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GENERAL APPEARANCE, GROWTH PATTERN, AND ANATOMICAL STRUCTURE OF CROWN-GALL TISSUE OF NICOTIANA TABACUM L. GROWN IN VITRO ON CULTURE MEDIA CONTAINING GLUCOSE OR SOLUBLE STARCH AS A CARBON SOURCE

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INTRODUCTION

A recent publication (KARSTENS and DE MEESTER–MANGER CATS, 1960) described the observation that substitution of glucose as a carbon source for soluble starch led to a striking alteration in the general appearance of the tissue cultures involved. This phenomenon was observed in a crown-gall strain of *Nicotiana tabacum* L. and in a strain derived from "cambium" (BAILEY, 1943) of *Rubus fruticosus* L. As to the origin of the strains used, that of tobacco was originally isolated by MOREL (1948), while the *Rubus*-strain was received in 1949 by the courtesy of Professor A. J. P. Oort who in 1948 isolated it in Gautheret's laboratory.

The present publication gives details concerning the external shape, growth pattern, and internal structure of tissue cultures of tobacco crown-gall. In addition to observations on tissues grown on glucose and starch media under the influence of light, the same experiments were done in total darkness. Particulars about conditions of culture can be found in the publication by KARSTENS and DE MEESTER–MANGER CATS (1960). In addition, some details will be given concerning the occurence of certain growth types in strains of *Cissus spec.*, *Crataegus monogyna* Jacq., *Daucus carota* L., *Rubus fruticosus* L., (KARSTENS and DE MEESTER–MANGER CATS (1960)) and of *Helianthus tuberosus* L. The strain of *Helianthus tuberosus* was derived from tubers of the cultivar White Jerusalem Artichoke. Details about the origin of the other strains can be found in the publication by Karstens and De Meester–Manger Cats. These are all normal strains and, therefore, to be cultivated on auxin-containing media.

It is remarkable that there is so little in the literature about how the general shape of tissue cultures comes into being, the more so because of the great and interesting variety in shape and growth habit. GAUTHERET (1959, p. 313) describes callus grown from the original explants as generally dense and consisting of firm tissue. Subcultured, spherical, hemi-spherical, or somewhat flattened colonies develop. This fleshy type may be maintained in the course of succes-

sive subcultures but quite often sooner or later other types develop. Gautheret distinguishes lamellar, compound, and discontinuous types of tissue cultures. In his conclusions on this subject (p. 344) the author states that "Les caractères des colonies tissulaires sont à la fois si nets et si variés qu'on pourrait être tenté de les utiliser pour établir une véritable classification des cultures *in vitro*." Only in a few cases has the development in shape of tissue cultures from the cubical fragments cut from the mass of tissue of the former "generation" been followed step by step (CAPLIN, 1947). More often, casual remarks base on "anatomical snapshots" are encountered.

GENERAL APPEARANCE AND SHAPE OF TISSUE CULTURES

In order to obtain callus formation in vitro from explants and subculture the newly-formed tissues to establish tissue cultures, media containing glucose or saccharose are nearly always used. This is done because of the simple fact that growth on glucose media, and less often, on media with saccharose, proved to be satisfactory. It is comprehensible, but in fact not reasonable, that there is a tendency to call "normal" all those phenomena which can be observed in tissue cultures grown on one or the other of these two media. The present writers will try to avoid the use of this term.

As has been mentioned in the introduction, crown-gall tissue of *Nicotiana* was cultivated under four different sets of conditions relating to the carbon source used and in relation to cultivation in darkness or in the light. Otherwise the conditions were identical. Plate 1*a* shows the difference in general appearance of glucose and starch cultures while details are presented in Table I.

It seems hardly necessary to comment on the details given in Table I. It is evident that the cultures on glucose media in both light and dark differ in nearly all respects from the starch types. The most important difference between the two starch types lies in the presence of considerable quantities of chlorophyll in the starch-light cultures.

The differences in general appearance, shape of the cultures, etc. were of considerable interest. Since microscopical examination of directional sections cut from tissue cultures is insufficient for an understanding of the development of such cultures, the authors have tried to obtain more information by another method. For this purpose, cubical fragments cut from cultures in the same way as is done for making subcultures were dusted on all sides with finely powdered sterilized charcoal before being put on fresh culture medium. The way in which the fragments develop into their specific shape can be followed to a certain extent with the aid of the charcoal particles. For instance, in certain strains, as growth proceeds the particles become dispersed specifically, more or less evenly, over the expanding surface of the culture. From sections made either by hand or by means of the usual microtechnical methods, the distribution of the charcoal particles can be easily studied. Using this method, several characteristic growth types could be established, some of which are represented in Fig. 1.

TABLE 1

CULTURE MEDIUM	LIGHT or DARKNESS	SHAPE of the CULTURE	GROWTH in relation to SUBSTRATE	COLOUR	CONSISTENCY
glucose 2 %	light	hemispherical	underside flat, culture on top of substrate	nearly white, opaque, downy	firm, massive
glucose 2 %	darkness	somewhat flattened	underside flat, culture on top of substrate	dirty white, opaque, somewhat fluffy	firm, massive
soluble starch 2 %	light	irregularly lobed	underside irregular, penetrating into substrate	variously shaded grass-green, somewhat translucent, fluffy	juicy, somewhat friable; cavities may be present
soluble starch 2 %	darkness	irregularly lobed	underside irregular, penetrating into substrate	pale brownish yellow, somewhat translucent, fluffy	very juicy and soft; cavities

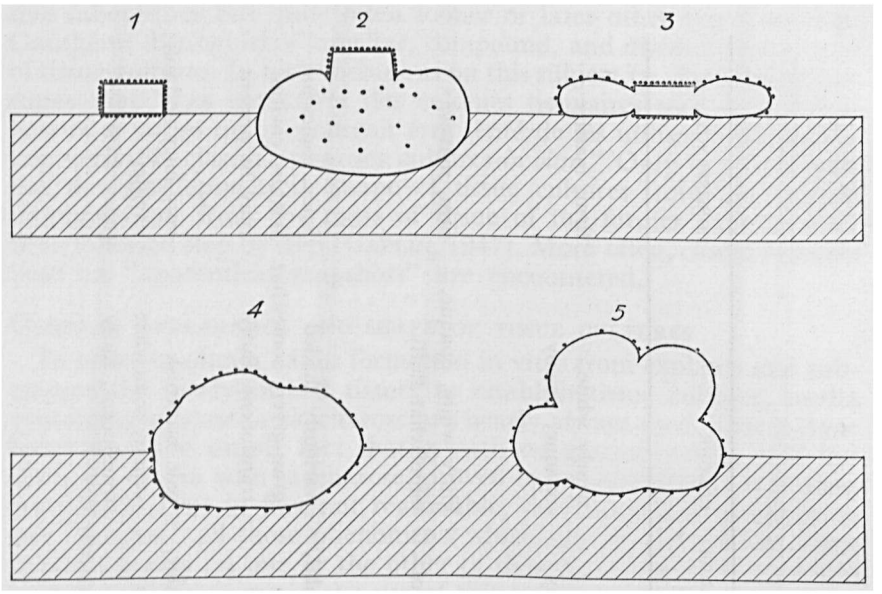

Fig. 1. Schematical representation of growth types. (median sections). The black dots represent charcoal particles with which the initial fragment of the culture was dusted. Culture medium hatched. 1, initial fragment; 2, *Rubus fruticosus*; 3, *Cissus spec.*; 4, *Helianthus tuberosus*; 5, *Daucus carota.*

Certain strains such as those derived from *Rubus fruticosus* L., *Crataegus monogyna* Jacq., and *Cissus spec.* always exhibit the initial fragment of the culture in some way or another. From Fig. 1, Nos. 1, 2, and 3, it is evident that growth can progress along very different lines. In *Rubus*, (Fig. 1, 2.) for instance, the principal growth takes place at the lower surface, while in *Cissus* (Fig. 1, 3.) development is always found along the sides. As a result, the cultures of *Cissus* grow on top of the culture medium, those of *Rubus* partialy penetrate into the substrate. A third difference lies in the fact that in the case of *Cissus* the original fragment always becomes necrotic, while that of *Rubus* remains alive and capable of growth. A fourth difference between the two strains consists of the distribution of the charcoal particles. In the case of *Cissus*, the particles are found at the outside of the growing and non-growing parts of the culture, whereas in *Rubus* the charcoal particles are found not only on the outside of the non-growing part of the culture but also along radiating lines in the interior of the growing part of the culture, starting at the site of the former lower surface of the original fragment. The observations made on *Cissus*-cultures need not to be further discussed since nothing unusual was seen. The case of *Rubus*, however, is much more interesting. The observed distribution of the charcoal particles must develop in such a way that some of the particles are grown over very soon while the rest are pushed forward by growing tissue and are only gradually grown over. This is of interest because it means that the

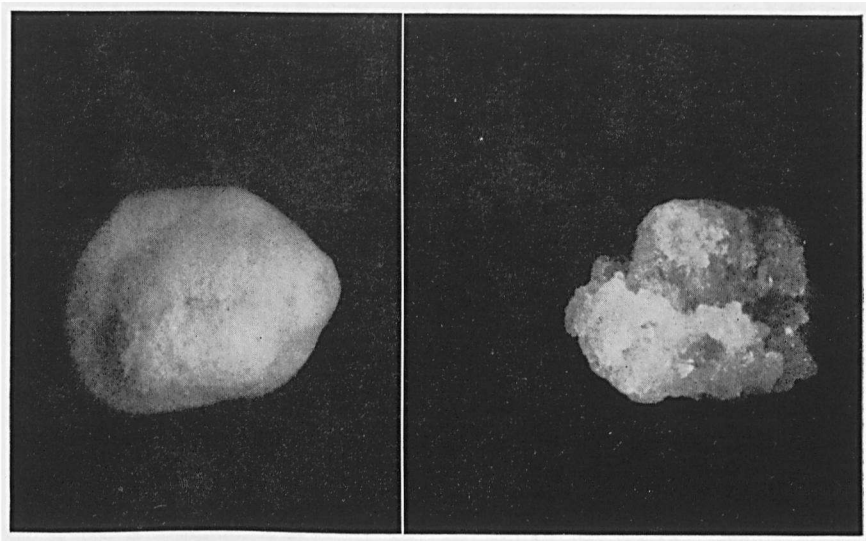

Plate 1*a*. *Nicotiana tabacum*. Crown-gall. Tissue cultures seen from above. To the left, a typical cushion-shaped tissue culture grown on glucose medium; to the right, a culture on starch medium exhibiting the characteristic knotty, somewhat translucent, appearance. (1.5 ×).

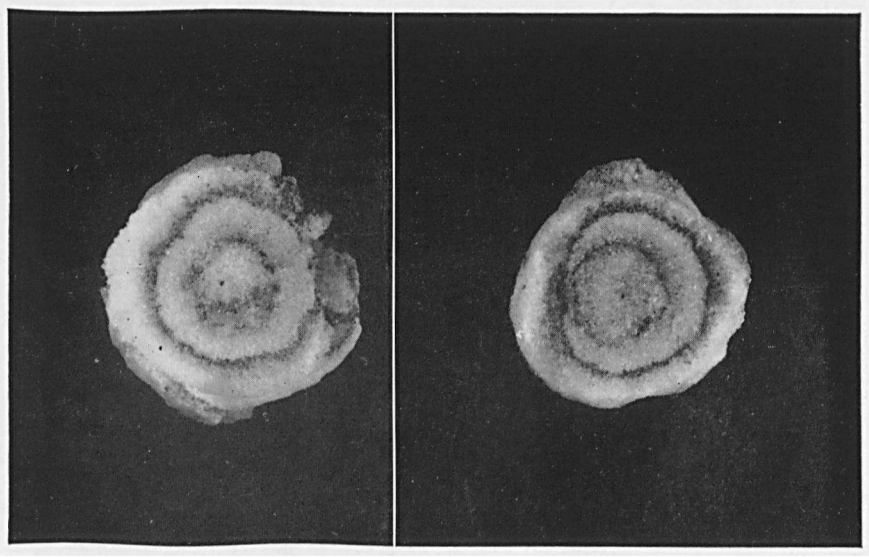

Plate 1*b*. *Nicotiana tabacum*. Crown-gall. Transversely cut cultures derived from cylindrical samples dusted with charcoal. After 26 days of cultivation a second dusting was applied. The photographs were taken 40 days after the second charcoal treatment. Two black zones can be distinguished. In the centre of each culture the little hole left by the stainless steel hook used to handle the material can be seen as a black point. (1.5 ×).

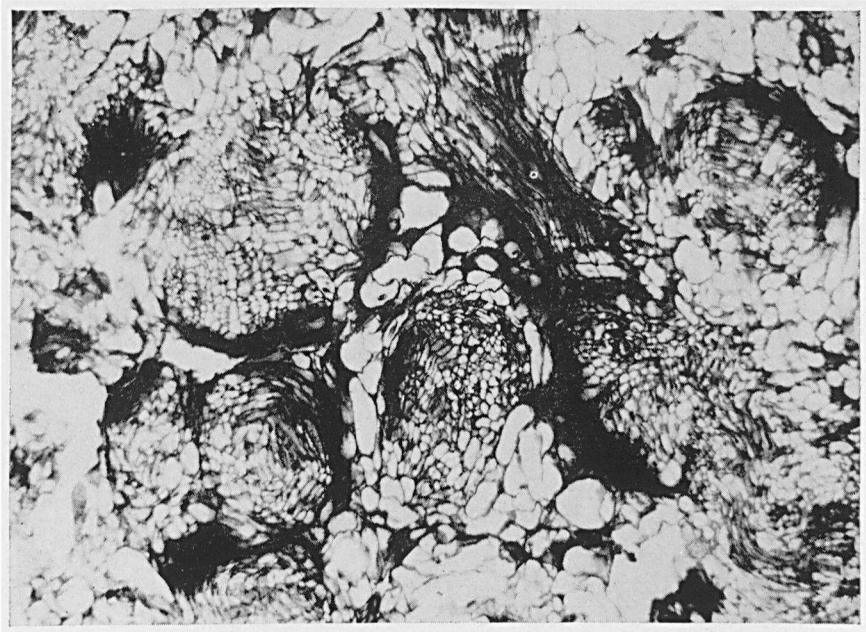

Plate 2*a*. *Nicotiana tabacum*. Crown-gall. Longitudinal section through a culture grown for about eight weeks on glucose medium in the light. The picture shows part of the initial fragment with vascular nodules and a strand of vascular tissue amidst parenchyma. The dark areas represent crushed cell masses. (30 ×)

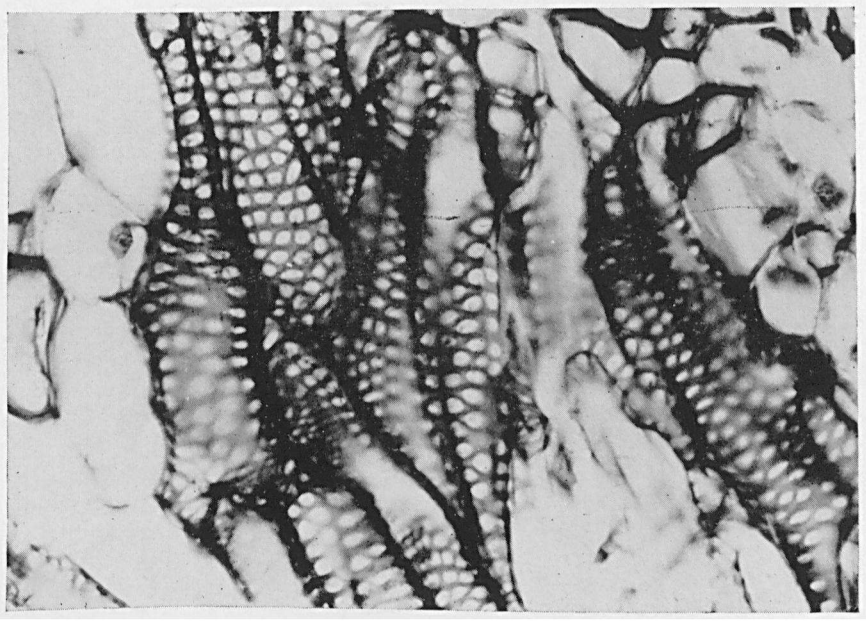

Plate 2*b*. *Nicotiana tabacum*. Crown-gall. Part of a vascular strand like that of Plate 2*a*, showing lignified reticular elements. (500 ×).

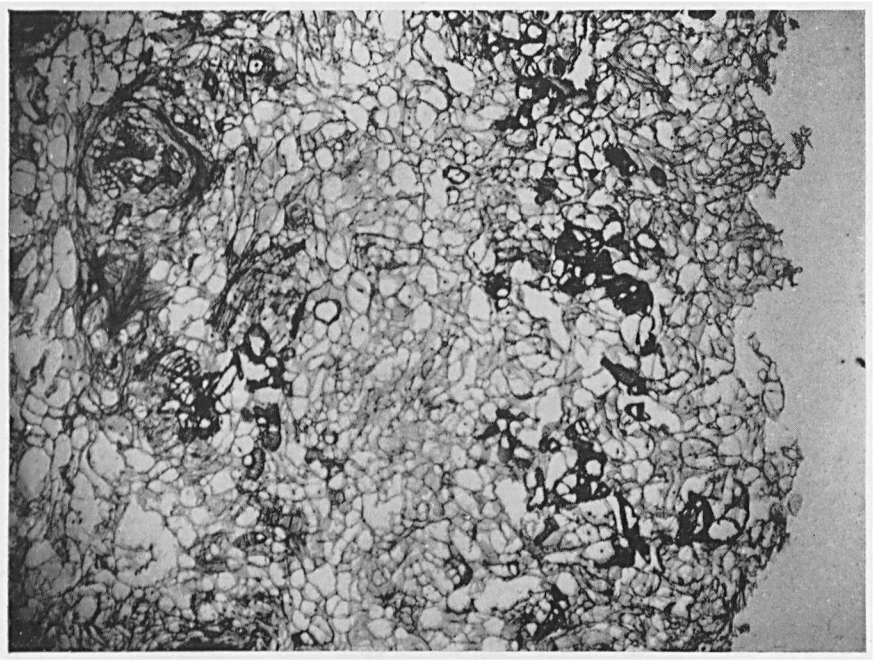

Plate 3*a*. *Nicotiana tabacum*. Crown-gall. Longitudinal section through a culture grown on glucose medium for about eight weeks in the light. To the left a part of the initial fragment with vascular nodules can be seen. All the rest in newly-formed tissue with scattered lignified elements. To the right the margin of the culture is visible. (30 ×).

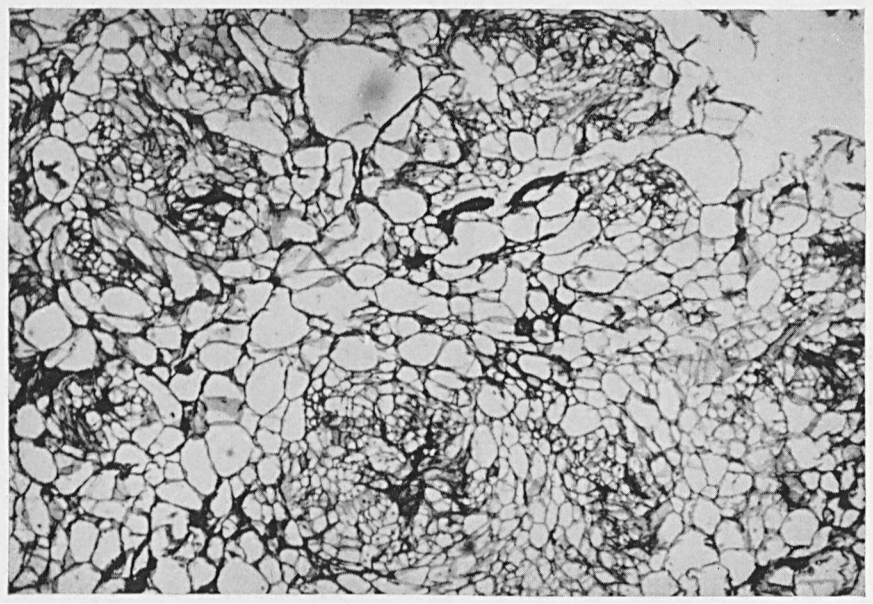

Plate 3*b*. *Nicotiana tabacum*. Crown-gall. Longitudina lsection through a culture grown for about six weeks on starch medium in the light. Among the large-celled parenchyma, scattered nodules formed by meristematic zones are present. Only a few lignified elements can be observed. (43 ×).

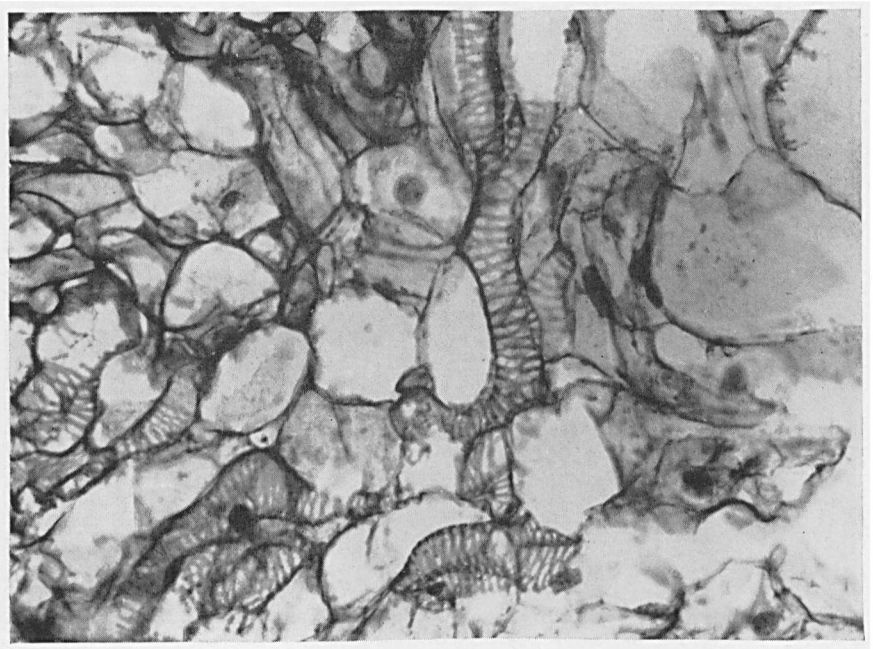

Plate 4*a*. *Nicotiana tabacum*. Crown-gall. Longitudinal section through a culture grown for about six weeks on starch medium in the light. Lignified reticular sometimes more or less scalariform elements. (300 ×).

Plate 4*b*. *Nicotiana tabacum*. Crown-gall. Longitudinal section through a culture grown for about six weeks on starch medium in the dark. The picture shows some lignified elements with the delicate, spiral bands characteristic for this tissue culture grown under the conditions described. (300 ×).

cells of the growing part of the culture do not form a closed front but are able to grow round obstacles.

Totally different growth types are exhibited by the strains of *Helianthus tuberosus* L. and *Daucus carota* L. Both were derived from the tuberous parts of the plants. It should be mentioned here that at the time of the experiments the strains were still of the fleshy type. Later, especially in the case of *Helianthus*, the strains changed more and more into the compound type. Such transformations have been observed before (see GAUTHERET, 1959, p. 319.) The growth types of these two strains are characterized by the pecularity that the original tissue fragment cannot be found in the tissue culture derived from it. (Fig. 1, nos 4 and 5). From the charcoal experiments it appeared that the charcoal particles become more or less uniformly dispersed on the expanding surface of the tissue culture. It is self-evident that the dispersion is greatest on the surface of the fastest growing parts of the cultures. The distribution of the charcoal particles all over the surface of the cultures is caused by the fact that the particles do not become enclosed: in this type of culture, therefore, the peripheral cells form a closed front. Apart from these similarities the strains differ in that the carrot cultures become more or less lobed, while the *Heliantus*-cultures expand more evenly.

The same charcoal method was used to study the growth pattern of the tissue cultures of tobacco crown-gall cultivated under the four

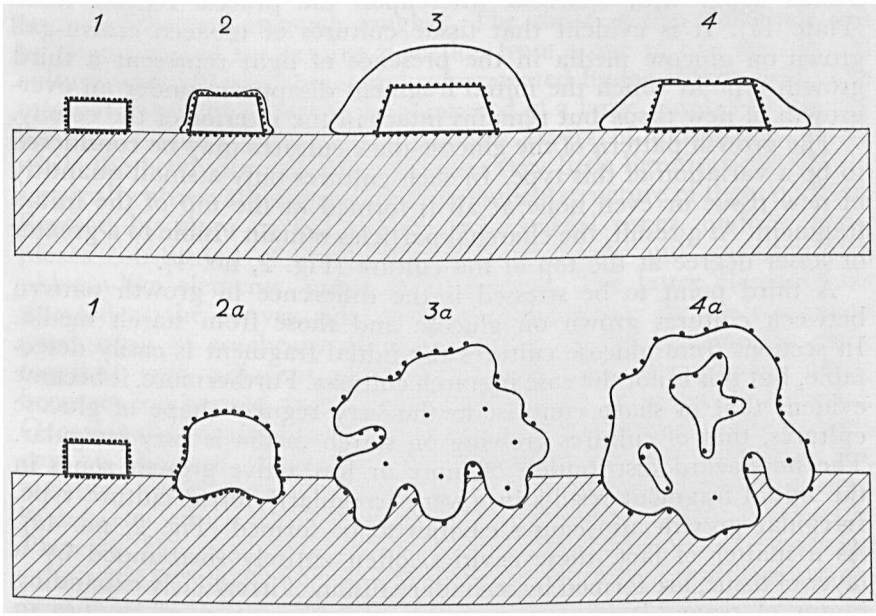

Fig. 2. Schematical representation of the differences in growth type of tobacco crown-gall cultivated *in vitro* (median sections). The black dots represent charcoal particles. Culture medium hatched. 1, initial fragment; 2, and 2*a*, young cultures on glucose and on starch media; 3, glucose-light culture; 4, glucose-dark culture; 3*a*, starch-light culture; 4*a*, starch-dark culture.

sets of conditions described above. The results are given somewhat schematically in Fig. 2.

The first conclusion to be drawn is at the same time a confirmation of the impression obtained by studying a vertical section from a culture grown on a glucose medium in the presence of light: such a section consists of a trapezium shaped area with a paler-coloured border along three sides, creating the impression that the darker area represents the original fragment while the border consists of newly formed tissue. The charcoal experiments leave no doubt that this is indeed the case. The layer of charcoal particles enveloping the initial fragment, with the exception of the lower surface, is broken on all sides by newly-formed peripheral cells growing around the charcoal particles which become, finally, wholy grown over. It must be emphasized that no charcoal particles are carried away, as was observed in the experiments with *Rubus* cultures; they remain together in the form of a flat black layer.

Another point of interest is the observation that the volume of the initial fragment as indicated by the coat of charcoal increases in size (Fig. 2, nos 2 and 3). In the section on internal structure more will be said about this point. During the development described, the charcoal particles disappear more and more under a layer of newly-formed tissue with the result that blackened initial fragments turn into white tissue cultures (Fig. 2, nos 1–3). These in turn can be dusted again with charcoal whereupon the process repeats itself. (Plate 1*b*). It is evident that tissue cultures of tobacco crown-gall grown on glucose media in the presence of light represent a third growth type in which the initial fragment disappears under an overgrowth of new tissue but remains intact in the interior of the colony.

The growth pattern of the glucose-dark cultures may be considered to be a variation of this type. In such cultures only a small quantitiy of new tissue or even none at all is formed at the top of the initial fragment. As a result, the charcoal particles remain visible to a greater or lesser degree at the top of the culture (Fig. 2, no. 4).

A third point to be stressed is the difference in growth pattern between cultures grown on glucose and those from starch media. In sections from glucose cultures the initial fragment is easily detectable, but this is not the case in starch cultures. Furthermore, it became evident that in sharp contrast to the very regular shape of glucose cultures, that of cultures growing on starch media is very irregular. The haphazard distribution of more or less active growth zones in the initial fragment results in a very irregularly lobed culture type. Irregular growth also causes cavities to be formed (Fig. 2, no. 4*a*). As a matter of fact, these cavities, often entirely surrounded by a layer of tissue, are formed by secondary fusion of irregularly expanding lumps of tissue. It is our impression that formation of cavities in cultures on starch media cultivated in light becomes increasingly evident. In early subcultures this tendency was much less in evidence. In dark cultures on starch medium, cavities were observed from the beginning. This irregular growth can be followed fairly well by

studying the distribution of charcoal particles. The haphazard distribution on the outside of the culture, in the interior of dense parts of the cultures, and enclosed in cavities, is only possible with a very irregular type of growth pattern.

ANATOMICAL DATA

It should be kept in mind in studying the internal structure of tissue cultures, that somewhere among areas of newly-formed tissue these cultures contain, either in concentrated or in dispersed form, remnants of the initial fragment. Furthermore, the anatomical structure of the initial fragment varies with the age of the subculture from which it originates and may vary according to its site in the mother culture. These considerations are based on the observation that the anatomical structure of a tissue culture undergoes a series of changes during each period of growth, from initial fragment to tissue culture ripe for the next subculture.

a. *The anatomical structure of glucose-cultures*

Attention must be drawn anew to the fact that in fresh and unstained sections of cultures grown on media with glucose as a carbon source, in both light and dark, the initial fragment and the newly-formed tissue are macroscopically easily distinguishable. Apart from a difference in colour, the consistency of the two parts is very different. The newly-formed tissue is firm but very easily cut, while the former original fragment is much tougher. The cause of this difference can be demonstrated by treating a section from a one to two months old culture with phloroglucinol and concentrated hydro-chloric acid. The original fragment proves to be composed of a large number of heavily lignified nodules separated by narrow layers of unlignified tissue, while the newly-formed tissue exhibits very little lignification. Microscopically observation reveals the anatomical structure of these nodules. Formed by more or less cup-shaped cambial zones, nodular complexes constituted of neatly arranged tracheary and probably also phloem-like elements, prove to be present. The latter elements are situated at the convex side of the cambial zones. By the continued production of tracheary elements, many of the phloem-like cells, together with adjacent parenchyma-cells from the ground tissue, become compressed into cup-shaped masses of cell-wall material. Occasionally, strand-like formations connecting several of the nodules can be observed (Plate 2*a*). Part of such a strand, exhibiting the reticular lignification characteristic of all tracheary elements present in tobacco crown-gall cultures grown on glucose media is given in Plate 2*b*. The newly-formed tissue consists mainly of parenchymatous cells with few scattered lignified reticular elements, sometimes gathered in small groups. (Plate 3*a*). There appear to be present concentric zones with more or less lignified elements.

b. *The anatomy of cultures grown on starch media*

In a preceding section it was mentioned that in tobacco crown-

gall cultures grown on media with soluble starch as a carbon source, in the light as well as in darkness, the initial fragment becomes dispersed all through the culture. Therefore, the initial tissue and newly-formed tissue cannot be distinguished. It is possible, however, that those parts which contain lignified elements in larger groups are parts of the initial fragments. Tobacco crown-gall tissue cultures grown on starch media under the influence of light or without any light at all have, apart from a few points of difference, the same anatomical constitution. In both cases a loosely built and very disorderly structure can be observed, consisting for the most part of parenchyma. Areas consisting of smaller cells are found amidst others characterized by the presence of much larger cells. In both light and dark cultures, cells of a diameter of 200μ can be often found. Cells of such a diameter are only rarely found in cultures from glucose media. As a matter of fact, the average diameter of cells from cultures of glucose media is much smaller. The small-celled areas in starch cultures can be considered to be of a meristematic character. Scattered throughout the tissue culture, lignified tracheid-like elements, solitary

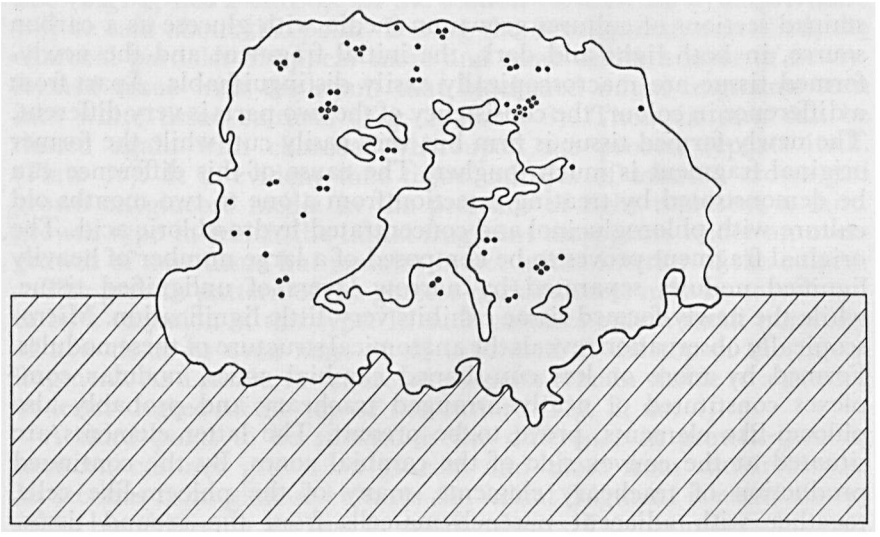

Fig. 3. Median section of a starch-dark culture of tobacco crown-gall. Lacunar growth type. Culture medium hatched. Each black dot indicates the location of a lignified element.

or in small groups, are present (Fig. 3). The observed structure is very much like that described by WHITE (1939) for his famous tissue culture derived from so-called genetical tumours of the hybrid between *Nicotiana glauca* ♀ and *N. langsdorffi* ♂. Here too, areas consisting of small cells alternate with large-celled zones. "It is not possible", according to White "to identify phloem, cambium, phelloderm, sclerenchyma, nor any other normal cell type except parenchyma, meristem, and an occasional isolated scalariform cell". Still, there

are some points of difference with our starch cultures. Starch cultures grown in the presence of light exhibit somewhat more differentiation because of the rare presence of formations comparable with the heavily lignified nodules in the initial fragment of glucose cultures (Plate 3*b*). However, lignification in this case is restricted to some few elements in the centre. In starch-dark cultures such formations have never been encountered.

White described the presence of scattered scalariform elements. In our starch cultures grown in the presence of light the lignification pattern is nearly always reticular. In addition to more or less rounded cells, much elongated tracheid-like elements are present (Plate 4*a*). Apart from the fact that such elongated elements are extremely rare in starch cultures grown in darkness, the lignification pattern is also quite different. In starch-dark cultures lignification takes place to a lesser degree and is nearly always present in the form of fine spiral bands (Plate 4*b*). Reticular elements are only rarely encountered. As far as the present authors are aware, the type of lignification described here is very seldom found in the literature concerning plant tissue culture. MUIR, HILDEBRANDT and RIKER (1958) present some illustrations of tracheid-like cells from a marigold tissue culture (*Tagetes erecta* L, var. "Sunset Giant") which clearly exhibit the same lignification pattern. From the description and illustrations, the marigold tissue culture seems to exhibit many points of resemblance with our tobacco culture cultivated on starch media in the dark. As a matter of fact, both tissue cultures are derived from crown-gall, both are cultivated in the dark, and both appear to have the same juicy consistency.

DISCUSSION

The anatomical study of crown-gall tissue cultures of tobacco grown on media with glucose or starch as a carbon source provides information that agrees very well with previously published results (KARSTENS and DE MEESTER-MANGER CATS, 1960). In that publication, attention was drawn to the fact that dry weight as a percentage of fresh weight in tobacco grown-gall tissue and in cambial tissue of *Rubus fruticosus* L. cultivated in vitro shows a much higher value on glucose media than on media with soluble starch as a carbon source. The data pertinent to the present publication are presented in Table II.

TABLE II

GROWTH CONDITIONS	DAYS OF CULTIVATION	DRY WEIGHT AS A PERCENTAGE OF FRESH WEIGHT	
		at the beginning of the experiment	at the end of the experiment
glucose 2 %, light	71	4.8 (4.4–5.4)	6.5 (5.9–8.0)
glucose 2 %, dark	71	6.3 (5.4–7.0)	7.2 (5.6–8.0)
soluble starch 2 %, light	49	2.6 (2.1–2.8)	2.4 (1.9–3.1)
soluble starch 2 %, dark	49	2.6 (2.1–2.9)	2.6 (2.1–3.2)

It is evident that the very low dry weight value on starch medium is caused by two factors. In the first place, tissues grown on starch media are composed of larger cells with a relatively higher water content. Secondly, the number of cells with thin cell-walls is much larger because the amount of lignification on these media is almost negligible.

Another interesting point is the difference in the percentage of dry weight at the beginning and at the end of the experiment for cultivation on glucose media. On starch media, in strong contrast, the percentages of dry weight of the tissues at the beginning and at the end of the culture period are identical.

These results fit in with the anatomical features observed. In the case of the starch cultures the initial fragment "dissolves" into the new tissue culture. Each fragment cut from such a culture has the same dry weight/fresh weight ratio, because the anatomical structure is statistically uniform throughout. Thus, the initial fragment has the same ratio as the whole culture from which the fragment is derived. Since the pattern of development is maintained during each subculture, the culture present at the end of a new culture period will possess the same properties as its predecessor.

The situation with the cultures on glucose media is totally different. Here, the initial fragment does not "dissolve" into the new culture. On the contrary, it follows a differentiation pattern of its own and exhibits considerable change through the formation of large amounts of lignified cells. The new culture, therefore, does not possess a uniform internal structure, but is composed of two areas of different character, i.e. the former initial fragment containing many lignified elements and a peripheral zone of newly-formed tissue with fewer, less lignified elements. Since the initial fragment is preferably cut from newly-formed tissue, it is obvious that the formation of large amounts of heavily lignified cells in this part of the new culture results in a higher dry weight/fresh weight ratio. The differences between glucose-light and glucose-dark cultures very probably depend on the fact that in the latter case, the zone of newly-formed tissue is thinner, so much thinner that it is not really possible to make subcultures from fragments consisting exclusively of newly-formed tissue. As a result, part of the initial fragment of the former generation will be present in the initial fragment of the new culture. This probably accounts for the higher initial dry weight/fresh weight ratio of the glucose-dark cultures. Finally, the changes which take place in the initial fragment, combined with the relatively poor development of new tissue, account for the higher ratio in "full-grown" glucose-dark cultures over glucose-light ones.

SUMMARY

Substitution of glucose as a carbon source for soluble starch can lead to a striking alteration in the general appearance of tissue cultures. This phenomenon was observed in tobacco crown-gall tissue and in a strain derived from the cambial zone of twigs of *Rubus fruticosus* L. (KARSTENS and DE MEESTER–MANGER CATS, 1960).

In the present paper details are given on the general appearance, growth pattern, and anatomical structure of tobacco crown-gall tissue cultures. In Table I differences of shape, colour, consistency, and manner of growth in relation to external conditions have been summarized. A method is described by which it is possible to study the growth pattern of tissue cultures: initial fragments or young tissue cultures are dusted with powdered charcoal under sterile conditions. After growth has taken place, the localization of the charcoal particles can be studied macroscopically, or microscopically in sections prepared according to routine microtechnical methods. Besides the tobacco cultures on glucose and starch media under varied external conditions, a number of other tissue cultures were tested. As a result, several growth types could be distinguished.

It is a matter of some interest that the tissue cultures of tobacco crown-gall on glucose and starch media possess totally different growth habits. In the glucose cultures the initial fragment remains intact and exhibits a very special differentiation while in the starch cultures the initial fragment "dissolves" into the growing culture. In glucose cultures new growth is strictly localized, i.e. in a meristematic zone formed at the outer surface of the initial fragment, the side in contact with the culture medium being excepted. In starch cultures, however, growth takes place by the activity of scattered meristematic loci present in the initial fragment and afterwards in the whole tissue culture. The initial fragments in glucose cultures, as mentioned before, exhibit a very special differentiation. In the initial fragment a great number of cup-shaped meristematic zones are initiated and give rise to complexes of heavily lignified cells. In the newly-formed tissue lignification takes place to a much lesser extent and in such a way that lignified elements are sparsely present in otherwise parenchymatic tissue.

In starch cultures cup-shaped meristems occur very rarely. They can only be found in light cultures. In addition, few lignified elements are formed by those meristems. On the whole, starch cultures grown either in the light or in the dark have a parenchymatic character with few and very scattered lignified elements. Finally, the lignification pattern of the lignified elements proved to be different in glucose and starch cultures. The anatomical features agree very well with the figures on dry weight/fresh weight ratios given in a previous paper (KARSTENS and DE MEESTER-MANGER CATS, 1960) and summarized in Table II.

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