1966

Study of Totipotency in the Leaf Cells of the Carrot Plant

Ch'I Sun

STUDY OF TOTIPOTENCY IN THE LEAF CELLS OF THE CARROT PLANT

BY

CH'I SUN

A thesis submitted
in partial fulfillment of the requirements for the
degree Master of Science, Department of
Botany, South Dakota
State University

1966

STUDY OF TOTIPOTENCY IN THE LEAF

CELLS OF THE CARROT PLANT

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable as meeting the thesis requirements for this degree, but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Thesis Adviser

Head of the Major Department

ACKNOWLEDGEMENT

I wish to take this opportunity to express my appreciation to Dr. David J. Holden, and to thank him for his suggestion of the problem and guidance throughout the investigation. I also thank Dr. Gerald A. Myers and all the members in the Department for the wonderful time I had during this study.

CS

LIST OF ABBREVIATION

Abbreviation	Term
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
NAA	alpha-naphthaleneacetic acid
2,4-D	2,4-Dichlorophenoxyacetic acid
2,4,5-T	2,4,5-Trichlorophenoxyacetic acid
C.M.	coconut milk
G.	glycine
N.	nicotinic acid
P.	pyridoxine hydrochloride
T.	thiamine hydrochloride
S.S.	sucrose solution
H.S.	Hoagland solution
M.S.	Murasahage & Skoog solution
S.	solid media
L.	liquid media
r.	root tissue
l.	leaf tissue
r.c.	root callus tissue
l.c.	leaf callus tissue
l.s.c.	leaf single cells
c.	callus tissue
s.	single cells suspension

Abbreviation	Term
r.l.	root like structure
p.e.	proembryo
y.p.	young plantlet
n.	nodule like structure
+	magnitude of growth response
-	no response

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Studies of the totipotency of cells of various parts of carrot tissues and organ formation by the cultivated carrot leaf cells have been made in this investigation. It is believed that each matured cell, even though appearances differ from cell to cell, still contains the whole set of genes as in the zygote. The differences in appearance are due to the functional genes. Theoretically, if certain stimuli were applied to force these nonfunctional genes to become functional, all these cells may behave the same way as the zygote. This potential of the matured cells to go back to the zygote stage is called totipotency.

F. C. Steward has studied the totipotency of the carrot root (34, 35, 36, 37, 38, 39). He started by cultivating a piece of carrot root tissue in media containing plant stimuli. He found that the tissue develops through several different stages and ends with an entire carrot plant, indicating that the cells in the carrot root tissues have totipotency. A question which may come to mind is whether the totipotency exists only in root tissues or can be found any place in the plant. If totipotency can be any place in a plant it can be expected that cells from all other tissues should have the ability to grow into a new plant.

Plant tissue culture was introduced by Haberlandt in 1902. Gautheret, who has summarized the work up to 1958, emphasized the work of early pioneers as well as his own contributions.

Within the plant kingdom, both gymnosperms and angiosperms have been used for tissue culture. For instance, Ginkgo biloba and certain species of conifers have been cultivated by E. Ball (3, 4), but angiosperms have more often been used (2, 6, 9, 17, 20, 31, 33). In the angiosperms most work is done with dicotyledonous plants.

Not all plants are easily cultivated. For example, aroids are difficult to cultivate as was mentioned by W. Tulecke (43).

There have been attempts to cultivate many parts of plant tissues from different plants. Roots have been reported on by William J. Robbins (27); P. R. White (45); Addison Lee (17); S. M. Caplin and F. C. Steward (9), and by Nirmal Arora, F. Skoog and O. N. Allen (2). Stems have been reported on by F. Skoog (29, 30). Leaves have been reported on by D. M. Bonner (7); S. Kuraishi and F. S. Okumura (16) and by T. A. Steeves, H. P. Gabriel and M. W. Steeves (32). Pith tissues have been reported on by Klaus Patau, Nirmal K. Des and F. Skoog (10, 25). Excised buds have been reported on by G. Morel and R. H. Wetmore (20) and by Khalil H. Al-Talib and John G. Torrey (1). Tubers and cotyledons have been cultivated by F. C. Steward, Marion Mapes and Joan Smith (37). Ovaries and ovules have been reported on by J. P. Nitsch (22) and by Nirmala Maheshwari (18). The excised portions of the embryo have been reported on by Robert W. Howell and F. Skoog (12) and by J. van Overbeek, Marie E. Conklin, and A. F. Blakeslee (23, 24). Endosperm and meristem tissues have been reported on by Taiki Tamaoki and Arnold J. Ullstrup (40). Tumorous tissues, as well as normal tissue, have been reported on by

A. J. Riker and Alice E. Gutsch (26) and by Denes De Torok and Kenneth V. Thimann (42). All these cultures can either maintain the metabolic activity in the media and enlarge cells or regain the dividing activity and form callus tissues.

The cultivating media usually contain three major components; carbohydrate, minerals and organic supplements. Sucrose is the best carbohydrate source for the tissue media. A concentration around 2% will give the best result.

There are several different formulae for minerals supplied in the media. Tukey's salts have been used for cultivating young embryos of carrot by J. van Overbeek, Marie E. Conklin and A. F. Blakeslee (23) and for young embryos of Ginkgo biloba by E. Ball (4). Knop's salts have been used for cultivating the young embryos of Ginkgo biloba (4). Modified Pfieffer's solution has been used by William J. Robbins (27). T. A. Steeves, et al., (32) used Heller's solution for leaf tissues cultivation. Nirmala Maheshwari (18) used Nitsch's medium for ovules. Morel and Wetmore (20) used Gautheret's medium for the cultivation of monocotyledons. However, modified White's medium is the most popular of all the formulae used.

Three groups of organic supplements are usually added to the medium for the purpose of stimulating cell metabolism. The first group consists of auxins and kinetin. The second group consists of vitamins, amino acids, organic acids and nucleic acids. The third group consists of unpurified organic compounds extracted from plant tissues or from other organisms.

IAA and 2,4-D are effective for the cultivating of carrot root and are reported on by S. M. Caplin and F. C. Steward (9). Beyond these, NAA, IBA and MCPA also have the stimulating effect shown by Khalil H. Al-Talib and John G. Torrey (1). Kinetin and gibberellic acid were used by Nirmala Maheshwari, T. E. Fox, Carlos Miller (18, 11) and many others.

Glycine was the first amino acid discovered to be effective in the tissue culture of tomato roots by P. R. White (45). Asparagine and proline are effective in radish leaf cultivation and arginine is effective in tobacco tissue as reported on by D. M. Bonner (7). Alanine, aspartic acid and glutamic acid are effective in sunflower culture reported on by L. G. Nickel and P. R. Burkholder (21), cysteine hydrochloride by S. C. Wiggans (44) glutamine, asparagine by A. C. Braum (8). Arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophane, tyrosine and valine as synthetic mixture applied to tissue culture by Robert Sandstedt and F. Skoog (28) and applied individually was also worked out in the same paper.

Purines, pyrimidines and their derivatives with vitamins have been used by D. M. Bonner (7), F. Skoog and Tsui (29), J. van Overbeek (24), Morel and Wetmore (20), S. C. Wiggans (44), Nirmala Maheshwari (18), Robert W. Howell and F. Skoog (12) and F. Bertossi, N. Bagni, G. Moruzzi and C. M. Caldarera (5).

As far as the unpurified organic compounds are concerned, coconut milk is believed to have powerful stimulating factors for young

carrot embryos and many other tissues (19, 23, 33, 34). Yeast extract has been used by Robbins (27), Khalil H. Al-Talib, John Torrey (1), and S. C. Wiggans (44). Casein hydrolysate has been reported on by A. J. Riker and Alice E. Gutsch (26), and Khalil H. Al-Talib and John G. Torrey (1). Malt extract, leaf extract, onion juice, watermelon juice and corn steep water have been reported on by Blakeslee and S. Satina (6), S. C. Wiggans (44), F. C. Steward and S. M. Caplin (33), Khalil H. Al-Talib and John G. Torrey (1), J. E. Fox and Carlos Miller (11). The effectiveness of these organic compounds to stimulate tissue growth is either by stimulating the cells metabolism or by cell division. As Bonner pointed out (7), arginine is very active in Nicotiana sylvestris but inactive in Raphanus. Coconut milk is believed to be a very powerful source of growth factors to most plants but is inactive in potato tuber growth (33, 34). IAA, coconut milk, malt extract, and casein hydrolysate are unsuccessful for Cocklebur tissue growth (11). In 1954 Wiggans (44) reported that yeast extract has no effect on the carrot plant on both callus and root formation.

An organic supplement may benefit one plant's growth, but that does not mean it would benefit all others. As a matter of fact, sometimes it will act as an inhibitor or toxin to other plants. For instance, glutamic acid is beneficial to tobacco tissue (28) but toxic to radish leaf growth (7). The inhibition by casein hydrolysate, peptone and yeast extract has been reported on by A. J. Riker and Alice E. Gutsch (26). The inhibition by coconut milk and yeast extract on bud expansion of Pseudotsuga taxifolia was pointed out by

Al-Talib and Torrey (1). All auxins which will inhibit the leaf expansion were also reported on the same paper.

The concentration of the organic supplement is very critical even in the same plant. It will stimulate the tissue growth in one concentration but act as an inhibitor in another. J. E. Fox and Carlos Miller (11) reported that kinetin will stimulate tissue growth at a lower concentration but inhibit it at a higher concentration. Khalil H. Al-Talib and John G. Torrey (1) reported that casein hydrolysate and watermelon juice have the same function.

The activity of the organic supplements may be enhanced by adding them as a mixture rather than separately. Coconut milk and adenine have a greater effect when added together (12). Robert Sandstedt and F. Skoog (28) also reported that some amino acids when added singly will cause inhibition.

That the behavior of cultivated tissue to the same medium may not be the same during different cultivating periods was reported on by Syono Kunihiko (13, 14, 15).

The requirement of organic compounds is not very specific in some plants. S. C. Wiggans (44) pointed out that IAA can be substituted for cysteine hydrochloride for callus formation of the carrot plant. Robert Sandstedt and F. Skoog (28) found that aspartic acid and glutamic acid can be substituted for the entire amino acid mixture and have the same result. He also found that if these two amino acids were omitted singly from the mixture there resulted no very serious loss of growth promoting activity. F. Bertossi, et al., (5) reported

that the ribonucleoprotein, spermine, can take the place of IAA in causing cellular division.

Organic compounds which can stimulate tissue growth have a common feature, as shown by Kenneth V. Thimann (41) to be a 6-membered ring and one acetic side chain. The nature of the ring is important. As a rule, a 5-membered ring does not confer the activity. The need of unsaturation in the ring is characteristic and cannot be replaced by unsaturation in the side chain. In 1950 F. Skoog (31) reported that auxin has functions in carbohydrate metabolism. In 1954 S. C. Wiggans (44) stated that IAA is effective in root and callus formation, NAA is effective in callus formation but causes little root formation and IBA has little effect on either.

MATERIAL AND METHODS

Roots of carrots, obtained from the market, and leaves of young carrot seedlings were used in this investigation. Seeds obtained from Northrup King & Co., were germinated in our laboratory and transplanted in vermiculite.

Two kinds of mineral solutions, Hoagland; Murasahage and Skoog, were used as shown in tables one and two.

Table One. Hoagland Salts

Chemical	Hoagland Stock Solution	Culture Medium
$Ca(NO_3)_2$	820.00 ppm	41.0000 ppm
KNO_3	505.00 ppm	25.2500 ppm
$MgSO_4$	240.00 ppm	12.0000 ppm
KH_2PO_4	135.00 ppm	6.7500 ppm
Fe	5.00 ppm	0.2500 ppm
Micrountrients		
H_3BO_3	5.72 ppm	0.2860 ppm
$MnCl_2 \cdot 4 H_2O$	3.62 ppm	0.1810 ppm
$ZnCl_2$	0.22 ppm	0.0110 ppm
$CuCl_2 \cdot H_2O$	0.10 ppm	0.0050 ppm
$Na_2MoO_4 \cdot 2 H_2O$	0.05 ppm	0.0025 ppm
	Total	85.7355 ppm

Two percent sucrose added to the media supplied the energy required for tissues metabolism.

Table Two. Murasahage & Skoog Salts

Chemical	Murasahage & Skoog Stock Solution	Culture Medium
NH_4NO_3	1650.000 ppm	82.50000 ppm
KNO_3	1900.000 ppm	95.00000 ppm
$CaCl_2.2\ H_2O$	440.000 ppm	22.00000 ppm
$MgSO_4.\ 7\ H_2O$	370.000 ppm	18.50000 ppm
KH_2PO_4	170.000 ppm	8.50000 ppm
NaFe.EDTA	50.000 ppm	2.50000 ppm
H_3BO_3	6.200 ppm	0.31000 ppm
$MnSO_4.\ 4\ H_2O$	22.300 ppm	1.11500 ppm
$ZnSO_4.\ 4\ H_2O$	8.600 ppm	0.43000 ppm
KI	0.830 ppm	0.04150 ppm
$Na_2MoO_4.4H_2O$	0.250 ppm	0.01250 ppm
$CuSO_4\ .\ 5\ H_2O$	0.025 ppm	0.00125 ppm
$CoCl_2\ .\ 6\ H_2O$	0.025 ppm	0.00125 ppm
	Total	230.91150 ppm

After coconut milk, obtained from market, was boiled and filtered, 10% was added to the media by volume.

Glycine at 300 mg, 10 mg thiamine hydrochloride, 10 mg pyridoxine hydrochloride and 10 mg nicotinic acid were dissolved in 100 ml. of distilled water to give a stock solution 100 times more concentrated than the vitamins and glycine required in tissue culture.

One hundred ppm 2,4-D, 2,4,5-T, and 1000 ppm IAA solutions were prepared separately as stock solutions.

Both solid and liquid media were used in this investigation. All the media used through this investigation are shown in table three. The liquid media are the same as the solid except that agar has been eliminated.

The primary culture used the media 1, 2, 3, 4, 7, 9, 10 and 11. The primary sub-culture used the media 1, 2, 3, and 4. The second and third sub-culture used media 1, 3, 5, 6, and 7.

For the solid culture 125 ml. Erlenmeyer flasks were used. Each flask contained 40 ml. of 0.8% agar media. For the liquid culture, both 125 ml. Erlenmeyer flask and T-tubes were used. Media were adjusted to pH approximately 5.6 before being poured into the flask and sterilized.

Microscopic studies of the callus colonies and leaf cells have been made both on fresh and preserved material.

The embryos and rooting callus colonies were transferred into solid media, and after a definite shoot was formed the plantlet was transferred to normal plant growth substrate.

Table Three. Composition of Various Media Used

Media No.	H.S.	M.S.	C.M.	2,4-D.	2,4,5-T.	IAA.	G.	N.	P.	T.	S.S.
1	H.S.	-	C.M.	2,4-D.	-	-	G.	N.	P.	T.	S.S.
2	H.S.	-	-	2,4-D.	-	-	G.	N.	P.	T.	S.S.
3	H.S.	-	C.M.	-	-	-	G.	N.	P.	T.	S.S.
4	H.S.	-	C.M.	2,4-D.	-	-	-	-	-	-	S.S.
5	H.S.	-	-	-	-	-	G.	N.	P.	T.	S.S.
6	H.S.	-	C.M.	-	-	IAA	G.	N.	P.	T.	S.S.
7	H.S.	-	C.M.	2,4-D.	-	IAA	G.	N.	P.	T.	S.S.
8	H.S.	-	-	2,4-D.	-	IAA	G.	N.	P.	T.	S.S.
9	H.S.	-	C.M.	-	2,4,5-T.	IAA	G.	N.	P.	T.	S.S.
10	-	M.S.	C.M.	-	2,4,5-T.	-	G.	N.	P.	T.	S.S.
11	-	M.S.	C.M.	2,4-D.	-	-	G.	N.	P.	T.	S.S.

H.S. = 5% Hoagland solution

M.S. = 5% Murasahage & Skoog solution

C.M. = 10% coconut milk

2,4-D. = 2,4, Dichlorophenoxyacetic acid, 2 ppm

2,4,5-T. = 2,4,5 Trichlorophenoxyacetic acid, 2 ppm

IAA = Indoleacetic acid, 50 ppm

G. = Glycine 30 ppm

N. = Nicotinic acid, 1 ppm

P. = Pyridoxine hydrochloride, 1 ppm

T. = Thiamine hydrochloride, 1 ppm

S.S. = 2% sucrose solution

RESULTS

The growth response and formation of callus tissue from cultures of leaf and root tissues of carrots are shown in table four, five and six. Fig. 1 shows tissues used in this investigation.

Leaves grew or formed callus tissue better in the liquid media (Fig. 2 and Fig. 3), but did poorly, and eventually died in solid media in the primary culture. Root tissues formed callus tissues only in the solid media (Fig. 4).

The sub-culture of single cell suspension can be maintained only on the liquid media, but sub-culture of callus tissues can be grown on solid media as well as in liquid media (Fig. 5, and Fig. 6).

The increase of density of the cell suspension together with the increase of the size and the population of small callus colonies is greatly enhanced by the presence of 2,4-D or aggregated along the flask wall but no further differentiation occurs.

Heart-shaped embryo clusters were found in aggregated cell masses in the medium in which 2,4-D had been completely used up or where it had been eliminated from the medium (Fig. 7, and Fig. 8). Isolated embryos were found in the same medium. All types of embryos will grow equally well in the same medium. Several isolated embryos in different stages of development are shown in Fig. 9. The transfer of those embryos into a new medium is not necessary until they become young plantlets in which definite roots and shoots appear (Fig. 10).

Callus from root culture rarely form isolated cells no matter whether it is transferred into a solid or a liquid media. It can

Table Four. Growth Response of the Explant on Various Media Used.

Media No.		Tissues Used	Expansion Cells	Callus Formation
1	S*	r*	-	+++
1	L	r	-	-
1	S	l	-	-
1	L	l		+++
2	S	r	-	+
2	L	l	-	+
3	S	r	-	-
3	L	l	-	-
4	S	r	-	+
4	L	l	-	+
7	L	l	++	+++
7	S	r	-	+++
9	S	r	-	++
9	L	l	-	++
10	S	r	-	++
10	L	l	-	++
11	S	r	-	+++
11	L	l	-	+++

* S = Solid (0.8% agar) media

L = liquid media

r = root tissue

l = leaf tissue

+ = magnitude of growth response

- = no response

Table Five. Response of Callus to First Sub-culturing

Media No.		Tissue Used*	Callus Formation
1	S	r.c.	+++
1	L	l.c.	+++
1	L	r.c.	+++
1	L	l.s.c.	+++
2	S	r.c.	++
2	L	l.c.	++
2	L	l.s.c.	++
3	S	r.c.	+
3	L	l.s.c.	-
3	L	l.c.	-
4	S	r.c.	+
4	L	l.c.	+
4	L	l.s.c.	-

* The tissues used in sub-culture are those which grew in media No. 1 for six weeks.

S = solid media

L = liquid media

r.c. = root callus tissue

l.c. = leaf callus tissue

l.s.c. = leaf single cells

+ = magnitude of growth response

- = no response

Table Six. Results of Continued Sub-culture
Second and Third Sub-culture .

Media No.		Tissues Used	Results	
1	S	r.c.	c.	+++
1	L	r.c.	c.	+++
1	L	l.c.	c. & s.	+++ **
1	L	l.s.c.	s.	+++ **
3	S	r.c.	r.l.	++
3	L	r.c.	r.l.	+++
3	L	l.c.	r.l.	++ **
3	L	l.s.c.	p.e.	+++ **
3	S	r.l.	y.p.	
3	S	l.c.	r.l.	++ **
3	L	p.e.	y.p.	
5	S	r.c.	n.	+
5	L	r.c.	n.	+
5	L	l.c.	n.	+
5	L	l.s.c.	n.	-
6	S	r.c. & l.c.	r.l.	+++
6	L	r.c. & l.c.	r.l.	+++
6	L	l.s.	p.e. & e	
7	S	r.c.	c. & n.	++
7	L	l.c.	c. & s. & p.e.**	

Table Six. (continued)

Media No.		Tissues Used	Results	
8	S	r.c. & l.c.	c.	+
8	L	r.c. & l.c.	c.	+

S, L, r.c., l.c., l.s.c., and + refer to same structure as stated in table five.

c = callus tissue

s = single cell suspension

r.l. = root-like structure

p.e. = proembryos

y.p. = young plantlet

n. = nodule-like structure

* r.c. & l.c. obtained from cultivated medium No. 1 to which several transfers had been made before second and third sub-culture.

**Later those tissues developed into embryo and young plantlet even through no further transfer has been made.

only increase the size or the population of callus colonies in the medium containing 2,4-D. Sub-culture to a new medium which eliminates 2,4-D is necessary for its differentiation.

In the medium without 2,4-D the cells in the center of the colony began to differentiate the vascular strands as shown in Fig. 11, and later on nodule-like structures were observed in the surface of those

colonies. Nodule bearing callus colonies were transferred into liquid and solid media and did equally well in the formation of root-like structures eventually on the opposite end a greenish shoot appeared (Fig. 12 and Fig. 13).

The plantlets formed either from an embryo or from a nodule bearing callus were transplanted to normal greenhouse conditions (Fig. 14).

This experiment has been run twice with five duplications for each single treatment.

Figure 1. Carrot root tissue and leaf tissue used in this investigation. Root obtained from market, and leaf from seedlings planted in our laboratory.

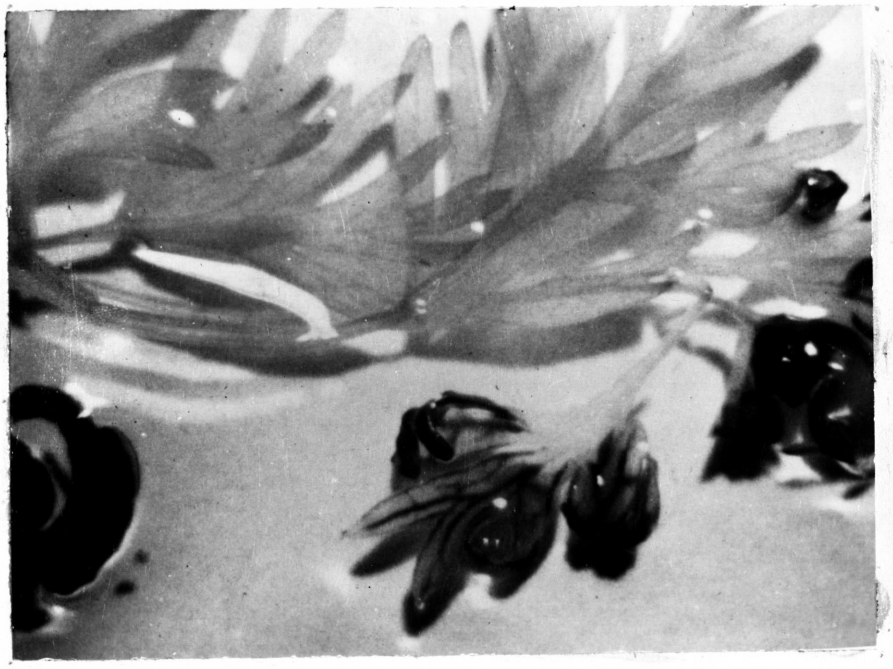

Figure 2. Cultivated leaf. Upper one shows the uncultivated leaf preserved in acetic acid and alcohol for six weeks, the lower one was grown in a medium containing 2 ppm 2,4-D. Callus tissues can be seen along the margin and midrib of the leaf.

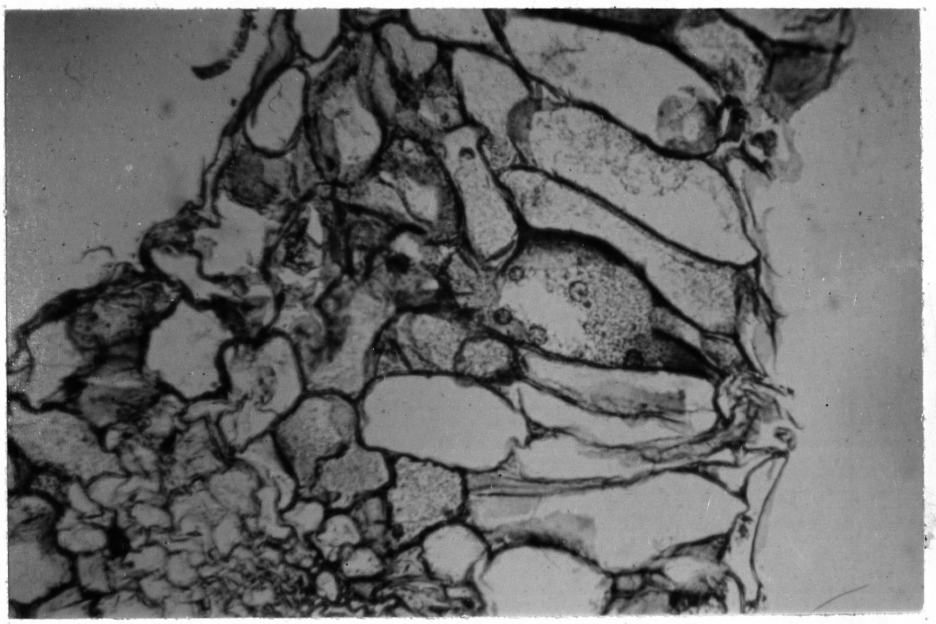

Figure 3. Enlargement of palisade parenchyma of cultivated leaf in the medium containing 2,4-D. A few cells in the lower right corner already have been discharged into the medium.

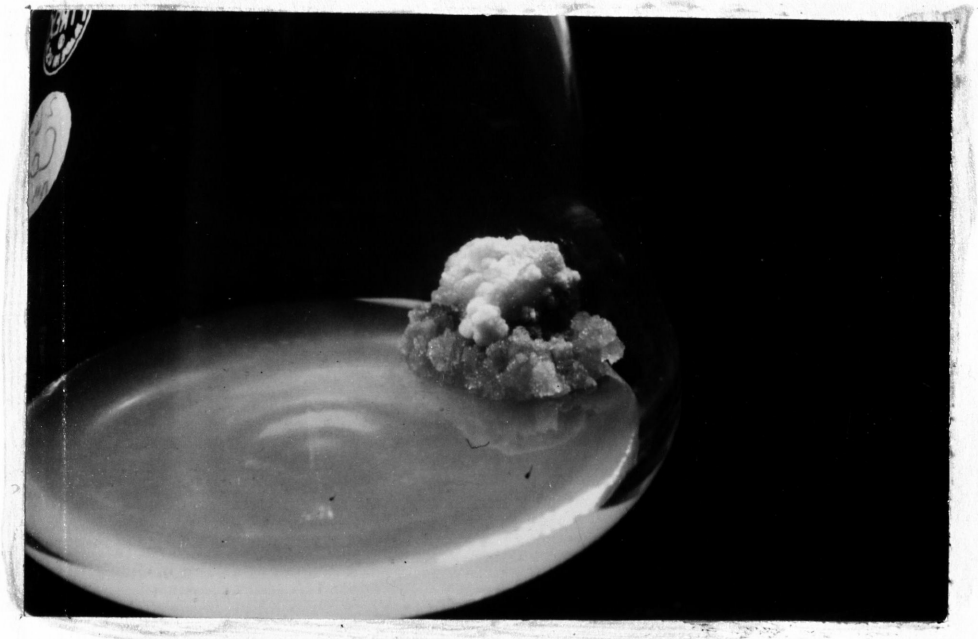

Figure 4. Callus tissue formed by cultivating carrot root tissue. The proliferated callus tissue is used for sub-culture.

Figure 5. Formation of cell suspension and callus colonies. Parts of the aggregate cell mass have fallen into the medium from the flask wall. It contains callus with heart-shaped embryos also. Isolated proembryos also can be seen in this picture.

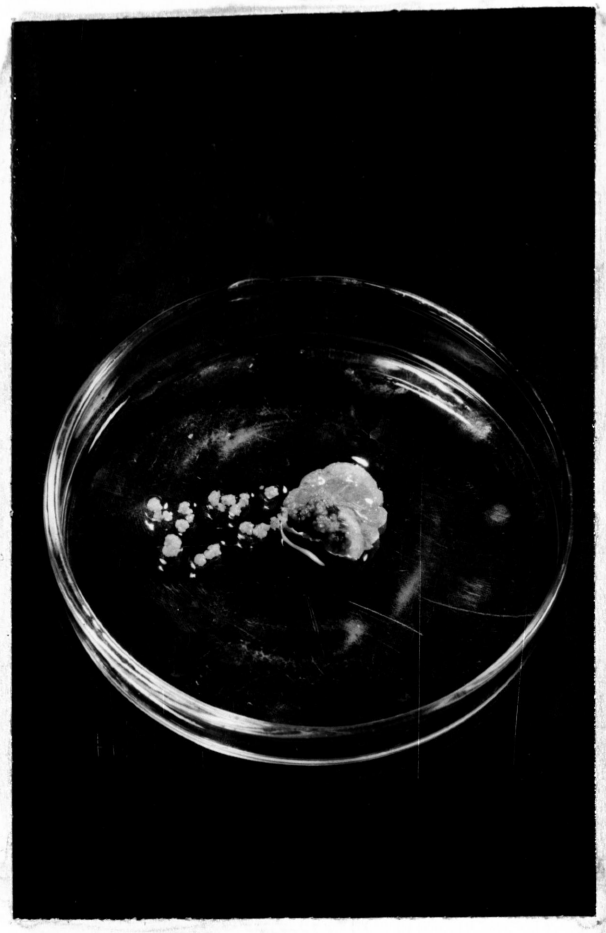

Figure 6. Callus colonies formed by sub-culture of callus tissue on the solid medium. The nodule-like structure can be seen on the surface of these colonies.

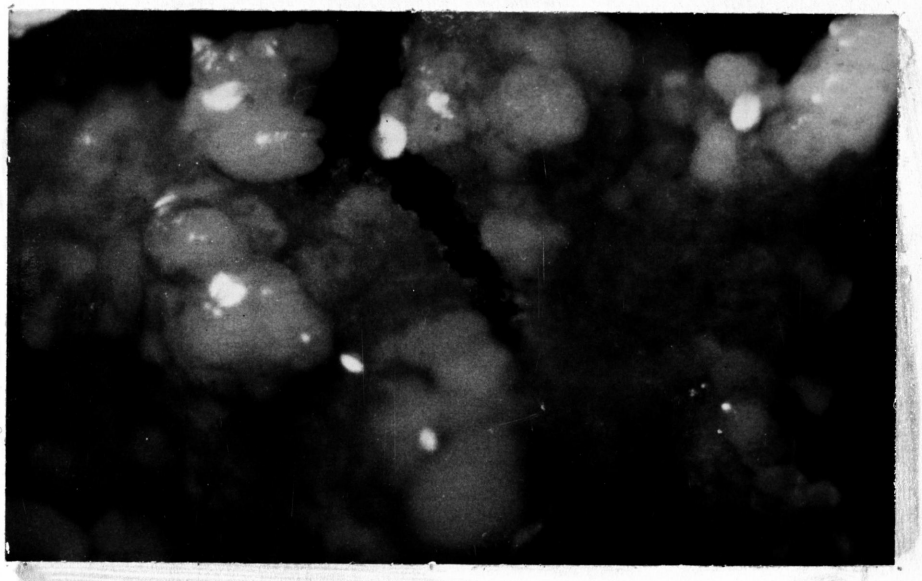

Figure 7. Heart-shaped embryos.

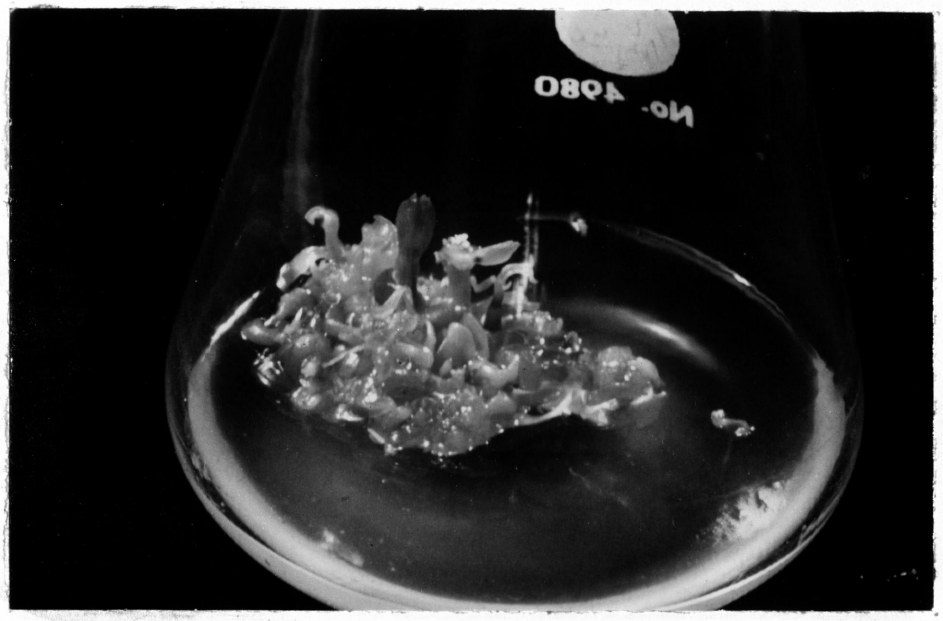

Figure 8. Young plants which have developed from heart-shaped embryos.

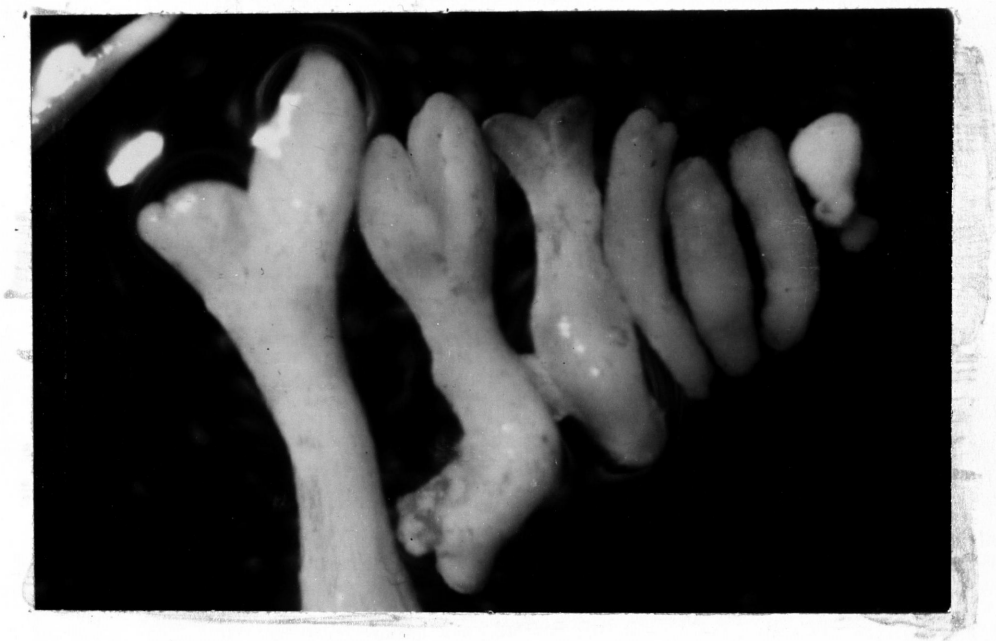

Figure 9. A series of developing young embryos found in the cell suspension.

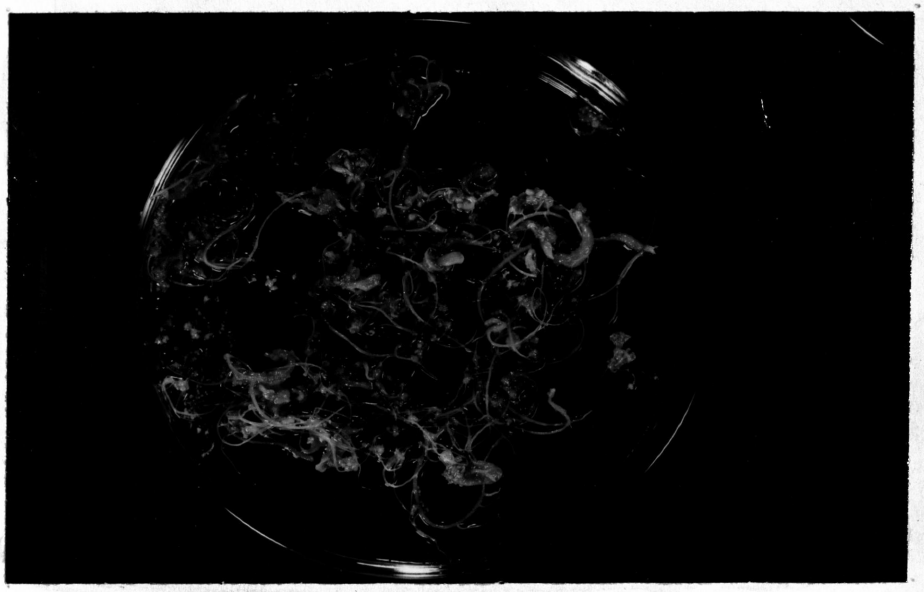

Figure 10. Isolated embryos from the cultivated cell suspension and callus colonies. The branching root and young embryos with cotyledons can be seen in this picture. These embryos developed from zygote-like cells. The upper end shows a piece of root bearing callus.

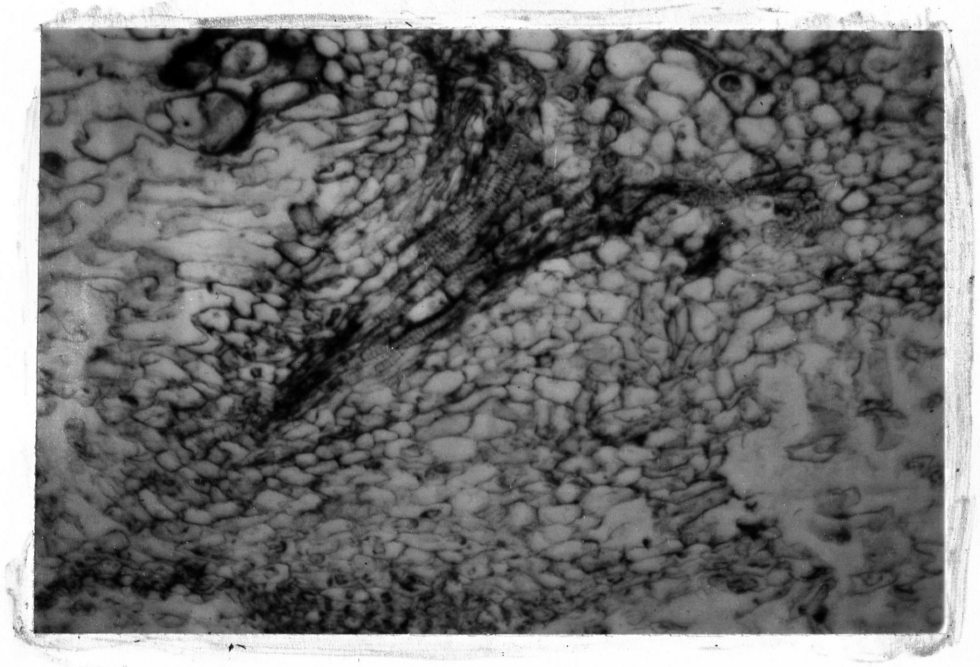

Figure 11. A cross section of a callus. It shows the central part of the callus, in which cells are beginning to differentiate into vascular elements. The further development of this tissue led to root formation.

Figure 12. A young plant formed from a cultivated carrot leaf.

Figure 13. An older plant formed from a cultivated carrot leaf.

Figure 14. The plants are transplanted into vermiculite where they continue to grow to maturity.

DISCUSSION

Due to its highly specialized function, the leaf tissue has been considered to be most difficult part of a plant to cultivate. However, some research work has been done with leaves resulting in a certain degree of growth, either by enlarging the leaf area or by forming the callus tissue. Little of the work carried on so far, however, has been intended to test the totipotency of the leaf cells.

In this investigation, we started with carrot leaves and root tissues, and the results were the formation of new plants. The development of these cultivated cells may be classified into five different stages. First of all, the matured cells lose their specificity and the metabolic activity was increased. This was followed by the second stage, the enlargement and division of the cells. The newly formed cells can either be present in the media as callus colonies or as a single cell suspension. The cultivated tissue will stay in this unspecialized stage as long as 2,4-D is present in the medium. However, most callus colonies are originally formed by the parenchyma cells located in the root vascular tissue or from leaf vascular tissue.

The start of differentiation of cultivated tissue is considered to be the third developing stage. In this stage, the two kinds of tissues behaved a little differently. In the callus colonies, each colony acted as a developing unit. The cells in the central part of the colonies began to differentiate as soon as the 2,4-D was eliminated from the cultivating media. These cells differentiated into vascular elements. The differentiation began from the center of the

colonies and spread toward the outside region, forming nodule-like structures. This was followed by the fourth developing stage in which organs were formed. The root-like structure was formed at one end and the shoot-like structures on the other end of the callus. As far as single cells are concerned, they appear either in the form of suspensions or aggregates as a mass of single cells, each single cell acting as a unit. When 2,4-D was used up or eliminated from the cultivating media many cells behaved like zygotes, each zygote forming an embryo and each embryo developing into a young plant which was independent of organic supplements.

The last developing stage is actually only a process of continued growth of the independent plant to maturity. All the further growth of root and shoot-bearing callus colonies or the dicotyledon-like embryos gave rise to a definite young plantlet, and those young plantlets in most aspects were similar to the plant which were raised from the seeds. The exception is that, in most cases, the tissue culture embryos are genetically identical.

The responses of cultivating tissues in various media are shown in the results. As we compared the minerals we used in the cultivating media, there were no distinguishable differences in responses. The comparison of Murasahage & Skoog solution versus Hoagland solution shows the following; K 39.20 ppm & 11.70 ppm gives difference of 27.50 ppm. Ca 6.05 ppm & 5.00 ppm gives difference of 1.05 ppm. Mg 1.84 ppm & 2.40 ppm gives difference of 0.56 ppm. P 1.92 ppm & 1.48 ppm

gives difference of 0.44 ppm. N 42.05 ppm & 10.50 ppm gives difference of 31.55 ppm.

Each element has two different concentrations both of which work equally well for cultivating tissues. The higher concentrations are not toxic and the lower concentrations are not limiting factors for the tissue growth. The range of mineral concentration for cultivating tissues is wider than the range of organic supplements. However, the nitrogen concentration shown in the above comparison refers to the nitrogen contained in the inorganic media.

The reasons for the differences in responses existing between the liquid and solid media to the primary cultivating of leaf and root tissues in this investigation is not known. It may be due to the fact that in cultivating leaves in the solid media there was only a small area of tissues attached to the media compared with the size of the tissues being cultivated. The absorbed water and nutrient from the media were not enough to maintain the tissues metabolism and evaporation requirement and resulted in the slower growth. However, these differences vanished in the sub-culture of both leaf and root tissues.

As we compare the effectiveness of various organic supplements to the cultivated tissues, 2,4-D is considered to be the key substance of dedifferentiation in matured cells of the carrot plant. Cultivated tissues will remain in this undifferentiated stage as long as the 2,4-D is present in the medium. Whether 2,4-D is metabolized or decomposed after long periods is not known. But the inhibition of the medium for

differentiation will finally vanish as shown in the results of this investigation. The transfer of a sub-culture of callus suspension to the new medium in which 2,4-D is contained required a much longer period if compared with the medium without 2,4-D for starting differentiation. And if we keep changing the 2,4-D medium, and use a short cultivating interval, tissues will remain in the undifferentiated stage indefinitely.

2,4,5-T shows a greater effect than 2,4-D in radish leaf cultivating but is not as powerful as 2,4-D for carrot tissues.

Vitamins and coconut milk can enhance the cell activity throughout all the cultivating stages but is not essential. Both vitamins and coconut milk have less effect on cell differentiation or dedifferentiation than 2,4-D.

IAA was believed to have effectiveness for the callus formation in root culture. However, in this investigation, it did not show any better result in callus formation as we compared it with 2,4-D in both root and leaf cultivating. It does effect the leaf expansion in the primary culture, which was not the case as Al-Talib and Torrey mentioned in their paper (1).

IAA and 2,4-D do not have the same effect in the plant tissue culture. One will enhance the differentiation, and the other will stimulate the cell to dedifferentiation.

The development of cultivated tissues is a continuous process. The end result of a former stage will be the cause of the later one. That the cultivated tissues respond differently to the same stimulus

at different periods of cultivation was known by Kunihiko (13, 14, 15). The tissues develop normally only with the right stimulus at the right time, otherwise abnormality will occur. In this investigation, the desire was to control and synchronize the culture and not end with abnormal embryos. If a synchronized culture could be established, much more detailed study of cell development and much more precise study of the effectiveness of organic supplements could be carried out.

Whether or not the totipotency generally exists in every plant within the plant kingdom is not involved in this investigation, but the assumption is greatly enhanced. If suitable media could be discovered, the media should contain the basic nutrients, both organic and inorganic, but the most important of all would be to discover the key substances which would turn on all the nonfunctional genes in each different plant. Those key substances might be, but do not have to be, some of the auxins. If these requests are met the duplicate of this experiment, i.e., starting with a matured cell and ending with an entire plant, could be expected.

CONCLUSION

a. The totipotency existing in the leaf and root cells of the carrot plant was tested. After removing the specialization of the matured cells by applied chemical stimuli unfunctional genes become functional. Many of these cells acted as zygotes. They developed through the embryo stages and ended as an entire plant. 2,4-D is a powerful tool, for dedifferentiation of matured carrot cells.

b. The development of cultivated leaf cells went through the following stages:

1. The matured cell lost its specialty and increased its metabolic and division activity.

2. The dedifferentiated cell either formed single cells or formed callus tissues.

3. Single cells acting as zygotes which developed into young embryos and callus cells differentiated into organ bearing callus colonies.

4. Both young embryos and organs bearing callus colonies developed into young plants.

c. The factors needed to bring about the change in each of the stages in tissue culture are:

1. Minerals, sugar, vitamin mixture and coconut milk are required through all cultivating stages.

2. 2,4-D is necessary to induce the formation of callus tissue of carrot plant. It is also a key substance for despecialization of matured carrot cells, and an inhibitor for differentiation of

callus colonies into organ-bearing colonies, and an inhibitor which prevents single cells from developing into young embryos.

3. IAA will enhance the differentiation of root-bearing callus and also enhance the developing of embryos from a single cell into a new plant.

4. The newly developed plants are independent of any organic supplements.

LITERATURE CITED

1. Al-Talib, K. H. and Torrey, J. G. 1959. The aseptic culture of isolated bud of Pseudotsuga taxifolia. Plant Physiol. vol. 34, pp. 630-631.

2. Arora, N., Skoog, F., and Allen, O. N. Kinetin induced Pseudonodules on tobacco roots. Amer. Jour. Bot. vol. 46, No. 8, 1959.

3. Ball, E. Differentiation in a callus culture of Sequoia sempervirens. 1950, Growth 14 pp. 295-325.

4. Ball, E. 1956. Growth of the embryo of Ginkgo biloba under experimental conditions. I. Origin of the first root of the seedlings in vitro. Amer. Jour. Bot. vol. 43, pp. 488-495.

5. Bertossi, F., Bagni, N., Moruzzi, G., and Caldarera, C. M. 1965. Spermine as a new growth-promoting substance for Helianthus tuberosus (Jerusalem Artichoke) in vitro. Experientia XXI/2 Nev. pp. 80.

6. Blakeslee, A. F. and Satina, S. 1944. New hybrids from incompatible crosses of Datura through culture of excised embryos on malt media. Sci. 99 pp. 331.

7. Bonner, D. M. 1939. Leaf growth factors, II The Activity of pure substances in leaf growth. Proc. Nat. Acad. Sci. U. S. 25, pp. 184-188.

8. Braum, A. C. 1958. A physiological basis for autonomous growth of the crown-gall turmor cell. Proc. Nat. Acad. Sci. 44, pp. 344.

9. Caplin, S. M. and Steward, F. C. 1948. Effect of coconut milk on the growth of explants from carrot root. Sci. 108: 655.

10. Das, N. K., Patau, K., and Skoog, F. 1956. Initiation of mitosis and cell division by kinetin and indoleacetic acid in excised tobacco pith tissue. Physiologia Plantarum vol. 9: 640-651.

11. Fox, J. E. and Miller, C. 1959. Factors in corn steep water promoting growth of plant tissues. Plant Physiol. vol. 34, No. 5, pp. 577-579.

12. Howell, R. W. and Skoog, F. 1955. Effects of adenine and other substances on growth of excised Pisum epicotyls cultured in vitro. Amer. Jour. Bot. vol. 42, No. 4.

13. Kunihiko, S. 1965. Physiological and biochemical changes of carrot root callus during successive cultures. Plant and Physiol. vol. 6, No. 3 (Japanese Society of plant physiologist) Tokyo.

14. Kunihiko, S. 1965. Changes in organ formation capacity of carrot root calluses during sub-cultures. Plant and cell Physiol. vol. 6 Tokyo.

15. Kunihiko, S. 1965. Changes in response to purine and pyrimidine derivatives of carrot root callus during successive culturing. Plant and cell physiol. vol. 16, No. 3 Tokyo.

16. Kuraishi, S. and Okumura, F. S. 1956. The effect of kinetin on leaf growth. Bot Mag (Tokyo) 69: 300-306.

17. Lee, A. E. 1958. The effects of various treatments in different genetics lines of excised tomato roots growth in culture. Bull. Tor. Bot. Club. vol. 85, No. 5.

18. Maheshwari, N. 1958. In vitro culture of excised ovules of Papaver samniferum. Sci. 127: 342.

19. Manney, J. R., Hillman, W. S., Miller, C. O., Skoog, F., Clayton, R. A., and Strong, F. M. 1952. Bioassay, purification and properties of a growth factor from coconut. Physiol. Plant 5: 485.

20. Morel, G. and Wetmore, R. H. 1951. Tissue culture of monocotyledons. Amer. Jour. Bot. 38: 138-140.

21. Nickell, L. G. and Burkholder, P. R. 1950. A typical growth of plants. II Growth in vitro of virus tumors of Rumex in relation to temperature, pH, and various sources of nitrogen carbon and sulfur. Amer. Jour. Bot. 37: 538.

22. Nitsch, J. P. 1951. Growth and development in vitro of excised ovaries. Amer. Jour. Bot. 38: 566-577.

23. Overbeek, J. van, Conklin, M. E., and Blakeslee, A. F. 1941. Factors in coconut milk essential for growth and development of very young Datura embryos. Sci. 94: 350.

24. Overbeek, J. van. 1944. Factors affecting the growth of Datura embryo in vitro. Amer. Jour. Bot. 31: 219.

25. Patau, K., Des, N. K., and Skoog, F. 1957. Induction of DNA synthesis by kinetin and indoleacetic acid in excised tobacco pith tissue. Physiologia Plantrum vol. 10.

26. Riker, A. J. and Gutsch, A. E. 1948. The growth of sunflower tissue in vitro on synthetic media with various organic and inorganic sources of nitrogen. Amer. Jour. Bot. 35: 227-238.

27. Robbins, W. J. 1922. Cultivation of excised root tips and stem tips under sterile condition. Bot. Gaz. 73.

28. Sandstedt, R. and Skoog, F. 1960. Effects of amino acid components of yeast extract on the growth of tobacco tissue in vitro. Physiologia Plantarum. vol. 13.

29. Skoog, F. and Tsui. 1948. Chemical control of growth and bud formation in tobacco stem segments and callus cultured in vitro. Amer. Jour. Bot. 35: 782-787.

30. Skoog, F. 1949. Chemical control of growth and organ formation in plant tissues. Colloqne International Du Centre National Dela Recherche Scientifique Sur La Morphogenese.

31. Skoog, F. and Robinson, B. J. 1950. A direct relationship between indoleacetic acid effects on growth and reducing sugar in tobacco tissue. Proc. Soc. Exp. Biol. Med. 74: 565-568.

32. Steeves, T. A., Gabriel, H. P., and Steeves, M. W. 1957. Growth in sterile culture of excised leaves of flowering plants. Sci. 126: 350-351.

33. Steward, F. C. and Caplin, S. M. 1951. Tissue culture from potato tuber: The synergistic action of 2,4-D and coconut milk. Sci. 113: 518-520.

34. Steward, F. C. and Caplin, S. M. 1952. Growth and metabolism of plant cells, IV. Evidence on the role of coconut milk factor in development. Amer. Bot. 16: 491-504.

35. Steward, F. C. and Caplin, S. M., and Miller, F. K. 1952. Investigations on growth and metabolism of plant cells I. New techniques for the investigations of metabolism, nutrition and growth of undifferentiated cells. Ann. Bot. 16: 58.

36. Steward, F. C. 1958. Growth and organized development of cultured cells. II. Organization in cultures grown from freely suspended cells. Amer. Jour. Bot. vol. 45, No. 10.

37. Steward, F. C., Mapes, M. O., and Smith, J. 1958. Growth and organized development of cultured cells I. Growth and division of freely suspended cells. Amer. Jour. Bot. vol. 45, No. 9.

38. Steward, F. C. 1963. The control of growth in plant cells. Sci. Amer. Oct.

39. Steward, F. C., Mapes, M. O., Kent, A. E., and Holsten, R. D. 1964. Growth and development of cultured plant cells, biochemical and morphogenetics studies with cell yield new evidence on their metabolism and totipotency. Sci. 143: 3601.

40. Tamaoki, T. and Ullstrup, A. J. 1958. Cultivation in vitro of excised endosperm and meristem tissue of corn. Bull. Tor. Bot. vol. 85, No. 4.

41. Thimann, K. V. 1951. The synthetic auxins: relation between structure and activity. Plant Growth substances Ed. by Skoog. University of Wisconsin Press.

42. Thimann, K. V. and DeTorok, D. 1961. The auxin requirement and the effect of an auxin-antagonist on tumorous and normal tissue of Picea glanca. Physiologia Plantarum vol. 14, pp. 543-547.

43. Tulecke, W. 1961. Recent progress and goals of plant tissue culture. Bull. Tur. Bot. vol. 88, No. 5.

44. Wiggans, S. C. 1954. Growth and organ formation in callus tissue derived from Dancus carota. Amer. Jour. Bot. 41:321.

45. White, P. R. 1939. Glycine in the nutrition of excised tomato roots. Plant Physiol. 14:527.