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Solenostemma argel Tissue Culture for Production of Secondary Metabolites

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ABSTRACT

The study attempted to establish tissue cultures from *Solenostemma argel* (family *Asclepiadaceace*) and to investigate the biosynthetic potentiality of the cultured tissues to produce alkaloids, cardinolides and flavonoids.Tissue cultures of *S. argel* were initiated on Murashige and Skoog (MS) media supplemented with auxins (IBA, NAA and 2,4-D) and cytokinins (BAP and Kinetin). Explants from cotyledonary leaves and hypocotyls tissues were used. *S. argel* showed a slow rate of callus formation when explants grown on MS media supplemented with 10mg/l IBA and 12.5 mg/l BAP, when 2 mg/l 2,4-D and 0.5 mg/l Kinetin were used, the explants initiated callus very rapidly, while media supplemented with 10 mg/l NAA and 12.5 mg/l BAP resulted in the least effect concerning callus initiation and growth. Anatomical study of callus cultures to investigate the degree of differentiation and organogenesis showed highly organized root-like structures and extensively lignified tracheids. Biochemical analysis showed that cultured tissues are capable to produce and accumulate alkaloids, cardinolides and flavonoids.

Key Words: *Solenostemma argel*, tissue culture, secondary metabolites.

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INTRODUCTION

Solenostemma argel, belongs to the *Asclepiadaceae* family. This family includes many wild growing medicinal plants (e.g. *Calotropis procera, S. argel, Leptadinea spp*). These plants are known to contain secondary metabolites such as alkaloids, cardinolides flavonoids etc., which are needed in manufacturing important pharmaceuticals.

Solenostemma is a monotypic genus with *S. argel* (Del.) Hayne, *S. oleifolium* Bullock et Bruce, *S. triste* (Nees) K. Muelli. *S. argel* is known in the Sudan as Hargel. It is widely spread in the Sudan *(El-Amin, 1990)* and commonly found in the northern region between Bar Bar and Abuhamed in Northern State *(El-kamali, 1996)*. Sudan is regarded now as the richest soruce of this plant *(Organgi, 1982; El-Ghazali, 1997 and Ahmed, 2003)*.

S. argel is considered to be medicinally important in the Sudan, Libya and Chad *(Ahmed, 2003)*. Argel leaves are used in herbal medicine for the treatment of some liver and kidney diseases and some allergies. It is an effective remedy for bronchitis and is used to treat neuralgia and sciatica *(Tharib et al., 1986)*. Also, it is used as incense in the treatment of measles and sometimes crushed and used as remedy for supporting wounds. The leaves are infused to treat gastrointestinal cramps, stomach ache, colic, cold and urinary tract infections and are effective as anti-syphilitic if used for prolonged period of 40-80 days *(Boulos, 1983)*.

Leaves possess purgative properties which may be due to the latex present in the stem parts. Also, *S. argel* is used for the treatment of diabetes and jaundice *(El-Kamali and Khalid, 1996)*.

Plant tissue culture is viewed as a potential mean of producing useful plant products. This is normally achieved under controlled conditions, according to demand and reduced cost and requirements. Despite the advantages of this technique, there are a variety of problems to be solved before it can be applied for large scale production of useful secondary compounds.

Also, tissue cultures have produced compounds previously undescribed and cultures of higher plant cells may provide an important source of new economically important compounds *(Butcher, 1977; Constabel and Tyler, 1994 and El-Tigani, 2008)* The relationship between the degree of tissue organization and the biosynthesis of secondary products is obscure. The spatial orientation of enzymes, compartmentalization of enzymes and substrates and the reservoir sites for product accumulation may be some of the factors involved in the biosynthesis of secondary products by specialized tissues *(Butcher, 1977 and Abbaro, 2002)*.

MATERIALS AND METHODS

Plant materials:
Source of seeds: *S. argel* seeds were collected from bushes growing wildly in northern Sudan (Marawy area, Northern State).

Chemicals:
General reagents used were obtained from the British Drug Houses (BDH), England.

Indole-3-butyric acid, 6-benzyl-amino purine, kinetin (6-furfurylamino purine), α-naphthalene acetic acid, 2, 4-dichlorophernoxy acetic aid, Murashige and Skoog medium were purchased from Sigma Chemical Company Ltd., England.

Methods:

Germination of seeds:

The selected seeds of *S. argel* were washed with water and germinated in sand: Clay mixture (2:1, v/v) contained in black polythene bags (20x10cm). The emergence of the cotyledonary leaves was taken as a criterion of successful germination.

Harvest of samples:

The seedlings were kept for a period of 2-6 months before harvesting the plant leaves and stems. The materials were air dried under laboratory conditions and were ground into a coarse powder and stored in closed sample tubes until used for analysis.

Callus initiation:

Surface sterilization of seeds:

The selected seeds were washed thoroughly with 70% ethanol as a wetting agent for one minute, then the seeds were immersed in hypochlorite solution obtained by the use of the filtrate of 10% calcium hypochlorite solution or the commercial "Clorox" solution. The treated seeds were allowed to stand for a period of 10 minutes in the sterilant solution and then washed 3-4 times with autoclaved distilled water. The surface sterilized seeds were allowed to soak in sterile distilled water for one hour before their transfer to the germination medium.

Germination medium:

Agar medium:

The medium was consisted of agar (Oxoid No. 3, bacteriological agar, 15g/l of medium). The germination medium was supplemented with 0.003 mg/l BAP.

Culture medium:

The standard culture medium for callus initiation and maintenance was the modified Murashige and Skoog medium *(Murashige and Skoog, 1962).*

Initiation and maintenance of callus cultures:

Seeds of *S. argel* were germinated under aseptic conditions and allowed to grow until the radicle had emerged (3-4cm) and the cotyledons expanded for a period of 3-4 days. Segments (1cm) from radicles, hypocotyls and cotyledons were excised under aseptic conditions and transferred to the culture medium.

Explants removed form mature plants from leaves and stem were washed thoroughly under running tap water to remove all the dust. The plant material was surface sterilized using "Clorox" and 0.1% mercuric chloride solutions for a period 15-20 minutes. Then, the material was washed several times with sterilized distilled water before transferring to the 9cm Petri dishes contained MS media.

Culture conditions:

All cultures were incubated at 28±2°C for 48-96 hours, after which the proliferated explants were aseptically transferred to the tissue culture jars (one explants/jar) containing the callus medium. The culture vessels were exposed to low light intensities from fluorescent lamp (Splendor, 40 W, cool and White) for 12 hours daily.

Callus multiplication:

The established calluses were subsequently sub-cultured to a fresh callus medium by aseptically transferring a piece of the callus into fresh medium. Calluses growth was determined qualitatively. Three to four-month-old calluses were harvested and investigated for their anatomical features and cell composition and 2) for their biosynthetic potentiality for alkaloids, cardinolides and flavonoids production.

Anatomy of *S. argel* callus cultures:

Established callus tissues were selected from the experimental materials for this study. The procedure used for tissue killing and fixation was that of *Sass (1958)*. The preserving fluid was formaldehyde (10 ml of 40%) and ethanol (50ml of 95%). Acetic acid was not used for callus tissues killing and fixation due to its swelling effect on the protoplasm. Preservation period was 7-10 days before sectioning. Staining has been carried out using safranin-fast green type.

Slides of permanent sections of different calluses were prepared and examined microscopically to study the internal structures.

Analytical Methods:

The general techniques for the sample preparation, extraction, separation, detection and identification of alkaloids, carolinolides and flavonoids adopted throughout the study were those described by *Harborne (1973 and 1998); Ahmed (2003) and El-Tigani (2008).*

RESULTS AND DISCUSSION

Tissue culture:

Callus initiation and growth:

Explants of *S. argel* cotyledonary leaves showed a low rate of callus initiation which took about 5 weeks and 8 weeks when grown on MS media supplemented with 10 mg/l IBA and 12.5 mg/l BAP. When the slow growing callus was subcultured to a fresh medium containing the same concentrations of IBA and BAP a higher growth rate was observed after 9 weeks.

The cotyledonary explants were grown on MS media supplemented with 10 mg/l NAA and 12.5 mg/l BAP a very slow rate of callus formation was observed.

On the MS media supplemented with 2 mg/l 2,4-D and 0.5 mg/l kinetin, the cotyledonary explants initiated callus very rapidly.

Hypocotyl explants grown on MS media supplemented with 10 mg/l IBA and 12.5 mg/l BAP showed a slower rate of callus formation when compared with that of the cotyledonary

leaves explants. Also, the effect of the addition of 10 mg/l NAA and 12.5 mg/l BAP to the MS media was investigated on callus initiation in *S. argel* hypocotyls explants. The callus imitation was very weak in this type of tissue.

On MS media supplemented with 2 mg/l 2,4-D and 0.5 mg/l kinetin, callus initiation from the hypocolyl tissues was very rapid and the callus growth was massive.

Morphlogy of *S. argel* calluses:
The morphology of the callus cultures differ from one plant to another *(Street, 1973; Butcher and Ingram, 1976 and El-Tigani and Ali, 2001).*

The calluses of *S. argel* grown on MS media supplemented with various combinations of auxins and cytokinins were characterized by a hard, compact, slow growing mass of tissues. The variations in the colour of the calluses depend on the changing environmental factors such as the length of photoperiod and in the quality of light used.

The callus which was grown in a long photoperiod (16 hours light/day) has shown green pigmentation in the early stages of growth which turned into a yellow colour when the callus has reached the age of 8 weeks. The colour of the callus changed to a dark brown when the callus reached the age of 12 weeks.

Decreasing the illumination period gradually until the incubation room has attained complete darkness during a period of 16 weeks, the callus changed form initial pale yellow in the young calluses to pale brown colour after 12 weeks (Figure 1).

Microscopic structure of *S. argel* callus:
Squash preparations of small pieces from the cotyledonary leaves callus were examined microscopically. The callus cells appeared as spherical to oval in shape (Figure 2A) and usually appear as in pairs (Figure 2B). Cells growing at the surface of the callus were elongated in shape having different forms.

Permanent slide preparations of callus tissue initiated from cotylednonary leaves showed a diversity of size and shape in the cells, from large vacuolated cells to smaller cells with dense cytoplasm. Generally the two types of cells remain parenchymatous cells. Figure (3) shows signs of organized tissues forming a lignified tracheid, (Figure 4A) shows the actively dividing cells forming groups at the periphery of the callus.

The hypocotyls callus showed various shapes of cells but the predominant one was the sausage-shaped cells. These elongated cells were observed as outgrowths near the surface of the callus. The callus of the hypocotyl is a friable callus and is composed of loosely arranged cells.

In few cultures thick root apices were observed in the central regions of the calluses. Figure (4-B), shows signs of organized tissues forming a lignified vascular element and in some calluses transverse sections of roots were recorded.

Secondary metabolites in *S. argel* tissues:
Detection of alkaloids in *S. argel* leaves and callus tissues:
According to the results shown in (Figure 5), the extract of leaves from mature plant seems to contain about 10 compounds, about 8 compounds seem to be the same based on their Rf values, but differ in their U.V. fluorescence, while the other compounds separated at Rf values 0.51 and 0.78 appeared in wave length 253 nm and 0.75 and 0.87 in wave length 356 nm are completely different. When the chromatoplate was treated with Dragenderff's reagent, 3 compounds from them seem to be similar to those compounds detected under UV light and the other 5 compounds were recorded and their Rf values were calculated.

The extract from the seedling leaves of *S. argel*_showed about 8 compounds, 5 compounds having Rf values 0.16, 0.27, 0.39, 0.45 and 0.77 were observed in the extract of the leaves from seedlings.

The same procedure was used to detect the occurrence of alkaloids in *S. argel* callus tissues, only one compound with Rf value 0.18 with purple fluorescence under UV light was obtained.

Detection of alkaloids in *S.argel* stem and callus tissue:
The same procedure for detection of alkaloids in *S. argel* leaves was followed for the detection of the stem alkaloids. Chromatographic properties of separated compounds are shown in (Figure 5-B).

After treating the separated compounds from stem tissue extract with Dragendorff's reagent about 5 compounds appeared most of them with orange coloration.

In the extract from the tissues from seedling stem, 7 compounds were observed, two compounds having Rf values of 0.30 and 0.64 similar compounds with the same Rf values that have been detected in the extract of leaves from mature plant but differ in their U.V. fluorescence.

Also, after treatment with Dragendorff's reagent about 5 compounds gave an orange and pale green coloration. The green coloration may be due to the green pigment which was extracted with alkoloids.

On the other hand 6 compounds were detected under UV light in the *S. argel* callus tissues initiated from the stem.

Cardinolides in *S. argel* leaves and callus tissues
Extract of leaves from mature plants seems to contain 3 compounds under UV light and when the chromotoplate was treated with Kedde's reagent one different compound appeared with Rf value 0.33. Seedling leaves extract showed two compounds, one of which (Rf. 0.23) was also detected in the leaves of mature plants.

The procedure used to detect cardinolides in *S. argel* callus tissue succeeded to separate one compound with Rf value 0.95 under UV light and with Kedde's reagent.

The literature concerning the production of cardinolides in this species is scarce and the authors consider this as the first report.

Flavonoids in *S. argel* leaves and callus tissues:

In the leaves from mature plants one compound with Rf value 0.40 was separated and the extract from seedling leaves showed 2 compounds. On the other hand one spot was revealed from the extract of the callus tissues.

The same procedure was followed for the detection of flavonoids in stem tissues and callus initiated from them. The results shown in (Figure 5) reveal that the extract from mature plant separated 2 compounds under UV light and the extract from seedling showed 3 different compounds based on their Rf values. The callus extract revealed about 5 spots under UV light. It can be concluded that various tissues of *S. argel* are responsive to growth regulators in respect to callus initiation and growth.

The morphological and anatomical studies showed that various forms of cells were detected in callus tissues and signs of accumulation of some products were obvious. Reducing cell division and enhancing cell enlargement seem to increase the productivity.

The callus tissues initiated from various organs of *S. argel* contained variable amounts of alkaloids, cardinolides and flavonoids as judged by the visual comparison of chromatographic separation on TLC plates. No quantitative determinations were carried out.

It can be suggested that, more research is needed using *S. argel* and related species from the Asclepiadaceae to screen the endogenous levels of secondary compounds of medicinal importance.

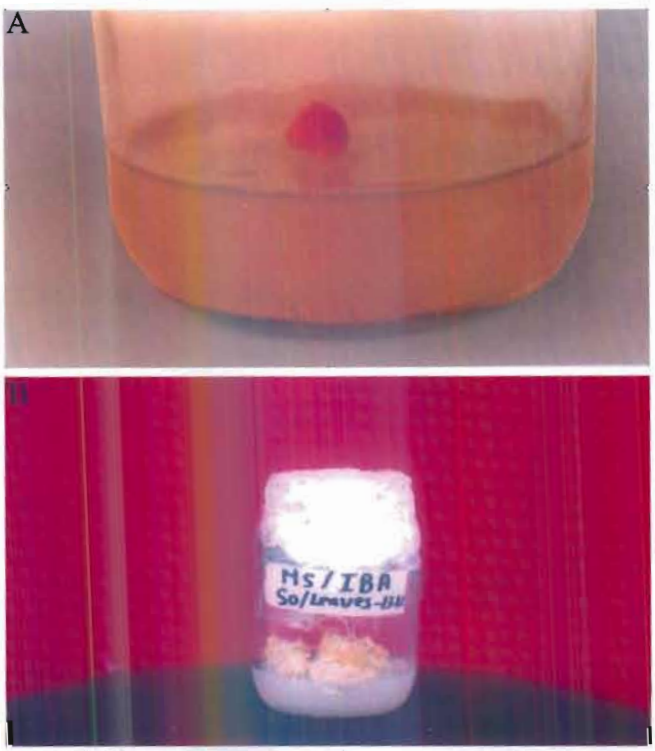


Figure 1: Tissue culture of *S. argel*.

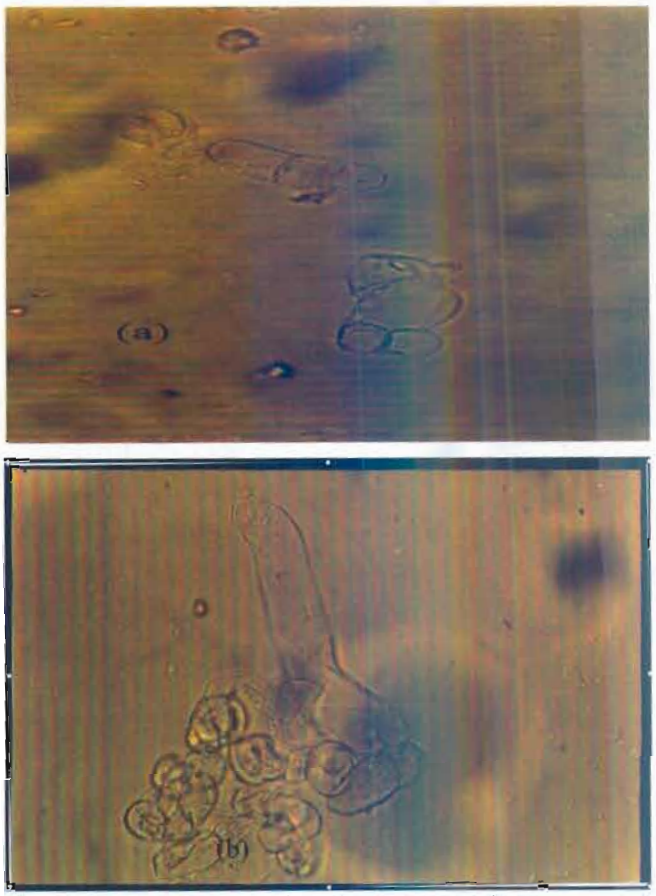


Figure 2: Squash preparation showing the cell structure and morphology of *S.argel* cotyledonary callus.

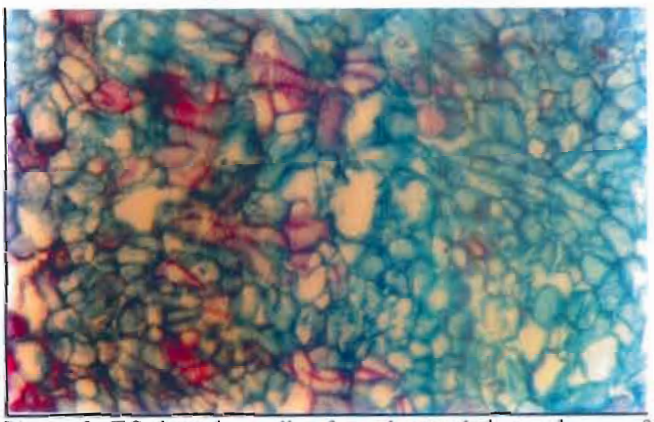


Figure 3: T.S through a callus from the cotyledonary leaves of *S.argel* showing tracheids (x 25).

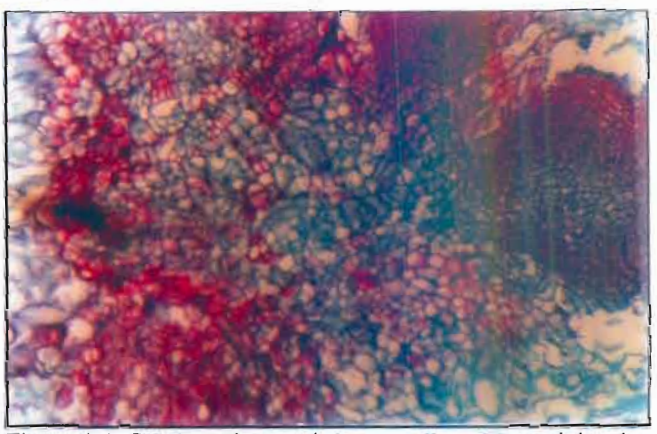


Figure 4-A: T.S through a cotyledonary callus of *S.argel* showing a large aggregate composed of cells at the periphery of the callus (x 10).

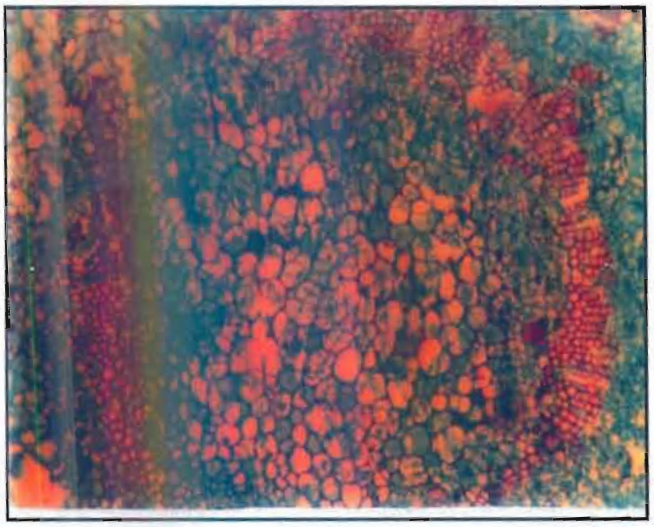
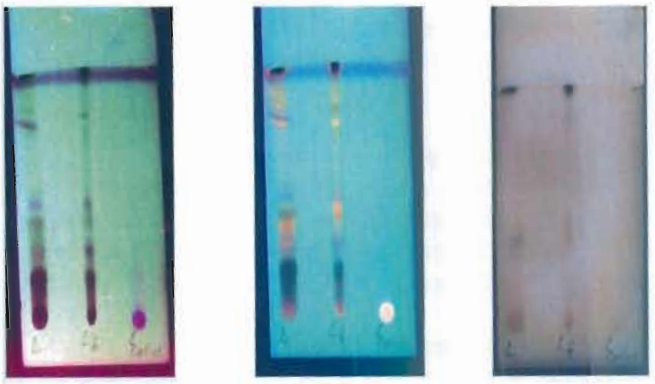


Figure 4-B: T.S. through through hypocotyls callus of *S.argel* showing a transverse section of root .



(A) (B) (C)

Figure 5: Thin layer plates of alkaloids extracts from *S.argel* leaves and callus tissue. Compounds separated on silica gel G precoated plates as appeared under U.V light.
A) At wave length 253 nm.
B) At wave length 356 nm.
C) after chemical treatment with Dragendorff's reagent.
L:Leaves from mature plant L6: Seedling leaves
L callus: From callus tissue.

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