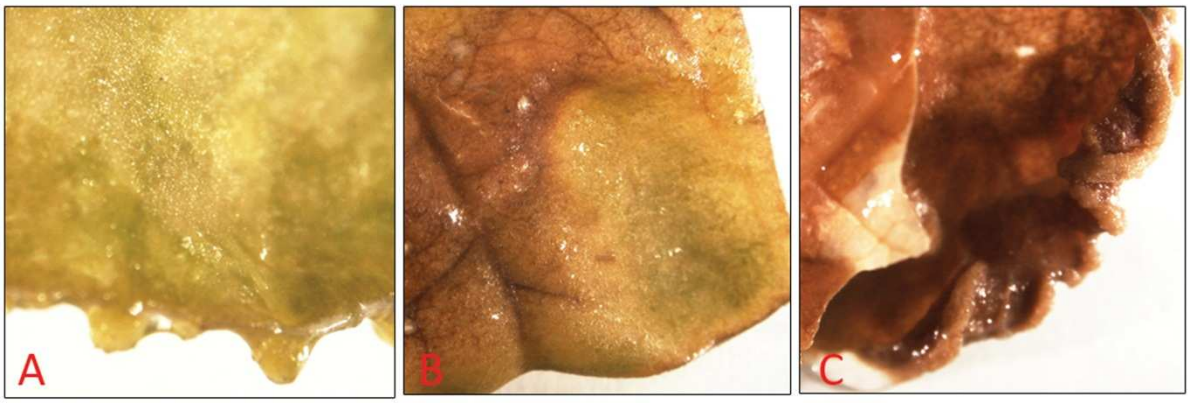

Figure 2: Browning effect observed on medium with dicamba and Kn

A. Green explants inducing callus at initial stage of culture on 2.0 mg/l dicamba + 1.0 mg/l Kn
B. Explants start to turn brown on 2.0 mg/l dicamba + 1.0 mg/l Kn
C. The callus induced explants completely turned brown on 2.0 mg/l dicamba + 1.0 mg/l Kn

Cultures were maintained on plain MS medium for 5 days in order to eliminate any contaminated cultures; the clean cultures were sub-cultured onto growth regulator media on the 6th day. In the previous study by Wee *et al.*, leaf explants of *S. androgynus* were maintained on MS medium for only 2 days, and then sub-cultured onto growth regulator medium to obtain maximum callus induction[8]. The difference in days of incubation both in previous [8] and the present study, suggests that age is a main factor in phenolic compound release and browning**.**

Proposed that leaves synthesize phenolic compound which are then distributed to the rest of the plant, hence leaves have high level of phenolic compounds compared to other parts[16]. This is in agreement with the current study where leaves were used and resulted in browning.

In the present study, browning occurred gradually and appeared to be accompanied by break down of chlorophyll pigment, since the phenolic compound may damage the chloroplasts. Similar observation was reported in an aquatic fern [17] where cultured material became brown and no further growth was observed, resulting in the death/necrosis of the explants. Similar observation was reported in the clonal propagation of *Beta vulgaris*[19].

A number of parameters, such as cultivar type, physiological conditions and time at which explants are collected, influence the degree of browning in explants . Antioxidants have the ability to control the browning phenomenon, including ascorbic acid, citric acid and caffeic acid. The content of phenolic compounds may be utilized as a biological quality indicator to estimate the quality of*in vitro* grown plants [20].

In the current study, browning of the tissues was common. The survival and growth of the explants rely on common factors including contamination by microbes, oxidation of phenolic compounds, physiological stage of mother plant and season at which the explants are collected. All these factors pertain to the browning phenomenon and successful establishment of axenic cultures.

CONCLUSION

The attempt to regenerate maximum callus from *S. androgynus* the reason might be due to the age of the explants while it was sub-cultured in the growth regulator medium. This caused browning effects in most of the cultures. Among the different growth regulator NAA and kinetin 2 mg/l and 1 mg/l resulted in maximum callus regeneration.
In future work on *Sauropus androgynus* should focus on overcoming browning in order to increase callus regenerated in NAA and kinetin media. Ultimately, tissue culture technology can be combined with molecular technologies to improve genetically this plant, which is medicinally and nutritionally important.

Acknowledgement
I thank the almighty whose blessings have enabled me to accomplish my Dissertation work successfully.I would like to express my sincere gratitude to The University of Nottingham and to my supervisor Dr. Peter G Alderson for the continuous support of my Master study and research

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