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Induction of Cell Division in Leaf Cells of Coconut Palm by Alteration of pH and its Correlation with Glyoxalase-I Activity

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ABSTRACT

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Cell division in suspension cultures obtained from leaf cells of coconut was influenced by pH of the culture media. A 3-fold increase in cell number was obtained at pH 7·0 compared to suspensions growing at pH 5·0. The pH of both cells and media changed after 48 h of growth. Internal cell pH showed a significant increase when cultures were grown at pH 7·0 and 8·0 and increased only slightly at pH 5·0 and 6·0. Glyoxalase-I activity of cells in suspension culture was found to be pH-dependent, showing maximum activity at pH 7·0. Glutathione, a co-enzyme for the substrate methylglyoxal for glyoxalase-I, produced a 2-fold increase in cell number at a concentration of 5×10^{-3} mol dm^{-3}. The polyamine, spermidine, promoted cell division maximally at a concentration of 10^{-6} mol dm^{-3}. Methylglyoxal-*bis* (guanylhydrazone), an inhibitor of spermidine biosynthesis, strongly inhibited cell division giving maximum inhibition at a concentration of 3×10^{-6} mol dm^{-3}. These results indicate a positive correlation between cell division and glyoxalase-I activity.

Key words *Cocos nucifera*, glyoxalase-I, pH, spermidine.

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INTRODUCTION

Coconut palm (*Cocos nucifera* L.) has remained largely recalcitrant so far as manipulation by *in vitro* cell culture techniques is concerned. Propagation of this tree in fields is often rendered difficult because of a root disease due to mycoplasma. Coconut is a major oil-yielding tree and also a supplier of many useful by-products. Thus, the development of techniques for clonal multiplication of high-yielding coconut varieties is extremely desirable. There are only a few reports of plantlet and callus formation in tissue culture (Eeuwens, 1978; Gupta, Kendurkar, Kulkarni, Shirgurkar, and Mascarenhas, 1984; Bhalla-Sarin, Bagga, Sopory, and Guha-Mukherjee, 1986; Branton and Blake, 1986), and there are no reports concerning the division and differentiation of vegetative tissues, which would be of most potential importance if elite trees are to be multiplied *in vitro*. Therefore, the aim of the present study was to achieve the first phase, i.e. induction of cell division in leaf cells of coconut.

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Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; GSH, glutathione; MGBG, methylglyoxal-*bis* (guanylhydrazone); PVP, polyvinylpyrrolidone; Spd, spermidine.

Our earlier studies on pea and *Datura* callus cultures indicated a clear correlation between cell division and glyoxalase-I activity (Ramaswamy, Pal, Guha-Mukherjee, and Sopory, 1983, 1984). In our previous report, pH was found to be a crucial factor in the control of cell division and glyoxalase-I in *Brassica* (Sethi, Basu, and Guha-Mukherjee, 1988).

Maintenance of cell pH at a certain value is characteristic of living systems of various levels of structural organization. A study of the effect of the pH of the medium on growth of cultivated higher plant cells is of great interest in this context (Butenko, Lipsky, Chernyak, and Arya, 1984). Investigations of this type may assist in the elucidation of processes occurring at the cellular level in the intact plant. From various animal studies there is increasing evidence that cytoplasmic pH plays an important role in the regulation of cellular metabolism, DNA synthesis and cell proliferation (Busa and Nuccitelli, 1984).

The present investigation was undertaken in order to gain a better understanding of the factors controlling cell division in coconut leaf suspension cultures. Cell suspensions are currently regarded as a convenient and useful system for such investigations (Gould, Bayliss, and Street, 1974; Bayliss, 1977; King, 1980). In the present study, the effect of pH on division of coconut leaf cells, changes in internal cell pH as well as medium pH as a result of division of cells and the correlation between pH and glyoxalase-I activity have been studied. In addition, the effect of glutathione, a co-enzyme for glyoxalase-I (Lohmann, 1932) and spermidine, a polyamine which is known to promote cell division (Palavan and Galston, 1982; Bagni, Fracassini, and Torrigiani, 1981), have been investigated.

MATERIALS AND METHODS

Plant material

One-year-old seedlings, leaves and nuts of the coconut variety West Coast Tall (WCT), procured from the Globe Nursery, Calcutta, were employed for this study. Leaf bases were used to obtain suspension cultures.

Isolation and culture of somatic cells

Y3 medium (Eeuwens, 1978) supplemented with sucrose (2%), kinetin (2 parts 10^{-6}), 2,4-D (2 parts 10^{-6}), casein hydrolysate (600 parts 10^{-6}) and PVP (200 parts 10^{-6}) was employed as the basal medium for growing somatic cells in suspension culture. The pH of the suspension culture medium was varied between 5·0 and 8·0 and was continuously monitored and adjusted in order to retain the original pH by the addition of appropriate buffer. An enzymatic method was employed to yield the cell suspension (Takebe, Otsuki, and Aoki, 1968). The leaf bases were cut into 1·0 cm^2 pieces, surface sterilized in 0·1% $HgCl_2$ for 10 min, rinsed with sterile water 3–4 times and placed in a 100 cm^3 Erlenmeyer flask containing 40 cm^3 of Y3 medium + 0·5% macerozyme R-10 and shaken for 6 h at 100 rev. min^{-1} in the dark at 25 ± 2 C. To remove the tissue fragments, the suspension was passed through autoclaved nylon mesh, centrifuged at 50 × *g* and the pellet resuspended in culture medium lacking macerozyme. After sustained growth had been obtained, suspension cultures were maintained by regular subculturing of a 1·0 cm^3 aliquot of cells (5×10^4) to 20 cm^3 culture medium in 100 cm^3 Erlenmeyer flasks. Cell number was determined before each subculturing. Spd, GSH and MGBG were filter-sterilized and added to the liquid media before starting a new subculture.

Cell number

Cell suspension growth was monitored by counting the cells using a haemocytometer under a light microscope. To improve the accuracy of cell counting, cell aggregates in the suspension culture were treated with chromic acid (Street, 1977). A known volume of suspension culture was placed on a haemocytometer slide, a mean of 10 field counts (100 × magnification) was obtained and the number of cells cm^{-3} was then calculated.

pH measurement

pH was measured using a Beckman model 70 pH meter. For measurement of internal cell pH, cells were pelleted by centrifuging 20 cm^3 of suspension culture at 4000 × *g* for 10 min, the pellet was

resuspended in 1·0 cm^3 double-distilled deionized H_2O and sonicated at 20 μ amplitude for 2 min in an ice bath, using a MSE sonicator. After sonication, the pH was measured as described above.

Enzyme assay

For the extraction and assay of the enzyme glyoxalase-I (*S*-lactoyl GSH methyl glyoxalase E.C.4.4.1.5), the procedure of Racker (1951) as modified by Ramaswamy *et al.* (1983) was followed. The extraction buffers used were citrate phosphate for pH 5·0 and 6·0, sodium phosphate for pH 7·0 and Hepes buffer for pH 8·0. The extract was centrifuged at 15 000 rev. min^{-1} with 50% $(NH_4)_2SO_4$ for 20 min in a Sorvall RC-5 centrifuge at 4 C. The supernatant was decanted and used for the assay.

The assay mixture contained, in a final volume of 1·0 cm^3, 0·5 cm^3 of 100 mol m^{-3} reaction buffer; 0·2 cm^3 of 3·5 mol m^{-3} methylglyoxal; 0·1 cm^3 of 1·7 mol m^{-3} GSH; 0·1 cm^3 of 16·0 mol m^{-3} $MgSO_4 . 7H_2O$ and 0·08 cm^3 H_2O. The mixture was incubated for 7 min before the addition of enzyme (0·02 cm^3). The formation of thioester, which shows maximum absorption at 240 nm, was measured using a Shimadzu UV-260 spectrophotometer. Protein was determined by the method of Bradford (1976). One enzyme unit (IU) is defined as the amount of enzyme catalysing the formation of 1·0 μmole of *S*-lactoyl-glutathione min^{-1} mg^{-1} protein in the standard assay system.

The data presented are the mean of at least three independent experimental values. Ten to 15 replicates were kept for control as well as for experimental sets.

GSH, MGBG, Spd and 2,4-D were obtained from the Sigma Chemical Company, U.S.A. All other chemicals were of analytical grade.

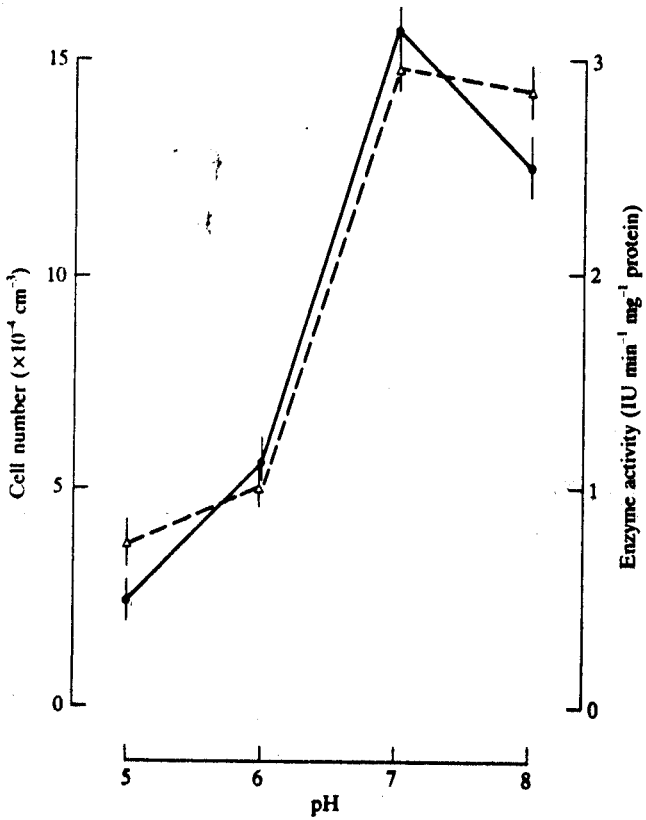

FIG. 1. Effect of pH on glyoxalase-I activity and cell number in suspension cultures 48 h after inoculation. The pH of the suspension culture medium was varied between 5·0 and 8·0 and was continuously monitored and adjusted so as to retain the original pH by adding appropriate buffer. (- - -▲- - -) Enzyme activity; (—◆—) cell number. Each experiment was repeated at least three times with a minimum of 10 replicates each. Bars at each point represent standard deviation.

RESULTS

pH optima for cell division in suspension cultures

Suspension cultures from leaf base cells were grown and maintained at pH 5·0, 6·0, 7·0 and 8·0. Total cell number had increased in all media 48 h after inoculation with a maximum value at pH 7·0, showing a more than 3-fold increase over the control (pH 5·0). Above pH 7·0 cell number decreased significantly (Fig. 1).

pH changes in cells and media

Since the pH of coconut leaf cells is between pH 5·0–6·0, it is apparent from Fig. 1, that unless there is a change in internal cell pH, there can be very little division of leaf cells. Therefore, the internal pH of cells grown in suspension culture over a range of pH was measured. As shown in Table 1, it was observed that the internal pH of cells in all media increased after 48 h. In media of pH 5·0–6·0, the internal pH of the cells increased by only 7%, whereas at pH 7·0 and 8·0, an increase of 17% and 26%, respectively, was registered. Thus, alteration of the internal pH of the cells depends upon the pH of the medium.

There was a simultaneous change in the pH of the culture medium after 48 h (Table 1). When the initial pH was 5·0 or 6·0, there was an increase in pH, whereas it decreased when the initial pH was 7·0 or 8·0.

pH values of different tissues in coconut

Since the pH of the suspension cultures appeared to affect cell division, a study was undertaken to assay the culture potential of different tissues of coconut. Table 2 shows the

TABLE 1. *Change in internal cell pH and pH of medium after 48 h of growth*

Original pH of culture medium	Internal pH of cell after 48 h of growth	pH of culture medium after 48 h of growth
5·0	6·0 ± 0·4	5·5 ± 0·2
6·0	6·1 ± 0·3	6·2 ± 0·4
7·0	6·5 ± 0·5	6·7 ± 0·3
8·0	7·0 ± 0·6	7·0 ± 0·5

pH was measured using a Beckman model 70 pH meter. Each experiment was repeated at least three times. Values are means and standard deviations of independent experimental values.

TABLE 2. *pH values for different tissues of* Cocos nucifera *L.*

Plant material	Source	pH
Green coconut water	3- to 6-month-old nuts	4·7 ± 0·4
Coconut roots	1-year-old seedlings	5·2 ± 0·5
Ripe coconut water	8- to 10-month-old nuts	5·5 ± 0·3
Coconut leaf bases	1-year-old seedlings	5·6 ± 0·4
Ripe coconut embryos	8- to 10-month-old nuts	6·5 ± 0·5
Ripe coconut endosperm	8- to 10-month-old nuts	7·2 ± 0·5

pH was measured using a Beckman model 70 pH meter.
Each pH value represents the mean of at least three independent measurements.

TABLE 3. *Effect of GSH, Spd and MGBG on cell number in suspension culture 48 h after inoculation*

Suspension cultures were raised from leaf bases and maintained in 100 cm^3 Erlenmeyer flasks containing 20 cm^3 of Y3 medium supplemented with sucrose (2%), kinetin (2 parts 10^{-6}), 2,4-D (2 parts 10^{-6}), casein hydrolysate (600 parts 10^{-6}) and PVP (200 parts 10^{-6}). GSH, Spd and MGBG were filter-sterilized and added before starting a subculture.

Treatment	Concentration (mol dm^{-3})	Cell number ($\times 10^{-4}$ cm^{-3})	% Increase
Control	—	16 ± 1·4	—
Glutathione	10^{-3}	16 ± 1·5	—
	3×10^{-3}	18 ± 1·1	13
	5×10^{-3}	28 ± 2·0	75
Spermidine	10^{-8}	38 ± 2·9	138
	10^{-6}	45 ± 3·2	181
	10^{-4}	30 ± 2·5	88
Methylglyoxal-*bis* (guanylhydrazone)	10^{-6}	5 ± 0·3	−69
	3×10^{-6}	5 ± 0·2	−69
	5×10^{-6}	3 ± 0·2	−81

% Increase in cell number was calculated as follows: (Experimental cell number − control cell number ÷ control cell number) × 100.

Each experiment was repeated at least three times with 10-15 replicates for each concentration. Values are means and standard deviations of independent experimental values.

pH of various tissues, which varied from highly acidic, as in green coconut water, to alkaline as in endosperm.

pH optima of glyoxalase-I in suspension cultures

Since glyoxalase-I activity has been shown to be related to cell division in *Datura* cells (Ramaswamy *et al.*, 1984), we determined whether glyoxalase-I activity is present in coconut cells. As shown in Fig. 1, it was found that the activity of the enzyme was very low at pH 5·0 and 6·0 but increased sharply by 3·5-fold over the control (pH 5·0) when the pH was raised to 7·0. With further increase in pH, the activity declined. GSH, which acts as a co-enzyme for the substrate for glyoxalase-I (Lohmann, 1932), was added at concentrations of 1×10^{-3}, 3×10^{-3} and 5×10^{-3} mol dm^{-3}, and yielded a 2-fold enhancement in cell division at a concentration of 5×10^{-3} mol dm^{-3} (Table 3).

Effect of Spd and MGBG on cell division

In order to increase cell division in coconut suspension cultures, spermidine, a polyamine which is known to promote cell division (Bagni *et al.*, 1981; Palavan and Galston, 1982), was added to the medium (pH 7·0) at concentrations of 10^{-8}, 10^{-6} and 10^{-4} mol dm^{-3}. After 48 h, a 3-fold increase in cell number was recorded at 10^{-6} mol dm^{-3} (Table 3). Methylglyoxal-*bis* (guanylhydrazone) (MGBG), which inhibits the enzyme *S*-adenosylmethionine decarboxylase (Slocum and Galston, 1985) resulting in the inhibition of Spd biosynthesis, when added to the culture medium, strongly inhibited cell division. Maximum inhibition was observed at a concentration of 3×10^{-6} mol dm^{-3} (Table 3).

DISCUSSION

The present investigation was undertaken to study the factors controlling division of somatic cells of coconut, which despite many attempts have remained recalcitrant. Since suspension cultures provide a relatively simple and convenient system for further study of the biochemical basis of cell division (Gould *et al.*, 1974; Bayliss, 1977; King, 1980), leaf cells of coconut were grown in suspension culture.

pH is known to be an important factor regulating cell division (Busa and Nuccitelli, 1984). Therefore suspension cultures were maintained over a range of pH values from pH 5·0 to 8·0 and cell division was recorded by counting cell numbers after 48 h. It was found that cell number increased by 3-fold at pH 7·0 as compared to pH 5·0. Thus it appeared that pH was a crucial factor controlling cell division, confirming an earlier report for *Brassica* suspension cultures from this laboratory (Sethi *et al.*, 1988).

In *Dictyostelium*, both protein synthesis and DNA replication are extremely pH-sensitive. An alkaline shift in intracellular pH occurs during the division cycle which peaks during S phase and mitosis (Aerts, Durston, and Moolenaar, 1985). *Physarum*, *Tetrahymena*, yeast and mouse lymphocytes also show a fairly close correlation between an increased pH and mitosis (Gerson and Burton, 1976; Gillies and Deamer, 1979; Hesketh, Moore, Morris, Taylor, Rogers, Smith, and Metcalfe, 1985; Ives and Daniel, 1987).

In the present study, the pH of the culture medium underwent a change 48 h after inoculation. There was an increase in pH when the initial pH was 5·0 or 6·0 and a decline in pH was observed when the initial pH was 7·0 or 8·0. In plant cell cultures, the transition to exponential growth appears to be associated with the development of an alkaline reaction in the culture medium (Wagner and Vogelmann, 1977). It is interesting that, to date, most investigators have employed medium at pH 6·0 in order to obtain cell division and differentiation in coconut. Our observation that maximum cell division occurs between pH 7·0 and 8·0 may be exploited in future studies. In addition, the results of this study suggest that plant parts which have a relatively high internal pH (embryos), if used as explants, may prove to be more amenable to culture. This hypothesis is substantiated by earlier reports (Bhalla-Sarin *et al.*, 1986) showing that coconut embryos could proliferate and differentiate *in vitro*.

It is of interest that different tissues of the coconut plant show variation in pH values. Embryonic tissue showed a higher pH value than the surrounding coconut water. It would thus appear that the highly acidic (pH 4·7) green coconut water may be inhibiting cell division and preventing precocious germination of the embryo under natural conditions.

Glyoxalase-I activity increased with an increase in pH. Maximum glyoxalase-I activity was obtained at pH 7·0, above which it showed a decline. Our studies indicate a correlation between cell division and glyoxalase-I activity. Glyoxalase-I probably influences cell division by acting on methylglyoxal, an inhibitor of cell division, as its substrate, thereby removing it from the system (Lohmann, 1932). GSH, which is a co-enzyme for glyoxalase-I (Lohmann, 1932) when added at a concentration of 5×10^{-3} mol dm^{-3}, also enhanced cell division by 2-fold. These results confirm earlier studies from our laboratory using *Brassica* cell suspensions (Sethi *et al.*, 1988).

The addition of 10^{-6} mol dm^{-3} Spd resulted in a 3-fold increase in cell number. MGBG, an inhibitor of Spd biosynthesis (Slocum and Galston, 1985), inhibited cell division markedly at a concentration of 3×10^{-6} mol dm^{-3}, indicating a crucial role for polyamines in cell division.

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