SPIRAL GROWTH AND SPIRAL STRUCTURE

III. WALL STRUCTURE IN THE GROWTH ZONE OF *PHYCOMYCES*

by

MAVIS J. MIDDLEBROOK AND R. D. PRESTON

Biophysics Subdepartment, Department of Botany, The University, Leeds (England)

INTRODUCTION

Spiral, or more strictly helical, structure has long been recognised as widespread in the elongated cells of plants and a good deal is now known concerning the details of structure in many different organisms. Thus the cellulose microfibrils are known to run spirally round the cell in cambial initials (PRESTON AND WARDROP[1]), conifer tracheids (see *e.g.* FREY-WYSSLING[2]; PRESTON[3,4,5]; WARDROP AND PRESTON[6]), vessels (PRESTON[7], phloem fibres (KUNDU AND PRESTON[8]; PRESTON[9]), sisal fibres (PRESTON AND MIDDLEBROOK[11]), bamboo (PRESTON AND SINGH[12]), and in a number of algae (ASTBURY AND PRESTON[13], PRESTON AND NICOLAI[14]) and in other cells. Any association of growth phenomena with spiral structure in isolated cells cannot therefore fail to have implications which may prove rather general. In the first paper of this series (PRESTON[15]) an attempt was made to give a quantitative expression of one possible way in which spiral growth in sporangiophores of *Phycomyces* might be associated with spiral structure in the growth zone, and in the second (PRESTON[16]), an explanation, again quantitative, was attempted of the phenomenon of spiral grain in conifers in terms of the structure of cambial cell walls. The statements made in the first paper involved a number of assumptions, and led to an expression of the course of spiral growth which was satisfactory in a quantitative sense as far as the data at that time available were concerned. During the past five years work has been in progress to test the validity of the assumptions made, to obtain further data against which the expression could be checked and to attempt a verification of some of the predictions to which it led.

One of these predictions has already been discussed elsewhere (PRESTON AND MIDDLEBROOK[10]). Among the assumptions made in the first paper the most serious was that the wall of the growth zone in the sporangiophore could be considered as composed of a series of "microfibrils" forming flat left-hand spirals round the wall, and it was found that an angle of some 12° between the spiral winding and the transverse—about the angle found in cambial cells—would fit adequately in a quantitative sense. In view of the criticisms of FREY-WYSSLING AND MÜHLETHALER[17,18] it should be emphasised that it was made clear even at that time that it was not at all necessary—nor was it envisaged—that the real microfibrils in the wall should be exactly organised in such a

References p. 48.

spiral. On the contrary, it was explicitly stated that these "*hypothetical*" fibrils were considered to have physical properties dependent upon the dispersion of the *real* microfibrils about this spiral direction. In other words the assumption was made only that the wall organisation was such as to make, for instance, the vector of maximum Young's Modulus spirally arranged round the cell. If then, the *hypothetical* fibrils turn out to be closely similar to the *real* set, which FREY-WYSSLING AND MÜHLETHALER[17,18] but not ROELOFSEN[19] doubt, so much the better. It is with this assumption concerning wall structure that the present paper is concerned.

Since the earlier paper was written, ROELOFSEN, who was jointly responsible for early work on *Phycomyces* wall structure (OORT AND ROELOFSEN[31]), while admitting that the expression which was derived did fit all the known facts, has attempted an explanation of different type and has tried to support it by a series of determinations of wall structure using studies under the polarising microscope and in the electron microscope ROELOFSEN[19,20,21]). Since the present paper is concerned only with structure, the structural side only of ROELOFSEN's work will be discussed here; his other criticisms of our attitude will be dealt with in later papers.

In view of the fact that ROELOFSEN's treatment of spiral growth demands a right-hand structural spiral in the growth zone, whereas ours demands a left-hand spiral, it is interesting first to note that ROELOFSEN reaches the conclusion that the microfibrils in the growth zone do indeed tend to be in a flat spiral but that this is right-handed (Z*) and not left-handed (S). There is, however, serious reason to question whether his observations can be reconciled with such a view, and it will be well to discuss the points raised by ROELOFSEN's work before proceeding to a statement of our own.

In a series of investigations on single wall fragments from the growth zone of *Phycomyces* sporangiophores, ROELOFSEN[19] measured the angle between the major extinction position and the cell axis as a value for the angle of orientation of the chitin fibrils known to be present in the walls. Before observation, the material had been cleaned in 5% NaOH and then stained in Congo red solution before mounting in glycerol. This procedure was adopted in order to increase the order of the birefringence. After measurement, some of these cells were subjected to hot 5% HCl and this led to a complete change in the major extinction position; from left-hand or right-hand spirals to transverse, or in one case even from left to right-hand spiral. Only one case examined retained its original spiral direction after treatment with acid.

The work of CASTLE[22] has already indicated the undesirable complications which arise when Congo red is used in this way. In cells treated by him using Congo red and KOH in cold aqueous solution, bright red streaks appeared and the birefringence was changed from negative to positive in sign. On heating the same cell, the stain showed up very much more clearly and the birefringence once more became negative. This was attributed by CASTLE to the presence of interfibrillar material, of possibly protein nature, which was axially oriented. Whatever the reason, however, this change in sign of birefringence after cold, then hot Congo red and alkali, indicates that care should be taken in interpreting wall appearances after such treatment.

The single wall fragments used by ROELOFSEN gave varying results. Out of 60 cells

* Throughout ROELOFSEN's papers, the spirals are referred to as S- and Z-spirals, this being the nomenclature of the American textile industry, which corresponds to our left-hand and right-hand spirals respectively. Our terminology follows the direction of right- and left-hand screws, as does that of CASTLE in his publications.

References p. 48.

examined, 31 gave "transverse" extinction, *i.e.* less than 3° from the transverse position, 16 cells showed left-hand spirals (average 6°), and 13 right-hand spirals (average 4°). From these data, ROELOFSEN concludes that right and left-hand spirals are about equally frequent in occurrence, but says, "in view of the constant right-hand rotation of the cells in the stage investigated, this is not possible". For this reason, he repeated the determinations with other specimens, but similar results were obtained. He also puts on record one observation of some axial orientation, and this leads him to doubt his method of staining the cells.

These observations would seem to us to indicate, therefore, that the methods used by ROELOFSEN lack precision, and that all chemical treatments should be avoided. If his results mean anything they would indicate the occurrence of right-hand spirals and left-hand spirals at about equal frequency, but in spite of his own data, ROELOFSEN still concluded that in the turgid cells right-hand spirals must predominate. It is difficult to see how such a conclusion can possibly be upheld.

Apart from any purely chemical effect of these various treatments there is also, as pointed out by ROELOFSEN, the very considerable shrinkage of the cells to be taken into account. The shrinkage of cells on drying he estimates to be about 15% of the length of the turgid cell in the top 2 mm of the growth zone. (CASTLE found a value for this shrinkage of 6% in 1938.) The treatment with Congo red and NaOH produced another 10% shrinkage, and further treatment with HCl caused yet another 10% shrinkage. As ROELOFSEN himself points out, this would tend to flatten the spirals in the material examined. His "transverse" cells, therefore, might well have had spirals oriented at a considerable angle in the turgid condition. It cannot be said that this admittedly most careful work adds very much to our knowledge of the structure of the growth zone. In our work, therefore, and in spite of very considerable technical difficulties, we prefer to use only material subjected to no chemical treatment whatever.

In ROELOFSEN'S 1951 paper[19], a number of electron micrographs are reproduced of the wall of the growth zone showing clearly sets of fibrils of 150–200 A diameter, which are said to lie predominantly in the transverse direction though it should be remembered that the cell axis is not easily defined exactly in electron micrographs. As one progresses downwards from the tip of the growth zone basipetally, the fibrils are seen to be less well oriented. Transverse folds are conspicuous in some of the photographs, which ROELOFSEN attributes to shrinkage in the cell wall because of (a) loss of turgor (b) chemical purification (c) drying out of the cell wall.

A point to notice here, however, is that some of the specimens used in the electron microscope had been pretreated with 5% NaOH and Congo red. In view of the very considerable shrinkage in the wall during such treatments it would again seem desirable that these observations should be repeated on untreated cells. The subjection of the specimen to drying, shadowing, and bombardment by electrons is sufficiently dangerous to the specimen, without also exposing it to additional severe chemical treatments.

It should be mentioned here, that the sporangiophores used earlier by FREY-WYSSLING AND MÜHLETHALER[17,18] were subjected to chemical treatments even more severe than those used by ROELOFSEN, since these were boiled 4–5 times in 10% KOH and water, oxidised with $KMnO_4$, followed by disintegration in a Waring blendor. These workers found a difference in size between fibrils in the tip and basal regions of the sporangiophore, an observation which was not, however, substantiated by ROELOFSEN. They also found an unusual "tennis-racket" structure, of crossed fibrils, encountered in

References p. 48.

no other cell type, which they alleged came from the growth zone also. Again, ROELOFSEN finds no confirmation of this arrangement of wall constituents.

The observations presented in this paper represent an attempt to determine wall structure in the growth zone of sporangiophores *Phycomyces*, using material subjected only to such treatments as are necessary in handling the material.

MATERIAL AND METHODS

Cultures of *Phycomyces nitens* + and — were kindly supplied by Dr R. K. S. WOOD of Imperial College, London, to whom our thanks are due. They were grown on malt agar at 20° C, and when sporangiophores began to appear, constant illumination was supplied from above. The petri dishes in which the cultures were grown were kept in glass vessels almost submerged in water in a glass tank, the temperature being kept constant by a Sunvic thermostat.

(i) *X-ray methods*

For X-ray photographs of the adult wall, stage 4 sporangiophores were plucked and laid parallel to each other on a microscope slide, the bundle being allowed to dry without flattening. For the growth zone region this procedure could not be followed, since the thin, delicate wall crinkles badly when it dries. The following procedure was therefore adopted. The sporangium was carefully removed with a sharp razor as soon as the cell was taken from the agar, and the cell immediately pressed down between two glass plates, the top one being weighted. When such cells had dried, they were examined under a microscope and perfectly flattened cells only were retained. These were placed in a pile, care being taken that the tips and sides should coincide, and cemented together with Durofix at the base of the growth zone, the tips remaining free. The block was then placed over a slit 1 mm wide by 10 mm long in a brass plate of the dimensions of a microscope slide. This was mounted on an ordinary mechanical stage which was so fitted in the X-ray spectrometer that the X-ray beam could be passed through the bundle of sporangiophores at any chosen distance from the tip of the growth zone. In this way, a series of photographs was taken at various distances up the growth zone block. Photographs were taken on a flat film using CuKα radiation, with a collimator 0.5 mm in diameter, and with a film-specimen distance of 3 cm.

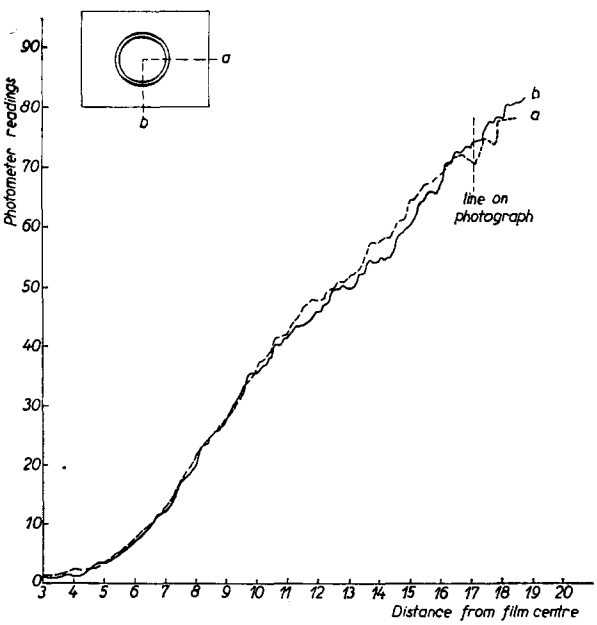


Fig. 1. Photometer readings across X-ray photograph of sporangiophore growth zone

(ii) *Optical methods*

Unlike the case of cellulose, the intrinsic birefringence of chitin is negative and this normally tends to cancel out the positive form birefringence so that the total effective birefringence of a chitinous cell wall is usually low. This makes for considerable difficulty in determining exactly the extinction direction and it is for this reason that previous workers (OORT AND ROELOFSEN[31], CASTLE[22], ROELOFSEN[21]) have chosen to increase the form birefringence by the addition of Congo red to the walls. In view of the undesirability of this procedure it was thought preferable to discard any such dangerous aids to measurement, but rather to follow SWANN AND MITCHISON[23] in an attempt to refine the methods for detection and measurement of low birefringence. As pointed out by SWANN AND MITCHISON, the minimum birefringence which can be detected and measured in a polarizing microscope depends, among other things, mainly on (a) the difference in intensity between the object and the ("dark") field when the nicols are crossed and (b) the general level of light intensity. To keep the field dark it is essential that internal reflections in the condenser, object

References p. 48.

carrier and objective should be reduced to a minimum. With the present method it was found sufficient to stop down the iris diaphragm severely, and to use immersion oil above and below the slide. The blooming of the lens components recommended by SWANN AND MITCHISON for extremely low birefringences was not necessary in this case. As regards intensity of illumination, and again as pointed out by SWANN AND MITCHISON, the minimum contrast which the eye can detect between object and field is lower the higher the general level of intensity. For this reason the usual microscope lamp is replaced by a carbon arc lamp, and this, together with the other precautions already mentioned, made it possible to determine the major extinction position on single walls of the growth zone without any treatment whatever.

The single walls were obtained by laying sporangiophores on a slide smeared with albumen fixative and allowing them to dry out at about 40° C. The albumen was hardened with alcohol and then the cell was scraped with a microtome knife so that patches of single wall were left. In the growth zone region it was sometimes found unnecessary to scrape the cell, because in removing the sporangium an oblique cut was sometimes made, so that a fragment of single wall was exposed, and this was easily measurable. For this reason it was possible to make a large number of determinations of the major extinction position in all regions of the sporangiophores.

RESULTS

(i) *X-ray investigations*

The adult wall of the sporangiophore, where secondary thickening is complete, yields a very highly oriented fibre diagram (Plate I, Fig. 2). Half the angle subtended by the equatorial arcs is about 9° and this gives therefore a rough (maximum) measure of the angle of inclination of the chitin chains to the cell axis. The spirals here therefore, are steeper than those found by HEYN[24], who gave a figure of 13.5° also obtained by X-ray methods. The two observations agree, however, in showing that the sporangiophore has chitin chains wound in a steep spiral in the secondary wall layers.

It was thought that the removal of protein from the wall should be attempted to ascertain their effect on the X-ray diagram. Therefore some fibre bundles were treated by immersion in a 0.1% papain and 1% sodium bisulphite solution, and kept in an incubator for 2 days at 40° C. After this, the bundle was washed and re-dried, the fibres being kept as straight as possible. A photograph is shown in Plate I, Fig. 3, and it is seen that the fibre diagram has been cleared up considerably, much of the diffuse ring in the picture having been removed. The main arcs seen originally, however, are unaltered.

In the growth zone region, a series of diagrams was obtained at different levels up the sporangiophore block, the lower ones showing chitin chains in the predominantly logitudinal direction, but having a degree of angular dispersion very much higher than those in the mature wall (Plate I, Figs. 4 and 5). Only at the tip of the growth zone did the diagrams show very wide arcs most intense in the meridional positions (Plate I, Fig. 6) indicating a tendency for the chains to adopt a transverse orientation. Photometric determination of intensities along the equator (Fig. 1a) and meridian (Fig. 1b) confirm the tendency towards transverse orientation of chitin chains. There is evidently, however, a very considerable angular dispersion. Some of this may have been induced, however, during specimen preparation.

(ii) *Optical properties*

The major extinction position of single wall layers in both adult and the growing zones of cells was measured on the preparations described above, and the results summarised in Table I. The mean value for the angle of orientation of chitin chains in the adult cell wall was found to be 5° from the vertical cell axis, in determinations on 23 cells which showed wide variation from 1.5° to 19°. Such variations are to be expected, from

References p. 48.

Plate I

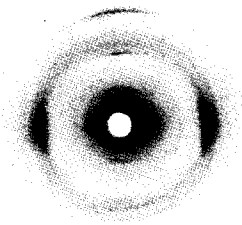


Fig. 2

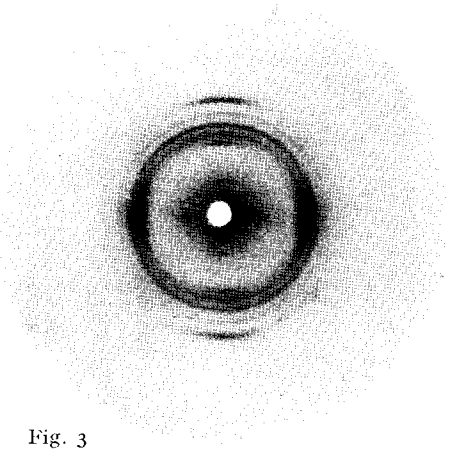


Fig. 3

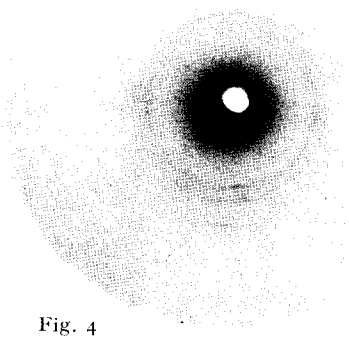


Fig. 4

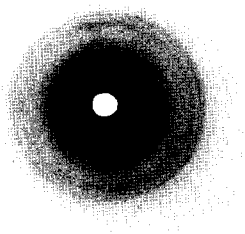


Fig. 5

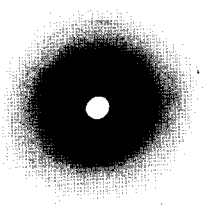


Fig. 6

Fig. 2. Untreated bundle of sporangiophores of *Phycomyces nitens*. X-Ray beam at right angles to vertical fibre axis. CuKα radiation. Film-specimen distance 3.1 cm.

Fig. 3. As Fig. 2, but bundle treated with 0.1% papain/1% sodium bisulphite solution for two days at 40° C.

Fig. 4. Flattened growth zone bundle, 2 mm from tip of cell. Equatorial arcs are still visible, indicating longitudinal orientation of chitin fibrils in the wall.

Fig. 5. Flattened growth zone bundle, 1 mm from tip of cell. Orientation of fibrils more random, but still shows a trace of equatorial arcs.

Fig. 6. Flattened growth zone bundle, 0.3 mm from tip of cell. Here arcs are slightly built up only in meridional position, see graph in Fig. 1 in text.

TABLE I

MAJOR EXTINCTION POSITIONS OF SINGLE WALLS OF PHYCOMYCES SPORANGIOPHORES (UNSTAINED)

Angle between the m.e.p. and the long axis of the cell (Degrees).

Adult wall regions	*Growth zones*	
	Stages 1 and 2	*Stage 4*
2.0	79.2	62.0
6.1	79.3	86.0
1.8	81.5	70.4
2.0	74.9	88.7
6.3	73.6	80.0
3.8	85.5	67.5
2.0		73.7
3.4		67.0
4.0		86.7
4.3		55.6
19.0		83.9
2.0		64.9
6.5		85.7
6.0		80.9
3.8		62.0
2.8		79.5
8.2		67.0
1.5		81.8
2.2		
10.0		
8.9		
2.0		
8.4		
Mean 5.01	78.99	74.63
	(*i.e.* 11.01° from transverse)	(*i.e.* 15.3° from transverse)

investigations on other cell types. On examination of the growth zone regions, flat spirals were found, 11° and 15° from the transverse in cells at different stages, again showing considerable variation.

The value found in the adult wall regions, mean 5°, is in fair agreement with the X-ray result of 9°, since in the latter method, the angle would be less accurately found, since (a) the X-ray angle includes any angular dispersion, (b) the sporangiophores in the fibre bundle used in the photograph may not be oriented absolutely parallel to each other, and (c) crinkling of the cell walls would give apparent dispersion of X-ray reflections. The measurement of the major extinction position clearly gives a better estimate of the angle of spiral. In all sporangiophores in which it was possible to be certain whether the single wall layer came from the upper or lower wall the spiral proved to be in the left-hand direction.

In addition to these values, it was confirmed that the secondary wall region consists of more than one layer, as found originally by OORT AND ROELOFSEN[31]. The spiral in the outer layer of the secondary wall was steeper, being in one case at an angle of 6.7°, when the inner layer of the wall was at 14.8° to the cell axis. These spirals were always in a left-hand direction, confirming the results of other workers in this field

References p. 48.

(CASTLE[25], OORT AND ROELOFSEN[31]). In view of the extreme thinness of the growth zone wall, it was not possible in every case to be certain whether an upper or lower wall was being examined, since both levels were in focus in the light microscope at the same time. This was especially the case in single walls which had been obtained by an irregular cut in the growth zone, leaving one wall exposed. However, since the spiral direction could definitely be identified in many cases, and these spirals were all left-handed, it is felt that this probably applies to all cells in our cultures.

It is known that cells have been found which normally rotate in an anti-clockwise direction (right-hand spiral). OORT[26] found several such cells, and in our observations a small percentage (about 2%) has been found. These may be associated with sporangiophores in which the growth zone shows a right-hand spiral. It is possible that sporangiophores showing this rotation are also found more frequently occurring in other strains.

Electron microscopy

In views of our criticisms of electron micrographs taken by other workers, attemps were made to isolate wall material without any chemical treatment. Turgid sporangiophores were plucked from their agar substrate and when handled quickly and firmly, induced to split axially along the growth zone. This phenomenon was reported earlier and is explained by CASTLE[27] who shows that the tensile stress in the wall is twice as great transversely as longitudinally. For this reason, when the cells burst the wall splits along its axis, leaving a single straight piece of wall material. The cells were then carefully washed with distilled water, and as much as possible of the cell contents removed by gently cleaning with a fine paint brush.

A series of electron micrographs is given (Plates II, III, Figs. 7–10*) at different intervals up the same growth zone, looking at the inside of the cell wall, from 0.4 mm to 2 mm from the tip. These pictures correspond closely with those obtained from X-ray photographs in showing that chitin fibrils are oriented predominantly in the axial direction in the lower regions of the growth zone, and that at about 0.5 mm from the tip, the underlying fibrils which are formed early in the life of the wall are oriented more or less transversely to the cell axis. It is probable that these transverse fibrils are obscured in the lower regions of the growth zone by the later-formed axially oriented fibrils. This layer of the wall in the growth zone must be thin, however, since the birefringence in this part of the cell is still negative.

When the specimens are shadowed so that the outside of the wall can be examined, no structure at all is visible, this probably being due to the presence of the cuticle on the outside.

The relation of wall to cytoplasm

(i) *The effect of vital dyes*

In attempting to determine the part of the growth zone in which new material is interpolated, a technique was devised to colour the wall without killing the cell, so that on further growth the new wall would be unstained. These, of course, followed NOLL'S[28] well-known experiments on algae. In his work, the aqueous environment of the plant was an advantage, since it was possible to surround the plants with a dye solution without adverse effect, but in this aerial sporangiophore with a thick waxy cuticle, the prob-

* Electron micrographs presented all show the inner wall layer of the growth zone wall. Arrow indicates cell axis. All are shadowed with gold/palladium.

References p. 48.

Plate II

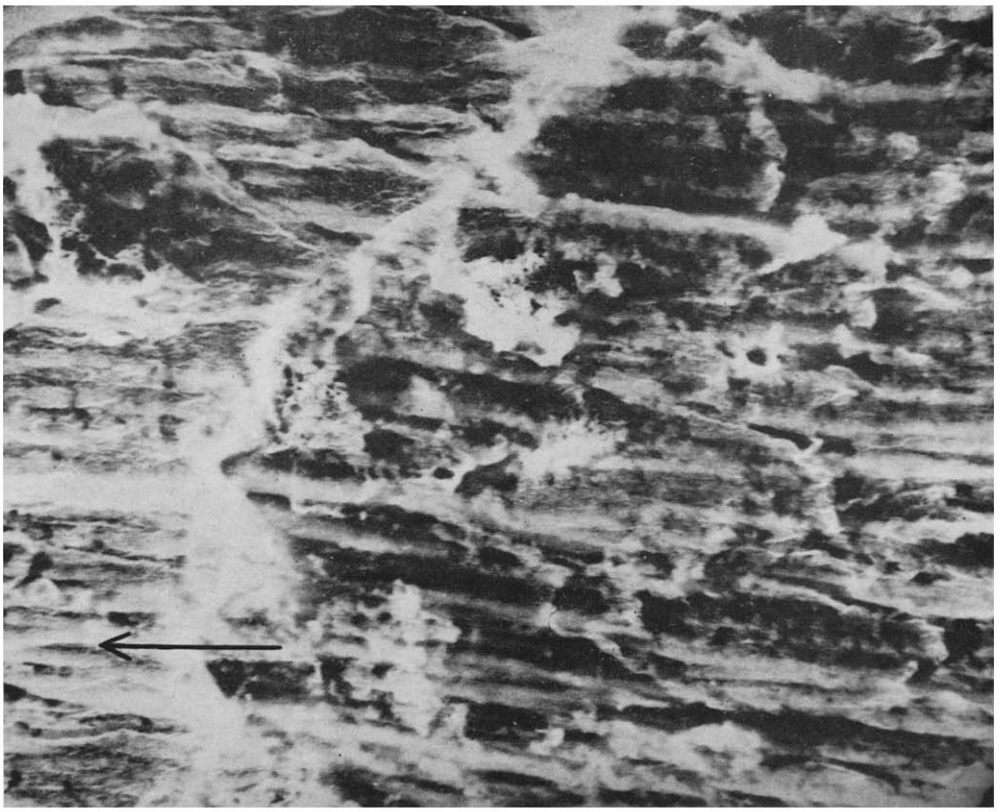


Fig. 7. 1.2 mm from tip of cell. Magnification × 15,000.

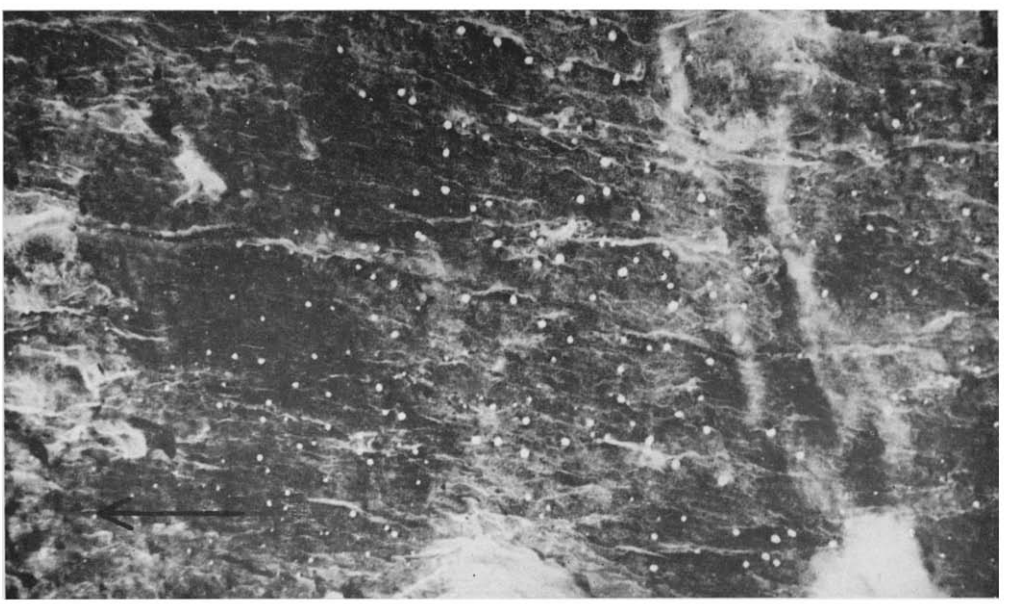


Fig. 8. 0.95 mm from tip of cell. Magnification × 15,000.

Plate III

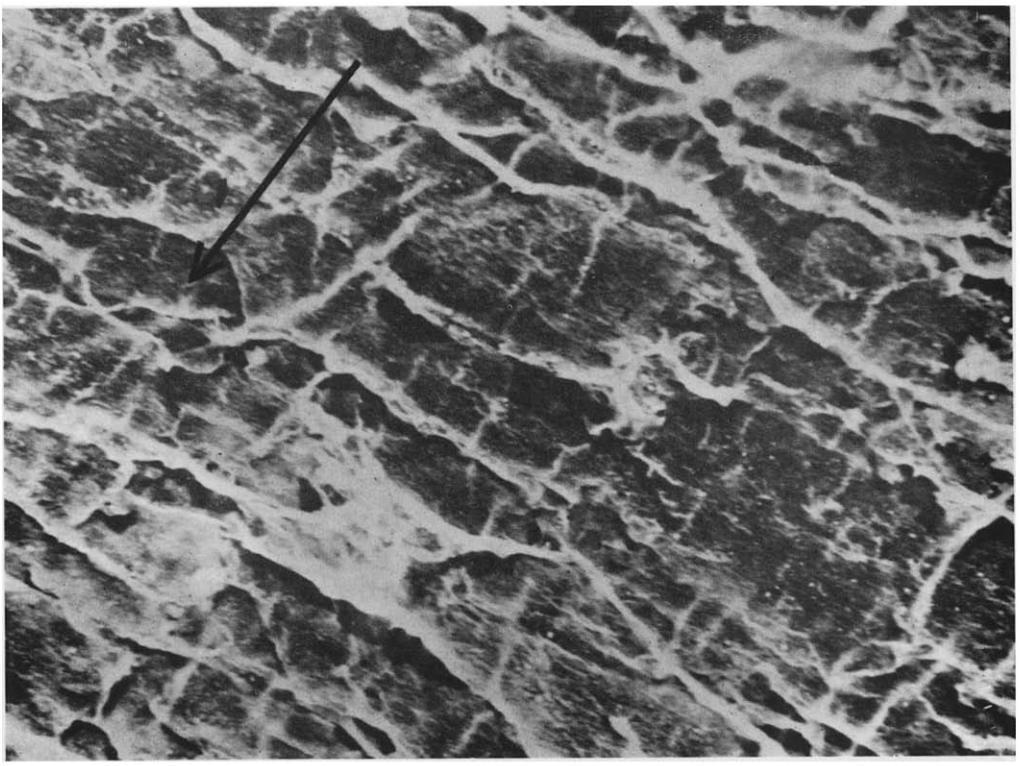


Fig. 9. 0.65 mm from tip of cell. Magnification × 15,000.

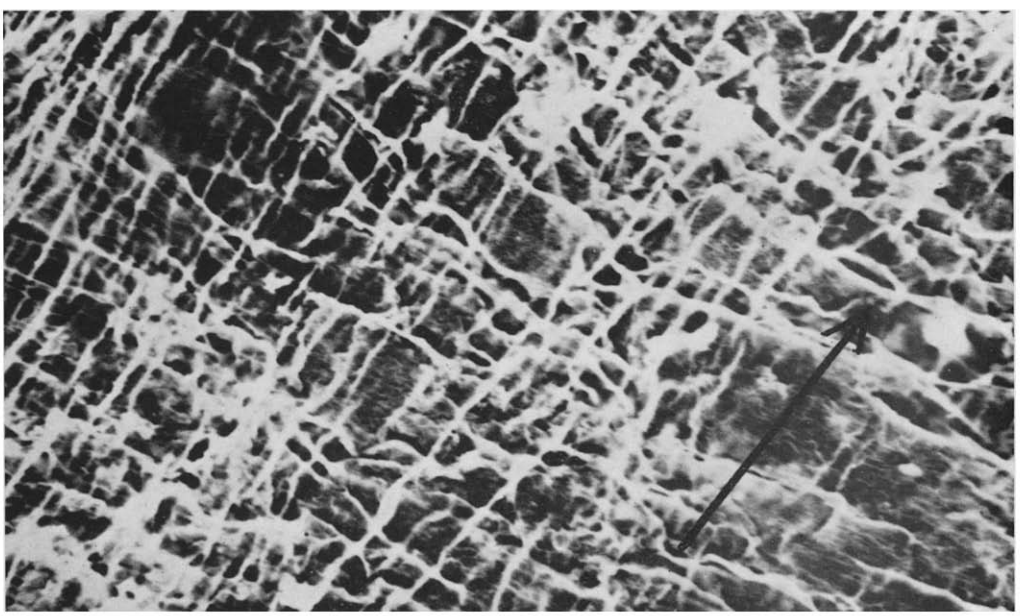


Fig. 10. 0.65 mm from tip of cell. Magnification × 7,500.

lem proved rather difficult. The sporangiophores were grown in small phials and then immersed for a short time in a dilute, aqueous solution of various vital dyes. The solutions were pipetted off, the cells meanwhile being supported along their lengths by a long cover slip so that they should not be pulled down with the liquid surface. The cells were allowed to go on growing for some time afterwards, and then examined under the microscope.

The prerequisite of a dyeing or other method of colouring the wall, is that it should impart an intense and even colour to the wall surface. A modification of NOLL's treatment first with 0.5% ferric chloride, then with potassium ferrocyanide, was used for some cells. The cell was first immersed in a ferric chloride solution in a very weak detergent, 0.002% dodecyltrimethylammonium bromide* for 15 minutes. After pipetting off the solution, the cell was replaced in the glass tank, where growth was resumed. After two hours, the cell was immersed in the potassium ferrocyanide solution and examined under the microscope, The number of cells which would continue to grow after the first part of the treatment was a small fraction of those tried, as there seems to be a wide variation in the tolerance of different cells. Some cells had taken up the stain rather patchily.

Other dyes were tried, Orange G giving a good, even colour, but, in the solutions which were weak enough not to kill the cells, the cells were too pale for a definite estimate to be made of coloured and uncoloured material. Of a range of other dyes, the number which penetrated the wall was small, and of these several gave patchy colouring and others killed the cells in even very dilute solutions. However, interesting results were given by malachite green and crystal violet, both of these stains causing abnormal growths of a type not previously reported. The observations to be discussed refer therefore to these two dyes and to the Prussian blue technique of NOLL.

When stage 4 sporangiophores were treated by ferric chloride followed by potassium ferrocyanide, with two hours of growth between treatments, it was seen that the adult region at the base of the growth zone was stained, but not the tip. The cell length had been measured at 15 minute intervals during the time interval between the two parts of the staining technique, and the growth in length was found equal to the length of the unstained region at the tip of the growth zone. When cells were left for longer periods, a clear line of demarcation developed between new, unstained wall material and the blue-coloured older wall. This evidence points to a region of very rapid wall growth at the extreme tip of the cell, which may or may not be accompanied by wall growth lower down the growth zone. If growth in girth and in length in these regions is taking place, it is not at any rate in isolated patches, such as those found by NOLL. If, however, new material is inserted very evenly and uniformly amongst the older wall regions, the general decrease in intensity of stain would escape notice.

In the case of cells treated by crystal violet and malachite green, the adult wall took up the stain well and evenly, but not so intensely as the growth zone. In the growth zone region too, the cytoplasm absorbed the stain very intensely, leaving a clear line of demarcation at the base of the thick plug of granular cytoplasm which occurs at the tip of the growth zone. This treatment did not usually kill the cell outright, and after the cells had been left to grow overnight, it was very often found in stage 1 cells that, from the base of the old growth zone, new ones had been pushed out, showing a rupturing of the old, coloured wall. This happened, but less consistently, in the cells which had been

* The effect of this detergent upon the cell growth was known from other investigations to be reported later.

References p. 48.

older when treated, *i.e.* in the early part of stage 4. Photographs of such cells are presented, (Plate IV, Figs. 11–14), but the coloured region does not show up clearly in these black-and-white photographs, since the yellow cell contents in the new and uncoloured regions appear as dark as the violet wall. Some cells were then cleared of colour with alcohol, and in some a new, thick cell wall had cut off the deeply-stained tip, but this was not so in all cases.

Another set of observations may be mentioned here, which also point to a region of greater sensitivity at the tip of the growth zone, at which a rapid wall growth takes place. Rotation and elongation in the normally growing sporangiophore were under observation for another series of measurements, to be reported later, and these involved the use of a marker on the tip of the cell, so that its rotation could be followed. For this purpose, a variety of small objects was used, Lycopodium spores, flour particles, small pieces of glass wool, etc. and for the work on stage 3 and 4 cells these proved very useful. When younger cells in stages 1 and 2 were being watched, however, it was found that if a marker were placed at the extreme point of the stage 1 cell, or on the tip of the sporangium in the stage 2 cell, the cell no longer grew normally, but temporarily ceased growth, and then formed a completely new tip at the side of the original one. The new growth zone usually had a diameter much smaller than that of the original cell at first (Plate V, Figs. 15 and 16). Later, however, the diameter of the new cell region usually became as great as that of the original cell, and only a slight constriction was left to indicate the change in the growth zone (Plate V, Fig. 17). This increase in diameter must, of course, be accounted for, in both normal and abnormal cell growth, but it is suggested that there is a much greater rate of wall deposition at the extreme tip of the sporangiophore than at any region further down the growth zone. Evidently this tip is very easily affected by the touch of any foreign body, since it is unlikely that this effect is produced by chemical means, as glass wool exhibits the same effect as other markers.

(ii) *Spirals found in the cytoplasm*

It has been observed in this laboratory on three occasions that, when cells are removed from the agar on which they are growing and watched as they dry out gradually, spirals are seen to form in the cytoplasm. One of these cells was photographed (Plate V, Fig. 18). and showed a left-hand spiral. This structure in the cytoplasm has not to our knowledge been seen before, and was only rarely found in our work. Its presence is, however, of great interest in relation to the mechanism of cell wall deposition.

DISCUSSION

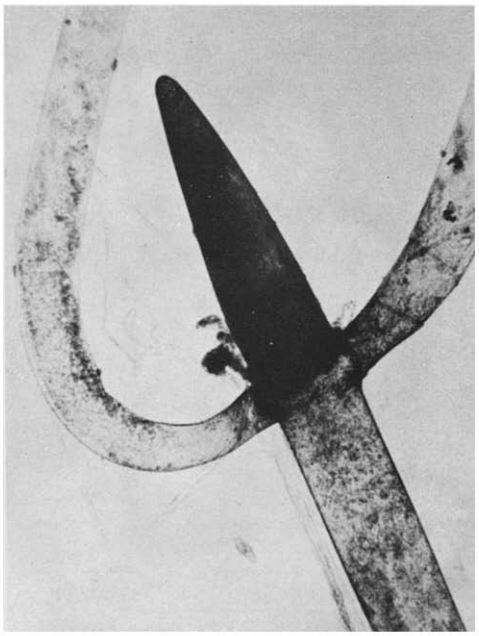
In the work above it has been confirmed that the cell wall of the sporangiophore of *Phycomyces* is wound by a set of chitin fibrils, which in the growth zone are inclined at an angle of about 15° from the transverse and, in the secondary wall region lower down the cell, run almost axially, the angle being about 5° from the vertical. Both spirals are left-handed in the majority of cells. These conclusions have been reached from work for the first time on cells which have been given no chemical treatment. This may explain differences between the work reported here and that of other workers.

Some further light has been shed on the formation of the primary wall, in that most of the evidence points to the presence of a small area at the very tip of the cell in which growth takes place. This is shown by the presence of axial orientation of chitin chains for

References p. 48.

Plate IV

Abnormal growths induced by chemical treatment or mechanical damage.



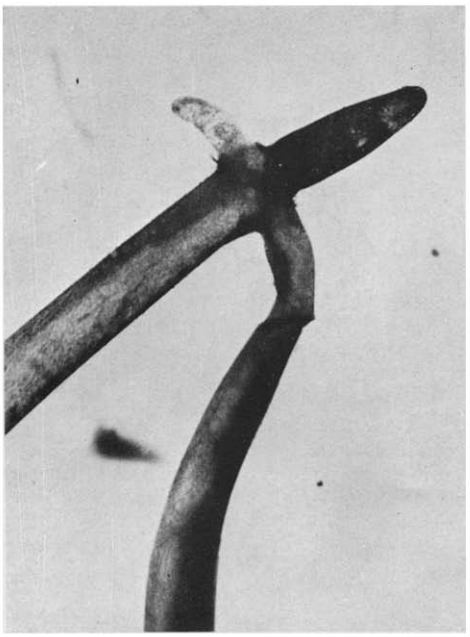


Fig. 11 and 12. Two sporangiophores treated when in Stage 1 by crystal violet solution for 5 minutes. The growth zone wall stained deeply, also the cytoplasm in this region. In each case two new growth zones have been pushed out from the base of the old one, and in Fig. 12 rupture of the old wall is visible.

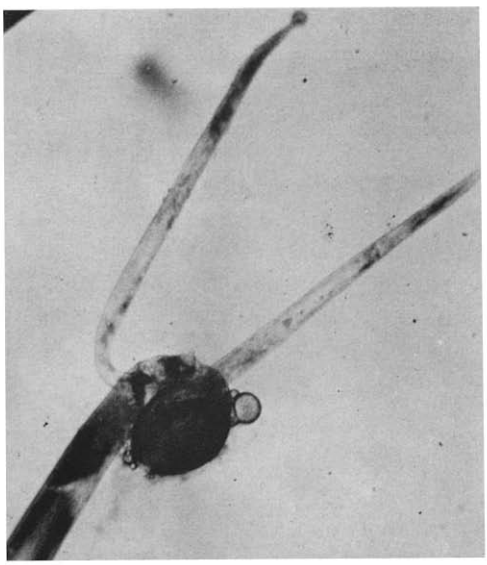


Fig. 13. Cell showing the similarity of the effect of mechanical injury. The sporangium was knocked over, after which two new growth zones formed below the sporangium, at the base of the region damaged.

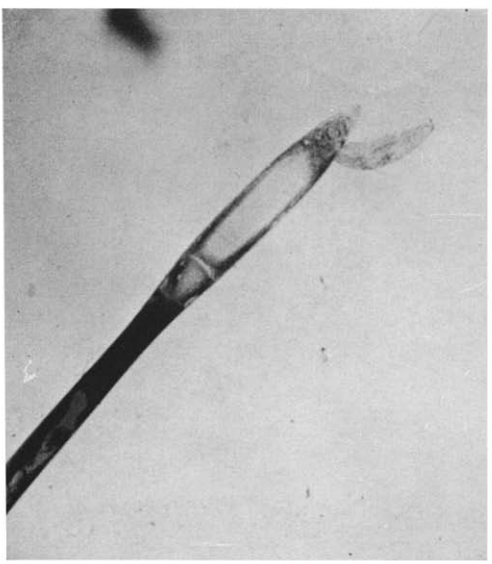


Fig. 14. Stage 1 cell treated with Malachite green for 5 mins, then left for 14 hours. In this case, the stain is not taken up so intensely by the growth zone wall and cytoplasm as by the adult wall. Here a new growing point is formed high up the old growth zone.

Plate V

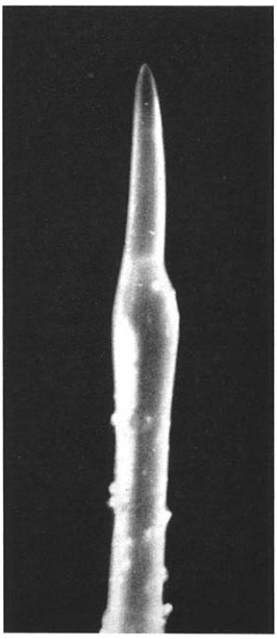


Fig. 15. Stage 1 sporangiophore on which a Lycopodium spore had been placed (spore visible as a white spot just below constriction of sporangiophore), then developed a new growing point.

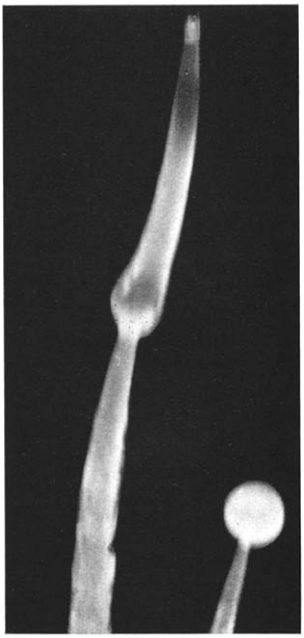


Fig. 16. Stage 3 cell which also grew abnormally after spore was placed on its tip, putting out a new growing point from the top of the sporangium.

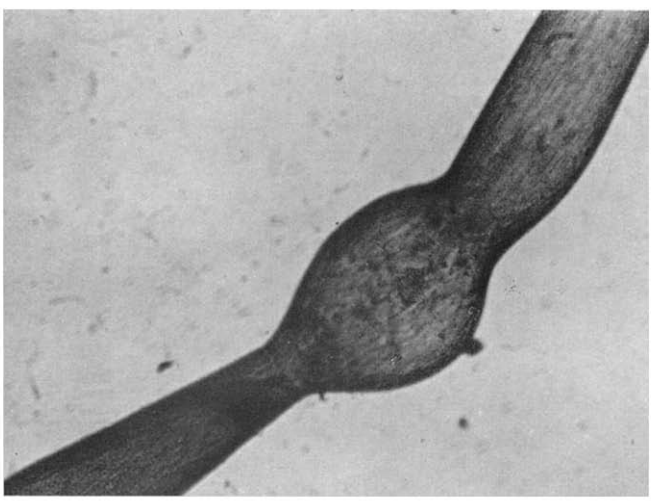


Fig. 17. Same cell as in Fig. 16, 12 hours later, showing the remains of the first-formed sporangium, now showing as an inflated region in the sporangiophore.

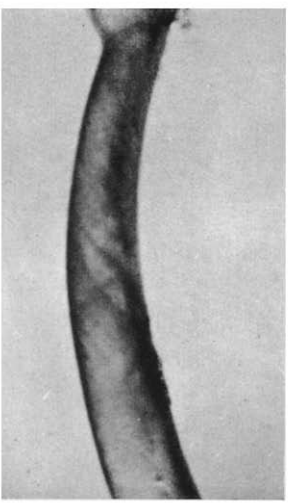


Fig. 18. Growth zone region of a Stage 4 sporangiophore, showing spirals in the cytoplasm.

some distance up the growth zone, and only at the extreme tip is there found only transverse orientation.

It is also found that abnormal growth occurs in the cell when the extreme tip is touched by a marker, and not when the marker is placed on any part of the growth zone further down. This indicates that a special area exists at the tip of the cell different from any other part of the wall. This is supported by GORTER's work[29], on the root hairs of *Lepidium sativim*. When soaked in 0.2% solution of 2.3.5. tri-iodobenzoic acid, longitudinal growth stops, but cellulose goes on being produced at the extreme tip of the cell only, plugs of this material appearing in irregular masses, which are without birefringence.

Although, however, it is shown that the extreme tip of the cell is the main region of growth in area of the primary wall, extension in area is known to take place in the whole of the growth zone, decreasing in rate as one goes further away from the tip (CASTLE[30]).

It has also been shown in this paper that the cytoplasm in the whole zone has different properties from that lower down the sporangiophore, in the differential uptake of various dyes. Especially in the case of crystal violet, a clear line of demarcation was left at the base of the growth zone in stage 1, where the dye was not taken up so intensively. This difference in properties of cytoplasm in different regions of the sporangiophore may reflect the various stages of wall deposition, *i.e.* the formation of new primary wall material in the growth zone, and the apposition of new secondary wall material in the lower regions of the cell. The well-known close association between cytoplasm and wall in the meristematic cell, which often cannot be plasmolysed, is another indication of the intimate connection between the two in rapidly growing regions, and here it is seen that in the growth zone the wall and cell contents take up the dye extremely intensely, while in lower regions of the cell, there is practically no assimilation of dye in the cytoplasm.

It is widely envisaged that primary wall growth is by the intussusception of new materials, involving some breaking and re-making of bonds as new material is inserted. This would necessitate properties in the cytoplasm involved in primary wall formation very different from those of cytoplasm active in forming new secondary walls by apposition of new chains.

The effect of the cytoplasm upon the orientation of materials forming the cell wall has been the subject of much speculation, but the appearance of spiral form in the cytoplasm of some cells would indicate that, at least in secondary wall deposition, this might be the chief factor causing orientation. The idea that the cytoplasm might act as a template, so that as fibrils are laid down they are also oriented, following the orientation of the cytoplasm, has been put forward. This fits in well with cells which show lamellae in sheets of alternating direction, as *Valonia*, *Cladophora*, etc.

The impact of the observations reported in this paper on current theories of spiral growth will be discussed more fully in a later paper when further data concerning the growth process will be presented. It may perhaps be pointed out here that the prevalence of left-hand spirals in chemically untreated material is in complete harmony with our explanation of spiral growth and not with ROELOFSEN's.

ACKNOWLEDGEMENTS

Our thanks are due to Mr B. KUYPER for his assistance in taking the electron micrographs in this paper, to Mr A. MILLARD for printing the X-ray photographs, and to Mr B. CLARKE for printing the photomicrographs published in this paper.

References p. 48.

We are indebted to the Department of Scientific and Industrial Research for a Grant in aid of a research programme of which this work forms a part.

SUMMARY

The wall structure of the growth zone of *Phycomyces* has been further investigated by means of X-rays, the polarizing microscope, and the electron microscope. Material has been used which has been subjected to no chemical treatment whatever, and it has been established that the flat spiral structure which occurs in the growth zone is sinistral. When the cells were treated with solutions of vital dyes, or subjected to mechanical injury, abnormal growths were obtained which appear to shed light on wall formation in the cell. Spirals in the protoplasm have been photographed.

RÉSUMÉ

Nous avons poursuivi nos études de la structure de la paroi de la zone de croissance de *Phycomyces* à l'aide des rayons-X, du microscope polarisant et du microscope électronique. Nous avons employé un matériel qui n'avait été soumis à aucun traitement chimique et nous avons établi que la structure en spirale plate qui se trouve dans la zone de croissance tourne à gauche. Lorsque lei cellules sont traitées par des solutions de colorants vitaux ou lésées mécaniquement, on observe des croissances anormales qui jettent de la lumière sur la formation de paroi dans la cellule. Nous avons photographié des spirales dans le protoplasme.

ZUSAMMENFASSUNG

Die Wandstruktur der Wachstumszone von *Phycomyces* wurde mit Hilfe von Röntgenstrahlen, des Polarisationsmikroskopes und des Elektronenmikroskopes weiter untersucht. Das verwendete Untersuchungsmaterial war keiner einzigen chemischen Behandlung unterworfen worden. Es wurde festgestellt, dass die flache Spiralstruktur, welche in der Wachstumszelle vorkommt, linksdrehend ist. Waren die Zellen mit Lösungen von Vitalfarbstoffen behandelt oder mechanisch lädiert, so wurden abnormale Wachstums-Erscheinungen beobachtet, welche auf die Wandbildung in der Zelle Licht werfen. Spiralen im Protoplasma wurden photographiert.

REFERENCES

1 R. D. PRESTON AND A. B. WARDROP, *Biochim. Biophys. Acta*, 3 (1949) 585.
2 A. FREY-WYSSLING, *Ber. deut. botan. Ges.*, 46 (1928) 444.
3 R. D. PRESTON, *Phil. Trans.*, B, 224 (1934) 131.
4 R. D. PRESTON, *Proc. Roy. Soc. B*, 133 (1946) 327.
5 R. D. PRESTON, *Proc. Roy. Soc. B*, 134 (1947) 202.
6 A. B. WARDROP AND R. D. PRESTON, *Nature*, 160 (1947) 911.
7 R. D. PRESTON, *Ann. Botany, N.S.*, 3 (1939) 507.
8 B. C. KUNDU AND R. D. PRESTON, *Proc. Roy. Soc., B*, 128 (1940) 214.
9 R. D. PRESTON, *Proc. Roy. Soc., B*, 130 (1941) 103.
10 R. D. PRESTON AND M. MIDDLEBROOK, *Nature*, 164 (1949) 217.
11 R. D. PRESTON AND M. MIDDLEBROOK, *J. Text. Inst.*, 40 (1949) T715.
12 R. D. PRESTON AND K. SINGH, *J. Exptl Bot.*, 1 (1950) 215.
13 W. T. ASTBURY AND R. D. PRESTON, *Proc. Roy. Soc., B*, 129 (1940) 54.
14 R. D. PRESTON AND E. NICOLAI, (presented to *Phil. Trans.*).
15 R. D. PRESTON, *Biochim. Biophys. Acta*, 2 (1948) 155.
16 R. D. PRESTON, *Forestry*, 23 (1949) 48.
17 A. FREY-WYSSLING AND K. MÜHLETHALER, *Schweiz. landw. Monatsh.*, 6 (1950) 1.
18 A. FREY-WYSSLING AND K. MÜHLETHALER, *Vierteljahrsschr. naturforsch. Ges. Zürich*, 95 (1950) 45.
19 P. A. ROELOFSEN, *Biochim. Biophys. Acta*, 6 (1951) 357.
20 P. A. ROELOFSEN, *Rec. trav. botan. néerland.*, 42 (1949–'50) 73.
21 P. A. ROELOFSEN, *Biochim. Biophys. Acta*, 6 (1950) 340.
22 E. S. CASTLE, *Protoplasma*, 31 (1938) 331.
23 M. M. SWANN AND J. M. MITCHISON, *J. Exptl Biol.*, 27 (1950) 226.
24 A. N. J. HEYN, *Protoplasma*, 25 (1936) 372.
25 E. S. CASTLE, *J. Cellular Comp. Physiol.*, 7 (1936) 445.
26 A. J. P. OORT, *Koninkl. Akad. Wetenschap. Amsterdam*, 34 (1931) 564.
27 E. S. CASTLE, *J. Cellular Comp. Physiol.*, 9 (1937) 477.
28 F. NOLL, *Abhandl. Senckenb. naturforsch. Ges.*, 15 (1884) 101.
29 C. J. GORTER, *Nature*, 164 (1949) 800.
30 E. S. CASTLE, *J. Cellular Comp. Physiol.*, 10 (1937) 113.
31 A. J. P. OORT AND P. A. ROELOFSEN, *Koninkl. Akad. Wetenschap. Amsterdam*, 35 (1932) 898.

Received September 3rd, 1951