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Effects of Position and Plant Growth Regulators on Morphogenesis and Growth Rate of Coconut Endosperm *In Vitro*

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ABSTRACT

Endosperm position (antipodal and micropylar) and plant growth regulators were investigated for their effect on morphogenesis and the growth rate of coconut endosperm cultured *in vitro*. This experiment was conducted at the Tissue Culture Laboratory, Horticulture Department, University of Hawaii at Manoa, USA. Embryo companion and plant growth regulators were not necessary to induce callogenesis. Coconut endosperm explants started to form callus after 3 wk of culture. Callogenesis occurred in 98.83% of all treatments on 31 wk of culture. Endosperm positions and plant growth regulators did not affect significantly the growth rate of the endosperm culture. The concentration of 2,4-dichlorophenoxyacetic acid (2,4-D) and 4-amino-3,5,6-trichloropicolinic acid (picloram) at 1×10^{-3} M affected significantly the growth rate after 9 wk of culture but did not inhibit thereafter. The growth rate of the control was the quickest on 31 wk of culture. Addition of 6-benzylaminopurine (BAP) did not affect significantly the growth rate of the endosperm culture. The growth rate of tissues increased substantially but decreased on 31 wk of culture. Embryo-like structures appeared from antipodal position calli treated with 1×10^{-6} M picloram. This study has provided the first report of embryo-like structures occurring on coconut endosperm cultured *in vitro*.

Keywords: coconut endosperm *in vitro*, endosperm position, plant growth regulators, morphogenesis, growth rate

INTRODUCTION

Coconut (*Cocos nucifera* L.) is grown in over 80 tropical countries on approximately 11 million ha (Thangaraj and Muthuswami, 1990). Most plantations belong to small holders who grow them for domestic use. All parts of the plant are very useful for humankind; consequently, various names have been given to coconut to reflect its usefulness; for example, tree of life, tree of abundance and tree of heaven (Green, 1991). It is also used as an ornamental plant in regions such as Hawaii and Florida. The plant is traditionally propagated by seed (fruit) which is recalcitrant and has a short storage life. The plant is long-lived but has a very long juvenile phase. It is generally cross pollinated and very heterozygous. Vegetative propagation *in vitro* of superior coconut is promising as a way to increase the productivity or homogeneity of plants. Coconut endosperm provides large and uniform explant tissue for experimentation. Endosperm culture would be advantageous since explants come from mature selected plants.

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The endosperm position of coconut fruit has been correlated with physiological age, with the solid endosperm starting to form in the antipodal end of the coconut fruit and growing gradually to the micropylar end (Tammes and Whitehead, 1969). 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 and Periyapperuma, 1989; Karunaratne *et al*., 1991). However, coenocytic (free-nuclear) endosperm from very young fruits did not survive in culture because of the lack of starch (Srivastava, 1982). Young endosperm at the celliferous stage was responsive to culture in *Citrus grandis* (Wang and Chang, 1978). Endosperm of coconut that was 6–7 mth postanthesis was responsive to culture *in vitro* (Kumar *et al*., 1985).

Whole plants are autonomous with regards 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 plant growth regulators (Christianson and Warnick, 1983), Skoog and Miller (1957) found that the balance of auxin and cytokinin in the culture medium governed morphogenesis. Auxins induced callogenesis, adventitious plantlets and roots in palms (Paranjothy, 1986). Among auxins, 2,4-dichlorophenoxyacetic acid (2,4-D) was the most effective for coconut culture (Karunaratne and Periyapperuma, 1989). Another auxin with properties similar to 2,4-D is 4-amino-3,5,6-trichloropicolinic acid (picloram). Picloram has been successfully applied for callogenesis in date palm (Omar and Novak, 1990), for embryogenesis in pejibaje palm (Valverde *et al*., 1987), and maintained regenerative callus over a long time in sugarcane (Fitch *et al*., 1983). The presence of cytokinins, auxins and high doses of sucrose (0.2 M) stimulated the growth of coconut and date callus (Eeuwens, 1978). Srinivasan *et al*. (1985) successfully induced somatic embryos of Christmas palm with 5–50 $\times$ 10^{-5} M 2,4-D and 50 $\times$ 10^{-5} M 6-benzylaminopurine (BAP).

The current research aimed to observe the effects of endosperm position (antipodal and micropylar) and plant growth regulators (2,4-D, picloram and BAP) on the growth rate of coconut endosperm cultured *in vitro*.

MATERIALS AND METHODS

Plant material

Fruits of 7 month-old post-anthesis coconuts were picked up freshly from coconut palm trees. Two fruits of one bunch were taken from one coconut palm tree cv. Samoan Dwarf grown in Manoa, Oahu, Hawaii. Another two fruits of one bunch were taken from a palm tree grown in Moanalua, Oahu, Hawaii. Fruits were surface disinfested with 95% ethyl alcohol, punctured to remove liquid endosperm and cut longitudinally with a sterile knife. Solid cylindrical plugs (8 mm in diameter and 2–6 mm thick) were aseptically cored with a cork borer and scooped with a spoon. Explants were taken from either the micropylar (upper half of fruit with embryo) and the antipodal regions (bottom half of fruit) of the endosperm. These plugs were used as explants. Single explants were placed into test tubes with the cut surface in contact with the medium.

Culture medium and methods

The basal medium of Branton and Blake (1986) was modified by the addition of putrescine (10 $mg.L^{-1}$) and the substitution of phytagel (1.7 $g.L^{-1}$) for agar. This was supplemented with 2,4-D or picloram at 0, 1 $\times$ 10^{-6}, 1 $\times$ 10^{-5}, 1 $\times$ 10^{-4} or 1 $\times$ 10^{-3} M. BAP at 1 $\times$ 10^{-5} M was added on 16 and 21 wk of culture. Activated charcoal (AC) at 2.5 $g.L^{-1}$ was added to all media. The pH levels of media were adjusted to 5.7 before they were autoclaved. Each sample was poured into 2.5 $\times$ 15 cm test tubes (14 mL) or into 125 mL Erlenmeyer flasks (40 mL) and autoclaved at

121 °C and 1 kg.cm^{-2} pressure for 15 min. After autoclaving, the medium was shaken every 10 min before gelling in order to disperse the AC. The medium was stored for about 1 wk before use as recommended by Ebert and Taylor (1990), in order to equilibrate growth regulators in the AC. The cultures were transferred in media that had had the auxin concentration decreased to 1×10^{-6} M on 9 wk of culture, to 1×10^{-7} M on 16 wk of culture, to 1×10^{-8} M on 21 wk of culture and without plant growth regulators on 26 and 31 wk of culture.

Statistical analysis

The experiment was laid out in a complete randomized block design (CRBD) with each fruit as a block. Treatments were factorial combinations of endosperm region (micropylar and antipodal), auxin type (2,4-D and picloram), their concentrations and cytokinin BAP (plus or minus) with 12 replications. Data were analyzed with the general linear model (GLM), procedure of the SAS statistical software (SAS Institute Inc., Cary, NC, USA). The average percentage callus formation was computed after 31 wk of culture. Callus growth was determined by subtraction of the initial fresh weight from the final fresh weight divided by the original weight. Growth rate was computed from callus growth divided by the number of weeks in culture. These data were analyzed using the GLM and Contrast procedures in the SAS software.

RESULTS AND DISCUSSION

Morphogenesis of coconut endosperm

Callus grew predominantly on the uncut surface of the explants and also from the side (Figure 1a). Coconut endosperm explants started to form callus after 3 wk of culture, which was earlier than reported by Kumar *et al.* (1985) at 4 wk and much faster than immature leaves that took 4–6 mth to produce callus (Jetsy and Francis, 1992). Callus was initially yellow-white and firm. Eventually, the callus covered the entire explants (Figure 1b) and became pale and slightly friable after 21 wk. Average callogenesis occurred with 98.83% of the explants for all of the treatments, including the control on 31 wk of culture (Table 1). This result was higher than that of Kumar *et al.* (1985) who reported about 30%, Fisher and Tsai (1978) who reported only one endosperm explant and Bhalla-Sarin and Bagga (1983) who reported no explants producing callus. The control produced a very high percentage (99.48%) of callogenesis (Table 1). Different results were reported by Bhaskaran (1985) where a combination of a low dose of cytokinin with a high dose of auxin was necessary for callogenesis of coconut embryos and by Perera *et al*. (2007) who reported that the concentrations of 2,4-D and AC were critical for callogenesis in coconut ovary culture. The high percentages of callogenesis happened because coconut endosperm contains endogenous hormones that have been widely studied and are

Figure 1 (a) Coconut endosperm starting to form callus on the top and the side of explants; (b) Callus covering the entire explants; (c) Embryo-like structure on endosperm calli of coconut.

known to be successful at promoting callogenesis (Dick and Van Staden, 1982; George, 1993; Yong *et al*., 2009).

The presence of an embryo in the endosperm explants was clearly unnecessary for callus initiation in coconut endosperm. This result did not agree with Kumar *et al.* (1985) who recorded that callus initiation in coconut endosperm occurred with a companion to the embryo. Endosperm tissue from the micropylar and antipodal position did not differ significantly in growth rate (Table 1). This result did not conform with the work by Abraham and Mathew (1963) where the endosperm from the micropylar region was more meristematic than that from the antipodal region.

An embryo-like structure appeared from the antipodal position of endosperm calli that was initially treated with 1×10^{-6} M picloram on 30 wk of culture (Figure 1c). This indicated that antipodal tissue had potential to produce embryogenesis/organogenesis. This result was contrary to Blake (1990) who concluded that long-term culture of coconut callus could not be developed further. Morphogenesis occurring in long-term cultures has been widely studied in several plants (Fitch *et al*., 1983; White, 1984; Gladfelter and Phillips, 1987) and indicated that picloram showed potential for inducing embryogenesis/organogenesis in long-term culture in coconut endosperm calli.

Growth rate of coconut endosperm

The growth rate of endosperm cultures increased substantially from 9 to 26 wk and decreased on 31 wk of culture. The position of endosperm between micropylar and antipodal tissues in coconut fruit did not affect the growth rates of the endosperm culture (Table 1). This result did not conform with work by Abraham and Mathew (1963) and Tammes and Whitehead

Table 1 Growth rate (milligrams per week) of coconut endosperm tissues on the duration of culture.

Source	Time (wk)					Average % Callogenesis
	9	16	21	26	31	
Endosperm						98.83
Position						
Antipodal	60 ± 6 ^{a}	149 ± 12 ^{a}	274 ± 27 ^{a}	439 ± 47 ^{a}	182 ± 24 ^{a}	98.87
Micropylar	55 ± 5 ^{a}	139 ± 12 ^{a}	262 ± 25 ^{a}	421 ± 46 ^{a}	183 ± 26 ^{a}	98.80
Auxin:						
2,4-D	61 ± 6 ^{a}	148 ± 12 ^{a}	265 ± 26 ^{a}	437 ± 46 ^{a}	187 ± 22 ^{a}	98.52
Picloram	54 ± 5 ^{a}	140 ± 12 ^{a}	271 ± 27 ^{a}	423 ± 46 ^{a}	178 ± 28 ^{a}	99.14
Concentration						
0 M	71 ± 8 ^{a}	132 ± 18 ^{ab}	261 ± 56 ^{a}	436 ± 98 ^{a}	473 ± 111 ^{a}	99.48
1×10^{-6} M	71 ± 8 ^{a}	156 ± 16 ^{ab}	263 ± 38 ^{a}	414 ± 65 ^{a}	150 ± 28 ^{b}	100.00
1×10^{-5} M	60 ± 7 ^{a}	166 ± 15 ^{a}	273 ± 37 ^{a}	457 ± 74 ^{a}	167 ± 23 ^{b}	99.48
1×10^{-4} M	66 ± 10 ^{a}	149 ± 16 ^{ab}	294 ± 43 ^{a}	436 ± 74 ^{a}	99 ± 11 ^{b}	98.33
1×10^{-3} M	21 ± 3 ^{b}	111 ± 24 ^{b}	246 ± 40 ^{a}	411 ± 70 ^{a}	168 ± 21 ^{b}	97.19
Cytokinin						
BAP 0 M		138 ± 10 ^{a}	261 ± 24 ^{a}	444 ± 46 ^{a}	200 ± 30 ^{a}	99.15
1×10^{-5} M		151 ± 14 ^{a}	277 ± 28 ^{a}	413 ± 46 ^{a}	136 ± 13 ^{a}	98.44

Mean values in the same factor followed by the same letter in each column are not significantly different at 0.05 probability level.

(1969) that showed the micropylar region of coconut endosperm was more meristematic and produced earlier formation than the antipodal region. When endosperm explants of coconut fruit older than 7 mth were used, the micropylar region was still meristematic and produced callus but the antipodal region may not have still been meristematic and so could not produce callus.

There was no significant difference in the growth rate of tissues treated with 2,4-D compared to picloram (Table 1). Different results reported that picloram was faster than 2,4-D for callogenesis, embryo induction and the final yield of embryos in *Gasteria* and *Haworthia* (Beyl and Sharma, 1983). On the other hand, picloram was slower than 2,4-D for callogenesis in sugarcane (Fitch and Moore, 1990) and 2,4-D was the most effective compared to other auxins in coconut culture (Blake and Eeuwens, 1982; Pannetier and Buffard-Morel, 1986; Karunaratne and Periyapperuma, 1989). These results could have been due to differences in the plants or explants.

The concentrations of 2,4-D and picloram influenced the growth rate of coconut calli. Auxins (2,4-D and picloram) at 1×10^{-3} M significantly reduced the growth rate on 9 wk of culture but the growth rate difference was not significant after being transferred to 1×10^{-6} M on 21 wk of culture. All growth regulator concentrations had similar growth dynamics in that the growth rate increased until 26 wk. Thereafter, the growth rate of cultures initially exposed to growth regulators declined, while the growth rate of the control continued to increase (Table 1). These results differ from those of other studies (Reynolds and Murashige, 1979; Tisserat and DeMason, 1980; Blake and Eeuwens, 1982; Branton and Blake, 1983; Gupta *et al*., 1984; Sharma *et al*., 1984; Zaid and Tisserat, 1984; Kumar *et al*., 1985; Karunaratne and Periyapperuma, 1989; Sugimura and Salvana, 1989; Jesty and Francis, 1992; Shah *et al*., 2003; Neibaur *et al*., 2008; Jahangir and Nasir, 2010) that have shown that auxin at high concentrations (1×10^{-5}–1×10^{-3} M) were necessary for callus induction, especially on medium supplemented with 1–3 $g.L^{-1}$ AC. This was because coconut endosperm has some auxin activity, which is increased by autoclaving and provides highly active natural cytokinin (George, 1993). Addition of AC in the media may gradually absorb and release plant growth regulators (Thomas, 2008) resulting in an increase in the effective amount of total plant growth regulators, which then inhibit the growth rate of the coconut endosperm.

The addition of BAP did not affect significantly the growth rate of coconut endosperm culture (Table 1). Different results have been reported that BAP could increase greatly the fresh weight of coconut callus (Eeuwens, 1978; Kuruvinashetti and Iyer, 1980) and date palm callus (Eeuwens, 1978; Sharma *et al*., 1984). These results could have been due to differences in the plants or explants.

Companions for the coconut embryo and plant growth regulators were not needed to induce callogenesis in the coconut endosperm culture *in vitro*. The success in achieving very high callogenesis of the coconut endosperm culture *in vitro* is noteworthy and could have been due to genotype, physiological age, protecting the explants from chemical disinfectants and media formulation. The successful formation of embryo-like structures on the coconut endosperm may be have been due to the media formulation, especially the inclusion of picloram. Picloram has also been reported to maintain regenerative callus lines in long-term tissue culture of sugarcane (Fitch *et al*., 1983) and to induce somatic embryos of immature zygotic embryos on *Phyla nodiflora* (Ahmed *et al*., 2011).

CONCLUSION

Combinations of embryo and plant growth regulators were not essential in inducing callogenesis in coconut endosperm culture *in vitro*.

Coconut endosperm tissues could produce 98.83% calli on 31 wk of culture. Endosperm position and plant growth regulators did not significantly affect the growth rate of endosperm culture. A concentration of auxins at 1×10^{-3} M significantly inhibited growth rate until 9 wk of culture but did not inhibit thereafter. The growth rate of the control was the quickest at 31 wk of culture. BAP did not significantly affect the growth rate of coconut endosperm culture. Embryo-like structures were produced from the antipodal position treated with 1×10^{-6} M picloram. This study has provided the first report of embryo-like structures occurring on coconut endosperm culture.

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