

EFFECT OF PHYSIOLOGICAL AGE AND GROWTH REGULATORS ON CALLUS BROWNING OF COCONUT ENDOSPERM IN VITRO CULTURE

LAZARUS AGUS SUKAMTO*

*Research Center for Biology - Indonesian Institute of Sciences
Cibinong, Indonesia*

ABSTRACT

The possibility of physiological age and growth regulators affecting callus browning of coconut endosperm was investigated. Solid endosperm explants of four coconut fruits from same branches of two coconut cultivars "Samoan Dwarf" were grown on modified Murashige and Skoog (MS) formula with addition of 10 mg l^{-1} putresine, 2.50 g l^{-1} activated charcoal (AC), 1.70 g l^{-1} phytigel, 0, 10^{-6} , 10^{-5} , 10^{-4} , 10^{-3} M 2,4-dichlorophenoxyacetic acid (2,4-D) or 4-amino-3,5,6-trichloropicolinic acid (Picloram) combined with 10^{-5} M 6-benzylaminopurine (BA). Callogenesis occurred on 98.83% of explants. Callus browning between different physiological ages (antipodal and micropylar tissues) of coconut endosperm at 9, 26 and 31 weeks of culture (WOC) was significantly different, but not at 16 and 21 WOC. Auxins of 2,4-D and Picloram did not affect significantly callus browning of endosperm cultures. Auxin doses at 10^{-6} , 10^{-5} , and 10^{-4} M decreased significantly callus browning at 9 and 16 WOC, respectively, but at 10^{-6} M browning was less significant compared to other doses at 21 WOC. Auxin dose at 10^{-3} M caused less significant browning compared to other doses at 31 WOC. The addition of BA decreased significantly callus browning at 9 WOC, but did not affect callus browning thereafter.

Key words: Coconut, *in vitro*, Picloram, 2,4-D, BA

INTRODUCTION

Coconut (*Cocos nucifera* L.) is a long-lived tree with a very long juvenile phase (3 - 5 years), generally cross-pollinated and very heterozygous. Vegetative propagation *in vitro* through the use of various explants has been attempted but the success is mostly limited to immature unselected plants and produced very few plants. Coconut endosperm is considered a mature tissue which provides large and uniform explants without damaging mother plant for *in vitro* culture.

* Corresponding author : lazarus_agus@yahoo.com

Physiological age is a critical factor for the success of *in vitro* culture (Ozyigit *et al.* 2007). The age of the endosperm at the time of culture is critical for growth *in vitro* (Chen *et al.* 1990). Explants from younger fruits responded better to culture (Karunaratne *et al.* 1991). However, coenocytic (free-nuclear) endosperm from very young fruits did not survive in culture because of lack of starch (Srivastava 1982). Young endosperm at celliferous stage was responsive to culture in *Citrus grandis* and apple (Mu and Liu 1978; Wang and Chang 1978). Endosperm of 6 - 7 months postanthesis in coconut which solid endosperm started to form in the antipodal end of coconut fruit (Tammes & Whitehead 1969) was responsive to *in vitro* culture (Kumar *et al.* 1985).

Whole plants are autonomous with regard to growth regulators but isolated tissues or cells require auxins or cytokinins to initiate and maintain growth until they become habituated or organized (Everett *et al.* 1978). Morphogenesis *in vitro* can be regulated by regulators (Christianson & Warnick 1983), Skoog and Miller (1957) found that the balance of auxin and cytokinin in culture medium governed morphogenesis. Auxins induced callogenesis, adventitious plantlets and roots in palms (Tisserat 1979; Paranjothy & Rohani 1982; Reynolds 1982; Paranjothy 1986). Among auxins, 2,4-D was the most effective compared to the others in coconut culture (Blake & Euwens 1982; Pannetier & Buffard-Morel 1986; Karunaratne & Periyapperuma 1989). Auxin at high doses (10^{-5} M - 10^{-3} M) was necessary for callus induction in palm, especially on medium supplemented with 1 - 3 g l⁻¹ AC (Jesty & Francis 1992). Another auxin with properties similar to 2,4-D is Picloram. Picloram has been successfully applied for callogenesis in date palm (Omar & Novak 1990), embryogenesis in pejobaje palm (Valverde *et al.* 1987), and maintained regenerative callus over long time in sugarcane (Fitch *et al.* 1983). Picloram was faster than 2,4-D for callogenesis, embryo induction, and final yield of embryos in *Gasteria* and *Haworthia* (Beyl & Sharma 1983). On the other hand, Picloram produced more phenolics from cut surfaces and was slower for callogenesis than 2,4-D in sugarcane (Fitch *et al.* 1983). The presence of cytokinins, auxins, and high doses of sucrose (0.2 M) stimulated growth of coconut and date callus (Euwens 1978). Combination of cytokinin at low dose with auxin at high dose was necessary for callogenesis of coconut embryos (Bhaskaran 1985). Srinivasan *et al.* (1985) successfully induced somatic embryos of Christmas palm with 5 - 50 x 10⁻⁵M 2,4-D and 50 x 10⁻⁵M BA. BA was more effective than kinetin in stimulating callus growth in longan culture (Litz 1988), increased greatly fresh weight of coconut callus and date palm callus (Euwens 1978; Kuruvinashetti & Iyer 1980; Sharma *et al.* 1984).

Explant browning is often associated with physiological age and failure of explants survival (Krishna *et al.* 2008; Guo *et al.* 2010). Browning in some explants may be very severe and causes inhibition or cessation of growth (Abdelwahd *et al.* 2008; Misra *et al.* 2010). Phenolics, tannins or oxidized polyphenols are synthesized through shikimic acid, phenylpropanoid, flavonoid, and terpenoid pathways. These substances are abundantly present in some plants and act as inhibitory agents (Preece & Compton 1991). Phenolics, especially the most common polyphenol cause oxidative browning in explants, which lead to discoloration of the culture medium (Forrest 1969; Davies 1972; Babbar & Gupta 1986; Tang & Newton 2004). Oxidized phenolic

compounds are frequently exuded into the medium by injured woody tissues causing lethal browning or blackening of explants (Alderson 1987; Bhat & Chandel 1991; Trautmann & Visser 1991).

Immature endosperms were more responsive in *in vitro* culture than mature ones (Cheema & Mehra 1982). This was partially due to oxidation products which were more abundant in the older explants (Sugimura *et al.* 1988; Preece & Compton 1991; Wu *et al.* 2010). Activated Charcoal can promote cell growth and development, it may be mainly due to its irreversible adsorption of inhibitory compounds in the culture medium and substantially decreasing toxic metabolites, phenolic exudation and browning (Zhu *et al.* 1997; Thomas 2008). Browning can be reduced or eliminated through the use of liquid media, AC, silver nitrate, ascorbic acid, citric acid, sodium hydrosulfite, cystein, diethyldithio-carbamate (DTT), potassium ethylxanthate, thiourea, benzimidazole, sodium bisulfite, polyvinylpyrrolidone (PVP), polyclar, glutathione, and bovine serum albumin, more frequent transfers, incubation with reduced illumination or in complete darkness, presoaking explants in sterile water, sealing the cut ends with paraffin wax, changing medium, avoiding high temperatures, discarding explants which show browning, reducing explants thickness, and choosing the most suitable stage (Chang *et al.* 2001; Wu & du Toit 2004; Mitsukuri *et al.* 2009).

Addition of auxins, particularly 2,4-D at high doses, led to browning of coconut leaf (Pannetier & Buffard-Morel 1986). Sugimura and Salvana (1989) observed that 2,4-D at 2.26×10^{-4} M - 4.52×10^{-4} M caused severe browning of tissues regardless of the stage and size of coconut inflorescence culture. 2,4-D at levels higher than 30×10^{-6} M inhibited callusing and enhanced browning of coconut embryos (Karunaratne & Periyapperuma 1989). Callus precociously isolated from explants also caused browning and necrosis in coconut inflorescence (Verdeil *et al.* 1993). Addition of cytokinin, such as kinetin also caused more browning than the use of auxin in palm cultures (Reynolds 1982).

The objective of the study is to know the effect of physiological age (antipodal and micropylar) and growth regulators (2,4-D, Picloram, and BA) on callus browning of coconut endosperm *in vitro* culture.

MATERIALS AND METHOD

Explant material

Plant materials of four seven-month old coconut fruits were taken from two bunches of two coconut trees cultivars "Samoan Dwarf" after opening inflorescences. These fruits immediately were disinfested by using alcohol 95%. The fruits were opened and their water decanted, cut horizontally with a sterile big knife.

Solid endosperms were aseptically cored with cork borer and scooped with a sterile spoon in laminar air flow. These endosperms were taken from either the micropylar region (upper half of fruit where embryo located) or the antipodal region (bottom half of fruit). Cylindrical endosperm shapes with 8 mm diameter and 4 mm thick, used as explants, were grown on various media treatments.

Media culture

Media cultures were a modification of Branton and Blake formula (1986) added with 10 mg l^{-1} putrescine, 2.50 g l^{-1} AC, 1.70 g l^{-1} phytigel, hormones 2,4-D or Picloram at 0 , 10^{-6} , 10^{-5} , 10^{-4} and 10^{-3} M concentrations, respectively, were combined with or without 10^{-5} M BA at 16 WOC. The pH of the media were adjusted to 5.70 before they were autoclaved. The media were poured into 2.5 x 15 cm test tubes (14 ml) and autoclaved at 121°C temperature and 1 kg cm^{-2} pressure for 15 minutes. Media were stored for one week before use. Single explant was placed into test tube with the uncut surface upright. Cultures were incubated at approximately 31°C in the dark room.

Experimental design

The experimental design was a randomized complete block design (RCBD) with each fruit as a block. Treatments were factorial combinations of endosperm region (micropylar and antipodal), auxins (2,4-D and Picloram) and their doses (0 , 10^{-6} M , 10^{-5} M , 10^{-4} M , 10^{-3} M) and cytokinin (with or without 10^{-5} M BA) with 12 replications. Callogenesis of coconut endosperm was counted as percentage on 31 WOC. The tissues browning were evaluated visually at every transfer using numerical scores ranging from 0 to 3 (0 : no browning, 1 : little browning, 2 : medium browning, and 3 : high browning) at 9, 16, 21, 26 and 31 WOC. Data were analyzed with the General Linear Models (GLM) and non parametric one way (Kruskal-Wallis test) procedure of Statistical Analysis System (SAS).

RESULTS AND DISCUSSION

Callogenesis of coconut endosperm

Coconut endosperm explants formed callus after approximately 3 WOC. Callus grew predominantly on the uncut surface and on the side of endosperms. Eventually, the callus grew and covered the entire explants. Callogenesis occurred on almost all explants with an average of 98.83% (Table 1). These results were better than those obtained by Kumar *et al.* (1985) i.e. 30% of coconut endosperms, Gmitter *et al.* (1990) i.e. 8 - 25% of *Citrus maxima* endosperms, Chen *et al.* (1990) i.e. 1.60 - 3.74% of *C. sinensis* endosperms; Pannetier and Buffard-Morel (1982) i.e. 20% of mature coconut leaves and 50% of juvenile coconut leaves, and Verdeil *et al.* (1989) i.e. 45% of coconut inflorescence. The good result could be due to genetic and sterilized factors. In addition, the plant materials used in this study were different in explants type, cultivars or plants than those used by other researchers. Sterilized method was different than of Kumar *et al.* (1985), this experiment only sterilized the outer skin of the fruits not the explants. Therefore, the explants were more responsive in promoting callogenesis.

Physiological age and calli browning

Oxidative browning (brown to black) of endosperm calli occurred during culture. Browning varied within or among treatments, ranging from 0: no browning, 1: slight

Callus Browning of Coconut Endosperm *in vitro* Culture - L.A. Sukanto

Table 1. Percentage of callogenesis of coconut endosperm after 31 weeks of culture

Source	Fruit 1	Fruit 2	Fruit 3	Fruit 4	Average
Endosperm	98.98 ± 0.71 ^a	99.44 ± 0.56 ^b	97.92 ± 1.30 a	98.98 ± 0.71 a	98.83
Position:					
Antipodal	98.89 ± 1.11 a	98.89 ± 1.11 a	97.69 ± 1.88 a	100.00 ± 0.00 a	98.87
Micropylar	99.07 ± 0.93 a	100.00 ± 0.00 a	98.15 ± 1.85 a	97.96 ± 1.40 a	98.80
Auxin:					
2,4-D	98.89 ± 1.11 a	100.00 ± 0.00 a	96.30 ± 2.54 a	98.89 ± 1.11 a	98.52
Picloram	99.07 ± 0.93 a	98.89 ± 1.11 a	99.54 ± 1.11 a	99.07 ± 0.93 a	99.14
Dose:					
0 M	100.00 ± 0.00 a	100.00 ± 0.00 a	97.92 ± 2.08 a	100.00 ± 0.00 a	99.48
10 ⁻⁶ M	100.00 ± 0.00 a	100.00 ± 0.00 a	100.00 ± 0.00 a	97.92 ± 2.08 a	100.00
10 ⁻⁵ M	100.00 ± 0.00 a	100.00 ± 0.00 a	100.00 ± 0.00 a	100.00 ± 0.00 a	99.48
10 ⁻⁴ M	97.50 ± 2.50 a	100.00 ± 0.00 a	95.83 ± 4.17 a	100.00 ± 0.00 a	98.33
10 ⁻³ M	97.92 ± 2.08 a	97.50 ± 2.50 a	95.83 ± 4.17 a	97.50 ± 2.50 a	97.19
BA					
0 M	99.00 ± 1.00 a	99.00 ± 1.00 a	99.58 ± 0.42 a	99.00 ± 1.00 a	99.15
10 ⁻⁵ M	98.96 ± 1.04 a	100.00 ± 0.00 a	95.83 ± 2.85 a	98.96 ± 1.04 a	98.44

^aMeans standard error of 12 measurements

^bMeans in the same group followed by the same letter in a column except for the average are not significantly different at the 5% level (based on a comparison of possible combinations between the averages of treatments)

Table 2. Browning levels of coconut endosperm callus (0 : no browning, 1 : light brown, 2 : brown 3 : dark brown)

Source		9 weeks	16 weeks	21 weeks	26 weeks	31 weeks
Average		1.18 ± 0.03 ^z c	1.75 ± 0.03 ^y b	2.25 ± 0.02 a	2.23 ± 0.02 a	2.18 ± 0.03a
Position	Antipodal	1.29 ± 0.04 a	1.73 ± 0.05 a	2.27 ± 0.03 a	2.32 ± 0.03 a	2.30 ± 0.04 a
	Micropylar	1.07 ± 0.04 b	1.76 ± 0.04 a	2.24 ± 0.04 a	2.14 ± 0.04 b	2.05 ± 0.04 b
Auxin	2,4-D	1.15 ± 0.04 a	1.73 ± 0.05 a	2.22 ± 0.03 a	2.21 ± 0.04 a	2.19 ± 0.04 a
	Picloram	1.21 ± 0.04 a	1.77 ± 0.04 a	2.29 ± 0.03 a	2.24 ± 0.03 a	2.17 ± 0.04 a
Dose	0 M	1.60 ± 0.06 a	1.94 ± 0.06 b	2.29 ± 0.05 a	2.21 ± 0.04 b	2.40 ± 0.07 a
	10 ⁻⁶ M	0.93 ± 0.06 b	1.47 ± 0.08 c	1.96 ± 0.05 b	2.25 ± 0.04 b	2.21 ± 0.05 a
	10 ⁻⁵ M	1.03 ± 0.06 b	1.58 ± 0.07 c	2.39 ± 0.05 a	2.42 ± 0.05 a	2.24 ± 0.06 a
	10 ⁻⁴ M	0.95 ± 0.06 b	1.64 ± 0.07 c	2.27 ± 0.06 a	2.09 ± 0.07 b	2.17 ± 0.07 a
	10 ⁻³ M	1.53 ± 0.07 a	2.15 ± 0.07 a	2.36 ± 0.05 a	2.18 ± 0.06 b	1.94 ± 0.07 b
	BA					
Cytokini n	0 M		1.96 ± 0.04 a	2.28 ± 0.03 a	2.24 ± 0.03 a	2.20 ± 0.04 a
	10 ⁻⁵ M		1.43 ± 0.05 b	2.22 ± 0.04 a	2.21 ± 0.04 a	2.15 ± 0.04 a

^zMeans standard error of 12 measurements

^yMeans in the same group followed by the same letter in a column except for the average are not significantly different at the 5% level (based on a comparison of possible combinations between the averages of treatments)

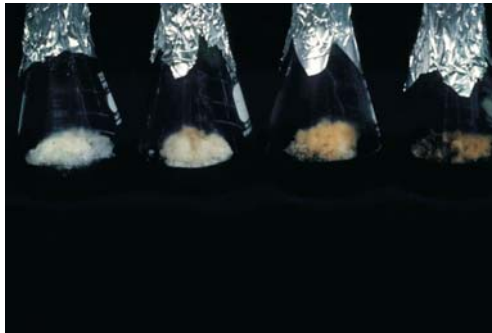


Figure 1. Browning of coconut endosperm callus at various levels i.e. 0, 1, 2, and 3 (from left to right)

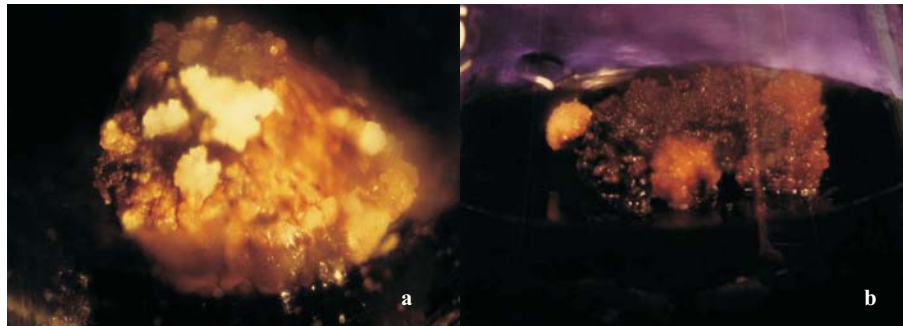


Figure 2. a). Varied colors of coconut endosperm callus, b). New yellowish white callus grown from the black callus of coconut endosperm

browning 2:medium browning, and 3: severe browning (Fig. 1). Tissue browning by all treatments was slight (score = 1.18) at 9 WOC. Tissue browning increased substantially (browning level = 1.75) after 16 WOC and reached maximal browning (browning level = 2.25) on 21 WOC. Thereafter browning decreased slightly on 26 WOC (browning level = 2.23) and 31 WOC (browning level = 2.18). Compton and Preece (1988) found that phenolic compounds exuded from excised explants were oxidized by peroxidases or polyphenolo-xidases, causing browning of both plant tissues and media. This might reduce growth or kill the tissues (Preece and Compton 1991). Severe browning of coconut endosperm still occurred even though in young explants which were not treated by disinfectants and incubated in the dark room. Whereas this condition was expected to prevent browning of *Geonoma gamiova* (Dias *et al.* 1994). The antipodal and micropylar tissues browning showed a steady increase at 21 WOC (Table 2). Statistical analysis showed that browning of antipodal tissues was more significant than in micropylar tissues at 9, 26, and 31 WOC. It could be due to antipodal tissues which were earlier formed (older) and thicker than micropylar tissues. These results support the findings of Murashige (1974) that the influence of

physiological age on explants responses was not significantly different at 16 and 21 WOC (Table 2). Explants thickness influenced browning, antipodal explants were thicker and showed more browning compared to micropylar explants. Similar results were obtained by Sugimura and Salvana (1989) in coconut inflorescence explants of 1 mm which had 32% browning compared to 11% browning in 0.5 mm thick coconut inflorescence explants.

Growth regulators and calli browning

The two types of auxins (2,4-D and Picloram) did not cause any significant difference in browning of endosperm tissues. Different results were reported by Fitch *et al.* (1983) that Picloram caused more browning than 2,4-D in *Saccharum spontaneum* cultures. The browning levels of tissues initially treated with 10^{-3} M auxin were significantly higher at 9 WOC, the highest at 16 WOC, and the lowest at 31 WOC than those of the other auxin concentrations (Table 2).

The browning levels of control were higher at 9 - 16 WOC than of those tissues treated with 10^{-6} M - 10^{-4} M auxins (Table 2). The tissues initially treated with 10^{-6} M auxin, showed significantly less browning than control or other auxin concentrations at 21 WOC. The tissues initially treated with 10^{-5} M auxin caused significantly more browning than of the other treatments at 26 WOC. This result disagreed with the findings of Pannetier and Buffard-Morel (1986), Karunaratne and Periyapperuma (1989), and Sugimura and Salvana (1989) that 2,4-D levels higher than 3×10^{-5} M caused more browning than lower concentrations in coconut explants. This result agreed with the findings of Phillips and Henshaw (1977) in *Acer pseudoplatanus* cell cultures. Addition of BA significantly decreased browning at 16 WOC. Similar result was reported by Herve *et al.* (2001) in *Eucalyptus gunnii* culture. However, those of addition did not affect significantly callus browning thereafter (Table 2).

Calli color changed progressively, from white to brown, to dark brown and to black (Fig. 2a). Then new yellowish white callus grew from the black callus and this sequence was repeated through many cycles (Fig. 2b). Severe browning did not inhibit the growth of endosperm cultures. Similar result was reported by Jones (1974) in oil palm and Ettinger and Preece (1985) in *Rhododendron* cultures. Coconut endosperm probably tolerated high level of 2,4-D or Picloram, even their browning were less than other treatments including control at 10^{-3} M on 31 WOC (Table 2), due to the presence of AC at 2.5 g l^{-1} in the medium and dark incubation. It agreed with the findings of Wang and Huang (1976), Fridborg *et al.* (1978), Tisserat (1979), Blake and Eeuwens (1982), Rao *et al.* (1987), Sugimura and Salvana (1989), and Krikorian (1994).

CONCLUSIONS

Callogenesis occurred on 98.83% of coconut endosperm explants. Calli browning increased significantly from 9 to 21 WOC but not thereafter. Browning of antipodal tissue-derived calli was more significant at 9, 26 and 31 WOC but not at 16 and 21 WOC compared to micropylar tissues. Treatments of 2,4-D and Picloram did not

affect calli browning. Auxins at 0 and 10^{-3} M produced significantly more callus browning than other doses at 9 and 16 WOC. Auxins of 10^{-6} M produced significantly less callus browning than other doses at 21 WOC. Calli browning was more intense at 10^{-5} M auxins at 26 WOC. Auxins of 10^{-3} M produced significantly less callus browning than other doses at 31 WOC. Addition of cytokinin BA produced significantly more callus browning at 16 WOC but not thereafter.

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