STUDIES ON THE GROWTH-FACTOR PRESENT IN COCONUT WATER *

By LOLITA B. SERRANO, PAZ T. MINA, LETICIA P. GOMEZ
BIENVENIDO C. SISON, JR.,[1] and AUGUSTO L. TENMATAY

ONE TEXT FIGURE

INTRODUCTION

Coconut water is the fluid endosperm that nourishes an immature embryo which later produces a spongy mass of cotyledony tissue that eventually fills the central cavity of the seed. This fluid was found to contain substances capable of supporting or stimulating growth, particularly growth by rapid and random division of otherwise mature cells of higher plants.(7)[2, 3]

One of the earliest workers who reported this phenomenon was Van Slyke, who in 1891, showed that coconut water is an excellent bacteriological culture medium. It was not until 1938, however, that this nutritional effect of coconut water attracted much attention. In this year, Blauvelt [4] determined the effect of coconut water on a number of organisms. He found that the addition of 10 to 25 per cent of noncooked, nonsterilized coconut water to ordinary nutrient media nearly doubled its cultural qualities in the case of *Stapylococcus aureus, Bacillus fecalis-alcaligenes*, and *Bacillus welchii*. Van Overbeck and co-workers [5] reported that cultivation *in vitro* of *Datura stramonium* embryo was stimulated by an admixture of coconut water and the usual medium.

Following this work of Van Overbeck, a number of investiga-

* This work was undertaken in the University of the Philippines and supported by the U. P. Natural Science Research Center under Grant No. 12 Series 1959—1960.

[1] Formerly Assistant Director, Industrial Research Center, National Institute of Science and Technology.

[2] Segretain, G., Compt. rend. **235** (1952) 1342—4.

[3] Nickell, L., Bot. Gaz. No. 2 **102** (1955) 2250228.

[4] Blauvelt, K., Jour. Lab. Clin. Med. **24** (1939) 420—423.

[5] Van Overbeck, J., et al. Amer. Jour. Bot. **29** (1942).

tions were undertaken which dealt with the correlation of the growth-promoting ability of coconut water with that of other known plant growth hormones, on a wide variety of plant and plant materials. It was found effective in promoting the growth of sunflower,(5) tobacco mosaic virus,(6) potato tissue culture,(2) *Pisum sativum* epicotyle,(4) and even orchid embryo.(3) On the other hand, Ultaman(9) and Ramakrishnan[6] in separate investigations found that coconut water was inhibitory to the growth of corn (maize) embryo. Hegarty(3) found a similar effect on the growth of cattleya. Wilson and Cutter(10) also reported that the liquid from the mature nuts are markedly inhibitory. According to them, inhibitory factors develop in the water and solid endosperm as the fruit matures and functions in initiating and maintaining the dormancy of the embryo.

Caplin and Steward(1) reported that heat-sterilized, filtered, water-clear coconut water obtained from mature nuts when added to an otherwise complete medium causes a marked increase in the growth *in vitro* of explants from carrots roots. Nickell[7] working on plant virus tumor tissues observed that the growth factor is stable to prolonged autoclaving and activity even increases on autoclaving. We suggested that possible presence of two growth factors, a thermolabile inhibitor and a thermostable growth stimulant.

In 1952, Shantz and Steward,(7) reported the isolation of three active substances from coconut water which stimulate the growth of carrot tissues. These substances were separated by differential solubility in various solvents and by partition chromatography on cellulose. Although active at low concentrations they failed to produce at any concentration a total response which approaches that given at the addition of whole coconut water. However, they also found that full response could be restored by adding an enzymatic hydrolyzate of casein and to a somewhat lesser extent by the addition of pure amino acid mixtures. Two of the substances have been identified as 1, 3-diphenylurea and indoleacetic arabinose.(7, 8)

MATERIALS AND GENERAL METHODS

Materials.—Young coconut water used was taken from fresh green nuts in which the solid endosperm is still in the gelatinuous,

[6] Ramakrishnan and Ramakrishnan, Curr. Sci. (9) **18** (1949) 242—344.
[7] Nickell, L., Bot. Gaz. No. 2 **102** (1955) 2250228.

jellylike stage. The coconut water from mature nuts was obtained from coconuts with hard meat or kernel. The coconut water was decanted from the nut, filtered off and the water-clear filtrate stored in the refrigerator until needed. All reagents used in the experiments are of C. P. grade.

GENERAL METHODS

a. *Microbiological.*—The microorganism used in this investigation is the mold *Aspergillus niger* NIST No. 11. This organism is of local origin obtained from Mr. Macario A. Palo, Director of the Biological Research Center of the National Institute of Science and Technology.

Stock cultures of the organism were maintained on solid nutrient Czapek's medium of the following composition:

	gm
$NaNO_3$	3.0
K_2HPO_4	1.0
$MgSO_4.7\ H_2O$	0.5
KCl	0.5
	gm
$FeSO_4.7\ H_2O$	0.01
Sucrose	30.0
Agar	15.0
Tap water	to make 1,000 ml

The mold was cultivated in 16- by 150-mm culture tubes on 6-ml plates of nutrient agar and incubated at 30°C. Stock transfers were made at 2-week intervals. The liquid medium on which the organism was grown has the same composition except that agar was omitted. After sterilization under 15 psig at 120°C each culture was aseptically innoculated with a spore suspension of the mold.

b. *Determination of growth-factor (G-F) activity.*—The growth-promoting activity of coconut water was determined by comparing the rate of growth of *Aspergillus niger* on standard Czapek's medium and on a medium containing coconut water.

Qualitative assay.—The analyses were carried out by substituting the liquid sample to be assayed (various concentrations of coconut water) in place of tap water in the standard Czapek's medium. The controls consist of unmodified Czapek's medium. The mold was grown on solid agar slants of the desired media.

The activity of the growth-substances is expressed as the observed rate of growth of the mold at 30°C for 40 hours after innoculation. On Table 1 is shown the results of a qualitative assay of the G-F.

TABLE 1.—*Qualitative assay of growth-factor activity in coconut water.*

Medium	Activity *
Regular Czapek's medium	x
Coconut water + Czapek's salts without sucrose	xxxx
Coconut water + Czapek's basic ingredients	xxxx
Coconut water + agar	xxx

* Number of x represents extent of growth of mold.

Quantitative assay.—Liquid media were used in these analyses. The innoculated media were incubated at 30°C for 48 hours after which time the mold mycelium were filtered on previously weighed filter papers and then dried at 40°C to constant weight. The difference in weight of the mycelium from the test sample and the control represent the G-F activity. In Fig. 1 is shown the linear relationships between the G-F (coconut water) concentration and weight of mold mycelium.

c. *Perevaporation.*—Fresh coconut water was evaporated to 1/10 to its original volume using flat-bottom shallow hard rubber trays and a 12-inch electric fan to produce air currents to enhance evaporation. The evaporation was performed at room temperature and 1 ml of toluene per liter of coconut water was added before evaporation to prevent microbial contamination.

d. *Dialysis.*—Cellophane dialyzing tubings used have a diameter of 1 5/8 inches. The ends of the dialyzing tubes were knotted twice to prevent leakage. One end was then atttached to a mechanical stirrer and suspended in 1-liter beakers containing distilled water. Stirring was continuous and the dialyzate changes after every 24 hours.

e. *Absorption.*—Pyrex glass tubes having an inside diameter of 5/8 inch and 24 inches long were used. One hundred ml of the solution were allowed to pass through 15 inches of column in 4 hours. Another method of absorption (batch method) used was by shaking the sample with 10 grams of absorbent continuously for 4 hours and then filtering off the absorbent.

RESULTS AND DISCUSSIONS

a. *The effect of coconut water on the growth of the mold.*—The growth of the mold on media containing pure coconut water (CW) from young and mature nuts were compared to that grown on standard Czapek's medium and also on media containing the Czapek's nutrients dissolved in the young and mature coconut water. The results are shown in Table 2. It may be seen that the mold grows on pure coconut water alone and in a somewhat

TABLE 2.—*Effect of coconut water on the growth of the mold.*

Medium	Activity (Weight of mat. mg)
Czapek's medium (control)	36.0
Czapek's + 100 ml young cw	142.7
Pure cw (young)	54.1
Czapek's + 100 ml mature cw	104.5
Pure cw (mature)	45.0

faster rate than on standard Czapek's medium. On the other hand the mold grows at a very much faster rate on a medium containing the basic Czapek's ingredients dissolved in coconut water. These results also confirm the data shown in Fig. 1 that the coconut water from young nuts seem to contain more growth-promoting substances or is more active than the water from mature nuts.

b. *Heating stability of the growth-factor.*—The sensitivity to heat of the G-F was studied using three kinds of samples: One was unheated coconut water taken directly from the young coconut water by means of a sterilized syringe and the other two were heated samples, one of which was boiled at atmospheric pressure and the other autoclaved at 15 psig both for 30 minutes. The results are shown in Table 3. It may be seen that the growth-promoting activity of the autoclaved coconut water is almost twice that of the unheated coconut water and also higher than that of the boiled coconut water. It may therefore be said that the G-F in coconut water is stable to heat treatment and as a matter of fact heating increases its growth-promoting activity.

c. *Acid and alkali stability of the growth-factor.*—The stability of the G-F to acid and base treatment was determined by mixing 100-ml coconut water samples with 20 ml each of 2.5 N HCl and

The activity of the growth-substances is expressed as the observed rate of growth of the mold at 30°C for 40 hours after innoculation. On Table 1 is shown the results of a qualitative assay of the G-F.

TABLE 1.—*Qualitative assay of growth-factor activity in coconut water.*

Medium	Activity *
Regular Czapek's medium	x
Coconut water + Czapek's salts without sucrose	xxxx
Coconut water + Czapek's basic ingredients	xxxx
Coconut water + agar	xxx

* Number of x represents extent of growth of mold.

Quantitative assay.—Liquid media were used in these analyses. The innoculated media were incubated at 30°C for 48 hours after which time the mold mycelium were filtered on previously weighed filter papers and then dried at 40°C to constant weight. The difference in weight of the mycelium from the test sample and the control represent the G-F activity. In Fig. 1 is shown the linear relationships between the G-F (coconut water) concentration and weight of mold mycelium.

c. *Perevaporation.*—Fresh coconut water was evaporated to 1/10 to its original volume using flat-bottom shallow hard rubber trays and a 12-inch electric fan to produce air currents to enhance evaporation. The evaporation was performed at room temperature and 1 ml of toluene per liter of coconut water was added before evaporation to prevent microbial contamination.

d. *Dialysis.*—Cellophane dialyzing tubings used have a diameter of 1 5/8 inches. The ends of the dialyzing tubes were knotted twice to prevent leakage. One end was then atttached to a mechanical stirrer and suspended in 1-liter beakers containing distilled water. Stirring was continuous and the dialyzate changes after every 24 hours.

e. *Absorption.*—Pyrex glass tubes having an inside diameter of 5/8 inch and 24 inches long were used. One hundred ml of the solution were allowed to pass through 15 inches of column in 4 hours. Another method of absorption (batch method) used was by shaking the sample with 10 grams of absorbent continuously for 4 hours and then filtering off the absorbent.

RESULTS AND DISCUSSIONS

a. *The effect of coconut water on the growth of the mold.*—The growth of the mold on media containing pure coconut water (CW) from young and mature nuts were compared to that grown on standard Czapek's medium and also on media containing the Czapek's nutrients dissolved in the young and mature coconut water. The results are shown in Table 2. It may be seen that the mold grows on pure coconut water alone and in a somewhat

TABLE 2.—*Effect of coconut water on the growth of the mold.*

Medium	Activity (Weight of mat. mg)
Czapek's medium (control)	36.0
Czapek's + 100 ml young cw	142.7
Pure cw (young)	54.1
Czapek's + 100 ml mature cw	104.5
Pure cw (mature)	45.0

faster rate than on standard Czapek's medium. On the other hand the mold grows at a very much faster rate on a medium containing the basic Czapek's ingredients dissolved in coconut water. These results also confirm the data shown in Fig. 1 that the coconut water from young nuts seem to contain more growth-promoting substances or is more active than the water from mature nuts.

b. *Heating stability of the growth-factor.*—The sensitivity to heat of the G-F was studied using three kinds of samples: One was unheated coconut water taken directly from the young coconut water by means of a sterilized syringe and the other two were heated samples, one of which was boiled at atmospheric pressure and the other autoclaved at 15 psig both for 30 minutes. The results are shown in Table 3. It may be seen that the growth-promoting activity of the autoclaved coconut water is almost twice that of the unheated coconut water and also higher than that of the boiled coconut water. It may therefore be said that the G-F in coconut water is stable to heat treatment and as a matter of fact heating increases its growth-promoting activity.

c. *Acid and alkali stability of the growth-factor.*—The stability of the G-F to acid and base treatment was determined by mixing 100-ml coconut water samples with 20 ml each of 2.5 N HCl and

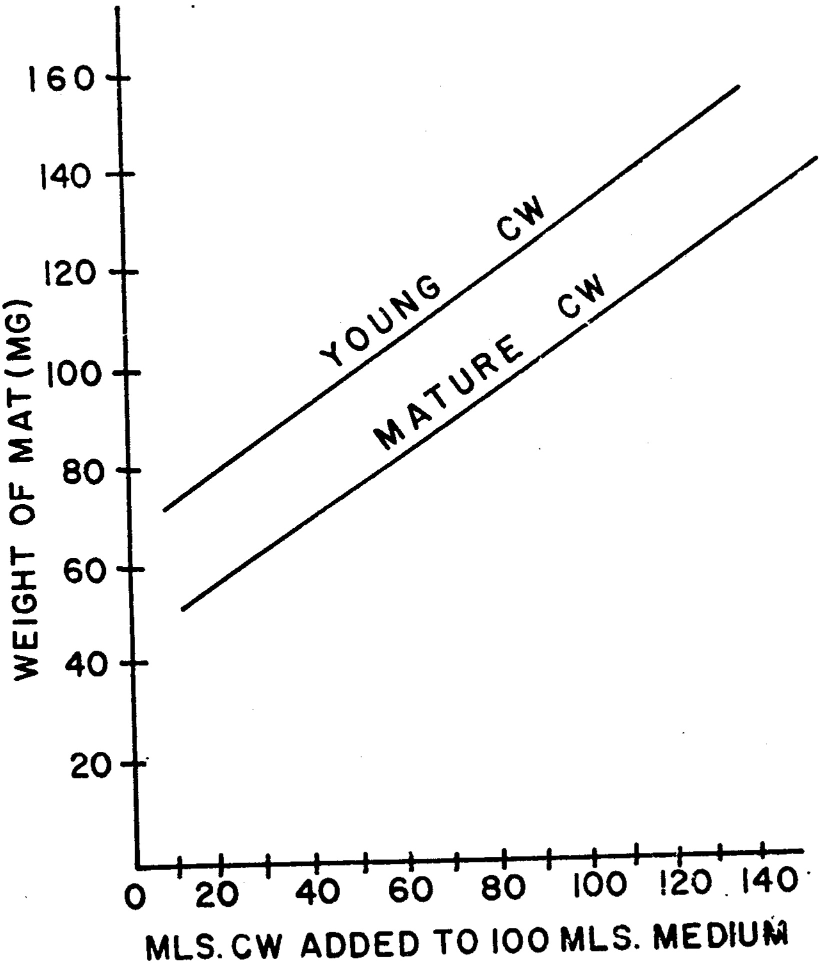

FIG. 1. Effect of coconut water (CW) concentration on the growth of the test mold.

TABLE 3.—*Heat stability of the growth factor.*

Medium	Activity (Weight of mat. mg)
Czapek's medium	27.2
Unheated coconut water	185.4
Boiled coconut water	309.7
Autoclaved coconut water	323.4

NaOH and the solution allowed to stand for 24 hours at room temperature. Separate samples were similarly treated but were autoclaved for 30 minutes at 150 psig and 120°C. These treated coconut water were then adjusted to pH 4.6 (the pH of the whole coconut water) concentrated to the original volume by perevaporation and then assayed. The results are shown in Table 4.

TABLE 4.—*Acid and alkali stability of the growth factor.*

Medium	Activity (Weight of mat. mg)
Coconut water control	142.7
Base-treated coconut water (autoclaved)	63.5
Acid-treated coconut water (autoclaved)	28.3
Base-treated coconut water (not autoclaved)	118.9
Acid-treated coconut water (not autoclaved)	164.8

These results indicate that the growth-promoting activity of coconut water which has been treated with acid and base and then autoclaved is destroyed. With heating, treatment with base causes a decrease in the growth-promoting activity while treatment with acid actually increases the activity.

d. *Dialyzability of the growth-factor.*—One hundred ml of concentrated coconut water (5 ×) was dialyzed with constant stirring The dialyzate was changed with fresh-distilled water after 48 hours and assayed. This was repeated four times and the results are shown in Table 5.

The highest growth-promoting activity is exhibited by the first dialyzate with the activity decreasing in the succeeding dialyzates. These results seem to indicate that the substances responsible for the growth-promoting activity of coconut water are small dialyzable molecules.

e. *Effect of fermentation on the growth-factor.*—Fresh coconut water was allowed to ferment at room temperature for 1 week.

TABLE 5.—*Dialysis of coconut water.*

Medium	Activity (Weight of mat. mg)
Czapek's medium (control)	80.7
Coconut water (control)	466.7
First dialyzate	228.3
Second dialyzate	153.8
Third dialyzate	143.6
Fourth dialyzate	112.9
Residue of dialysis	92.2

The pH of the fermented coconut water was readjusted to 4.7 (the original pH) and then assayed. The results are shown in Table 6. It may be seen that fermentation of the coconut water brings about a decrease in the G-F activity.

TABLE 6.—*Effect of fermentation on growth factor.*

Medium	Activity (Weight of mat. mg)
Czapek's medium (control)	60.20
Coconut water unfermented (control)	276.10
Fermented coconut water pH 3	154.50
Ether extract of fermented coconut water	84.60
Ethanol extract of fermented coconut water	139.90

f. *Behavior of the growth-factor with organic solvents, adsorbents, and salts of heavy metals.*—Equal volumes of various organic solvents were added to concentrated coconut water (10 × original concentration). The mixtures were continuously stirred for 12 hours and in the case of immiscible solvents, the two layers were separated and the aqueous layer assayed. The solvent was evaporated off from the organic solvent layer as in the case of miscible solvents and the residue dissolved in water and assayed.

It was found that the G-F is not precipitated by acetone or ethyl alcohol. It is also not soluble in most organic solvents such as benzene, carbon tetrachloride, chloroform, and toluene. Some components of the G-F were, however, found to be extractable by acetone and ether. The G-F components extract by ether affects (stimulates) mold sporulation more than the formation of mold mat.

The results of adsorption experiments using both the column and batch methods are shown in Table 7. The G-F is not ad-

TABLE 7.—*Adsorbability of the growth factor by various adsorbents.*

Adsorbent	Activity of effluent (wt. of mat. mg)	Decrease in activity (mg) (diff. in activity between control and effluent)
Zeolite	117.0	16.3
Cellulose acetate	128.3	7.0
Amberlite	115.5	17.8
Kaolin	131.9	1.4
Young coco water (control)	133.3	—
Czapek's medium (pH 5)	53.9	—

sorbed by the various adsorbents used under the conditions of these experiments.

Separate portions of concentrated coconut water (10 × original concentration) were treated with an excess of 10-per cent solution of lead acetate, mercuric acetate, and cupric acetate. The precipitates were filtered and suspended in water. The suspension treated with ammonium sulfide, the metal sulfide filtered off and the aqueous solution evaporated to dryness. The long, needlelike brownish crystals formed were dissolved in water and assayed. The results are shown in Table 8. The crystal obtained by lead and mercuric acetate precipitation were found to be

TABLE 8.—*Precipitation of the growth factor by salts of heavy metals.*

Medium	Activity (Weight of mat. mg)
Czapek's + young cw	139.3
Czapek's + Pb ppt.	93.3
Czapek's + Pb filtrate	209.2
Czapek's Cu ppt.	84.4
Czapek's Cu filtrate	184.6
Czapek's Hg ppt.	75.8
Czapek's Hg filtrate	122.6
Czapek's medium (pH 5)	53.8

similar to that obtain by ether extraction in the manner in which it stimulates the sporulation of the mold more than the mat formation.

SUMMARY

1. The activity of the supposed growth-factor on coconut water is measured in terms of the growth response of the mold culture of *Aspergillus niger*.

2. This growth-promoting factor is present in coconuts of varying stages of maturity. The growth-promoting activity decreases with the age of the nut.

3. The growth-promoting factor is stable to heat and the activity increases in heating.

4. The active substance or substances are resistant to cold acid but only slightly resistant to cold alkali. The growth-promoting activity is destroyed by hot acid and alkali.

5. The growth-promoting factor is dialyzable.

6. The growth-promoting activity is uneffected by the fermen-

tation of coconut water.

7. Under the condition of the experiments, the growth-factor is not adsorbable on any of the adsorbents employed; namely, activated charcoal, alumina, cellulose acetate, permutite, silicic acid, amberlite, and kaolin.

8. The growth-factor is insoluble in benzene, carbon tetrachloride, chloroform, and toluene. It can be extracted by ether and acetone and to a lesser extent by ethanol.

9. Certain components of the growth-factor can be precipitated by salts of lead and mercury.

REFERENCES

1. CAPLIN, S. M., and F. C. STEWARD. Effect of coconut milk on the growth of explants from carrot root. Science **108** (1948) 655–657.
2. CHAPMAN, H. W. Potato-tissue cultures. Amer. Potato Jour. **32** (1955) 207–210.
3. HEGATRY, C. P. Germination of orchid seed. Am. Orchid Soc. Bull. (1955) 457–64.
4. HOWELL, ROBERT. Phosphorus nutrition of soy-beans. Amer. Jour. Bot. **42** (1955) 360.
5. LEVINE, MICHALE. Response of fibrous roots of sunflower and tobacco tissue cultures to plant growth substances. Bot. Gaz. **112** (1951) 281–289.
6. SEGRETAIN, H. W. Action of nitrogenous substances on multiplication of tobacco mosaic virus in tissue culture. Pasteur Inst. Paris **18** (1954) 209.
7. SHANTZ, E. M., and F. C. STEWARD. Coconut milk factor: The growth-promoting substances in coconut milk. Jour. Amer. Chem. Soc. **74** (1952) 6133–35.
8. SHANTZ, E. M., and F. C. STEWARD. The identification of compound A from coconut milk. Jour. Amer. Chem. Soc. **77** (1955) 6351–3.
9. ULTAMAN, P. The effect of coconut water on the growth of immature embryos of corn (maize). Curr. Sci. (7) **18** (1949) 251–252
10. WILSON, K. S., and V. M. CUTTER, JR. The distribution of acid phosphatases during development of the fruit of Cocos nucifera. Amer. Jour. Bot. **39** (1952) 57–58.