A Basic Study of Mulberry Cell Culture for Silkworm Feeding

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Callus was induced from the veins and young petioles of mulberry leaves on modified Linsmaier and Skoog medium with the addition of 3% sucrose, 10^{-8} M of auxin and 10^{-7} M of kinetin. The growth rates of both solid and liquid cultures were nearly the same. The best growth rate was 3~4 fold during a 2 week culture period in a jar-fermenter.

To use cultured mulberry cells instead of fresh leaves as the major ingredient in artificial silkworm food, cells needed to be cultured under light to produce a small amount of chlorophyll. IBA was the most effective auxin for obtaining slightly green cells while 2, 4-D was ineffective.

Silkworms fed artificial food which included these cultured mulberry cells, spun good cocoons.

For thousands of years the natural food source for the silkworm has been mulberry leaves. The disadvantages in using this material are; it is available for only a short period during a year, mulberry plantations require much space, and they have become more expensive with each passing year. Therefore, to simplify the breeding process several projects have been undertaken to develop an artificial nutrient for the silkworm.^{1,2)} One promising technique is to culture mulberry tissue on a large scale in an artificial medium and to use this material directly, or to mix it with other components, as food for the silkworm.

The aim of our investigation was to culture mulberry cells in an artificial medium and to determine whether the silkworm can be fed on this material. Culture condition for producing callus from mulberry cells and the results of feeding experiments with the silkworm are described here.

MATERIALS AND METHODS

Plant. Material was taken from a mulberry tree (*Morus bombycis* Koidz, Kairyo Ichinose) grown in an open field.

Medium. The medium used was that of Linsmaier and Skoog³⁾ containing a 3% carbon source, 10^{-4} M~ 10^{-6} M of auxin and 10^{-5} M~ 10^{-7} M of kinetin.

Callus induction. Calluses were induced from the veins, petioles, stems and roots of this plant on agar basal mediums with 10^{-5} M of auxin.

Cell line. Calluses were maintained on the above agar medium for about one year, after which they were transferred to a liquid culture to produce a fine suspension culture and to obtain basic information for large scale culture. The cell line in the suspension was subcultured by transferring one third of the cells to fresh medium of the same composition every 2 weeks. Cells were incubated at 30°C in the dark. Liquid suspension cultures were made on a 110 rpm reciprocal shaker.

Cultivation under light. Callus was also cultivated under light to improve its character. An incubator with fluorescent lamps was used in this experiment and the light intensities are shown in Table V.

Feeding experiment with silkworms. The breed of silkworm used was *Bombix mori*, "Hi 124×Shi 124." The breeding room was kept at 29°C with a humidity of 70~80%. Callus from mulberry tissue was used instead of green mulberry leaves as one of the components of the artificial food.

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Abbreviations: IAA, indole-3-acetic acid; 2, 4-D, 2, 4-dichlorophenoxyacetic acid; IBA, indole-3-butylic acid; NAA, α -naphthaleneacetic acid.

RESULTS

Effect of growth regulators and basal media on callus induction

We examined differences in the callus inducing ability of various tissues (the vein, petiole, stem and root) monthly from April to July.

The medium used was the Linsmaier and Skoog basal medium containing one of four auxins and kinetin. The four auxins were IAA, IBA, 2, 4-D and NAA. Table I shows

medium was more effective than any of the others tested.

Effect of growth regulators on the subculture of callus

We investigated the best concentration and combination of auxins and kinetin for producing callus during subculture. The auxins used were IAA, IBA, 2, 4-D and NAA each at concentrations of 10^{-4} M, 10^{-5} M or 10^{-6} M. Kinetin was also combined with each auxin at each of the three levels. Table III shows

TABLE I. EFFECT OF GROWTH REGULATORS ON CALLUS INDUCTION

In this table "LS" stands for the Linsmaier and Skoog medium. A, B, D, N stand for IAA, IBA, 2, 4-D and NAA respectively, and K for kinetin. Numbers 5 and 7 mean that the hormone concentrations were 10^{-5} M and 10^{-7} M.

Inoculation material	Weight of the inoculation material	Medium	Average weight of callus after 3 weeks of culture			
			April	May	June	July
Vein	0.04 g	LSA5K7	0.17 g	0.10 g	0.10 g	0.06 g
		LSB5K7	0.38	0.39	0.36	0.33
		LSD5K7	0.50	0.45	0.38	0.37
		LSN5K7	0.20	0.14	0.15	0.14
Petiole	0.03	LSA5K7	0.09	0.12	0.06	0.03
		LSB5K7	0.03	0.33	0.30	0.20
		LSD5K7	0.33	0.42	0.40	0.30
		LSN5K7	0.10	0.08	0.08	0.04
Stem	0.05	LSA5K7	0.07	0.09	0.11	0.04
		LSB5K7	0.13	0.31	0.14	0.04
		LSD5K7	0.15	0.30	0.20	0.05
		LSN5K7	0.10	0.06	0.06	0.04

the results of callus induction from mulberry tissue with combinations of each of the four auxins and kinetin. Callus was induced frequently on the medium containing 10^{-5} M of IBA or 2, 4-D, while IAA and NAA were less effective. The vein and petiole of the leaf were the most effective callus inducing tissues.

An agar medium, containing 10^{-5} M of IBA or 2, 4-D and 10^{-7} M of kinetin, produced an abundant growth of callus. We next determined the most effective basal medium, using the vein as the inoculation material.

Table II shows the result of callus formation in different basal media: (Linsmaier and Skoog³⁾; Murashige and Skoog⁴⁾; White⁵⁾; and Heller.⁶⁾)

In this experiment, the Linsmaier and Skoog

TABLE II. EFFECT OF BASAL MEDIA ON THE CALLUS INDUCTION

Basal medium	Growth regulator	Callus formation
Linsmaier & Skoog	B5K7	+++
Murashige & Skoog	B5K7	++
White	B5K7	+
Heller	B5K7	±
Linsmaier & Skoog	D5K7	++
Murashige & Skoog	D5K7	++
White	D5K7	±
Heller	D5K7	±

Growth of callus; +++, very excellent growth; ++, excellent growth; +, slight growth; ±, no growth.

that IBA was slightly better than 2, 4-D.

TABLE III. EFFECT OF GROWTH REGULATORS ON THE GROWTH OF THE SUBCULTURED CELLS

	Concentration of auxins	Callus growth concentration of kinetin			
		10^{-6} M	10^{-8} M	10^{-7} M	OM
IAA	10^{-4} M	+	+	+	+
	10^{-5} M	+	++	++	+
	10^{-6} M	+	+	+	+
IBA	10^{-4} M	++	+++	+++	++
	10^{-5} M	+++	+++	+++	++
	10^{-6} M	+	++	++	++
2, 4-D	10^{-4} M	+	++	++	++
	10^{-5} M	++	++	++	++
	10^{-6} M	+	++	++	++
NAA	10^{-4} M	++	++	++	++
	10^{-5} M	+	+	+	+
	10^{-6} M	±	±	±	±

Effect of nitrogen and carbon sources on the growth of callus cells during subculture

A few studies on the nitrogen and carbon sources for cell growth have been published.⁷⁻⁹⁾ These show that for the nitrogen sources in the Linsmaier and Skoog basal medium, the best ratio of the nitrate type (NO_3^-) to the ammonium type (NH_4^+) is 2:1. In contrast, in Heller's medium the nitrogen source used is the nitrate type only. As these basal media produced quite different callus growth, we examined the growth rate by varying the ratio of the nitrate and ammonium types of nitrogen sources, using KNO_3 and NH_4Cl . The total nitrogen amount was the same in all cases.

Figure 1 shows growth in a stationary culture. This indicates that to maintain good callus growth, both types of nitrogen source were necessary. The best growth was obtained when the ratio of the nitrogen sources was 2:1; the same ratio as that for the Linsmaier and Skoog medium.

We also examined the growth of callus using several different carbon sources; sucrose, glucose, and fructose in a 1~5% concentration range. Figure 2 shows the results of this experiment. As seen in this figure the best growth was obtained with a 3% sucrose addition.

FIG. 1. Effect of the Nitrate and Ammonium Types of Nitrogen in Basal Medium on the Growth of Cultured Cells.

1: 0 and 0: 1 indicate, respectively, nitrate and ammonium type only in the medium.

FIG. 2. Effect of Different Sugars as a Carbon Source on the Growth of Cultured Cells.

Effect of organic materials in the basal medium on the growth of callus cells during subculture

Having determined that the best medium for mulberry callus culture is the Linsmaier and Skoog basal medium with 3% sucrose, 10^{-6} M of IBA and 10^{-7} M of kinetin, we next examined the effect of adding organic materials to the basal medium. The organic materials used and the results of their addition are shown in Table IV. Yeast extract, ascorbic acid and an amino acid mixture were slightly effective in improving growth but no remarkable effects were observed.

TABLE IV. EFFECT OF ORGANIC MATERIALS IN THE BASIC MEDIUM ON THE GROWTH OF THE SUBCULTURED CELLS

	Materials	Concentration	Callus growth
Natural organic material	Yeast extract	0.2%	+++ (+)
	Malt extract	0.2%	++
	Milk casein	0.2%	+++
	Corn steep liquor	0.4%	±
	Ascorbic acid	0.01 mg/100 ml	+++
	Ca-pantothenic acid	0.1 mg/100 ml	++
Vitamin	Riboflavin	0.01 mg/100 ml	++
	P-amino benzoic acid	0.01 mg/100 ml	±
	Folic acid	0.01 mg/100 ml	++
	Biotin	0.001 mg/100 ml	++
	Vitamin B ₁₂	0.0001 mg/100 ml	±
Amino acid	Amino acid mixture (20 kinds)	0.2%	+++
	Each simple amino acid	0.01%	all ±
	Mulberry leaf extract ^{a)}	1% 5%	all ±

^{a)} Fresh green mulberry leaves were homogenized with the same weight of 1/30 M of P-Buffer (pH 6.5). Extracts were sterilized with a membrane filter.

Effect of physical conditions on the growth of callus cells during subculture

Physical conditions which influence the growth of cells, *i.e.* the initial pH of the medium and the culture temperature, were examined. The effect of the initial pH of the culture medium on growth is shown in Fig. 3. In this experiment the initial pH was measured before autoclaving. The figure shows that the growth of the cells did not vary in the range of pH 5~pH 8. The effective culture temperature was also examined. Results are shown in Fig. 4. In this experiment the best temperature was 30°C; above 35°C the callus turned brown and growth decreased.

Effect of light intensity and wave-length on the growth of callus cells during subculture

The light source was a Toshiba fluorescent lamp, type FLR-40S. Light intensities were measured just under the source. The light intensity in the culture vessel was reduced to approximately 50% that on the outside. In intact plants, the amount of chlorophyll is known to vary with the wavelength. Thus, the effect of wavelength on growth and the degree of green color were studied. Different wavelengths were examined by filtering white light through red, yellow, blue, green and violet

FIG. 3. The Effect of the Initial pH of Culture Medium on the Growth of Cultured Cells.

FIG. 4. The Effect of Culture Temperature on the Growth of Cells.

TABLE V. EFFECT OF LIGHT INTENSITY AND WAVE-LENGTH ON THE GROWTH OF THE SUBCULTURED CELLS

Cellophane filter and the wave-length		The growth and color of callus			
		9000 lux	4000 lux		
No cellophane		+++	Green	+++	Green
Violet cellophane	420 nm	+++	Green	+++	Green
Blue cellophane	480 nm	++	Green	++	Yellow
Yellow cellophane	580 nm	+	Brown	++	Yellow
Green— cellophane	550 nm	+	Brown	++	Yellow
Red cellophane	660 nm	+	Brown	+	Brown

cellophane. The wavelength range of the light transmitted through each cellophane filter, was measured with a spectrophotometer. After 20 days of culture, white light and white light passed through a violet cellophane filter promoted the greening of callus. The growth rate was not reduced when compared to a dark culture. The light intensity was in the range of 4000~9000 lux. In all the experiments, with pH, temperature and the addition of organic materials, almost the same results were obtained for both callus with IBA or 2, 4-D. But this occurred only when using callus that had been subcultured with IBA. The callus subcultured with 2, 4-D did not become green, as the results presented in Table V show.

Liquid culture

To obtain a large amount of callus for silkworm food, a jar-fermenter culture was necessary. A small scale liquid suspension culture was first examined. The liquid suspension culture was made in a 300 ml Erlenmeyer flask containing 50 ml of liquid medium at 30°C on a reciprocal shaker operated at 110 rpm for 2 weeks. This experiment showed that cells of the mulberry could be cultivated not only on an agar medium but also in a liquid suspension. The growth rates of both cultures were nearly the same. Next, jar-fermenter cultures were made, using an Iwashiyama MB-type fermenter. We examined the optimum conditions for aeration volume and agitation speed.

In these experiments 100 ml of a cell suspension that had been pre-cultured for 2 weeks was aseptically transferred to 1000 ml of

TABLE VI. EXAMINATION OF THE JAR-FERMENTER CULTURE CONDITION FOR MULBERRY CELLS

These figures show the fresh weight of cells after 2 weeks of jar-fermenter culture. Inoculation weight was about 45 g.

Aeration volume	Agitation speed			
	0 rpm	150 rpm	300 rpm	450 rpm
0	12 g	33 g		
3/10	110 g	166 g	120 g	
6/10		108 g	86 g	69 g
12/10		82 g	59 g	

fresh medium in a jar-fermenter. Results are shown in Table VI. The best conditions for this experiment were 300 ml of aeration/min. (about 1/3 V.V.M.) and an agitation speed of 150 rpm. Under these conditions, the best growth rate was 3~4 fold over a period of 2 weeks. This growth rate was higher than that for a suspension culture in a flask, which was due to the limited aeration volume in the flask culture.

Silkworm feeding experiment

Silkworms have recently been bred with artificial food containing corn, sucrose, corn-starch, green mulberry leaves and other elements.^{1,2)} The content of the green mulberry leaves is about 10~40% of the total weight. Artificial food without green mulberry leaves, however, is not sufficient for silkworms. Thus, we examined whether silkworms could be bred with artificial foods containing the two kinds of cultured cells instead of green mulberry leaves.

The components of the artificial food used are listed in Table VII.

TABLE VII. THE COMPONENTS OF ARTIFICIAL FOOD USED AS A STANDARD

Mulberry leaf power	20.0%
Defatted soybean	30.0%
Starch	10.0%
Sucrose	10.0%
Cellulose powder	19.0%
Ascorbic acid	2.0%
Salt mixture	1.0%
Citric acid	1.0%
Choline chloride	0.5%
β -Sitosterol	0.1%
Vitamin mixture	0.2%
Antiseptics ^{a)}	1.0%
Agar powder	5.0%
Distilled water	3 ml/g dry diet

^{a)} Sorbic acid: chloramphenicol.

In the feeding experiment, we examined 4 types of food:

- (1) One containing the components given in Table VII.
- (2) The same as (1) but the content of green mulberry leaves was 0%.
- (3) The same as (2) but with an addition of 20% mulberry cells that had been cultured in the dark.
- (4) The same as (2) but with an addition of 20% mulberry cells that had been cultured under light.

TABLE VIII. SILKWORM FEEDING TEST OF THE ARTIFICIAL FOOD CONTAINING MULBERRY CELLS CULTURED IN THE DARK AND UNDER THE LIGHT CONDITION

	(1)	(2)	(3)	(4)
Mulberry leaf powder	20%	0%	0%	0%
Culture cells				
(dark culture)	0%	0%	20%	0%
Cultured cells				
(light culture)	0%	0%	0%	20%
No. of larvae used	100	100	100	100
Cocooning worm				
percentage	90	74	86	91
Cocoon shell weight				
(mg)	405	320	360	407
Cocoon shell				
percentage	22	20	21	22
Cocoon weight (g)	1.84	1.60	1.74	1.85

Results are shown in Table VIII. We concluded that the cultured cells grown under

light provided an adequate silkworm food, and could be used as a substitute for mulberry leaves. The chlorophyll content of the cultured cells needed was only 1/100 that of native green mulberry leaves for good results, as measured by Arnon's method.¹⁰⁾

DISCUSSION

Previous papers have reported that cultured mulberry cells are very unstable and the growth rate is very poor^{11,12)} but we succeeded in keeping the growth rate at a high level by using IBA as the auxin and the veins of the leaf as the inoculation material. The growth rates were almost the same for dark and light cultures. The best growth rates, 3~4 fold over 2 weeks, was obtained in a jar-fermenter culture.

To use the cultured cells instead of green mulberry leaves in our artificial silkworm food, we had to culture the cells under light to obtain a small amount of chlorophyll. A chlorophyll content 1/100 that of native green leaves was adequate. No cells subcultured with 2,4-D turned green under any of the experimental conditions; only the subculture with IBA was effective.

Silkworms can be fed food containing the cells cultured in the dark but prefer the food containing cells cultured under light. This phenomena indicates that the green cultured cells may contain some important attractive factor(s) than the white cultured cells. Furthermore, Hayashiya and his co-workers^{13~15)} have been reported that there is a red fluorescent protein (RFP), having anti-viral activity for nuclear polyhedra virus, was produced from a protein from the mid-gut of the larvae, chlorophyll a and a basic protein of green leaves. From these points, to use green culture cells as the component substituted for the mulberry leaves, is more suitable than to use white cultured cells.

We observed that silkworms fed with the artificial food containing the green cultured cells instead of mulberry leaves, showed normal growth and spun cocoons as good as

spun by worms those fed with common artificial food containing mulberry leaf powder.

The use of an artificial food containing green cultured cells should make it possible to reduce breeding costs, as costly plantation space and the cost of harvesting leaves will be saved. It should also be possible to breed the silkworm throughout the year under aseptic conditions.

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