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# FOOD PRODUCTION BY TISSUE CULTURE TECHNIQUES







Interim Report
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PROJECT: Food Research
7X84-13-002

TASK: Unconventional Means of Food Production

PHASE: Growth system studies of different varieties of plant tissue

# FOOD PRODUCTION BY TISSUE CULTURE TECHNIQUES

Interim Report

by

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# FOOD PRODUCTION BY TISSUE CULTURE TECHNIQUES

## SUMMARY

The feasibility of producing large amounts of plant tissue in submerged culture was established. Alteration of the proximate composition of a tissue by variation of the media appears possible. Glycerol appears to be a good source of organic carbon for carrot tissue in submerged culture. Casein hydrolysate is equivalent to coconut milk as a source of growth factors.

# FOOD PRODUCTION BY TISSUE CULTURE TECHNIQUES

## INTRODUCTION

Exploratory studies were initiated in 1960 to determine the usefulness of tissue culture techniques for food production and the feasibility of producing large amounts of undifferentiated plant tissue in submerged culture.

Cultures of normal edible tissues (potato, carrot, and the stems of grape, tomato, and tobacco) growing as callus tissue on agar, were obtained from the Plant Pathology Department of the University of Wisconsin. In addition, cultures of sweet potato, cress, rhubarb, broccoli, cabbage, and asparagus were isolated, grown, and maintained in this laboratory.

## PROCEDURE

The "tobacco" medium of Hildebrandt et al. (1) with 15 percent coconut milk and 0.1 ppm α-naphthaleneacetic acid (C medium), and with 6 ppm 2,4-dichlorophenoxyacetic acid (D medium) was used (Table 1). The medium without agar was used for submerged cultures, and with 0.6 percent agar for isolation and maintenance of callus tissue cultures.

To scale-up liquid cultures to large volume, we used Erlenmeyer flasks (300 ml), then Fernbach flasks (3 liters), and finally either 12-liter Florence flasks or 14-liter fermentor jars. The inoculum for the large flasks and jars was prepared by transferring a piece

Table 1

Composition of Basic Mineral Salt-Sucrose Medium or "Tobacco" Medium (1)

| Nutrient Ingredient | |
|---|---|
| $Na_2SO_4$ | 800.0 mg |
| $Ca(NO_3)_2 \cdot 4H_2O$ | 400.0 mg |
| $MgSO_4 \cdot 7H_2O$ | 180.0 mg |
| $KNO_3$ | 80.0 mg |
| KCl | 65.0 mg |
| $Na_2H_2PO_4 \cdot H_2O$ | 33.0 mg |
| $Fe_2(C_4H_4O_6)_3$ | 40.0 mg |
| $MnSO_4 \cdot 4H_2O$ | 4.5 mg |
| $ZnSO_4 \cdot 7H_2O$ | 6.0 mg |
| $H_3BO_3$ | 0.375 mg |
| KI | 3.0 mg |
| NAA | 0.1 mg |
| Ca Pantothenate | 2.5 mg |
| Glycine | 3.0 mg |
| Thiamine | 0.1 mg |
| Sucrose | 20.0 g |
| Agar | 6.0 g |
| Water | 1000.0 ml |

1. 6.0 mg 2. 4-dichlophenoxyacetic acid are added for "D Medium."

of tissue from the agar to 100 ml of liquid medium in a 300-ml Erlenmeyer flask. This culture was agitated on a rotary shaking machine. When sufficient growth occurred, the contents were transferred to a Fernbach flask containing one liter of medium. This culture was also agitated on a shaker. After another period of growth, 200 to 300 ml of the culture were inoculated into six liters of medium in a 12-liter flask, or 10 liters of medium in a fermentor jar on the Fermentor Drive Assembly. The tip of a 100-ml volumetric pipette was cut off before sterilization to facilitate this transfer of tissue.

To aerate and agitate the culture in the 12-liter flask during growth, sterile compressed air was continuously sparged into the culture (2). The culture in the fermentor was agitated by a variable speed stirrer, and air was metered and filtered before being introduced into the culture through a sparger.

The cultures on the shaking machine were incubated at 28° to 30°C. while the culture in the 12-liter flask was of necessity subjected to the variations of room temperature. The fermentor jar was partially submerged in a 28°C. thermostatically controlled water bath.

Table 2 shows yields of tissue obtained from some six liter cultures in 12-liter flasks aerated by sparging compressed air into the culture, and yields from 10-liter cultures grown on the fermentor assembly under controlled conditions of temperature, aeration, and agitation. All the cultures were grown in basic mineral salt-sucrose medium with 15 percent coconut milk.

Table 2

| Tissue | Days in Culture | Yield of Tissue (Dry Wt.) Total g | g/l |
|---|---|---|---|
| **6-Liter Cultures in 12-Liter Flasks** | | | |
| Carrot | 4 | 24.6 | 4.1 |
| Carrot | 5 | 27.5 | 4.6 |
| Carrot | 5 | 24.3 | 4.0 |
| Rose Stem | 5 | 22.9 | 3.8 |
| Carrot | 6 | 30.0 | 5.0 |
| Tomato Stem | 6 | 29.1 | 4.8 |
| Carrot | 6 | 28.6 | 4.7 |
| Carrot | 7 | 33.6 | 5.6 |
| **10-Liter Cultures in 14-Liter Fermentors** | | | |
| Carrot | 4 | 60.4 | 6.0 |
| Carrot | 5 | 75.1 | 7.5 |
| Carrot | 6 | 79.0 | 7.9 |
| Carrot | 6 | 82.6 | 8.2 |
| Sweet Potato | 7 | 80.0 | 8.0 |
| Sweet Potato | 7 | 71.4 | 7.1 |
| Sweet Potato | 8 | 76.6 | 7.6 |
| Carrot | 10 | 79.0 | 7.9 |
| Tomato Stem | 14 | 79.0 | 7.9 |

## CONTAMINATION

At first, contamination with mold and bacteria was a problem in the cultures growing in the large flasks and fermentors. Sorbic acid at various concentrations did not inhibit mold in the cultures but Mycostatin (25 units/ml) and/or Fungizone (2 mcgm/ml) were effective antifungal agents. Tylosin (20 ppm) or chlorotetracycline (25 mcgm/ml) controlled bacterial contamination. None of these antibiotics has produced any apparent toxic effect on the growth of tissue.

## GROWTH STIMULATORS

All of the tissues grew well on agar but when the cultures were transferred to the first liquid medium the lag period for most was long (three to four weeks or longer). Table 3 lists substances used in an attempt to stimulate or initiate growth of the tissues in the first liquid culture.

Coconut milk is used routinely in media by most investigators in the plant tissue culture field as a source of growth factors, some of which are yet unknown (13, 14).

Comparisons were made of batch cultures of tissues grown in the fermentors in 10 liters of broth with coconut milk, casein hydrolysate, adenine sulfate, yeast extract, dried skim milk or combinations of these substances. Table 4 gives the results.

Table 3

Growth Stimulators

| Additive | Replaced | Effect |
|---|---|---|
| 2% glucose (3, 4, 16) | 2% sucrose | none |
| 1% glucose + 1% levulose (5) | 2% sucrose | none |
| Urea (6, 7) | Nitrates | none |
| Kinetin (0.2 ppm) + Indoleacetic acid (8, 9) | | Stimulated callus-no effect liquid |
| Gibberellic acid (5 ppm) (10, 11) | | Inhibition |
| Algae hydrolysates | Coconut milk | none |
| 2% extract of corn seedlings | Coconut milk | Stimulated carrot and sweet potato |
| 1% cottonseed flour | Coconut milk | none |
| Adenine sulfate (9, 12) | | None in first liquid but stimulator in 10L cultures |

## COMPOSITION OF TISSUE

Proximate analyses were determined by the Analytical Chemistry Laboratory. The results showed a rather wide variation and were apparently dependent on the medium in which the tissue was grown and the conditions of aeration or agitation. Table 5 gives the results of the chemical analyses

Table 4

Yield of Tissue from 10 Liter Fermentor Cultures with Different Supplements in Basic Sucrose-Mineral Salt Medium

| Tissue | Supplement | Days in Culture | Yield of Tissue-Dry Wt. Total grams | g/l |
|---|---|---|---|---|
| Carrot | CM[1] (15%) | 6 | 82.6 | 8.26 |
| Carrot | CM (15%) | 6 | 79.0 | 7.90 |
| Carrot | CM (15%) | 7 | 74.5 | 7.45 |
| Carrot | CM (15%) | 10 | 79.0 | 7.90 |
| Carrot | CH[2] (0.2%) | 5 | 75.1 | 7.51 |
| Carrot | CH[2] (0.2%) | 6 | 80.0 | 8.00 |
| Carrot | CH (0.2%) | 6 | 77.2 | 7.72 |
| Carrot | CH (0.2%) | 6 | 78.6 | 7.86 |
| Carrot | CH (0.2%) + Ads[3] (40ppm) | 7 | 104.7 | 10.47 |
| Carrot | YE[4] (0.2%) | 6 | 81.0 | 8.10 |
| Carrot | SM[5] (0.2%) | 7 | 69.1 | 6.91 |
| Carrot | SM (0.5%) | 7 | 85.0 | 8.50 |
| Carrot | SM (1%) | 7 | 120.4 | 12.04 |
| Carrot | SM (1%) | 7 | 100.5 | 10.05 |
| Carrot | SM (1%) | 7 | 102.6 | 10.26 |
| Carrot | 11.25% CM;0.15% CH;0.75;SM[6] | 6 | 122.0 | 12.20 |
| Carrot | 5% CM;0.066% CH;0.33% SM[7] | 6 | 95.8 | 9.58 |
| Carrot | CH (0.2%) | 14 | 72.5 | 7.25 |
| Carrot | SM (1%) | 10 | 100.6 | 10.06 |
| Sweet Potato | CM (15%) | 7 | 80.0 | 8.00 |
| Sweet Potato | CM (15%) | 7 | 58.6 | 5.86 |
| Sweet Potato | CH (0.2%) | 7 | 71.4 | 7.14 |
| Sweet Potato | CM (15%) | 8 | 76.6 | 7.66 |
| Tomato Stem | YE (0.2%) | 14 | 87.4 | 8.74 |
| Tomato Stem | CH (0.2%) | 14 | 94.0 | 9.40 |

1. CM = Coconut milk
2. CH = Casein hydrolysate
3. Ads = Adenine sulfate
4. YE = Yeast extract
5. SM = Dried skim milk
6. Combination was equal to 3/4 the usual amounts of CM, CH, and SM.
7. Combination was equal to 1/3 the usual amounts of CM, CH, and SM.

Proximate Analyses Calculated on % Dry Weight Basis

| Tissue | Supplement | Apparatus | Aeration | Protein | Fat | Ash | Crude Fiber |
|---|---|---|---|---|---|---|---|
| Carrot | CM (15%) | F | Stirring only | 36.05 | 14.34 | 8.98 | 14.81 |
| Carrot | CM (15%) | F | Air only | 26.44 | 15.85 | 6.26 | 16.68 |
| Carrot | CM (15%) | F | Stirring + Air | 14.64 | 33.17 | 4.22 | 26.26 |
| Carrot | CM (15%) | F | Stirring + Air | 12.86 | 41.76 | 3.39 | 24.00 |
| Carrot | CM (15%) | 12LF | Air only | 31.59 | 6.53 | 8.66 | |
| Carrot | CM (15%) | 12LF | Air only | 28.53 | 7.42 | 6.18 | |
| Carrot | CM (15%) | 12LF | Air only | 23.17 | 6.46 | 6.61 | 22.62 |
| Carrot | CM (15%) | 12LF | Air only | 28.91 | 5.05 | 6.41 | 22.10 |
| Carrot | CM (15%) | 12LF | Air only | 23.16 | 13.00 | 6.55 | 26.00 |
| Carrot | CH (0.2%) | F | Stirring + Air | 17.32 | 26.67 | 3.20 | 25.09 |
| Carrot | CH (0.2%) + AdS | F | Stirring + Air | 15.74 | 30.63 | 2.09 | 24.46 |
| Carrot | YE (0.2%) | F | Stirring + Air | 16.02 | 29.80 | 2.82 | 27.50 |
| Carrot | SM (1%) | F | Stirring + Air | 30.10 | 9.00 | 3.96 | 17.87 |
| Sweet Potato | CM (15%) | F | Stirring + Air | 18.33 | 32.18 | 7.72 | 30.53 |
| Sweet Potato | CH (0.2%) | F | Stirring + Air | 23.26 | 27.51 | 1.73 | 29.42 |
| Sweet Potato | CM (15%) | F | Stirring + Air | 17.42 | 17.89 | 9.13 | 26.18 |
| Tomato Stem | YE (0.2%) | 12LF | Air only | 36.79 | 10.33 | 4.10 | 26.86 |
| Rose Stem | CM (15%) | 12LF | Air only | 18.32 | 8.33 | 12.01 | 11.18 |
| Grape Stem | CM (15%) | 12LF | Air only | 30.36 | 2.40 | 11.10 | 11.81 |

CM = Coconut milk; CH = Casein hydrolysate; SM = Skim milk; YE = Yeast extract
AdS = Adenine sulfate F - 14 liter fermentors with 10 liter cultures
12LF = 12 liter flasks with 6 liter cultures

TABLE 5

## CONTINUOUS CULTURE

Several experiments were attempted using continuous nutrient feed and automatic harvest. Ten liters of broth were inoculated with approximately 2.0 grams dry weight of carrot tissue, and the cultures were allowed to grow for three to four days before continuous feed-automatic harvest was begun. Table 6 shows the results of these studies.

Difficulties were encountered with the fermentor assembly when continuous culture studies were attempted. The tissue grew in compact masses between the baffles and the jar walls, and in the opening of the tubing. The air and nutrient inlet tubes and the harvest outlet tube became plugged with tissue. Calculations of growth rates from the continuous harvest did not reflect actual growth because of these static masses.

Table 6

Continuous Nutrient Feed-Automatic Harvest

| Days in Culture | Days on Continuous | Total Harvest (liters) | Yield of Tissue-Dry Wt. Total grams | g/l |
|---|---|---|---|---|
| 6 | 3 | 4.9 | 26.2 | 5.3 |
| 11 | 8 | 22.9 | 152.5 | 6.6 |
| 12 | 8 | 22.4 | 142.9 | 6.3 |
| 17 | 12 | 33.3 | 151.6 | 4.5 |

The above experiments were terminated for one or more of the following reasons:

1. Failure to the feed tube from nutrient reservoir.

2. Air inlet and nutrient tube clogged with tissue.

3. Harvest tubes were completely obstructed with solid masses of tissue.

4. Fermentor jar broke over week-end in water bath.

5. Pressure in fermentor blew cotton plugs out of orifices and tissue was pressured into water bath.

A semi-continuous culture was grown for 38 days. Ten liters of broth were inoculated with 2.0 grams dry weight of carrot tissue. After allowing five days for growth, three liters of the culture were harvested and three liters of fresh nutrient were added every two to three days. A total of 492 grams dry weight of tissue was produced from 54 liters of medium. This is equal to 9.1 grams/liter or approximately 13 grams dry weight of tissue per day.

It was thought that by using a larger inoculum the initial three to four days growth period could be eliminated, and the culture could be attached immediately to a slow nutrient feed and automatic harvest. It was hoped that by this procedure the large static masses of tissue would not develop. The actively growing liter carrot tissue cultures in four Fernbach flasks were aseptically pressured into five liters of CM broth in a fermentor jar. This amount of inoculum was calculated to be approximately 26 grams dry weight of tissue or 2.9 g/l in the total nine liters in the fermentor. The continuous nutrient feed and automatic harvest were started but within the first 24 hours the harvest line became plugged up and on the third day the nutrient inlet tube was obstructed with tissue growing under the head assembly and in the tube. These masses were aseptically blown out by air pressured through the

tubing. On the fifth and sixth days the inlet and outlet tubes were again obstructed. The culture was harvested on the eighth day. Table 7 shows the results.

Table 7

Harvest

| Day | | Volume-liters | Dry Weight-grams | g/l |
|---|---|---|---|---|
| 1 | | 2.0 | 8.5 | 4.1 |
| 2 | | 1.2 | 9.5 | 7.9 |
| 3 | | 0.7 | 5.6 | 8.1 |
| 4 | week- | feed & harvest | | |
| 5 | end | tubes plugged | | |
| 6 | | 0.5 | 3.6 | 7.2 |
| 7 | | 0.9 | 5.9 | 6.3 |
| 8 | culture harvested | 8.6 | 77.4 | 9.0 |
| Totals | | 13.9 | 110.5 | 7.9 |

## ALTERNATE CARBON SOURCES

Investigators have obtained large amounts of plant material by growing specific tissues of higher plants in liquid media for the production of relatively high cost metabolic products (17, 18, 19, 20). Sucrose has been used as a standard carbon source in many laboratories (15, 21, 22, 23). We, also, have been able to produce manyfold increase of tissue in large volume submerged cultures, but the practicability of

using agricultural products (e.g., sucrose, coconut milk, dried skim milk) in the media for food production is questionable.

Attempts were made to grow carrot tissue in media with non-edible chemical compounds substituted for the sucrose. Batch cultures were grown in the fermentors for six days in media containing glycerol as the carbon source (15, 24, 25). Cultures were run with sucrose + CM, sucrose + CH, and with glycerol + CM and glycerol + CH as controls. Table 8 shows the results of the experiments.

## SUMMARY AND CONCLUSIONS

We have established the feasibility of producing large amounts of plant tissue in submerged culture.

It may be possible to alter the proximate composition of the tissue produced for food by varying the chemical composition of the media.

From the limited experiments reported, glycerol seemed to be a good source of organic carbon for carrot tissue growing in submerged culture; casein hydrolysate was equal to coconut milk as a supplement for growth factors.

Substances were tested for their growth stimulating ability but few were found to be active in liquid cultures.

Table 8

Cultures in Fermentors with Glycerol as Carbon Source

| Medium Basic Mineral Salts + | Volume of Culture in Liters | Total Moles C in Culture | Moles C per liter | Yield of Tissue - Dry Wt. Total grams | g/l | g/Mole C |
|---|---|---|---|---|---|---|
| 2% sucrose + 0.2% CH | 10 | 7.0 | 0.7 | 78.6 | 7.8 | 11.1 |
| 2% sucrose + 15% CM* | 10 | 9.5 | 0.9 | 80.8 | 8.0 | 8.8 |
| 2% sucrose + 0.2% CH | 6 | 4.2 | 0.7 | 55.0 | 9.1 | 13.0 |
| 5% glycerol | 6 | 9.7 | 1.6 | 13.8 | 2.3 | 1.4 |
| 5% glycerol + 0.2% CH | 6 | 9.7 | 1.6 | 48.6 | 8.1 | 5.0 |
| 2.5% glycerol + 15% CM | 6 | 6.4 | 1.0 | 60.4 | 10.0 | 10.0 |
| 2.5% glycerol + 0.2% CH | 6 | 4.8 | 0.8 | 57.3 | 9.5 | 11.8 |

*Carbon content calculated on 5% total carbohydrate in coconut milk (Circular #549 USDA Proximate Composition of Foods)

CH = Casein hydrolysate

CM = Coconut Milk

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The feasibility of producing large amounts of plant tissue in submerged culture was established. Alteration of the proximate composition of a tissue by variation of the media appears possible.

Primary Field: Unconventional Foods
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