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Full Length Research Paper

Induction and multiplication of callus from endosperm of *Cycas revoluta*

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The usage of medicinal plants in traditional medication has gained the attraction from global and local markets, mainly to cure diseases or simply for health maintenance. Callus cultures were initiated from the endosperm of the medicinal plant *Cycas revoluta*, cultured on half-strength Murashige and Skoog (MS) medium supplemented with 30 g/L sucrose and various concentrations (5, 10 and 20 µM) of 2,4-dichlorophenoxyacetic acid (2,4-D), 1-naphthalene acetic acid (NAA) and 4-amino-3,5,6-trichloropicolinic acid (picloram). Explants treated with various auxins formed calli with different morphologies. In the induction studies, 20 µM picloram was the most efficient formulation for callus formation. The callus was formed after 17.8 ± 0.5 days in the medium. However, callus was not formed in the control medium (MSO) and medium supplemented with 20 µM 2,4-D. Calli were successfully maintained in 10 µM picloram at normal photoperiod (16 h light, 8 h dark). The calli treated with10 µM picloram that incubated in 24 h dark condition was found to exhibit less browning effects. Addition of 1 g/L polyvinylpyrrolidone (PVP) aided in overcoming the browning effects by absorbing the phenolic compounds in the medium. The combination of auxin (10 µM picloram) and cytokinin (10 µM kinetin) was able to multiply more friable and yellowish-green calli.

Key words: *Cycas revoluta*, callus cultures, phenolic compounds, medicinal plant.

INTRODUCTION

Cycas revoluta or commonly known as Japanese sago palm belongs to the family of Cycadaceae. It can be described as evergreen, palm-liked, leaves with pinnately divided, glossy green, narrow leaflets with a sunken midrib and the margins rolled downward (Gilman, 1999). This plant, once found world-wide, is today restricted to warm climates. *C. revoluta* produces leaves all year round, flower from May to July, and the seeds ripen from October to November. Its flowers are dioecious, meaning that individual flowers are either male or female, but only one sex is to be found on any one plant. The pollinators are restricted to insects and wind as the plant is not self-fertile. Besides, it can fix nitrogen in light (sandy) and medium (loamy) soils or even well-drained soil. It can survive in acid, neutral and basic soils (Brenner et al., 2003).

C. revoluta is known to be one of the medicinal plants that has been used natively in several countries. The Cycas leaves are used traditionally in the treatment of cancer and hepatoma (Hill, 1993). The terminal shoot is an astringent diuretic. However, the most significant part that contributes to the main chemical compounds is located in the seed. It can be eaten raw or cooked (Chang, 2004). Besides, the seed can also be dried and ground into powder then mixed with brown rice and fermented into "*date miso*" or "*sotetsu miso*" (Brenner et al., 2003). However, many researches carried out until recently were focused on the compounds present in the

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Abbreviations: MS, Murashige and Skoog medium; **2,4-D**, 2,4-dichlorophenoxyacetic acid; **NAA**, naphthaleneacetic acid; **picloram**, 4-amino-3,5,6-trichloro picolinic acid; **PGRs**, plant growth regulators.

seeds as this extract can be used to treat certain tumours by inhibiting the growth of maglinant tumours (Kowalska et al., 1995).

Apart from limited research being carried out on *C. revoluta* nowadays, several species from this family are classified as rare and endangered. Therefore, cell and tissue culture techniques can be developed to overcome the problems such as slow-growing, rapid loss of seed viability, and low morphogenesis potentials. Thus, the plant tissue culture were carried out in this study in order to observe the effects of different auxins at various concentrations on the induction of callus from the endosperm explants of *C. revoluta*, as well as to examine the appropriate auxin and/or cytokinin and their concentrations for callus maintenance. Apart from that, the callus cultures obtained in normal photoperiod and dark conditions as well as with the presence of 1 g/L PVP were also compared.

MATERIALS AND METHODS

Plant materials

The seeds of *Cycas revoluta* were collected from the campus of University Malaysia Sabah (UMS) in December 2005.

Surface sterilization

Seeds were washed under running tap water for about 20 min to remove soils and other contaminants on the outer surface of the seed coats. Secondly, 30% Clorox® containing 1 to 2 drops of Tween-20 (Amresco, USA) was prepared and later used to incubate the seeds for further sterilization. Then, the explants were thoroughly rinsed with sterile distilled water for about 5 - 10 min to remove the remaining Clorox® solution. Additionally, the seeds were immersed in 70% ethanol for 5 min, followed by rinsing twice with sterile distilled water for approximately 5 min. Endosperms from the seeds were cut to a size of 1 X 1 cm and used to induce the callus.

Basal medium

Medium used to induce callus from these explants was half-strength of MS medium (Murashige and Skoog, 1962). Besides, 3% (w/v) of sucrose was added. The pH was adjusted to 5.7 ± 0.1 using pH meter (Mettler Toledo) prior to autoclaving at 121°C for 15 min in an autoclave (Hirayama, Japan). Lastly, 0.8% (w/v) of Vietnam agar (Duchefa, Netherlands) was added to provide the solid support for the explants to grow. After autoclaving, approximately 25 mL of medium was poured into each of the Petri dish.

Callus induction

Callus induction studies were carried out by culturing the sterile endosperm explants in the half-strength MS medium containing 2,4-D (Sigma, USA), NAA (Duchefa, Netherlands), or picloram (Duchefa, Netherlands), at concentrations of 5, 10 and 20 µM. The control medium was MSO, without any addition of plant growth regulators. A total of five explants were cultured in each treatment and the experiments were repeated thrice. The morphologies, degree of callogenesis and days of callus formation were observed and results were recorded. All the cultures were incubated at room temperature of 23 ± 1°C, 16 h light and 8 h of darkness.

Callus maintenance

In order to determine the best medium formulation for callus maintenance, the initiated calli were separated from the explants and subjected to several studies. In determining the effects of plant growth regulators on callus maintenance, the callus initiated in the callus induction studies were transferred to a fresh new half-strength MS medium containing 5, 10 and 20 µM picloram. Effects of the combination of auxin and cytokinin on callus maintenance were also carried out whereby the calli were treated with the combination of 5 or 10 µM picloram and 5 or 10 µM kinetin. In addition, cultures were incubated in normal photoperiod (16 h light; 8 h dark) and dark (24 h) conditions. In order to reduce the browning effects, the calli were also treated with 1 g/L PVP. A total of 10 calli were cultured in each treatment and the experiments were repeated thrice.

RESULTS AND DISCUSSION

Callus induction

Endosperm explants showed negative response in the control medium which was lacked plant growth regulators (Figure 1a). The explants cultured in the control medium turned brownish in colour and died after one month of culture. This situation is supported by Corredoira et al. (2002) who observed that no embryogenic callus was formed via zygotic embryos in medium without plant growth regulators. Furthermore, Das et al. (1999) reported that no sign of callus formation was observed when leaf and petiole explants from *Tryphonium trilobatum* were cultured in media without auxin and cytokinin.

For the callogenesis from endosperm explants of *C. revoluta*, calli were initiated from the cut margins of the explants in the presence of suitable medium. Three types of plant growth regulators were tested; 2,4-D, NAA, and picloram. Among these, picloram showed the best response, whereby 2,4-D and NAA were not found suitable in callus induction (Table 1). The assessments of callus formation were evaluated through the percentage, day as well as the degree of callus formation. Even though the explants showed different characteristics under the influence of plant growth regulators, to certain extent, initiation of the callus could be seen faster in suitable medium whereas in the less appropriate medium, the induction rate was slower. This basically could be due to the fact of plant specificity towards plant growth regulators.

In this study, the earliest callus initiation was observed in half-strength of MS medium supplemented with picloram. 20 µM picloram gave the best induction response by forming callus in the shortest time (Figure 1d); calli were formed after 17.8 ±0.5 days of culture (Table 1). Besides, explants in the MS medium supplemented with 5 (Figure 1b) and 10 µM picloram (Figure 1c) induced calli after 23.8 ±1.0 and 19.8 ± 1.0 days of culture, respec-

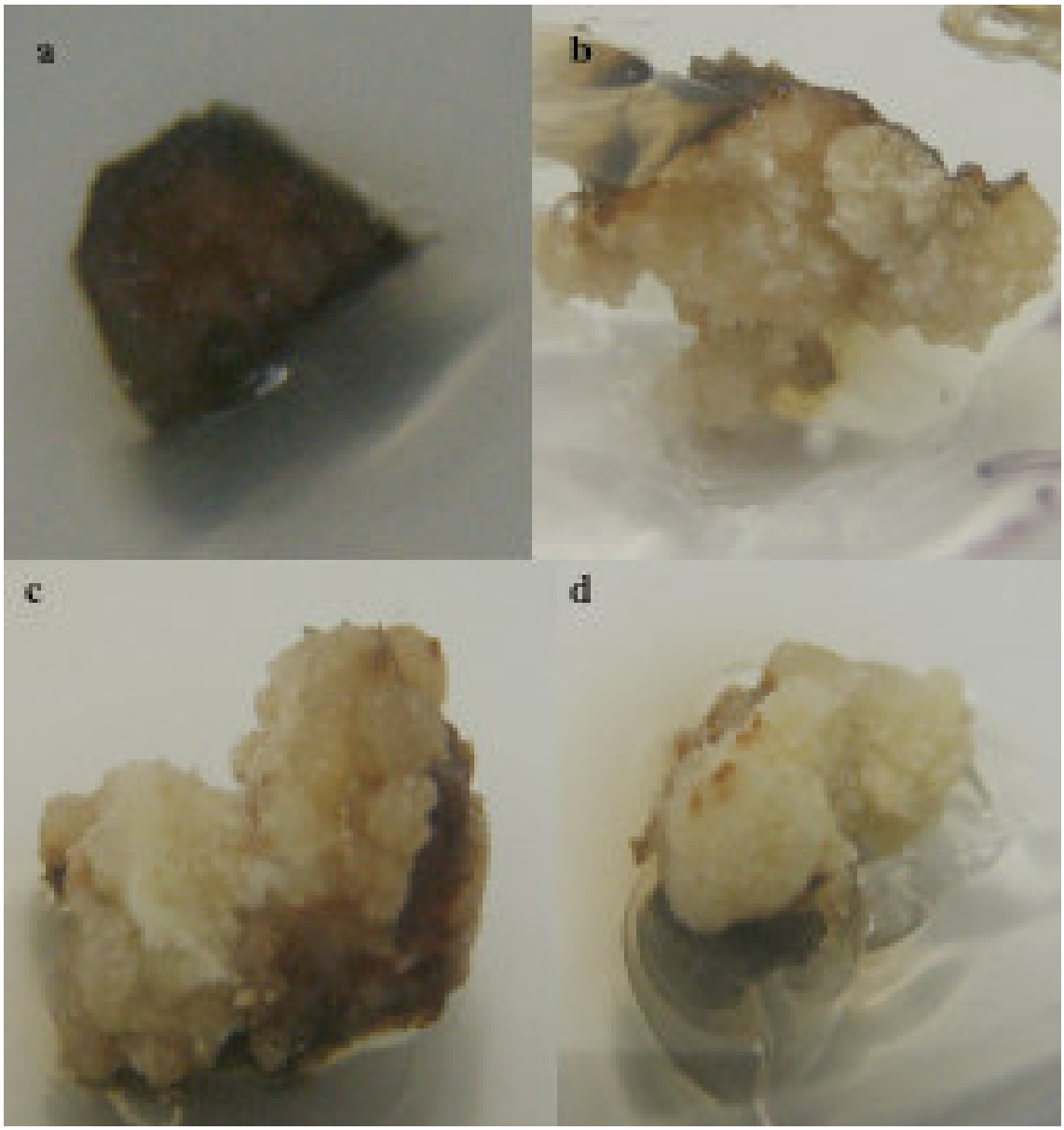

Figure 1. Callogenesis from the endosperm explants of *Cycas revoluta* after one month of culture in half-strength MS medium supplemented with different concentrations of picloram: (a) control; (b) 5 µM picloram; (c) 10 µM picloram; and (d) 20 µM picloram.

tively. The maximum percentage of callus formation (100%) was achieved in half-strength MS medium supplemented with picloram at the concentration of 20 µM (Figure 1d). At lower concentrations (5 and 10 µM), the callus induction percentage decreased to 80%; the explants could not reach 100% due to the accumulation of phenolic compounds in the medium that caused the explants to turn brownish after 2 weeks. In subsequent observation, calli formed in the presence of picloram were larger whencompared to NAA and 2,4-D supplementation. Calli induced from different plant growth regulators were distinct from one another in terms of the morphologies. The healthiest calli with yellowish-white in colour were those cultured in the medium supplemented with 20 µM picloram.

Picloram was used in the study carried out by Cardoza and D'Souza (2001) where somatic embryos were induced from callus in *Anacardium occidentale* L. (cashew) cultured in 0.5 µM picloram and supplement with 20 g/L sucrose. Bordallo et al. (2004), in another study, reported the induction of calli from stem explants of potatoes in medium supplemented with 1.65 µM picloram. Bach and Pawlowska (2003) proved that picloram could also be used to induce embryogenic callus from leaf explants of *Gentiana pneumonanthe* in the medium containing 8 µM, whereby embryogenic callus of various

Table 1. Effects of MS medium supplementation with various auxins at different concentrations on callus induction from the endosperm explants of *Cycas revoluta*.

Plant growth regulator	Conc. (µM)	Percentage Of callus formation (%)	Day of callus formation (Day ± SD[a])	Degree of callus formation[b]	Morphological changes
Control	0	0	-	-	Explants turned to brown in colour after 2 weeks of culture
2,4-D	5	20	30.5±0.6	+	Formation of yellowish-white, friable callus. Growth of callus was retarded as browning effect could be seen on the cultures.
	10	10	39.5±2.4	++	
	20	0	-	-	
NAA	5	20	26.3±1.0	++	Yellowish-white and friable callus could be seen from the cultures but the stages of callus growth were affected by the phenolic compounds.
	10	40	20.5±2.4	+++	
	20	60	19.0±0.8	++++	
Picloram	5	80	23.8±1.0	++++	Healthy and more friable callus in yellowish-white colour was observed. Cultures were also affected by browning effects.
	10	80	19.8±1.0	+++++	
	20	100	17.8±0.5	+++++	

[a]SD: Standard deviation.
[b]Degree of callus formation (cm): (-) absence of callus; (+) 0.1-0.2; (++) 0.2-0.3; (+++) 0.4-0.5; (++++) 0.6-0.7; (+++++) 0.8-0.9.

appearances such as yellow, white or crystalline was formed. In fact, 84% of leaf explants, and 90% of the zygotic embryo explants from two major rattan species, namely, *Calamus merrillii* and *Calamus subinermis* produced friable embryogenic calli after 6 weeks of culture in the media supplemented with picloram, regardless of the concentrations used (10.4, 20.7, or 31.1 µM) (Goh et al., 2000).

On the other hand, explants cultured in the half-strength MS medium supplemented with NAA at 5, 10 and 20 µM, showed relatively slower response to initiate callus than picloram but faster than 2,4-D. Calli formed after 26.3 ± 1.0 days, 20.5 ± 2.4 and 19.0 ± 0.8 days in 5, 10 and 20 µM NAA, respectively. In this study, calli formed in the presence of 20 µM NAA was yellowish-white and friable but the growth was eventually interrupted by the browning effects after 2 weeks. Among these three treatments, maximum percentage of callus formation (60%) was achieved in half-strength MS medium supplemented with NAA at the concentration of 20 µM, followed by 40 and 20% in 10 and 5 µM NAA, respectively.

In the research carried out by Sukuzi and Nakano (2000), two types of calli were initiated from the leaf explants of *Muscari armeniacum*: fast-growing, yellow nodular callus and white friable callus which could only be obtained on the medium containing 54 µM NAA with a frequency of 58% after eight weeks of culture. Besides, Kumar et al. (2002) revealed that petiole explants from woody legume, *Calliandra tweedii*, responded best in 0.5 µM NAA, where 30% of the explants produced normal embryos after 90 days. Martin (2004), on the other hand, stated that embryogenesis callus initiated from leaf explants of *Centella asiatica* in suspended half-strength MS medium supplemented with 2.69 µM NAA developed a mean of 204.3 somatic embryos per 100 mg of callus. In that experiment, 88% of the embryos underwent maturation and conversation to plantlets upon transferred to half-strength MS medium containing 0.054 µM NAA.

In the treatments using 2,4-D, degree of callus was also estimated (Table 1). Calli formed in 5 µM 2,4-D was white in colour and non-friable. Somehow, the callus formation was retarded when the concentration of the hormone increased from 10 to 20 µM. The duration taken to initiate callus was 39.5 ± 2.4 days in 5 µM and 30.5 ± 0.6 days at the concentration of 10 µM 2,4-D. The explants turned into brownish in colour due to the formation of phenolic compounds accumulated around the explant after 2 weeks. Percentages of callogenesis were relatively low in these three treatments as 20% of calli were formed in 5 µM 2,4-D, 10% in 10 µM 2,4-D, and no callus was formed in 20 µM 2,4-D.

2,4-D is relatively a useful plant growth regulator as several callus induction studies were developed by using different concentrations of this hormone. Yang et al. (2003) proved that large-scale propagation of *Phragmites communis* by somatic embryogenesis from the stem segment explants produced hard white callus on MS medium supplemented with 9.05 µM 2,4-D for 4 weeks. Plants were regenerated to form root when the embryogenic callus was transferred to medium at 0.45 µM 2,4-D. Additionally, Martin (2003) reported that the highest number of somatic embryos derived from friable calli were developed from leaf, internodes and root explants on ½ or ¼ MS medium supplemented with 1.0 µM 2,4-D. Another study conducted by Fernando and Gamage (2000)

initiated more than 70% of nodular calli from zygotic embryos of *Cocos nucifera* (coconut) by using 24 µM 2,4-D within 2 - 3 weeks of culture.

Effects of different concentrations of picloram

The calli induced from the endosperms were eventually subcultured into medium containing picloram at 5, 10, and 20 µM. This was because picloram gave rise to the best responses towards callus initiation, especially at the concentration of 20 µM. However, calli maintenance were not suitable in 20 µM picloram; the colour changed from yellowish-white to brown, indicating necrosis. Calli no longer survived in this medium composition due to a phenomenon called habituation. Habituation refers to the natural occurring event whereby callus cultures, upon continue passage, lose their requirement for hormone-dependence (Chawla, 2002). Habituation occurred for the reason that the plant cell itself was able to produce the hormone needed for the growth and development, or that the tissue became sensitive if the plant growth regulators ever present in the culture media. Hence, lower level or elimination of regulator level might be an ideal way to maintain the callus culture (Pischke et al., 1997). Therefore, several attempts were taken to subculture the callus culture into 5 and 10 µM picloram in 3 weeks interval. 10 µM picloram was found to be the best regulator to maintain the callus culture.

Effects of culture conditions

In general, physical factors like light and dark culture conditions play an important role in the growth of callus tissues and development of somatic embryos (Smith, 2000). The entire cultures maintained in the presence of normal photoperiod (16 h light, 8 h dark) hastened the browning effects which resulted in the necrosis of the calli. Calli maintained in the light condition turned to brown and eventually died. On the other hand, cultures maintained in the dark showed slower browning effects in the medium containing 10 µM picloram. The survived calli were more friable and yellowish-green in colour. Kintzios et al. (2000), likewise, studied of the effects of light on the induction of callus and somatic embryogenesis from leaves of chilli pepper *(Capsicum annum L.)*. They reported that more calli and somatic embryos were induced from the callus in darkness compared to explants exposed under normal photoperiod.

Effects of PVP

Basically, PVP is an insoluble, a polyphenol adsorbent which is normally used to remove phenolic substances. PVP hydrogen bonds absorb polyphenol, thus reducing their amount (Layer, 2003). 1 g/L PVP was added in the media to prevent necrosis of the calli due to the formation of phenolic compounds. The addition of PVP to the media to absorb phenolic compounds led to the requirement of increased concentrations of picloram because PVP also absorbed the plant growth regulators, decreasing its amount in the media.

Ogita (2005) reported that addition of 0.25 g/L PVP to a medium was effective in overcoming the browning of the culture medium and promoted the growth of somatic embryos in *Dendrocalamus strictus* (bamboo). In this study, addition of 1 g/L PVP into the media containing 5, 10, 20, 25, 30 and 35 µM picloram, revealed that only the calli in the medium supplemented with 10 µM picloram managed to survive from being affected by the browning effects. Calli in this medium were the healthiest, having a friable texture and yellowish-white in colour. When the concentrations of picloram were increased to 25, 30, and 35 µM, these treatments still failed to maintain the calli, which turned brown and became necrotic after one month.

Effects of combinations of auxin and cytokinin

Instead of using auxin alone to maintain the callus culture, a combination with cytokinins was also used for further development of the cultures. The ratio of the auxin to the cytokinin is important to determine the type of culture established (Slater, 2003). In most of the dicot, if both are too low, there is no growth or development (Ashmore, 1997). The benefits of combination were showed by several studies. Hamama et al. (2001) developed a protocol for the induction, maturation, and germination of somatic embryos of jojoba (*Simmondsia chinensis L.*) by culturing the leaf tissue in darkness on half-strength MS medium supplemented with the combination of 2.26 µM 2,4-D and 2.22 µM BAP. This gave rise to 66 - 80% of callus after 21 days of *in vitro* culture. On the other hand, Mauri and Manzanera (2003) reported that medium supplemented with 10 µM BAP and 10 µM NAA was used to induce somatic embryos from immature zygotic embryo of Holm oak (*Quercus ilex*), followed by embryo development and maturation. Mauri and Manzanera (2003) also stated that the combination of auxin and cytokinin could be used to stimulate cell division in proembryogenic masses of the oak species.

Efforts were also taken to subculture calli into combination media consisting of picloram and kinetin at the concentrations of 5 or 10 µM. Only the medium with 10 µM of picloram mixed with 10 µM of kinetin cultured in the dark showed positive result. The survived calli in this medium were yellowish-green in colour and more friable in their morphologies. The calli also exhibited less browning effects as compared to medium containing 5 µM of picloram mixed with 5 µM of kinetin. Combination medium of 5 µM of picloram with 5 µM of kinetin formed less friable and unhealthy calli. Initially the cultures were observed to be yellowish-white calli, but after prolonged maintaining in this medium for one month, calli eventually died due to the accumulation of phenolic compounds.

Perspectives

This study can be furthered by carrying out activities such as induction of somatic embryogenesis which can develop into whole plant. Besides, organogenesis can help to introduce an intact plant. Optimization of medium formulation for callus maintenance can be improved by testing with different concentrations of PVP in order to overcome the browning effects. Extraction of secondary metabolites from the callus culture can also be carried out to isolate the chemical compounds for the production of useful pharmaceutical products. Lastly plant transformation by *Agrobacterium*-mediated method can be applied to introduce the pest-resistance plant as well as to produce bigger seeds or more chemical extracts.

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