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Full Length Research Paper

Control of contamination and explant browning in *Curculigo latifolia in vitro* cultures

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Curculin, which is extracted from fruits of *Curculigo latifolia* has been found to have a sweet taste with sweetness-modifying characteristics of natural sweeteners and has been shown to be a good low-calorie sweetener. *In vitro* growth performance of *C. latifolia* cultures is limited by contamination and browning. This study was aimed to minimize the effect of phenolic components, eliminate contamination and identify suitable explants for *in vitro* regeneration of *C. latifolia*. Our results showed that the use of ascorbic acid, citric acid, polyvinylpyrrolidone (PVP), bavistin and chloramphinicol (0.1%) for 9 h significantly reduced the shoot tip explants browning. This pre-treatment was followed by 70% ethanol for 90 s, 30% Clorox® and Tween 20 for 15 min, mercuric chloride (0.1%) for 5 min, and 10% Clorox® for 10 min for eliminating contaminants. This technique reduced contamination (62.5% aseptic) with high survival rate (100%) of shoot tip explants. In petiole explants, pre-treatment with ascorbic acid, citric acid, PVP, bavistin, chloramphinicol and streptomycin (0.1%) for 9 h followed by the same sterilization technique significantly reduced browning and contamination (83.3% aseptic), but the percentage survival of explants was only 49.2%. Beside, rejuvenation of explants from petiole was difficult when compared to shoot tip cultures. The results showed that this technique was more efficient in sterilization of shoot tip tissue than petioles.

Keywords: *Curculigo latifolia, in vitro* cultures, phenolic components, sterilization technique, plant preservative mixture (PPM).

INTRODUCTION

Lemba (*Curculigo latifolia* Dryand) is a member of the Hypoxidaceae family. It is a perennial herb with modified stem. This plant species is wide spread in forests and is found in Malaysia, parts of Kalimantan, Indonesia and Singapore. Curculin and neoculin contained in the fruit are unique sweet proteins because they exhibit both sweet-tasting and taste-modifying properties (Suzuki et al., 2004). These proteins have been proven to be 500 to 9000 times sweeter than sucrose by weight (Yamashita et al., 1990; Nakajo et al., 1992; Kurihara and Nirasawa, 1994; Yamashita et al., 1995). This makes it a desirable plant for low calorie sweetener based industries. Besides, it is also considered as a valuable medicinal plant in having anticancer properties (Ismail et al., 2010), antidiabetic properties (Kant, 2005) and inhibiting hepatitis B virus (Mohamed et al., 2007). Therefore, *C. latifolia* has great potential for the pharmaceutical industry. In addition, this important plant which is native to Malaysia is abundantly wild and not cultivated. The seed does not germinate easily and propagation through rhizomes and corms is still uninvestigated. Standard tissue culture protocols for *C. latifolia* including sterilization, regeneration and acclimatization have also not been established.

Plant micropropagation is an *in vitro* technique of growing “aseptic” plant cells, tissue or organs separate from the mother plant in an artificially prepared nutrient medium. It is particularly important for mass propagation especially when seed propagation and conventional

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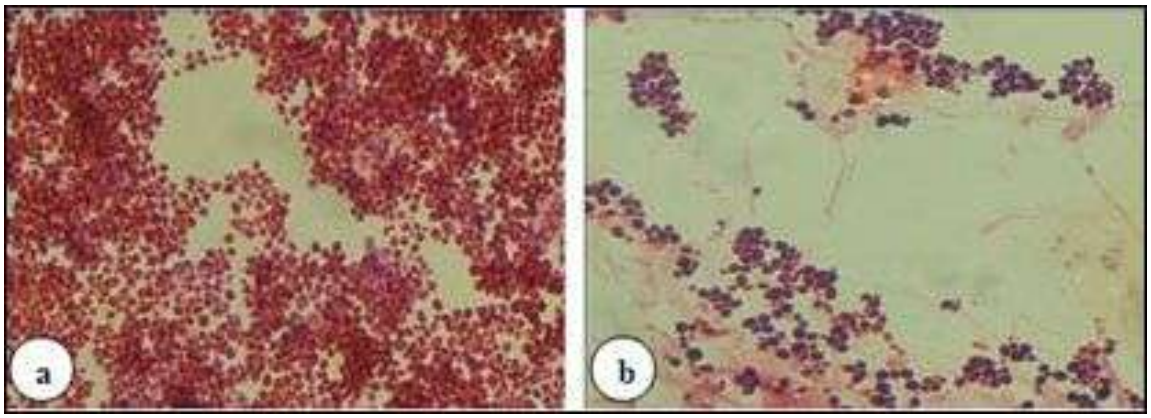

Figure 1. Presence of (a) gram-negative and (b) gram-positive bacteria in *in vitro* cultures of *C. latifolia.*

vegetative techniques are difficult or unsuccessful.

Although aseptic conditions are usually employed, *in vitro* contamination of tissue cultures by microorganisms is often the most serious problem in plant tissue culture (Omamor et al., 2007). Contamination is not always seen at the culture establishment stage; some internal contaminants become visible at later subcultures and are difficult to eliminate (Reed et al., 1998). Chemicals such as antibiotics, fungicides, alcohols, mercuric chloride and sodium hypochloride are normally used to remove contaminants. Isothiazolones are a class of industrial biocides that have been used in the form of plant preservative mixture (PPM) in tissue culture media to control microbial contamination (Niedz and Bausher, 2002).

Apart from contamination, browning of excised plant tissues and nutrient media occurs frequently and remains a major basis for recalcitrance *in vitro*. The severity of browning has varied according to species, tissue or organ, developmental phase of plant, age of tissue or organ, nutrient medium and other tissue culture variables (Huang et al., 2002).

The browning phenomenon is usually imputed to oxidized phenolic compounds by polyphenol oxidase (PPO) and pre-treating with polyvinylpyrrolidone (PVP) and antioxidants like ascorbic acid and citric acid are ways to remove phenols or reduce their accumulation in tissue culture media (Krishna et al., 2008). Phenol oxidation enzymes could be influenced by environmental factors as well. Presence of light and high temperature raise browning rate by increasing the enzyme activity (Dobránszki and Teixeira, 2010).

A study was carried out to control contamination and browning of cultures during the mass propagation of *C. latifolia* using various concentrations of plant growth regulators. This paper presents findings of the study on effective methods for eliminating contamination and reducing browning in shoot tip and petiole cultures of *C. latifolia*.

MATERIALS AND METHODS

Source of plant materials and explants

Plants were collected from Beranang, Negeri Sembilan and maintained in polybags under shelter with 70% shade and average of 295.83 µmol $m^{-2}s^{-1}$ light intensity. Petiole and shoot tip samples for establishment of explants were obtained after 6 months of planting.

Culture media conditions

Petiole and shoot tip tissues in all experiments were cultured in 100 ml flasks containing 40 ml medium consisting of Murashige and Skoog (MS) basal salt and vitamins, 30 gl^{-1} sucrose, 2 gl^{-1} gelrite and 2 mgl^{-1} thidiazuron (TDZ). The pH of medium was adjusted to 5.8 prior to autoclaving at 121°C for 15 min at 15 p.s.i. Cultures were incubated in the dark for the first 2 weeks of establishment at 25 ± 2°C followed by 16/8 h light/dark conditions and the light intensity of 40 µmol $m^{-2}s^{-1}$ provided by cool white fluorescent lamps at the same temperature.

Pre-treatment for eliminating contamination and browning

Since both gram-positive and negative bacteria were observed to be present in cultures disinfected by alcohol and sodium hypochloride (Figure 1a and 1b), two efficient antibiotics for both gram-positive and negative bacteria were used as pre-treatments in the this experiment.

Shoot tips and petioles were washed under running tap water and Teepol for 35 min, and then given two types of pre-treatments. Pre-treatment 1 contained 0.1% (v/v) bavistin, 0.1% (w/v) chloramphinicol, 0.1% (w/v) PVP, 0.1% (w/v) ascorbic acid and 0.1% (w/v) citric acid. Pre-treatment 2, was the same as pre-treatment 1, but with addition of 0.1% (w/v) streptomycin. In both pre-treatments, explants were emersed for 6, 9, 12 or 15 h and all treatments were agitated on an orbital shaker (150 rpm). The explants were then washed with sterile distilled water, followed by 70% ethanol for 90 s and 30% Clorox® and Tween 20 for 15 min. Finally, the explants were washed with sterile distilled water for five times and cultured on solid media. This experiment consisted of eight treatments arranged in factorial based on completely randomized design with each treatment containing 15 flasks. Each

Table 1. Rating scores for browning in petiole and shoot tip explants of *C. latifolia*.

Rating	Remark
0	Explants completely brown
1	Browning along cut edges
2	Slight browning along cut edges in contact with media

experiment was conducted in three replications. Contamination and browning data were collected after 30 days of culture. Browning was rated on an arbitrary scale over a period of 4 weeks (Table 1).

Eliminating contamination

In all treatments, the explants were washed under running tap water and Teepol to remove soil from the surface for 35 min. Explants were surface sterilized with 70% (v/v) ethanol for 90 s, 30% Clorox® and Tween 20 for 15 min and rinsed with sterile distilled water, and then pre-treated (with earlier mentioned pre-treatment), surface sterilized with 70% (v/v) ethanol for 90 s, and 30% Clorox® and Tween 20 for 15 min. Samples were then exposed to one of four treatments (I, II, III and IV) and cultured (Table 2).

This experiment consisted of four treatments conducted in completely randomized design. Each treatment in this experiment consisted of eight flasks, where each flask contained one explant, and each experiment was repeated three times. Contamination data were collected after 45 days. Explants with no visible contaminant growth after 30 days on culture medium were placed into individual flasks with the same constituents for an additional 15 days to determine any undetected contaminants before subculturing.

Data analysis

Data were analyzed using the analysis of variance procedure in the SAS statistical software, and the Duncan's new multiple range test (DNMRT) was used for comparison among treatment means.

RESULTS

Pretreatment for eliminating contamination and browning

Analysis of variance revealed that factor pre-treatment had a significant effect on all recorded parameters (Table 3). Result also indicated that factor period of time was highly significant on score of browning for both explants and percentage of survival for shoot tip explants. However, it was not significant for other parameters (Table 4). The interaction of pre-treatment x period of time was significant on score of browning in petiole explants and percentage of aseptic shoot tips and percentage of survival shoot tips (Table 5). Mean comparison among two pre-treatments showed that pre-treatment with bavistin, chloramphinicol, streptomycin, PVP, ascorbic acid and citric acid was more effective than another pre-treatment for petiole explants. Nevertheless, using bavistin, chloramphinicol, PVP, ascorbic acid and citric acid for pre-treatment was desirable for shoot tip explants (Table 3). It was also observed that pre-treatment for nine hrs was the best period for both petioles and shoot tip explants (Table 4). All periods of pre-treatments were not effective for elimination contamination. However, contaminants were reduced to 46.7% of aseptic culture using bavistin, chloramphinicol, streptomycin, PVP, ascorbic acid and citric acid as pre-treatment for 9h. Nevertheless, browning was reduced effectively and explants survival rate was increased to 93.3% using this pre-treatment (Table 5). The petiole explants that survived were green in colour, while those that did not survive turned to brown and black in colour.

Results also indicated that pre-treatments at all periods were unable to effectively reduce contamination in shoot tip cultures, but using bavistin, chloramphinicol, PVP, ascorbic acid and citric acid as pre-treatment for 9h was the best for reducing contamination (33.3% clean) and browning (1.47), with a high survival rate (93.3%) (Table 5). The surviving shoot tip explants were observed to be yellowish in colour, whereas non-surviving explants turned to brown and black in colour. Browning was reduced and shoot tips showed slight browning along cut edges in contact with media after pre-treating for 9 h (Figure 2a), while shoot tips became completely brown when treated with pre-treatments for 6 h (Figure 2b). Based on these results pre-treatments with or without streptomycin for 9h were used in the subsequent experiment with petiole and shoot tip explants respectively.

Eliminating contamination

Treatment I resulted in 25 and 54.2% aseptic shoot tip and petiole cultures, respectively, whereas treatment II resulted in 62.5 and 83.3% aseptic explants, respectively, where 49.2% of aseptic petiole explants were necrotic. Utilizing PPM at both concentrations (1 and 2 ml^{-1}) was not as effective as using mercuric chloride for microbial elimination in both explants. However necrosis in petiole explants was also observed in higher rates when PPM was used (Table 6).

DISCUSSION

The use of field grown plants as a direct source of explants for the establishment of aseptic *in vitro* cultures is generally considered a major setback, especially with

Table 2. Treatments used to sterilize petiole and shoot tip explants.

Treatment	Protocol
I	Cutting explants to 1 cm and surface sterilizing with 10% Clorox® for 10 min and rinsing three times with sterile distilled water.
II	Disinfecting explants with 0.1% (w/v) mercuric chloride for 5 min, cutting into 1 cm, transferring into 10% Clorox® for 10 min, and rinsing tree times in sterile distilled water.
III	Cutting explants to 1 cm, dipping into 10% Clorox® for 10 min, rinsing three times with sterile distilled water, and culturing onto medium containing 1 ml l^{-1} plant preservative mixture (PPM).
IV	Cutting explants to 1 cm, dipping into 10% Clorox® for 10 min, rinsing with sterile distilled water, and culturing onto medium containing 2 ml l^{-1} plant preservative mixture (PPM).

Table 3. Effect of pre-treatments on percentage of aseptic explants, browning scores and percentage of survival explants after 30 days of culture.

Pre-treatment	Mean					
	Petiole			Shoot tip		
	Aseptic explants (%)	Browning score	Survival (%)	Aseptic explants (%)	Browning score	Survival (%)
P1	23.3^{b}	1.40^{b}	86.7^{a}	25.0^{a}	1.12^{a}	80.0^{a}
P2	41.7^{a}	1.68^{a}	93.3^{a}	10.0^{b}	0.95^{a}	65.0^{b}
F- test	*	**	*	*	*	*

Table 4. Effect of different period of time on percentage of aseptic explants, browning scores and percentage of survival explants after 30 days of culture.

Period (h)	Mean					
	Petiole			Shoot tip		
	Aseptic explants (%)	Browning score	Survival (%)	Aseptic explants (%)	Browning score	Survival (%)
6	33.33^{a}	1.03^{c}	93.3^{a}	16.67^{a}	0.80^{b}	80.0^{ab}
9	36.67^{a}	1.93^{a}	93.3^{a}	20.00^{a}	1.30^{a}	90.0^{a}
12	33.33^{a}	1.73^{a}	86.7^{a}	20.00^{a}	1.23^{a}	66.7^{bc}
15	26.67^{a}	1.47^{b}	86.7^{a}	13.33^{a}	0.80^{b}	53.3^{c}
F- test	ns	**	ns	ns	**	**

leaf canopy close to the ground (Webster et al., 2003). The higher percentage of contamination in shoot tip cultures was due to nearer position of shoot tip to the ground, while petioles from the leaf basal region is further from the soil and hence further from source of contamination. In this study, contamination was successfully reduced in both shoot tip and petiole explants using the modified sterilization protocol. However, petioles became more easily necrotic and rejuvenation of explants from petiole was more difficult when compared to shoot tip cultures. It appears therefore that shoot tip culture was more suitable for *C. latifolia* micropropagation.

Microbial contaminants could be affected by rainfall by enhancing humidity. The petiole phylotaxy of *C. latifolia* overlap one another which causes rain water to collect between leafstalks, making it a suitable place for pathogen growth. This mechanism of pathogen contamination was minimized as the collected plants were cultured in polybags under rain shelter for at least 6 months to restrict microbial development. A postulate for successful tissue culture is the establishment of a sterilization technique. A common method of sterilizing explants is by using alcohol and sodium hypochloride. However, optimizing the technique or even opting for another technique is often executed for contaminant free explants when explants are prone to high contamination. The presence of both gram positive and negative bacteria emphasises the need for the use of chloramphinicol and streptomycin which have been known to restrict both

Table 5. Effect of pre-treatments in different period of time on percentage of aseptic explants, browning scores and percentage survival of explants after 30 days of culture.

Treatment	Mean					
	Aseptic petiole (%) (± S.E.)	Browning score of petiole (± S.E.)	Survival petiole (%) (± S.E.)	Aseptic shoot tip (%) (± S.E.)	Browning score of shoot tip (± S.E.)	Survival shoot tip (%) (± S.E.)
P1T1	20.0 ± 0.00^{bc}	0.87 ± 0.067^{e}	86.7 ± 6.67^{ab}	13.3 ± 6.67^{bcd}	0.93 ± 0.067^{bcd}	93.3 ± 6.67^{a}
P1T2	40.0 ± 0.00^{ab}	1.87 ± 0.13^{ab}	93.3 ± 6.67^{ab}	33.3 ± 6.67^{a}	1.47 ± 1.13^{a}	93.3 ± 6.67^{a}
P1T3	20.0 ± 11.55^{bc}	1.60 ± 0.0^{bc}	80.0 ± 0.00^{ab}	26.7 ± 6.67^{ab}	1.20 ± 0.20^{abc}	66.7 ± 6.67^{ab}
P1T4	13.3 ± 6.67^{c}	1.27 ± 0.17^{cd}	86.7 ± 6.67^{ab}	26.7 ± 6.67^{ab}	0.87 ± 0.24^{bcd}	66.7 ± 13.33^{ab}
P2T1	46.6± 6.67^{a}	1.2 ± 0.11^{de}	100.0 ± 0.00^{a}	20.0 ± 0.00^{abc}	0.67 ± 0.18^{d}	66.7 ± 17.64^{ab}
P2T2	46.7 ± 6.67^{a}	2 ± 0.0^{a}	93.3 ± 6.67^{ab}	13.3 ± 6.67^{bcd}	1.13 ± 0.67^{abcd}	86.7 ± 6.67^{a}
P2T3	33.3 ± 6.67^{abc}	1.87 ± 0.67^{ab}	93.3 ± 6.67^{ab}	6.7 ± 6.67^{cd}	1.27 ± 0.18^{ab}	66.7 ± 6.67^{ab}
P2T4	40.0 ± 11.55^{ab}	1.67 ± 0.18^{ab}	86.7 ± 6.67^{ab}	0.0 ± 0.00^{d}	0.73 ± 0.67^{cd}	40.0 ± 0.00^{b}
F- test	ns	ns	*	**	ns	*

ns, Non-significant; *, significant at $p < 0.05$; **, significant at $p < 0.01$. Means within columns followed by the same letters are not significantly different at p<0.05 (DNMRT). P1, pre-treatment with bavistin, chloramphinicol, PVP, ascorbic acid and citric acid; P2, pre-treatment with bavistin, chloramphinicol, streptomycin, PVP, ascorbic acid and citric acid; T1, T2, T3 and T4, pre-treatment in 6, 9, 12 and 15 h respectively.

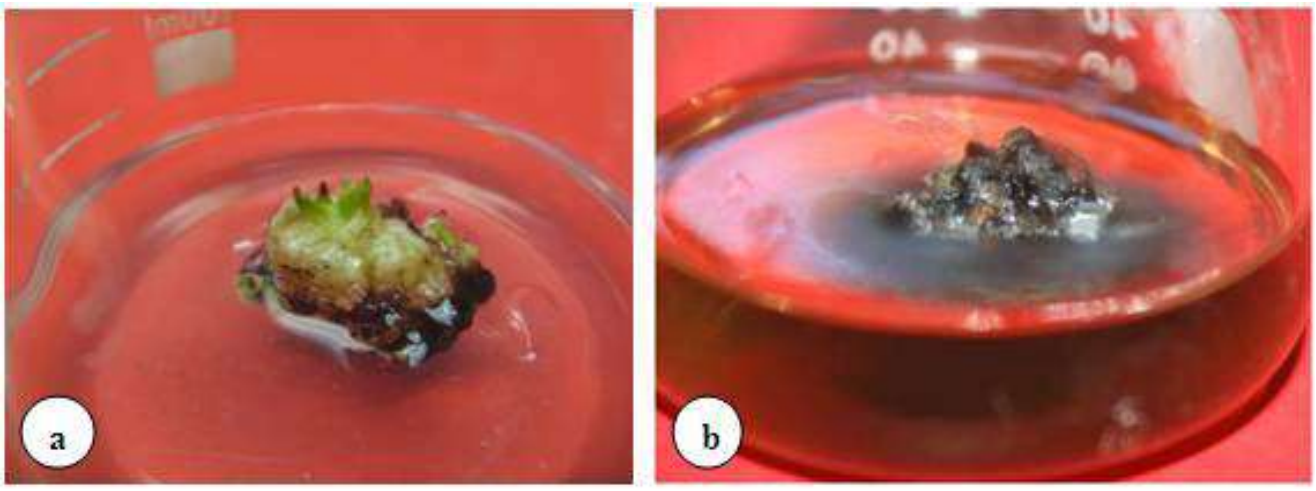

Figure 2. Occurrence of browning after one month of culture: (a) Pre-treated explants with bavistin, chloramphinicol, ascorbic acid, citric acid and PVP (0.1%) for 9 h; and (b) for 6 h.

Table 6. Effect of sterilization method on percentage of aseptic explants and percentage of survival.

Treatment	Mean		
	Petiole		Shoot tip
	Aseptic explants (%) (± S.E.)	Survival (%) (± S.E.)	Aseptic explants (%) (± S.E.)
Control	54.7 ± 4.17 c	76.7 ± 1.67 a	25.0 ± 7.22 b
$HgCl_2$	83.3 ± 4.17 a	49.2 ± 7.94 b	62.5 ± 7.22 a
PPM (1 ml l^{-1})	62.5 ± 7.22 bc	46.7 ± 3.33 b	33.3 ± 4.17 b
PPM (2 ml l^{-1})	75.0 ± 0.00 ab	22.2 ± 5.55 c	45.8± 11.02 ab
F- test	**	**	*

*, Significant at $p < 0.05$; **, significant at $p < 0.01$. Means within columns followed by the same letters are not significantly different at p < 0.05 (DNMRT).

gram positive and negative bacteria, while the presence of fungi requires the use of bavistin, a fungicide.

A serious limitation in *C. latifolia* micropropagation is oxidation of polyphenols exuded from cut surfaces of explants and their release into the culture media causing browning and necrosis of explants. Browning has been described as enzymatic oxidation of phenolic substances by PPO (Chitbanchong et al., 2009). PPO is mainly in the vacuoles, while the enzyme is localized in plastids or chloroplasts. They do not come in contact with each other, but during excision cells are injured, and the browning reaction is initiated (Murata et al., 1997). Besides PPO, phenylalanine ammonia lyase (PAL) and peroxidase (POD) are also responsible for browning arising from wound as a catalyser of polyphenol biosynthesis (Krishna et al., 2008). Shoot tips which are considered as etiolated tissue, since they are covered by petioles, should show less browning than petioles. Wounded surface in shoot tips emit higher exudates than petioles, as the cut surface of explants exude polyphenols that are easily oxidized and cause explant necrosis and medium darkening (Abdelwahd et al., 2008).

The results showed that pre-treatment with streptomycin, chloramphinicol, bavistin, ascorbic acid, citric acid and PVP for 9h reduced microbial contamination in petioles, while pre-treatment with chloramphinicol, bavistin, ascorbic acid, citric acid and PVP for 9 h was better for eliminating contaminants in shoot tips. Although these were not efficient methods for contaminants elimination, it was observed that microbial contaminants were reduced in these treatments. These pre-treatments decreased browning significantly in both explants. Pre-treatment of explants for 6 h did not effectively reduce browning and contamination. Augmenting the pre-treatment for more than 9 h did minimize browning, but when explants were stirred on an orbital shaker for more than 9h the occurrence of necrosis was evident. It could be summarized that pre-treatment with antioxidants for nine hours minimized browning effectively, even with the high polyphenols exudation from wounded explant surfaces.

In spite of the synergistic effect of PPO and POD in browning (Tang and Newton, 2004; Krishna et al., 2008), PPO, POD and PAL play a vital function in plant defence against bacteria, fungi and other pathogens (Krishna et al., 2008; Houssien et al., 2010). Another interesting observation made in this experiment was significantly reduced contaminants level on pre-treatments 1 and 2 for 9 h in shoot tip and petiole explants respectively. It can be assumed that since these pre-treatments were able to reduce phenol leaching efficiently in shoot tip and petiole explants, it thereby, helped maintenance of sufficiently high phenolics level in plant (explant) system to restrain the growth of pathogens (Krishna et al., 2008).

Of the four protocols using three anti-microbial agents, mercuric chloride proved to be more effective in reducing contamination in cultures in both explants. However, some contaminations were still observed after sub culturing on new medium after 30 days. This is attributed to latent pathogen effect (Omamor et al., 2007). Using mercuric chloride at a concentration of 0.1% for 5 mins resulted in minimal phytotoxicity to shoot tip explants and presented acceptable control of contamination. However, mercuric chloride was phytotoxic to petiole explants. PPM has been reported to prevent microbial contamination in tissue culture (Guri and Patel, 1998). In media supplied with PPM, contaminants grew on the upper surface of explants, but none was found on or in the media. This indicates that direct contact with PPM containing media could reduce contaminants effectively. The present results suggest that contamination can be reduced by adding PPM (1 and 2 $ml l^{-1}$) to the medium, but this was not as effective as disinfecting using mercuric chloride. PPM at 2 $ml l^{-1}$ clearly resulted in phytotoxicity to explants and caused necrosis to petiole cultures. However, this concentration (2 $ml l^{-1}$) was not phytotoxic to shoot tip explants, but contamination was relatively high.

Conclusion

Contamination by microbes has been a persistent problem for *in vitro* propagation of *C. latifolia*. Another serious constraint of micropropagation of this plant is the presence of secondary metabolites which are oxidized after wounding and cause subsequent browning and necrosis of explants. It was easier to eliminate contamination and browning from petioles, but petioles were also easily damaged by PPM and mercuric chloride sterilization procedures. Pre-treating with bavistin, chloramphinicol, streptomycin, PVP, ascorbic acid and citric acid (0.1%) for 9 h followed by dipping in ethanol (70%), surface sterilizing with 30% Clorox®, disinfecting with 0.1% (w/v) mercuric chloride for 5 min, cutting explants into 1 cm, and transferring into 10% Clorox® for 10 min was the best technique for reducing browning and contamination in shoot tip explants.

Shoot tips were thus more suitable for *C. latifolia* tissue culture as it showed higher survival percentage and better regeneration than petioles and an acceptable percentage of non-contaminated cultures. This protocol could also be an efficient procedure to eliminate contamination and browning in monocot plants with higher potential for microbial contamination due to shoot apices being close to the ground.

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