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Effective Surface Sterilization and Callus Induction Protocol for Copper Leaf *(Acalypha Wilkesiana)*

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Abstract

Acalyphawilkesiana (*Euphorbiaceae*) is a plant of medical and economic importance, which has been little utilized by researchers and farmers. An effective surface sterilization and callus induction protocol has been developed to enable future studies on the physiology and genetic improvement of this plant. Five surface sterilization treatments were used to sterilize leaf and stem segments of *A. wilkesiana*. MS medium with 3% sucrose, 0.2% phytagel was used to establish aseptic cultures. None of the treatments achieved 100% decontamination of explants. Surface sterilization of the stem segments with 15% rifampicin and 15% benomyl for 4 hours followed by 70% ethanol for 1 minute and 30% clorox for 30 minutes gave the highest incidence of clean cultures 71%. In contrast leaf segments could not withstand such intense surface sterilization treatment. Difference between surface sterilization protocols were significant ($P<0.05$). The clean cultures after 5days were transferred to medium containing growth hormones for callus induction. For induction of callus, auxin and cytokinin were supplemented to MS basal medium at 0.5 to 2.0mg/l alone or in combination. Callus production was highest on the control medium and on medium with 1.0mg/l 2,4-D and 0.5mg/l of kinetin. The induced callus was pale green and friable. For callus induction, the media treatments show significant differences in size of callus induced.

Keywords: *Acalypha wilkesiana*, *Euphorbiaceae*, Callus induction, Surface sterilization.

1. Introduction

*Acalypha wilkesiana*is an evergreen shrub in the *Euphorbiaceae* family. The genus is closely related to *Euphorbia, Croton* and *Phyllanthus*. Plants are found mainly in tropical region of Africa and Asia. *Acalypha wilkesiana* family was originally imported from Asia to Africa. Weed species usually grow anywhere rapidly, whereas ornamental plants are grown in all parts of the world and some are cultivated as foliage plants in greenhouses and gardens. Some species of *Acalypha* are known for their medicinal properties and many are listed in pharmacopoeia (H.P. India 1971) for controlling abnormal sodium and potassium metabolism (Ikewuchi *et al.* 2005). They are usually used as antibiotic, bacteriostatic as well as fungistatic, as painkillers and for treating naso-pharyngeal infections (Burkill*et al.* 1985). In some parts of Nigeria they use leaf extracts to treat fungal infections such as jedi-jedi and also to treat malaria. The leaves are boiled in water to form a red liquid which is added to bathing water for treating skin infections. It's also good for treating swellings, colds and headaches (Burkill *et al.* 1985).

Phytochemical studies of *Acalypha wilkesiana* have shown the presence of sesquiterpenes, monoterpenes, triterpenoids and polyphenols (Akinde, 1986) plus alkaloids, flavonoids, saponins and tannins in trace amounts (Akinyemi *et al.* 2006). Bussing *et al.* (1999) reported that aqueous and ethanolic extracts of *Acalypha wilkesiana* have cytotoxic and immunomodulating properties. Water extract shows significant antibacterial and antifungal properties (Jekayinfa *et al.* 1997). Water and ethanol leaf extracts were shown to be very active against strains of MRSA (methicillin resistant *Staphylococcus aureus*) and also effective against food borne pathogens (Akinyemi *et al.* 2006).

Plant tissue culture is considered as an alternative to agricultural processes for producing valuable phytochemicals. *Acalyphawilkesiana*is now considered to be an important plant for its medicinal and chemical attributes, but it is not yet utilized commercially. Many underutilized plants have received little attention from farmers and consumers, because of their less competitive growth compared to other crops. In order to increase our knowledge of the physiology and biochemistry of this plant, it is desirable to establish procedure for the axenic culture of cells and tissues *in vitro*. Only a few studies have been carried out on the *in vitro* culture of *Acalypha wilkesiana,* hence the present study of callus regeneration for mass multiplication. This study has focused on *in vitro*callus regeneration using different types of auxins.

2. Materials and Methods

2.1 Mother plants and their maintenance

The explants used for this study were collected from Broga field and then transferred to The University of Nottingham, Malaysia campus shade house. The Mother plant were grown in plastic pots which contain 2 part perlite and 1 part peat. Benomyl fungicide was sprayed every two weeks to reduce the incidence of fungal contamination.

2.2 Tissue culture protocol

2.2.1 Aseptic conditions

To maintain the aseptic state of cultures, all the experiments were carried out in laminar flow clean air cabinet, which was equipped with ULPA (Ultra low penetration air) and certified by ESCO, USA. Prior to use, surfaces of the flow chamber were wiped with 70% ethanol and subjected to ultra violet radiation for 20 minutes. The forceps and scalpels used in all the experiments were sterilized at 250°C using Steripot 350 glass bead sterilizer. All glassware, media, equipment and filter papers used in this study were autoclaved at 121°C for 15 minutes at 15 psi.

2.2.2 Preparation of culture media

The basal medium for the culture of *Acalypha wilkesiana* was MS medium enriched with 3% (w/v) sucrose and 0.2% (w/v) phytagel. Before autoclaving at 121°C for 15 minutes the pH of MS medium was adjusted to 5.8 by adding 0.1 N NaOH (Sodium hydroxide) or HCl (Hydrochloric acid). For induction of callus, growth regulators were supplemented with the culture medium.

2.2.3 Preparation of stock solutions of growth regulators

50mg of 2,4-D and Kn powder were added individually to the glass beaker and dissolved using few drops of 0.5 N NaOH. Then the solution was makeup to 50ml by adding sterile distilled water. If needed heat was provided to dissolve the hormones completely. The stock solution was sterilized using 0.2 µm syringe filers and stored at 4°C in dark.

2.2.4 Fungicide stock solution

Stock solutions of benomyl and carbendazim were prepared at 1mg/ml by dissolving 50mg of fungicide powder in 50ml sterile water. The solutions were sterilized using filter (0.2 µm syringe filers) and stored at 4°C.

2.2.5 Antibiotic stock solution

For preparation of a stock solution 50mg of rifampicin was dissolved in a few drops of Dimethyl sulfoxide (DMSO) and makeup to 50ml using sterile water. The stock solution was sterilized using filter (0.2 µm syringe filers) and stored at 4°C in darkness.

2.3 Surface sterilization

It is important to establish an aseptic culture condition for *in vitro* studies of *A. wilkesiana,* using different types of surface sterilization treatment and duration. Juvenile leaf and stem segments were used as explants. Antibiotic (rifampicin) and fungicide (benomyl and carbendazim) were employed in surface sterilization process. Antibiotic and fungicide stock solutions were prepared and sterilized using filter syringe (Section 2.2.4 and 2.2.5). The collected explants were washed under running tap water for 10 minutes and then subjected to surface sterilization. The concentration of sterilants was varied for each treatment (Table I).

Table I: Treatments with antibiotics and fungicides prior to surface sterilization	
Treatment	**Protocol**
T0	*Surface sterilization:* 70% (v/v) ethanol for 1 min, 30% (v/v) Clorox for 30 min
T1	1% Rifampicin + 1% Carbendazim for 24 h and followed by surface sterilization.
T2	2% Rifampicin + 2% Carbendazim for 24 h and followed by surface sterilization.
T3	2% Rifampicin + 2% Carbendazim for 48 h and followed by surface sterilization.
T4	15% Rifampicin + 15% Benomyl for 4 h and followed by surface sterilization.

T0-Treatment 0: The explants were rinsed with sterilized water and surface sterilized with 70% ethanol for 1 minute, then 30% Clorox for 30 minutes. Following ethanol treatment explants were washed with sterile distilled water. After the Clorox treatment, explants were washed 3 times with sterile distilled water.

T1-Treatment 1: The explants were washed under running tap water for 10 minutes. Then the washed explants were transferred to sterile schott bottle containing 1% rifampicin and 1% carbendazim, and placed on a shaker for 24 hours at 200 rpm. After 24 hours the explants were surface sterilized as stated above in treatment 0 (T0).

T2-Treatment 2: The procedure was the same as treatment 1 (T1) except that explants were treated with 2% of rifampicin and 2% carbendazim for 24 hours.

T3-Treatment 3: The procedure was the same as treatment 2 (T2) except that the duration was increased 48 hours.

T4-Treatment 4: The explants were washed in running tap water for 10 minutes and then treated with 15% rifampicin and 15% benomyl for 4 hours.
The edges of surface sterilized explants were trimmed and dried in sterile tissue paper before transferring onto the culture medium.

2.4 Effect of growth hormones on callus induction of *A. wilkesiana*
To study the effect of growth hormones on callus culturing of *A. wilkesiana*, 2,4-D and Kinetin were used individually and in combination. The best surface sterilization treatment from the above experiment was followed for treating explants. Surface sterilized explants were cultured in hormone free MS medium for 5 days, and those without any contamination were sub-cultured onto callus induction medium containing combinations of 2,4-D (2,4-dichlorophenoxyacetic acid) at 0, 1.0 and 2.0mg/l and Kinetin (N^6-furfuryladenine) at 0 and 0.5mg/l. After inoculation, the explants were kept in culture room with 3000 lux intensity of light with 16 hours light and 8 hours dark photoperiod and 26±2°C. The explants were evaluated after 25 days of culturing. All the experiments were carried out in randomized block design. All the treatments were repeated 4 times with 20 explants/replicate.

3. Results

None of the surface treatments achieved 100% decontamination of explants (Figure I). The contaminating agents included bacteria and fungi. Use of fungicides and antibiotic raised the decontamination level from 0% to 71% (Table II). Stem segments responded better to surface sterilization treatment than the leaf explants, the latter died following antibiotic and fungicide treatments.

Stem explants treated with rifampicin and benomyl showed better result when compared to rifampicin and carbendazim. Without antibiotic and fungicide 100% contaminations occurred, whereas with rifampicin and carbendazim the contamination was reduced to 63.3% (Table II). However, the mortality of explants was very high, due to the duration of the treatment. By increasing the concentration of rifampicin and benomyl and by decreasing the time duration, in treatment T4 the mortality and contamination of explants was decreased to 29%.

The callus induction varied with concentration of auxin and cytokinin. The media without any growth hormones and with auxin and cytokinin at low concentration were required for callus induction. Callus browning was observed in the media with the higher auxin concentration and the cytokinin. Data were recorded after 25 days of incubation (Table III).

3.1 Effect of MS medium

Callus induction was noticed on stem segments cultured on plain MS medium after 10 days. The amount of callus induced was very high and it was soft and friable. The callus induction was noticed from the cut end of the explants. Most of the explants cultured on plain MS medium produced callus (Figure II). The percentage of callus response was 80%, which was the highest of all treatments. The size of callus formed was also comparatively high, which ranges from 2-3 cm (Table III).

3.2 Effect of 2,4-D

The stem segments induced callus from the cut ends in all concentrations of 2,4-D and percentage of initiation ranged from 65-70 % and their size was about 1-2 cm in average (Table III). The maximum response was observed at 1.0mg/l 2,4-D (Figure II) and callus was green and friable. The time taken for callus induction varied between 10-15 days.

3.3 Effect of Kinetin

70% of cultures initiated callus on medium containing 0.5mg/l kinetin (Table III) within 10-12 days. The callus was friable and pale green. Shoot and root formation were observed in the culture medium containing kinetin alone, and some roots were produced directly from the callus. Some explants developed into whole plantlets with 3-4 leaves.

3.4 Effect of 2,4-D and Kn

High amount of callus initiation (80%) was observed in 1.0mg/l of 2,4-D and 0.5mg/l of Kn and their size was also comparatively high (2-3 cm) (Figure II). This percentage was higher than that for auxin alone, whose average was 70%. Moderate amounts of

callus were produced on medium containing 2.0mg/l of 2,4-D and 0.5mg/l of Kn (Table III). The callus was friable, non-organized and highly vacuolated. Shoots and roots were also observed in the cultured material.

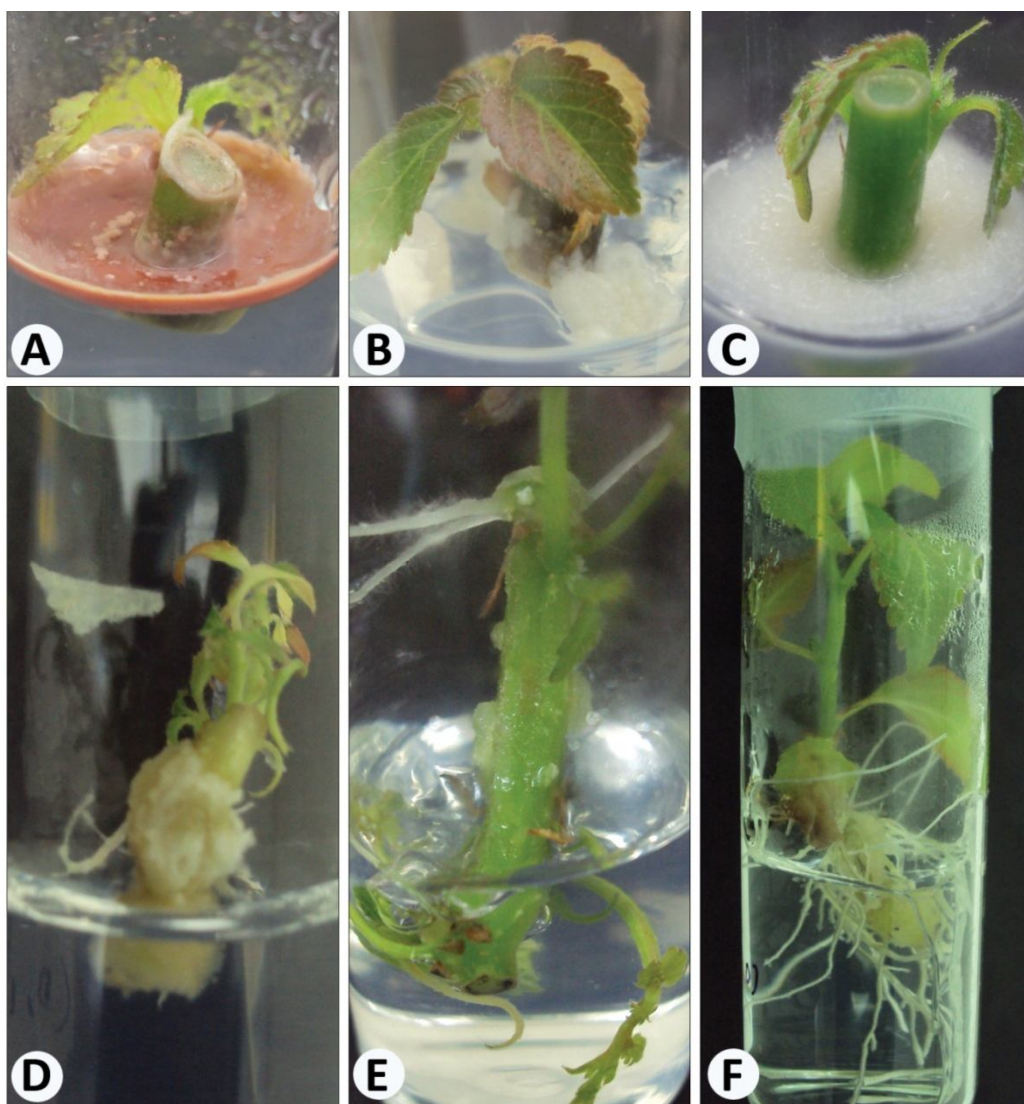
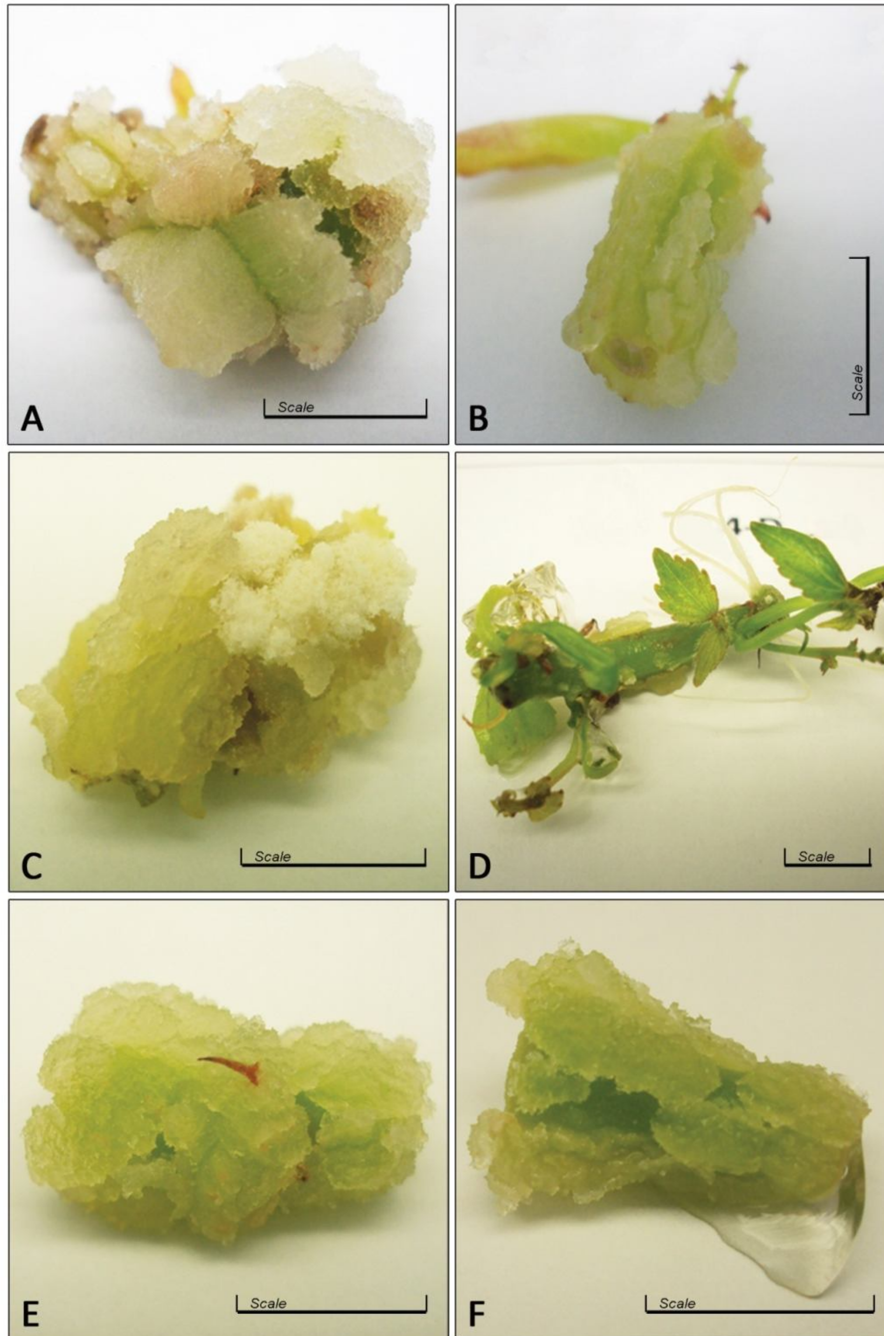


Figure I Contaminants and plant regeneration observed on nodal segments

A. Bacterial growth after 9 days in MS medium without any growth regulators
B. Bacterial contamination observed after 17 days in 1.0mg/l 2,4-D medium
C. Bacterial contamination observed after 20 days in 2.0mg/l 2,4-D medium
D. Multiple shoot induction from callus in 1.0mg/l 2,4-D and 0.5mg/l Kinetin medium
E. Direct root development from callus inoculated in 0.5mg/l Kinetin medium
F. Shoot and root initiation in medium containing growth hormone 0.5mg/l Kinetin



** Images of the second replica; (Scale=100mm)*

Figure II Effect of growth hormones on callus induction

A. High amount of callus produced from stem segments on MS medium
B. Pale green friable callus produced on MS medium containing 1.0mg/l 2,4-D
C. Pale green callus formation in MS medium containing 2.0mg/l 2,4-D
D. Multiple shoots and root formation from internodal explants on 0.5mg/l Kn medium
E. Friable callus after 25 days on MS medium containing 1.0mg/l 2,4-D and 0.5mg/l Kn
F. Less callus initiated on MS medium containing 2.0m/l 2,4-D and 0.5mg/l Kn

Table II: Effect of surface sterilization treatments on incidence of contamination in cultured stem explants after 10 days.

Treatment	Clean cultures (%)	Contaminated (%)	Fungal (%)	Bacterial (%)
T0	0	100	71.6	28.4
T1	20	80	56.2	43.7
T2	26.6	73.3	63.6	36.3
T3	36.6	63.3	65.7	34.2
T4	**71**	**29**	**68.2**	**31.7**

(Note: Each treatment consisted of 2 replicates with 30 explants in each)

Table III: Effect of 2,4-D and Kn on growth of stem explants after 25 days

PGR (mg/l)		Contamination & Response (%)	Shoot (%)	Root (%)	Callus (%)	Size of callus
2,4-D	Kn					
0	**0**	**15**	**5**	**-**	**80**	+ + +
1.0	0	25	5	-	70	+ +
2.0	0	25	20	10	65	+ +
0	0.5	20	40	20	70	+
1.0	**0.5**	**15**	**5**	**10**	**80**	+ + +
2.0	0.5	15	15	-	70	+ +

Intensity of callus: +, <1cm; + +, 1-2cms; + + +, >2cms *(Note: Each treatment consisted of 4 replicates with 20 explants /replicate)*

4. Discussion

Explants treated with Clorox and ethanol (Treatment 0 – T0) alone did not produce any clean culture. Hence, the antibiotic and fungicide were employed in treating the explants to get contamination free culture. Use of rifampicin and carbendazim showed positive response in reducing the microbial contamination. As treatment duration was increased, the mortality of explants also increased. At the same time a small increase in antibiotic and fungicide concentrations did not show much difference in reducing contamination. The low concentration of antibiotic and fungicide solution failed to reduce the contamination level (T3 - 63.3%) and the high duration of treatment (24 and 48 hours) resulted in high mortality. Carbendazim was not as effective as benomyl for reducing fungal contamination.

Leaf explants could not withstand the surface sterilization treatments. In treatment 1, 100% leaf segments survived, but were contaminated heavily within 3 days. In treatments 2 and 3, all the leaf segments produced pigmentation and had a bleached appearance in 2 days without any visual contamination. Hence, it was concluded that leaf explants were not suitable to withstand antibiotic and fungicide treatments.

In the present study, contamination by fungi was mostly observed and neither of the fungicides could control the contamination completely. Wittenbach and Bukavoc (1999) reported that in cherry plant micropropagation, fungal contamination was controlled by application of benomyl at 2%. In the culture tubes mostly white cottony fungus, formation of black layer and white spore's type fungus was observed. Such contamination was identified within 2-3 days of incubation and was mostly found in the cultures of stem segments.

The frequency of bacterial contamination was comparatively low. Reporting the bacterial contamination percentage is inappropriate, since some of the bacterial growth might remain unseen when in the presence of active fungal growth. There may be formation of cloudiness at the base of the explants and green, black colour molds over the explants. Bacterial contaminants may be external to the explants or endophytic. There are two types of bacterial contaminants found in cultures, namely gram + ve and gram – ve. *Bacillus and Staphylococcus*is Gram - ve bacteria, whereas *Enterobacter and Pseudomonas* are Gram + ve bacteria. Gram – ve bacteria appear in cultures at an early stage, whereas Gram + ve bacteria tend to appear in older, well established cultures (Leifert *et al.* 1989). Rifampicin showed positive response in controlling bacterial contamination but still we did not provide 100% decontamination. Ying *et al.* (2006) conducted a comparative study of meropenem, a novel antibiotic, with four commonly used antibiotics, i.e. cefoperazone, cefotaxime, rifampicin and carbenicillin. Meropenem showed the highest suppression of bacterial growth. Similar results have been reported by Bipna *et al.* (2010) for *Vandaarbuthnotiana* and Amanda *et al.* (2009) for *Citrus sinensis* plant regeneration.
In the case of *A. wilkesiana,* the plant material itself may be the source of contamination (Omamor *et al.* 2007). The plant tissues may contain endophytic non-pathogenic bacteria. These bacteria will enter through natural openings of the plant but won't grow in broth culture and also cannot be identified by eye.
Shoot, leaves and roots were initiated from the clean cultures and callus was also initiated in hormone-free MS medium after 10 days (Figure I). This suggests that *A. wilkesiana* plant material contains natural growth hormones.

In this study, explants responded to different combinations of plant growth regulators. This differential response of explants to callogenesis upon the addition of auxins and cytokinins demonstrated the phenomenon of tissue sensitivity. Narayanaswamy (1990) reported that each tissue type requires a different formulation for callogenesis. The results obtained indicated that the type of plant growth regulators, the ratio between auxins and cytokinin and the type of explants had an influence on callus formation and morphogenesis. Similar observations have been made in the *in vitro* culture of *Tagetesaccumina* (Kothari and Chandan 1990).
In *A. wilkesiana*, callus was induced on stem segments on MS medium alone and on

medium provided with 2,4-D and Kn. The presence of the cytokinin appeared to be essential for callus induction. In other related species such as *Phyllanthusamarus Schum*, David *et al.* (1991) reported that 1.0mg/l 2,4-D and 0.5mg/l BA were required. For *Phyllanthusacidus Skeels*, Duangporn *et al.* (2009) reported that 1.0mg/l 2,4-D and 1.0mg/l Kn were required for callus induction. In *Passiflorafoetida*, Rassol *et al.* (2011) reported that for callus initiation 2.0mg/l 2,4-D with 0.5mg/l Kn were required. Previous studies by Pang (2009) and Hang (2010) concluded that, for callus induction in *A. wilkesiana,* MS medium without any growth hormones gave the best result.

Vasana (1985); Huang *et al.* (1989) and Huang (1988) indicated that for callus induction 2,4-D was an essential requirement. It can be assumed that higher level of auxins proved more toxic to cells in culture.

Mitra and Chatarvedi (1972) reported that the callus of different species may vary in texture, friability, coloration and presence of chlorophyll or anthocyanin pigments. Stem segments of *Citrus grandis* gave callus strains that were greenish-white, compact, nodular and slow growing. The juvenile green friable callus induced in *Adeniahondala* and *Baliospermummontanum* consisted of uniform parenchymatous cells with chlorophyll pigments. The texture and the colour of the callus depended on the source of the cells, the ingredients in the nutrient medium and the duration of subculture. In the current study, the MS medium alone and with growth hormones influenced the mass, the colour and the texture of the callus. Synergistic effect of auxin and cytokinin was also observed on callus induction.

Conclusion

An effective surface sterilization and callus culturing protocol has been established for *Acalypha wilkesiana*. A two-step surface sterilization protocol with treatment of explants with 15% rifampicin and 15% benomyl followed by 70% ethanol for 1 minute and 30% Clorox for 30 minutes could achieve acceptable control of bacterial and fungal contaminants. None of the treatments achieved 100% decontamination. In further research it is proposed that the antibiotic meropenem be tested to improve the suppression of bacterial contamination.

Sub-culturing of clean cultures onto the MS medium with 1.0mg/l 2, 4-D and 0.5mg/l Kn gave high amount of callus. However the same amount of callus was also induced in medium without any growth hormones. At high concentration of auxin, callus induction was comparatively low. Medium supplemented with 0.5mg/l of Kn promoted shoot induction. There was significant effect in size of callus formation using different growth regulators ($p<0.05$).

On considering the medical importance of *Acalypha wilkesiana*, plant tissue

culture provides a means for fast *in vitro*propagation. Further research should concentrate on rapid multiplication of *Acalypha wilkesiana* through indirect plant regeneration, by focusing on shoot and root induction from callus cultures. The advancement of *in vitro* plant regeneration procedures will allow the application of gene transfer technologies for improvement of secondary metabolites.

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