SOME MISCELLANEOUS EXPERIMENTS WITH COCONUT EMBRYO CULTURE

By

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ABSTRACT

The growth and development of coconut embryo cultures can be manipulated by various methods, including the alteration of the carbon source in the basal medium. Shoot growth was favoured when glucose was used as the carbon source, but root growth was stimulated when sucrose was used at equimolar concentrations. Both fructose and glucose were found to, stimulate vitrification and mannitol was inert. BAP had no effect on the growth and development of coconut embryo cultures up to a level of 316 M. Ethylene and carbon dioxide built up in the culture flasks to biologically active concentrations but did not affect growth in the time period measured. The build-up of these gases could be alleviated through altering the sealing mechanisms of the culture flasks. In vitro, coconut embryos germinate faster in sealed culture flasks and it is assumed that this is to do with the gaseous composition of the headspace. Genotype of the embryo has perhaps the greatest influence on coconut growth and development in vitro, with significant differences being found in the growth rates of the 10 Pacific genotypes tested. This issue should be taken into account when recommendations are made on the use of a general protocal for the use of coconut embryo culture for the collection and conservation of germplasm.

INTRODUCTION

Cutter and Wilson (1954) first attempted coconut embryo culture to study the role of the 'coconut milk factor' in the growth and development of excised embryos. De Guzman and co-workers became interested in the method to propagate makapuno coconuts which fail to germinate in vivo. (Guzman and Rosario 1964; Guzman 1970; Balaga and Guzman 1970; Guzman et al, 1971). However, coconut embryo culture has wider applications than propagating makapuno coconuts, foremost of which is for the collection and exchange of germplasm (Assy Bah et al, 1987; Sossou et al 1987; Ashburner et al, 1996). Embryo culture also provides the potential for conserving germplasm both in the short-(Assy-Bah and Englemann 1993) and long-term through cryopreservation (Assy-Bah and Englemann 1992). Coconut embryo culture has also shown potential for in vitro selection for such traits as drought resistance (Karunaratene et al. 1991) and embryo cloning (Karunaratne and Periyapperuma 1989). The first coconut embryo protocol that described collection, culture and acclimatization was published by Assy Bah et al. (l 989)

Despite the long history of coconut embryo culture, a number of issues remain to be resolved (Ashbumer and James 1994). There is confusion in the literature regarding the sugar type and concentration of sugar which should be used in the basal media. Other areas that have received little attention are the effects of the in vitro gaseous environment and genotype on the growth of the embryo culture. Genotype has been shown to affect growth of embryo cultures in coconut palms. (Assy Bah 1986) but requires further study. This paper investigates and discusses these issues.

MATERIALS AND METHODS

In the present study, some coconut embryos were imported from the Cocoa and Coconut Research Institute, Kerevat, Papua New Guinea and prepared using a previously described method

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(Ashburner et al 1996). Others were derived from dehusked fruit purchased locally in Melbourne, Australia after container shipment from either Western Samoa or the Solomon Islands. Locally-purchased coconuts were prepared using the method described by Ashburner et al (l 99 1). Each separate experiment was conducted with embryos from the same source.

Unless otherwise stated, the germination medium consisted of MS macro and micro-elements (Murashige and Skoog 1962), vitamins (Morel and Wetmore 1951), 6% sucrose and 0.2% activated charcoal. Each embryo was placed into an autoclave 80 m long x 25 mm diameter cylindrical polycarbonate flask containing 10 ml of germination medium. The growth medium used in post-germination culture consisted of BMY_3, (Eeuwens 1978) with 0.2% activated charcoal, 0.8% agar (Difco-Bacto) and 4% sucrose. Growth medium in aliquots of either 10, 20 or 40 ml was dispensed into either 80 nun long x 25 mm diameter or 105 mm long x 42 mm diameter cylindrical polycarbonate flasks and then autoclaved for 20 min at 121^0C.

Cultures were grown at 30-3l^{0}C with a 16 h photoperiod provided by a cool-white fluorescent tubes (90 µmol $m^{-2}S^{-1}$) for 24-28 weeks. Cultures were transferred from the germination medium to 10 ml of growth medium at germination or at week 8 after culture initiation in the case of slow-germinating embryos. Germination was indicated by the emergence of the plumule from the embryo. Plantlets were transferred to fresh media at weeks 12 and 20 after culture initiation. At the same time, the volume of the medium was increased to 20 and 40 ml, respectively, to allow for growth of the embryos.

Unless otherwise stated, the following measurements were made on each plant at the completion of each experiment; shoot length, number of leaves, length of primary root, number of adventitious roots and number of secondary roots (Ashburner et al. 1993).

Effect of carbohydrate source

Various sugars were tested individually, or in combination, to determine their effect on the growth and development of coconut embryo cultures. Those tested singly were sucrose (6% w/v); glucose (3.2% w/v); fructose (3.2% w/v) and mannitol (32% w/v), which represented equimolar proportions. A mixture of sucrose, glucose, fructose and mannitol (11.4%, 9%, 6.5%, 10% w/v) was also tested and represented the ratios that occur naturally in mature liquid endosperm (Raghavan 1976). The treatments were applied to both germination and growth media. Each treatment had 15 replicates and the results were analyzed by analysis of variance. The binary data arising from the germination and mortality study were analyzed using a generalized linear model employing a logit lonk function.

Effect of BAP

The effect of N^6-benzylaminopurine (BAP) on the growth and development of cultured embryos was investigated by growing germinated coconut embryos on growth medium supplemented with BAP on a logarithmic concentration scale at either: 0, 10, 31.6, 100 or 316 µM. Each treatment contained 25 replicates and the results were analyzed by analysis of variance.

Effect of the gaseous environment

The effect of the gaseous environment on development of coconut embryos was investigated by germinating and growing embryos in flasks with screw-top lids but no additional sealing, of flasks sealed with either polyester (PE) or polyvinyl chloride 9 PVC) films that were sterilized by immersion in 70% (w/v) ethanol. A control treatment was measured and consisted of an unsealed vessel containing basal medium but no plant. The embryo dimensions, number of embryos germinating,

growth of plantlets and concentrations of ethylene (C_2H_4), carbon dioxide (CO_2) and oxygen (O_2) after 4 and 8 weeks of culture. C_2H_4 concentration was determined by gas chromatography (9C-3A, Shimadzu) using flame-ionization detection after passage through an alumina F1 (Alltech) column. Oxygen and CO_2 levels were also determined by gas chromatography (9C-3A, Shimadzu) using thermal conductivity detection after passage through a MP-l (Alltech) column. The design was fully randomized and each treatment was replicated 15 times. Embryo dimensions and gas concentrations were analyzed by analysis of variance and germination data were analyzed by a generalized linear model employing a logit link function.

Effect of genotype

The influence of genotype on growth of embryo cultures was investigated by testing various genotypes imported from two collections: Olomanu (Western Samoa), arid Lever Solomon Ltd. (Solomon Islands. The embryos were sterilized in 2% sodium hypochlorite (NaOCl) for 15 minutes arid imported in germination medium solidified with 0.8% agar. The genotypes tested from the Solomon Islands collection represented many different accessions from the south Pacific, including Kiribati Tall, Kiritamati Tall, Rangiroa Tall, Rennell Tall arid Solomon Islands Tall. The genotypes tested from Western Samoa were indigenous to the area arid were Niu'Afa, Niu Ati, Niu Samatao, Niu Vai and Samoan Tall.

RESULTS

Effect of carbohydrate source

Embryo survival arid growth was significantly affected by the type of sugar in the basal medium (Table 1). Shoot growth significantly ($p=0.05$) greater with fructose, glucose arid the mixture of sugars than with sucrose. Conversely, sucrose promoted significantly ($p=0.05$) greater root growth compared with other sugars. Those plants that grew on sucrose had a significantly lower mortality rate compared with other substrates. Death of embryo cultures appeared mostly to be a result of vitrification of the shoot.

The type of sugar also significantly ($p=0.025$) affected germination rate of embryos (Fig. 1). Sucrose arid glucose promoted faster germination than the mixture arid those in media containing fructose were the slowest to germinate. Embryos failed to germinate when mannitol was the primary carbon source. One hundred percent of the embryos germinated within 8 weeks on all sugar sources except mannitol which may be regarded as an inert sugar for stimulating growth mi coconut embryo cultures.

Effect of BAP nutrition

BAP up to 316 μM did not significantly affect the growth and development of coconut embryos in culture (Fig. 2).

Effect of the gaseous environment

The type of sealing on embryo culture flasks greatly affected the gaseous environment in vitro (Table 3). Those vessels that were sealed with PVC film accumulated significantly higher levels of C_2H_4, and CO_2 in the head space of the flasks dm those vessels with other types of sealing. (No detectable levels of CO_2 or C_2H_4, accumulated in the head space of flasks with no embryo cultures). Germination was significantly faster in treatments sealed with plastic; films as measured after 4 weeks (Fig. 2) but sealing had no significant effect on subsequent growth (data not shown). Embryos in

culture flasks which had developed a high C_2H_4, concentration showed brown surface discoloration where they were in contact with head-space gases.

Effect of genotype

The different Pacific tall accessions grew at significantly (p=0.05) different rates (Table 4). Rangiroa and Solomon Tall grew well in embryo culture, Rennell and Tarawa Talls were intermediate and Kiritamati Tall was the slowest growing accession.

The named coconut variants from Western Samoa also showed differences: mi performance in vitro (Table 5). Niu Ati had low growth rates, Niu Vai and Niu Sarnatao had moderate growth rates and Niu' Afa and Samoan Tall had high growth rates.

DISCUSSION

Coconut embryo cultures derive their carbon nutrition from the basal medium in the form of sugar. No consensus currently exists on which particular form is superior. In the present study, high root to shoot ratios were evident in embryo cultures using sucrose as a sugar source. This may result from an increase mi osmolarity due to catabolism of sucrose into fructose and glucose by the action of the activated charcoal during autoclaving (Druart and De Wulf 1993). Although glucose and fructose resulted in enhanced shoot growth, they should not be used sole carbon sources due to the risk of embryo death, particularly through vitrification. Mixed sugar basal media do not appear to give any improvement, in growth rate over single sugar media. Autoclaved fructose has been shown to be inhibitory to the mi vitro growth of some plants (Drew et al. 1994) and the catabolism of sucrose into fructose and glucose may have the same effect. Therefore use of filter-sterilised sucrose should be investigated mi future research.

In vitro manipulation of root and shoot growth may be achieved with the use of exogenous growth regulators. Previous experiments on coconut embryo cultures with growth regulators include the use of auxins (Sajise and Guzman 1972; Guzman and Manuel 1977; Tonian 1978; Ashburner et al 1993), cytokinins (Guzinan et al 1971; Fisher and Tsai 1978) and gibberellins; (Guzman 1970) but, with the exception of the systematic study of the NAA response (Ashburner at al 1993), no plant response patterns were evident. In the present study, BAP had no effect on growth of coconut embryo cultures, although high concentrations were used. This cytokinin also failed to produce any observed effect when applied to coconut palms in vivo (Fisher and Theobold 1989), indicating that coconuts may not be sensitive to exogenous applications; of BAP, even though it is used extensively in coconut tissue culture (Branton and Blake 1983; Verdeil et al 1994). Coconut seedlings are sensitive to another cytokinin, kinetin, which causes stunting when applied in vivo (Remison 1984). Future research on the use of cytokinins for manipulation of coconut embryo cultures should perhaps concentrate on kinetin rather than BAP, although the use of cytokinins is probably limited because of the species' monopodial growth habit.

Sealing tissue culture flasks is necessary to prevent, desiccation and infection of the cultures, but this sealing may result in altered growth as a result of accumulation of carbon dioxide and ethylene, depletion of oxygen (Jackson et, al 1991) and the accumulation of other volatile components whose effects: are unknown (Thomas and Murashige 1979). The accumulation of these gases were also evident in the present study, even in vessels without extra sealing (unsealed). Ethylene accumulation in vitro is important because it decreases shoot growth, root production and chlorophyll biosynthesis as well as causing vitrification (Biddington 1992), cyntokinins, sugar and inorganic nutrition, and stress in the plant (Pengelly and Su 1991) and through ethylene autocatalytic action (Yang and Hoffman 1984). The production of such high levels of ethylene may be due to stresses such

as poor basal medium aeration. The development of pneumatathodes in coconut embryo cultures is further evidence that the basal medium may need more substantial aeration. The accumulation of ethylene in coconut embryo culture flasks is of concern since it is present at biologically-active levels (Yang and Hoffman 1984) even in loosely sealed containers, especially since the activated charcoal in the basal medium would be adsorbing much of the ethylene produced. The phenolic oxidation evident by the browning of coconut embryos appeared to be a direct result of the action of the ethylene. Carbon dioxide accumulation should not be a concern since it may induce leaves with greater autotrophic capacity in vitro (Kozai 1990) and has been shown not to affect growth at these levels in other systems (Jackson et al 1991). Jackson et al (l991) also found that depletion of oxygen concentration at these levels also had negligible effects on growth.

One of the major causes of variability of growth on tissue culture is caused by the genotype of the plant being cultured and this is particularly true for coconut embryo cultures (Assy Bah 1986). The variability displayed both within and between ecotypes gives an indication of why it is difficult, if not impossible, to prescribe a standard embryo culture system for coconut palms. Why different ecotypes and individuals within an ecotype should behave differently mi culture is probably genetic in origin and too difficult to explain. Assy Bah (1986) found that a greater proportion of individuals from dwarf ecotypes developed shoot and root systems mi embryo culture than those of tall ecotypes (96.3% versus 64.3%), and tall proportions ranged from 33.3% in West African Tall to 93.3% in Rennell Tall, although this does not appear to be a clue to the degree of domestication displayed by the ecotype.

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Table 1: Effect of sugar source in the basal medium on average shoot and root growth and plant survival of coconut embryo cultures				
Sugar Type	Shoot length	Leaf number Per plant	Root length (mm)	Mortality
Fructose	120	5.3	16	80
Glucose	123	5.2	7	60
Mannitol	No germination			
Mixed	87	4.6	7	53
Sucrose	35	3.6	47	0
LSD (p=0.05)	41	0.9	22	-

Table 2: Effect of BAP concentration on average root and shoot Growth of coconut embryo cultures				
BAP Concentration (μM)	Shoot length	Leaf number Per plant	Root length (mm)	Number of roots Per plant
0	64	4.4	15	0.92
10	52	4	14	0.91
31.6	58	3.3	13	0.89
100	69	3.9	18	0.76
316	72	4.2	16	0.79
LSD (p=0.05)	28	0.8	8	0.22

Table 3. Effect of sealing culture flasks on the gaseous composition of The head space of coconut embryo cultures after 8 weeks of culture			
Sealing	C_2H_4 Concentration (ppm)	O_2 Concentration (5)	CO_2 Concentration (%)
Control	0	19.1	1
Unsealed	1.19	19.4	0.19
Polyester	1.38	18.9	0.41
Polyvinyl chloride	6.42	18.2	2.54
LSD (p=0.05)	3.6	1.2	1.06
Normal atmospheric CO_2 concentration was below the detection level of the assay methods.			

Table 4: Effect of genotype on average growth of coconut embryo cultures Derived from the Solomon Islands				
Ecotype	Shoot length (mm)	Leaf number Per plant	Root length (mm)	Secondary root Number per plant
Kiritamati Tall	42.5	4.5	16.5	3.9
Rangiroa Tall	81.3	6.4	25.6	5.9
Rennell Tall	51.1	5.1	12.9	2.4
Solomon Tall	71.5	6	26.4	7
Tarawa Tall	67.2	5.2	31.8	7
LSD (p=0.05)	21.1	1.1	18.4	5.6

Table 5: Effect of genotype on average growth of coconut embryo Cultures derived from Western Samoa				
Ecotype	Shoot length (mm)	Leaf number Per plant	Root length (mm)	Secondary root Number per plant
Niu' Afa	53.4	3.7	8.5	1.2
Niu Ati	26.7	2.9	4.4	0.4
Niu Samatao	43.2	3.3	4.1	1.9
Niu Vai	36.5	3	1.8	0.8
Samoan Tall	28	4.5	13.4	1
LSD (p=0.05)	19.4	1	6.1	1.5

Figure 1. Effect of sugar source in the basal medium on speed of germination of in vitro coconut embryos.

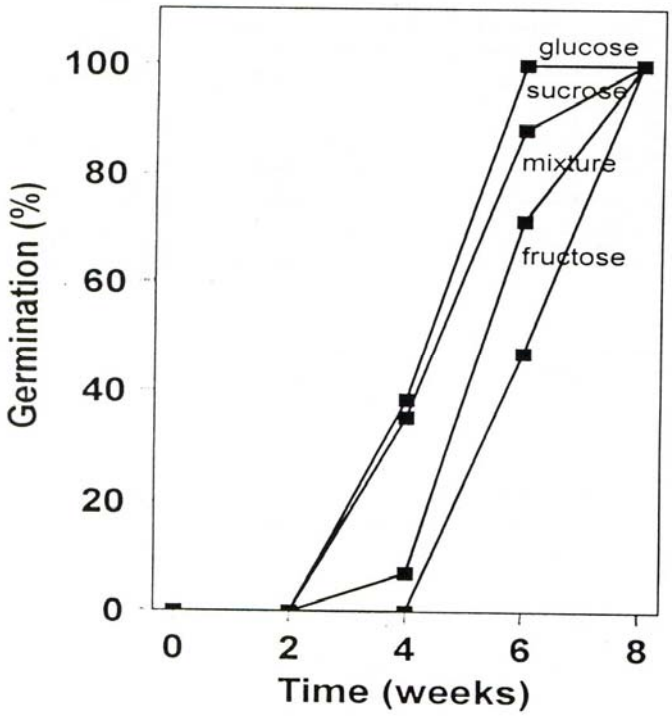

Figure 2. Effect of sealing the culture vessael woth polyvinyl chloride (PVC) or polyester (PE) filns on the rate of germination of coconut embryo cultures.