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THE CULTIVATION OF PLANT TISSUES IN VITRO WITH STARCH AS A SOURCE OF CARBON

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INTRODUCTION

The literature on plant tissue culture shows few reports on the use of starch as a source of carbon. GAUTHERET (1945), considering the action of chemical substances on living systems, points out the necessity of putting three questions: *a*, is the substance to be studied indispensable? *b*, what is the optimal concentration? *c*, is substitution by other substances possible? In plant tissue culture, sugars (monosaccharides or disaccharides) proved to be necessary to growth, and after studying the optimal concentration Gautheret turned to the third question and investigated a number of substances for their value as a sugar substitute. These last investigations proved starch to be valueless as a substitute with the tissue tested, i.e. his well-known strain of carrot root tissue.

HILDEBRANDT and RIKER (1946, 1949) investigated a large number of substances for the same purpose. The strains used were derived from tumours of *Tagetes erecta* L. var., *Chrysanthemum frutescens* L. var., *Vinca rosea* L., *Helianthus annuus* L. var. and of a hybrid of *Nicotiana glauca* Grah. ♀ × *N. langsdorffii* Weinm. ♂. According to these authors, little or no growth took place on culture media containing 1 % of soluble starch. Their data indicate, however, that the cultures of *Vinca* and *Nicotiana* tissues grew better than the others. The same results were obtained in experiments on the use of series of concentrations other than 1 %. (HILDEBRANDT and RIKER, 1950, 1953; RIKER and HILDEBRANDT, 1953, 1955). Neither Gautheret nor Riker and Hildebrandt mention attempts to continue their culture on starch media.

The results communicated by NICKELL and BURKHOLDER (1950) are entirely different. These authors state that soluble starch is "a surprisingly good source of carbon" for a tissue strain derived from virus tumours of roots of *Rumex acetosa* L. (BURKHOLDER and NICKELL, 1949). The growth value, i.e. the ratio between the fresh weights of the cultivated tissues at the beginning and at the end of a certain culture period on a starch medium is about 55 % of that on an optimal glucose medium: 8.56 on glucose against 4.70 on starch medium. Furthermore, prolonged cultivation on starch media by means of subcultures proved to be possible.

The fact that with the iodine reaction a colourless zone around the cultivated tissues becomes apparent led to the presumption that virus tumour tissue of *Rumex acetosa* is able to digest starch by means of

an extra-cellular enzyme. A possibly related phenomenon was discovered by GALL (1948). During an investigation of the influence of 2–4-dichlorophenoxyacetic acid on the transformation of reserve starch in callus-growing fragments of bean stems, he discovered a positive correlation between the intensities of callus formation and of starch hydrolysis. BRAKKE and NICKELL (1951) thoroughly investigated the transformation of starch by *Rumex*-tissue and arrived at two important conclusions. In the first place they were able to show that the starch-splitting enzyme is an α-amylase. Secondly, it seems hardly to be doubted that the enzyme enters the culture medium as a result of a true secretion by intact cells. Some further publications of BRAKKE and NICKELL (1952, 1955) and NICKELL (1953) contain complimentary data. The data given by NICKELL and BRAKKE in 1954 are important for the present investigation. These authors compared the growing properties of their *Rumex*-strain with tissue cultures derived from crown-gall of *Nicotiana tabacum* L., *Helianthus annuus* L. and *Vinca rosea* L. The growth values of *Nicotiana* and *Vinca* tissue were 50.7 and 46.6 %, respectively, of that of *Rumex* tissue, while two sunflower strains exhibited a growth value of only 11 %. This shows that *Nicotiana* and *Vinca* tissues grow tolerably well on starch medium. Therefore, the present writers do not agree with the statement made by Nickell and Brakke that "reports in the literature that other plant tissues studied grow very little or not at all on starch is confirmed here for 4 different crown-gall tissues". Furthermore, no attempts at prolonged culture seem to have been undertaken. LAMPTON (1952) and STRAUS and LA RUE (1954) reported that tissue cultures derived from endosperm of *Asimina triloba* Dunal. and *Zea mays* L. are able to use starch as a source of carbon. After completion of this manuscript, an article by CONSTABEL (1960) was published. This author mentions the fact that tissue cultures derived from *Juniperus communis*, *Crataegus monogyna* and *Pyrus communis* exhibit some growth on starch-containing media. Very good growth was exhibited by a fast growing, auxin autotrophic strain of *Juniperus*. From the description it appears that both α- and β-amylase are secreted although the author does not stress this point, nor is it clear whether prolonged cultivation on starch-containing media was attempted. Finally, attention is drawn to the survey done by GAUTHERET (1955) on the nutrition of plant tissue cultures.

Unaware at the time of the work of Nickell and his co-workers, [1]) the present investigation was started in 1953.

MATERIAL AND METHODS

Seven strains were tested for their ability to use starch as a carbon source. Particulars about the strains used are assembled in Table 1. All strains used were well established, and are still in cultivation at our laboratory.

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[1]) The senior author first became acquainted with Nickell's work at the Colloquium on Plant Tissue Culture at Briançon (1954).

TABLE I

Strains derived from:	Isolated by:	No. of passage used:	Particulars:
Cissus spec.	Oort [1]) (1948)	32	cambial zone, twigs
Crataegus monogyna Jacq.	Karstens (1949)	19	cambial zone, twigs
Daucus carota L.	Gautheret (1939)	96	cambial zone, root
Daucus carota L.	Karstens (1952)	18	cambial zone, root
Nicotiana tabacum L.	Morel (1948)	45	crown-gall
Rubus fruticosus L.	Oort [1]) (1948)	31	cambial zone, twigs
Rubus fruticosus L.	Oort [1]), Karstens (1951)	32	mutant "accoutumé"

In the culture media used, glucose was replaced by soluble starch. For the first subculture on starch medium a concentration of 5 % soluble starch (Brocades and Stheemann) was used. However, this brand proved to contain an appreciable amount of reducing sugars. It was therefore replaced by soluble starch "Analar" from B.D.H., initially in a concentration of 0.5 % to obtain a clear culture medium. Growth was considerably improved, however, by increasing the concentration to 2 %.

Autoclaving was at first omitted for fear of the production of sugars and other substances by partial hydrolysis of the starch. It was found, however, that under the conditions used no hydrolysis of starch occurs. NICKELL and BURKHOLDER (1950) mention the same experience.

The culture tubes were placed at 25° C in specially constructed incubators. [2]) By means of a small electromotor in these incubators, platforms with culture tubes fixed in a vertical position were slowly rotated. Parallel to the axis of rotation Philips fluorescent tubes were fixed in order to provide a uniform amount of illumination for the cultures.

CULTIVATION ON A STARCH MEDIUM

It very soon became apparent that the seven strains used for this investigation reacted very differently to the transfer from the glucose medium to the starch-containing media. The tissues of *Cissus spec.* and *Crataegus monogyna* exhibit so little growth that a second subculture could not be effected. The five remaining strains showed better growth, so that a second subculture could be made. Gautheret's carrot strain and the *Rubus* "accoutumé" strain showed hardly any growth at all, while our own carrot strain exhibited a slightly better development. The results with the normal strain of *Rubus* were much better, especially with the strain derived from tobacco crown-gall. The experiments were continued with these two tissue cultures. This does not imply that the discarded strains are absolutely unable to develop on starch

[1]) Professor A. J. P. Oort (Wageningen) very kindly presented me with tissue cultures isolated by him during his stay in 1948 in Gautheret's laboratory (Paris).

[2]) We should like to thank Dr. W. Kruyt, Research laboratory of the Amsterdamse Chininefabriek (Amsterdam Quinine Factory) for his kind permission to use the idea of rotating platforms in the construction of our incubators.

media. As a matter of fact, it is our impression that many strains are able to adapt themselves sooner or later to this type of culture medium.

As mentioned before, the crown-gall strain of *Nicotiana* in particular, grows very well on starch. Substrains started in 1953 and cultivated since then on starch, have grown through 42 and 35 passages. Normal tissue of *Rubus fruticosus* can also be grown on starch medium, but the growth is very much slower than is the case with tobacco. Started at the end of 1953, it has grown through only 28 passages. This is partly due to the fact that in the beginning 0.5 % starch was used. In that period growth was so slow that subculturing was only necessary once in four months. Since July 1956, 2 % starch has been used with the result that growth has improved considerably as may be seen from the fact that subcultures now have to be made every three months. In comparison with the tobacco strain, this is still very slow, since the latter has to be transferred to new culture medium every six or eight weeks. This is remarkable because the growth rate of both strains on glucose media is about the same and such that subcultures have to be made every two months. In addition to culture on starch medium in the light, it was investigated whether growth is possible on starch medium in the dark. For this purpose starch substrains of *Nicotiana* were used. Culture proved possible, and two substrains were obtained which grow very well under the conditions described. Subculturing takes place every 2 to $2^1/_2$ months. Started in September 1954, one of these substrains has been cultivated through 31 passages.

Growth on starch medium in comparison with the development on glucose medium

To evaluate the development of tissue cultures on starch medium compared with that on media with glucose, fresh weight and dry weight determinations were carried out on tissue cultures at the beginning and at the end of certain growth periods. To obtain as much uniformity in growth as possible, great care was taken to start with uniform tissue fragments. As material, *Nicotiana* and *Rubus* starch strains were used which had gone through a considerable number of passages. In the case of *Nicotiana*, in addition to experiments in light a few series of dark experiments were done. The results are shown in Table II.

Several points of interest present themselves. In the case of *Nicotiana* it is clear that the growth rate on starch media both in light and darkness is very high, taking into account the fact that the culture period is only 49 days as against 71 days for the cultures on glucose media. Furthermore, it is apparent that growth on starch medium in darkness is very uniform. A second point of interest lies in the fact that the dry-matter content of the tissues grown on starch media is considerably lower than that of tissue cultivated on glucose media. The high water content and the rapid development on starch media are certainly interrelated. A third point to be considered is the striking difference in dry-matter content at the beginning and at the end of

TABLE II

	Number of days cultivation	Growth value [1]		Dry weight as a percentage of fresh weight			
				at the beginning of the experiment		at the end of the experiment	
Nicotiana tabacum							
crown-gall							
glucose 2 %, light	71	16.4 ± 0.8	n = 20	4.8 (4.4–5.4)	n = 6	6.5 (5.9–8.0)	n = 7
glucose 2 %, dark	71	11.4 ± 1.3	n = 15	6.3 (5.4–7.0)	n = 6	7.2 (5.6–8.0)	n = 7
starch 2 %, light	49	14.2 ± 1.2	n = 24	2.6 (2.1–2.8)	n = 6	2.4 (1.9–3.1)	n = 7
starch 2 %, dark	49	11.0 ± 0.2	n = 24	2.6 (2.1–2.9)	n = 6	2.6 (2.1–3.2)	n = 6
Rubus fruticosus							
glucose 2 %, light	61	13.7 ± 0.6	n = 23	5.5 (5.1–5.8)	n = 6	5.5 (5.2–6.0)	n = 8
starch 2 %, light	61	5.9 ± 0.4	n = 24	3.3 (2.1–3.9)	n = 6	4.3 (3.7–4.9)	n = 8

[1]) Ratio of fresh weights at the beginning and at the end of the experiment.

the experiments in tissues cultivated on glucose media. No difference in this respect could be observed in the starch cultures. In the next section this point will be discussed further.

As to the *Rubus* cultures, the low growth value on starch medium is apparent. The difference in dry-matter content between glucose and starch cultures is less spectacular than in *Nicotiana* but is clearly evident.

Growth pattern on glucose and starch media

On transfer of tissue cultures of both *Nicotiana* and *Rubus* from glucose to starch media, striking changes in growth occured. To the best of our knowledge, no mention of this phenomenon has been made in literature. It is, however, so striking that, especially in the case of *Nicotiana*, it is hardly believable that the cultures on glucose and starch belong to the same strain. Crown-gall tissue in vitro from *Nicotiana* appears on glucose medium and cultivated in light as white, semiglobular, downy, solid masses of tissue, growing with a smooth underside on the surface of the culture medium. Grown in darkness, the cultures appear somewhat less regular in shape but otherwise show no differences. Cultivated on starch medium in the presence of light, the colour of the cultures is grass-green and the shape is very irregular. Furthermore, the cultures very often contain cavities, grow to a considerable extent into the culture medium, and are very juicy. Brought back on glucose-containing media, the original growth picture sooner or later returns. Cultivated in darkness, the cultures are pale brownish-yellow in colour and of a very soft substance.

The glucose and starch cultures of *Rubus* show a different picture. On glucose media the cultures are green and show a semiglobular underside that grows well into the medium. Furthermore, the cultures are of a firm consistency. On starch-containing media, however, the cultures are creamy-white in colour, grow on but not into the culture medium, and are of a much softer consistency. More detailed information concerning growth pattern and internal structure will be given in a subsequent paper (Van Lith–Vroom, Gottenbos, and Karstens, 1960).

The cause of the growth observed on starch media

It is obvious that the positive results obtained with substitution of starch for glucose would lead to an investigation of the cause of this phenomenon. It is also obvious that the presence and activity of starch-hydrolyzing enzymes had to be considered.

In our survey of the literature, the work of Brakke and Nickell (1951) is mentioned. Their investigations were able to prove that tissue cultures derived from virus tumours of *Rumex*-roots developed very well with starch as a carbon source as a substitute for glucose because of the activity of an α-amylase actively secreted by the tissue into the culture medium. In the discussion following the papers given by Dr. Nickell at the colloquium at Briançon (Brakke and Nickell, 1955) the senior author stated that in the case of the tissue cultures

of *Nicotiana* and *Rubus* not only α-amylase but probably also β-amylase played a part in the hydrolysis of starch.

Some details of the present research may be given here. Under the influence of the tissues under cultivation the starch present in the culture media disappears in the area close to the tissue. This could be demonstrated by the iodine test for starch. In order to learn more about the agents present, a series of experiments was started using WIJSMAN's diffusion method (1889), applied in more recent time by VAN KLINKENBERG (1931) and MEEUSE (1943, 1952). The principle of Wijsman's test method is very simple. On the surface of a starch solution solidified by means of agar a drop containing starch-hydrolizing enzymes, e.g. malt-extract, is put. In the course of time, the surface is treated with an aqueous iodine solution. According to the presence of one or more types of amylases, a specific colour pattern develops. In the case of α-amylase, for instance, starch is quickly hydrolised into products that do not give any colouration with iodine. The result is a colourless circular area in the middle of a blue field, the colourless area being caused by enzyme activity, the blue surrounding parts showing where starch is still intact. If, however, only β-amylase is present in the test solution, starch hydrolysis is slower and products are formed which give a reddish violet colouration with iodine. In the case of β-amylase, a reddish violet spot surrounded by blue results. In the case of a mixture of α- and β-amylases, a combination of the described results becomes apparent. Around a colourless area caused by the action of α-amylase, first a reddish violet ring (β-amylase activity) and then a blue field (indicating intact starch) become visible.

The material to be tested for amylase activity was used in three ways: as living tissue, as tissue killed by various means, and as press sap. Living tissue was also used in three forms: freshly cut cubes, cubes washed with sterile water to remove the contents of cut cells, and cubes after a period of cultivation. In the last case the cubes were covered with a layer of newly formed cells. By means of small stainless steel hooks implanted into the cubes during the manipulations for making subcultures, it was possible to handle the young cultures without damage. The intact upper side was finally brought into contact with the test plate, with the hope that the cells in question had remained undamaged.

Three methods were used to kill tissue: freezing at a temperature of minus 5° C, treatment with ether vapour, and heating in a water bath at 100° C. Press sap was prepared by forcing a piston into a perforated hollow cylinder containing the material. The sap obtained was used without further treatment or treated with different temperatures. For the tissue-test, *Nicotiana* tissue was used, while press sap was prepared from *Nicotiana*, *Rubus* normal and *Daucus carota* Gautheret's strain, all tissues being cultivated on glucose medium. Amylase appeared to be active in the case of living tissue and of untreated press sap.

In order to obtain more information about the amylases present,

TABLE III

Material used	Unheated control	Heated to 70° C	Heated to 80° C	Heated to 100° C
Nicotiana tabacum crown-gall	large colourless centre reddish violet ring	smaller colourless centre broader reddish ring	pale violet centre reddish violet ring	no decolouration
Daucus carota strain Gautheret	colourless centre reddish violet ring	yellow brown centre reddish violet ring	diffuse, pale violet spot	no decolouration
Rubus fruticosus normal	brownish violet centre reddish violet ring	diffuse, pale violet spot	no decolouration	no decolouration

a number of experiments were done on temperature sensitivity. In addition to untreated press sap, sap was tested after treatment in a waterbath for 10 minutes at 70, 80, and 100° C. The results are collected in Table III.

It is apparent that quantitative and qualitative differences in the constitution of the press sap samples affect each other. Furthermore, it might be possible that certain press saps contain interfering impurities. From our data it seems that the activity of α-amylase decreases considerably with the 70° C treatment while that of β-amylase remained the same. This is in contradiction to the results of VAN KLINKENBERG (1931) who using purified preparations came to the conclusion that β-amylase is the less stable enzyme. This author investigated the pH influence on the activity of amylases both in a mixture of α-amylase and β-amylase and separately. α-Amylase appears to be active in a pH range of 4–8, while β-amylase shows a much wider range of activity i.e. from 3–11.5.

A plate method was used to test the pH sensitivity of amylase present in tissue cultures. In Petri dishes starch-containing agar plates of the same thickness were prepared. Dilute hydrochloric acid or sodium hydroxyde were used to obtain a series of plates to cover a wide pH range. The pH was measured with the aid of a glass electrode. Two plates were available for each point.

From well-grown tissue cultures of *Nicotiana*, uniform slices were cut by means of a special apparatus; from these slices cylinders were punched out and placed on the test plates. At the end of 48 hours at 25° C the plates were developed with the aid of an iodine solution. The results are summarized in Table IV.

TABLE IV

pH	Colourless central area, diam. in mm.	Pale violet coloured central area, diam. in mm.	Reddish violet ring, thickness in mm.
2.18	—	—	—
2.53	—	—	—
3.03	—	5	—
3.70	—	13	2
4.12	—	17	5
5.13	31	—	4.2
5.33	32	—	3.5
6.27	36	—	4
6.88	35.5	—	4
7.89	36	—	5.5
8.25	—	21	7
8.67	—	30	11.5
9.66	—	21	7
10.23	—	10	2
10.37	—	21	7
10.94	—	14.5	4
11.48	—	9	1

The authors are very well aware that these data do not give much information as to the specific pH ranges of α-amylase and β-amylase

activity in the material tested. The data in columns 2 and 3 possibly point to the combined activity of both α- and β-amylase since the values for the colourless area start and stop very abruptly. It seems probable, however, that the data in column 4 must be ascribed to the presence of β-amylase.

It is necessary to draw attention once more to the problem of whether the presence of one or more amylases in the culture medium is due to a true secretion of these enzymes by intact cells of the cultivated tissue or whether these substances have penetrated accidentally from cells damaged by growth or other processes. The fact that much larger quantities of amylases were found in the culture medium than were present in the tissue itself was a strong argument for BRAKKE and NICKELL (1951) to draw the conclusion, that the observed situation could only be explained as a veritable secretion of the enzymes by intact cells. This point was discussed after the paper read by Nickell at the Briançon colloquium in 1954 (BRAKKE and NICKELL, 1955). On that occasion Gautheret gave it as his opinion that as a result of irregular growth certain cells are pressed and become damaged. From such cells enzymes will penetrate into the culture medium. In his survey on the physiology of plant tissue cultures (GAUTHERET, 1955), however, the point is no longer stressed. It is our opinion that it will be very difficult to exclude the possibility of accidental penetration of enzymes from damaged cells. The results reported by Brakke and Nickell, however, are at least an indication that real secretion can be very important. The problem of true secretion of enzymes by tissue cultures is also discussed by REINERT, SCHRAUDOLF and TAZAWA (1957).

Another question is the "reasonableness" of the presence of amylase in culture media. This cannot be discussed before the presence of notable quantities of amylases in culture media without starch is established. The experiments summarized in Table III make such presence very probable. The Wijsman plate method was used to settle this point. Cylinders of starch or glucose-containing culture media on which tissues had been cultivated were put onto starch-containing agar plates. At the end of equal periods of incubation, identical and positive results as to the presence of amylases were obtained. This result makes such "reasonableness" very doubtful. This phenomenon was described long ago in the microbiological literature, among others for *Aspergillus niger* v. Tiegh. (FUNKE, 1922).

SUMMARY

Experiments on the possibility of cultivating tissue cultures on media containing starch instead of glucose have led to starch strains derived from a crown-gall strain of *Nicotiana tabacum* and from a normal cambium strain of *Rubus fruticosus*. Started in 1953, to date 42 and 28 passages respectively could be accomplished by uninterrupted cultivation on starch media. A substrain of *Nicotiana* on starch medium has been cultivated in darkness through 31 passages. Cultivation on starch media could be accomplished because of the activity of amylases given off by the cultivated tissues. The presence of an α-amylase and possibly a β-amylase could be established. The phenomenon has been described that growth habits on glucose

and starch media are strikingly different. Transfer back to glucose medium results sooner or later in the restoration of the glucose growth habit. In a separate paper, the changes in growth habit, the observed changes in growth pattern, and particulars about the internal structure will be discussed. Biochemical aspects of the phenomenon described are under investigation.

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