THE UNIVERSITY OF MANITOBA

A CYTOLOGICAL STUDY OF VACUOLES IN FUNGAL HYPHAE

A Thesis

Submitted to

The Faculty of Graduate Studies and Research

In Partial Fulfilment of

the Requirement for

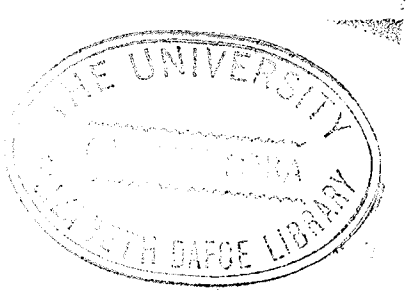
The Degree of Master of Science

---

by

Soo Huah Koay

April, 1966



ACKNOWLEDGEMENTS

The author is greatly indebted to Dr. P. K. Isaac for suggesting this study as well as for the encouragement and instruction in the course of the work. The author also wishes to express her thanks to Miss Maria de Geus for her assistance with electron microscopical work. The investigation was supported by a graduate assistantship from the National Research Council.

ABSTRACT

The development, structure and function of vacuoles in some filamentous fungi, particularly Rhizoctonia solani were studied by several cytological methods.

The development and general form and behaviour of vacuoles were followed with the phase contrast microscope, and the electron microscope was used to study the fine structure.

The relationship between the contents of the vacuole and the surrounding cytoplasm was studied by micro-interferometry and found to be variable and complex.

The accumulation of neutral red was investigated and was shown to be markedly pH dependent.

No clear measurement of the osmotic pressure of the vacuoles could be made.

TABLE OF CONTENTS

	PAGE
INTRODUCTION	1
LITERATURE REVIEW	2
FUNCTIONAL ROLE OF VACUOLES	4
DETERMINATION OF CYTOPLASMIC AND VACUOLAR CONCENTRATIONS BY THE INTERFERENCE MICROSCOPE	6
OSMOTIC RELATIONSHIPS	8
MATERIALS AND METHODS	11
ORGANISM AND CULTURE METHODS	11
DYE ACCUMULATION	12
STUDIES OF THE PHYSICAL FORM OF VACUOLES	13
DETERMINATION OF DRY MATTER CONCENTRATION	14
DETERMINATION OF DIFFUSIBLE CONTENTS	16
DETERMINATION OF OSMOTIC PRESSURE	16
DETERMINATION OF THE MEAN MOLECULAR WEIGHT OF VACUOLAR CONTENTS	17
EXPERIMENTAL RESULTS	18
FORM AND STRUCTURE	18
(a) Vacuolization of Hyphae	18
(b) The Effect of Cytoplasmic Streaming on Vacuoles	19
(c) Fine Structure of Vacuoles	19
(d) Interferometric Studies	21
FUNCTION STUDIES ON THE BEHAVIOUR OF VACUOLES	24
(a) Uptake of Neutral Red	24
(b) Osmotic Pressure	25
DISCUSSION	27
FORM AND STRUCTURE	27

TABLE OF CONTENTS CONTINUED

	PAGE
FUNCTION	30
SUMMARY	32
BIBLIOGRAPHY	33
FIGURES AND TABLES	38

LIST OF FIGURES

FIGURE		PAGE
1-10	Micrographs of hyphae of R. solani growing in slide culture on a plain agar medium. Positive phase contrast. Magnification 1000 x.	
	Figure 1	38
	Figure 2	38
	Figure 3	39
	Figure 4	39
	Figure 5	40
	Figure 6	40
	Figure 7	41
	Figure 8	41
	Figure 9	42
	Figure 10	43
11	Electron micrograph of vacuoles fixed in $KMnO_4$ showing tonoplast, flocculent contents and a thin layer of cytoplasm between the vacuole and the plasmalema	44
12	Electron micrograph of vacuoles fixed in osmium showing fine vesicular contents	44
13	Electron micrograph of R. solani fixed in osmium showing a septum between full and empty parts of the hypha	45
14	Electron micrograph of Alternaria tenuis fixed in glutaraldehyde and $KMnO_4$ showing a large central vacuole containing complex bodies	45
15	Similar to Figure 14 showing membrane bound vesicles	46
16	Similar to Figure 15	46
17	Similar to Figure 14	47
18	Electron micrographs of young hyphae of A. solani fixed in glutaraldehyde and $KMnO_4$ showing the low density of membranes	47
19	Diagram showing the relationship between the concentration (g/100 ml) of vacuoles and surrounding cytoplasm. R. solani, slide culture, plain agar	48

LIST OF FIGURES CONTINUED

FIGURE		PAGE
20	As in Figure 19, but culture grown on 1/50 N sucrose-peptone medium ----------	49
21	Diagram of the relationship between vacuolar concentration and distance of vacuole from apex. R. solani, slide culture, plain agar ----------	50
22	As in Figure 21, but relationship between cytoplasmic concentration and distance from apex---	51
23	Diagram showing the relationship between vacuolar and cytoplasmic concentrations before and after treatment with 4% formalin. R. solani, slide culture, plain agar ----------	52
24	Drawings of representative portions of hyphae showing differing types of relationship between vacuolar and cytoplasmic concentrations -------	53
	(a) Medium vacuolar concentration ----------	53
	(b) Low cytoplasmic concentration----------	53
	(c) Low vacuolar concentration High cytoplasmic concentration----------	54
	(d) High vacuolar concentration High cytoplasmic concentration----------	54
	(e) Emptying side branch-low vacuolar concentration ----------	55
	(f) Medium vacuolar concentration Low cytoplasmic concentration ----------	55
25	The same portion of hypha of R. solani showing the process of accumulation of neutral red during a period of 1.1.2 hours.	
	(a) The appearance of "neutral red granules"---	56
	(b) A later stage of uptake ----------	56
	(c) As in Figure 25 (b) ----------	57
	(d) As in Figure 25 (c) showing coalescence of vacuoles with increasing dye accumulation--	57
26	Accumulation of neutral red at pH 9.0 ----------	58
27	Micrograph showing the loss of neutral red after transfer to an acid medium (pH 4.0);compare Figure 25 (d) ----------	58

LIST OF FIGURES CONTINUED

FIGURE		PAGE
28	Micrograph showing coalescence of vacuoles at an early stage of neutral red accumulation. "Neutral red granule" stage ----------	59
29	A series of micrographs showing the effect of $CaCl_2$ solutions of increasing concentration. (a) 0.02 M, (b) 0.04 M, (c) 0.06 M ----------	59
30	A series of micrographs showing the effect of sucrose solutions of increasing concentration. (a) water, (b) 0.1 M, (c) 0.5 M, (d) 1.0 M.----	60

LIST OF TABLES

TABLE		PAGE
I (a)	Values of diameter (μ) of vacuoles (D_v) and hyphae (D_h) and of concentration (g/100 ml) of vacuoles (C_v) and cytoplasm (C_c) in hyphae of R. solani (plain agar medium - 3 day old slide culture). ----------	61
I (b)	Same as Table I (a) replicate experiment------	64
II	Values of D_v, D_h, C_v and C_c for a mycelium of R. solani growing in slide culture on 1/50 N sucrose-peptone agar ----------	67
III (a)	Values of D_v, D_h, C_v and C_c for a hypha of R. solani at various distances from the apex (plain agar medium,3 day old slide culture) ----------	69
III (b)	Same as Table III (a) (7 day old culture)-----	72
IV	Values of C_c for hyphal apices and portions of non-vacuolate old hyphae (7 days)-------	75
V	Values of C_c and C_v in water and 4% formalin. I and II = replicate slides, a-d = adjacent vacuoles ----------	76

INTRODUCTION

INTRODUCTION

Observation of the cytoplasm of living fungi with the optical microscope reveals structures, spherical to ovoid in shape, that appear to be devoid of contents and similar in this respect to the vacuoles seen in mature plant cells. They are also invariably called vacuoles, but unlike those in plants they can be seen to be attached to the hyphal wall and are frequently flattened in the area of contact. An alternative explanation for their existence might be that there is insufficient cytoplasm to fill the hyphae and the so called vacuoles are indentations in the cytoplasmic membrane. The experience of mycologists that it is difficult to demonstrate plasmolysis in filamentous fungi tends to support the latter possibility.

In recent years many physical techniques have been developed that were not available to physiologists in the first half of the century when most of the studies on water relations of plants were conducted, and the purpose of this study is to describe attempts to clarify the function of the vacuoles in filamentous fungi through the application of some of the newer methods.

LITERATURE REVIEW

LITERATURE REVIEW

Vacuoles are regarded as one of the cytoplasmic inclusions in cells. They are considered to be characteristic of mature plant cells where they may occupy more than half the total cell volume and are filled with liquid cell sap, they are rarely found in the meristematic tissue. They are not generally conspicuous in animal cells, however, in protozoa, contractile vacuoles act as the excretory organ by which waste products are expelled to the outer medium. The size and shape of vacuoles depends on a number of factors such as the age and the activity of the cytoplasm, they tend to be spherical in quiet cytoplasm, and may be rod-shaped, elongate or irregularly shaped in streaming cytoplasm (Zirkle, 1937).

In fungi, relatively small, globular vacuoles are first seen adhering to the walls of the young hyphae. As the hyphae become older, the vacuoles increase in size and may occupy a total of more than 50% of the volume between adjacent septa. Buller (1933) observed that as fungal fruiting bodies begin to develop many small vacuoles fuse to form a large vacuole occupying most of the basidium. He considered that the growth of the vacuoles was due to the presence of osmotic substances in the vacuoles and to water absorption from the medium. In the mycelium of Fimetaria fimicola, Buller noticed that the vacuoles moved with the streaming cytoplasm and were capable of being carried with the protoplasm from cell to cell passing through the pores in the septa. While passing through the pores the vacuoles became constricted and many divided into two or more small vacuoles which often

fused together again. In the highly vacuolated hyphae of Pyronema confluens, Buller (1935) observed that the vacuoles were more firmly attached to the cell wall and even the pressure of rapidly streaming protoplasm was not sufficient to free the vacuoles of their attachment, but their shapes were changed by the pressing of the protoplasm. Arthur (1897) observed the vacuoles of coenocytic hyphae and noticed that when streaming in a branched trunk hypha the large vacuoles tended to divide into two or more parts, with one part going into the branch hypha, while the other went on in the trunk hypha.

The streaming of protoplasm in fungi has been considered by some authors to be due to vacuolar pressure. Buller (1938) studied translocation in fungi, and thought that the vacuoles probably play a role in forcing the protoplasm to the young hyphal tips, also Corner (1948) considered that the vacuole of the basidium has the function of forcing the protoplasmic contents into the basidiopores. Well (1964) studying the features of developing and mature basidia and basidiospores of Schizophyllum commune, showed that small vacuoles appear in the basal position of the basidium after the sterigmatal initiation. The small vacuoles tend to coalesce and may force the cytoplasm and nuclei into the basidiospores. Vacuolation proceeds until it reaches a stage in which a thin peripheral layer of cytoplasm is left in the basidium.

From electron microscopic studies, I. Manton (1962) observed that some young vacuoles showed an obvious electron dense precipitate which was thought to be due to a reaction of the vacuolar solutes

with the $KMnO_4$ used as a fixative. Also, Well (1965) observed that vacuoles were frequently irregularly shaped in the dikaryotic basidium, and that the tonoplast appeared as an electron dense line, presumably consisting of a unit membrane; it is possible that the irregular shape was due to poor fixation.

THE FUNCTIONAL ROLE OF VACUOLES

In plants, vacuoles play a significant role in accumulating ions into the vacuolar sap. The ionic concentration in the vacuole is generally higher than that of the external medium. The vacuolar concentrations of potassium and chloride in the giant internodal cells of Nitella transluscens are higher than that of the medium. MacRobbie (1964) suggested that two separate processes are involved in the active uptake of salts by the vacuoles; an active accumulation in the cytoplasm and a subsequent transfer of salts actively across the tonoplast into the vacuoles. Several authors, e.g. Bath and Sponsler (1942), have suggested that the vacuoles might be the site of reactions of physiological interest, but published papers have concentrated on the uptake process, for instance, many studies have been made in connection with vital staining of vacuoles. Vital staining involves the uptake of dye by living cells and because most vital stains are more or less toxic cells seldom survive in them for long. Although several dyes have been proposed as vital stains, practically all work has been done with neutral red. One characteristic of the uptake of this dye is the frequent appearance

of concentrated droplets or "neutral red granules" in the cell after treatment with dilute neutral red solution. This was observed by Cicklhorn (1929) who noted that neutral red granules not only occurred in the cytoplasm of animal cells, but also in the vacuoles of plant cells. According to this author the neutral red granules disappeared after plasmolysis, but reappeared at deplasmolysis. The droplets at first appeared to be fluid, but later solidified and became hard and brittle. Baker (1945) considered that the appearance of neutral red granules indicated that there were pre-existing bodies in the vacuoles. However, other investigators such as Yoshimi Nagatani (1960) and Singh (1937) have explained the presence of the neutral red granules on the basis of reaction to the dye and not to the pre-existing nature of the vacuoles. Singh concluded that the pre-existing nature of the vacuoles may induce the neutral red granules formation.

Neutral red is a weakly basic dye, entirely in the associated state in an alkaline medium, but dissociating in an acidic medium. Owing to this property, the penetration of the dye into cells and vacuoles depends upon the pH difference between the cell interior and the exterior medium,and the degree of dissociation of the dye. By using sea urchin eggs, Chamber (1930) demonstrated that the vacuoles of the eggs take up neutral red, when placed in alkaline sea water; but when transferred to acidified sea water they become colorless. Chambers and Chambers (1961) indicated that the neutral red diffuses rapidly from the medium through the acidic cytoplasm and into the more acidic vacuoles.

Scarth (1926) showed that Spirogyra and Tradescantia zebrina could be stained by neutral red and other basic dyes. As the dye accumulated, granular precipitates occurred and aggregated together into droplets in the cytoplasm and appeared to pass into the vacuole. The formation of drops of stain, which appeared to be a coacervate, could be increased by the addition of alkali (ammonia) or an alkaloid (caffeine). The stain droplets increased in size as the ammonia penetrated the cell. The amount of accumulation of neutral red in the vacuoles also depended upon the alkalinity of the medium, the more alkaline the medium the more accumulation.

DETERMINATION OF CYTOPLASMIC AND VACUOLAR CONCENTRATIONS BY THE INTERFERENCE MICROSCOPE

Until the development of the interference microscope no method was available that would enable measurement to be made of the concentration of solute in the vacuoles of living cells.

The interference microscope has the advantage in that it can be used to measure the mass, concentration, or thickness, of single cells and other tissue elements. In practice, it has been the unknown thickness of most cells which has been the main difficulty with the technique, but in the case of cells that are approximately spherical or cylindrical in shape, the thickness can be estimated by measuring the diameter.

The concentrations of dry substance in Amoeba, different stages of development of pollen grains, nuclei of cells and sperm heads have

all been determined using the interference microscope, With the original Dyson (1950) microscope, Davies et al (1954) determined the changes in dry mass during development of pollen grain of Tradescantia bracteata. They found that the dry mass was increased about ten times during the development of mature pollen grains, and that the total dry weight increased during the formation of vacuoles. They observed that increase in the cell volume is accompanied by a decrease in the average concentration of dry substance. The larger cell with large vacuole was likely to have the lower average concentration of protoplasm. With the Baker-Smith interference microscope, the concentration of dry substance in nucleus and cytoplasm of living sea urchin eggs, the changes during fertilization and the first division have been determined by Mitchison and Swann (1953). The concentration of dry substance in the cytoplasm of the unfertilized egg is about 25 g/100 ml while that in the nucleus is about 16 g/100 ml.

Barer (1956) showed that the concentration in the cytoplasm of most tissue cells is generally between 10 and 25 g/100 ml. More than 55 g/100 ml in concentration was recorded for some bacterial cells, fungal spores, cilia and some sperm heads. The concentration of human red blood cells has been shown to be about 28 - 34 g/100 ml.

The concentrations of biological materials most often vary with the change of physiological conditions. For example, the change in concentration of dry substance in cytoplasm of chick fibroblast depends upon the extent of toxicity of the medium (Barer and Dick, 1955).

In none of the studies mentioned was an attempt made to measure the concentration of solute in the vacuole, in fact in most cases material was deliberately selected that was non-vacuolate to avoid errors due to uneven distribution of dry mass within the cell.

OSMOTIC RELATIONSHIPS

Osmotic phenomena have been studied in detail in the higher plants, and animals and to a much lesser extent in the fungi. De Vries (1884) introduced a method for determining the osmotic value of plant cells by immersing them in solutions of known concentration of cane sugar or potassium nitrate and noting the concentration in which the cells show the first signs of plasmolysis. This work has been elaborately studied since then by Dixon and Atkin (1916), Ursprung and Blum (1916), Weber (1926) and many others and found to give adequate data for the estimation of the osmotic value of the cell sap. A more accurate and versatile method is the plasmometric method of Hofler (1920). The osmotic pressure is found to vary widely in different plants and even in different varieties of the same species. Bennet-Clark, Greenwood and Barker (1936) illustrated three varieties of beetroot which differed markedly in osmotic pressure and sugar content. They also showed that the value of osmotic pressure determined by the plasmolytic method is generally greater than that determined by cryoscopic methods. A fact that was interpreted by them to imply that cytoplasm is capable

of secreting water into the vacuole against an osmotic gradient. The measurement of osmotic pressure in fungi presents considerable difficulty owing to the uncertainty of detecting plasmolysis, however, the phenomenon has been studied in some parasitic fungi. The osmotic pressure of various parasitic fungi was found to be greater than that of their hosts by Thatcher (1939, 1942) and by Lonsdof (1934) who determined the osmotic pressure by means of the plasmolytic method. The higher osmotic pressure in the parasitic fungi than in their hosts was regarded as characteristic of the host-parasite relationship by Lilly and Bernett (1951). Thatcher (1942) considered that the higher osmotic pressure in the parasite was necessary to enable them to absorb water from the host plant. According to Hawker (1950), a high osmotic pressure in a parasite is not necessary for penetrating the host cuticle.

Several fungi have the ability to grow in solutions with extremely high osmotic pressures. Some data presented by Hawkins (1916) are shown in the following table.

Lilly and Barnett (1951) suggest that these data show that the limiting osmotic pressure is dependent upon both the fungus and the compounds used, and so the effect upon the fungus cannot be regarded as a simple physio-chemical process.

Species	Glucose* (atm.)	Sucrose (atm)	Potassium Nitrate (atm)	Calcium Nitrate (atm)
Plenodomus destruens	58.3	47.4	54.5	33.6
Diplodia tubericola	63.2	42.1	58.8	33.6
Rhizopus nigricans	663.2	42.1	27.5	15.9
Botrytis cinerea	63.2	47.4	54.5	27.7
Ceratostomella fimbriala	63.2	47.4	54.5	19.5

* Limiting concentrations not used.

MATERIALS AND METHODS

MATERIALS AND METHODS

ORGANISM AND CULTURE METHODS

Rhizoctonia solani Kuhn was used in the experimental part of this investigation. This fungus was obtained from the stock culture collection (No. R-3) of the Department of Botany of the University of Manitoba. In addition, use was made of the collection of micrographs of this and other fungi held by the Electron Microscope Section of the Division of Biological Sciences.

The stock cultures were stored on potato-carrot agar medium (Dade, 1960). The medium is composed of 20 gm each of potato and carrot and 15 gm of agar per litre of distilled water. The fungus was transferred from the above medium and grown on sucrose-peptone agar medium at 20-25°C for use in the experiments.

Sucrose-peptone agar medium:

Sucrose	10 gm
Peptone	5 gm
KH_2PO_4	2 gm
$MgSO_4 \cdot 7H_2O$	0.5 gm
Agar	15 gm
Distilled H_2O	1000 ml

For microscopic observations of undisturbed hyphae, it was necessary to grow the fungus on slide culture (Gow, 1958; King, 1964). Standard 1" x 3" microscope slides were sterilized in a plain agar medium with a composition of 15 gm agar per litre of distilled water

in a pyrex storage dish. After sterilization in an autoclave for 15 minutes at 15 lb pressure, the microscope slides were taken out with the aid of sterile forceps. The excess plain agar was allowed to drain off and each slide was placed in a petri plate on the surface of a solidified layer of 1.5% agar. After the thin film of plain agar had solidified, a disc cut with a No 2 cork borer (4 mm diameter) was taken from the edge of a one week old culture of R. solani grown on sucrose-peptone agar. The inoculum was placed one end of the slide, and the petri plate was placed in a dessicator jar lined with filter paper moistened with a dilute copper sulphate solution to prevent bacterial growth. The method ensured that a high humidity was maintained throughout the growing period of the fungus.

The 5-7 day old slide cultures of R. solani were taken out from the petri plates and a thin glass strip, cut with a diamond tipped slide marker from a 22 x 50 mm cover glass was placed on either side of the hyphae which were then mounted with drops of distilled water under a 22 x 50 mm cover glass which rested on the strips. This enabled the mounting solution to be changed by adding drops of the new solution at one end while the old solution was drawn off with filter paper at the other.

DYE ACCUMULATION

The uptake and accumulation of neutral red was studied by the classical technique as used by Chamber (1930), Scarth (1926), Singh

(1937) and Taylor and Hayes (1942). The following neutral red solution was used:

Neutral Red Chloride (B.D.H. Standard Stain) ---- 0.05 gm
Distilled water ---------- 1000 ml

The pH of the solution was adjusted to a suitable level by the addition of drops of 0.01 M NH_4OH.

STUDIES OF THE PHYSICAL FORM OF VACUOLES

All routine observations were carried out with a positive phase contrast microscope, and unless otherwise stated, the micrographs in this thesis are photographed through such a microscope. Most of the micrographs have been printed for maximum contrast to reveal the outline of the vacuoles most clearly.

An electron microscope was used to study the fine structure of the vacuoles. Portions of 7-day old cultures of R. solani growing on sucrose-peptone agar were cut into small sections approximately 1 mm square and fixed with 1.2% unbuffered aqueous $KMnO_4$ solution for about half an hour at room temperature. Dehydration was carried out using a graded ethyl alcohol series for about 15 minutes at each change. The material was embedded in cross-linked methacrylate consisting of 0.5% of the cross-linking agent divinyl benzene in butyl methacrylate and with 0.8% benzoyl peroxide as a catalyst. Polymerization of the blocks was brought about under vacuum at 58°C for 24 hours (Pease, 1960). The blocks were sectioned by means of a

glass knife on the LKB ultramicrotome. Thin sections were mounted on carbon films and observed under a Philips EM-75 B electron microscope.

DETERMINATION OF DRY MATTER CONCENTRATION

Both the dry matter concentrations of the vacuoles and of the cytoplasm were determined by using the Dyson interference microscope (Vicker Instruments, Model No. M 2595). The measurements were made on the slide cultures mounted in distilled water, using Kohler illumination with white or monochromatic (546 mμ) light. The diameters of the hyphae and of the vacuoles were measured with the aid of a filter eyepiece (6x).

The measurements were made after rotating the wedge plates of the interference microscope to the parallel position, i.e. until the field was uniformly illuminated at the maximum fringe width (Hale, 1958).

The dry matter concentration of the cytoplasm was calculated from the optical retardation and the depth of the hyphae with the following formula:

$$Cc. = \frac{Rh \cdot \lambda \cdot 100.}{Dh. \ M. \ X.} \quad g/100 \text{ ml} \quad \text{---------- (1)}$$

Where Cc = dry matter concentration (g/100 ml) of the cytoplasm

Rh = optical retardation, in micrometer units, of the hyphae (no vacuole)

λ = wavelength of light used (0.546 μ)

Dh = depth of hypha (μ)

M = micrometer constant (= 130 units/ λ)

X = 0.17 = 100 α , where α is the specific refractive increment (Armstrong, Budka, Morrison, and Hassom, 1947).

The depth of the hyphae and of the vacuoles was estimated by assuming they had a circular section and measuring the diameter.

In calculating the dry matter concentration of the vacuoles allowance had to be made for the depth of cytoplasm and wall above and below the vacuole. This was achieved by measuring the retardation through the vacuole (Rhv) and through the cytoplasm immediately adjacent to the vacuole (Rh). Then the retardation of the vacuole (Rv) alone, was calculated:

$$Rv = Rhv - \frac{(Dh - Dv) \cdot Rh}{Dh} \qquad (2)$$

Where Dv = diameter of the vacuole (μ)

The calculated value for the retardation due to the contents of the vacuole was then used to calculate the dry matter concentration of the vacuole (Cv):

$$Cv = \frac{Rv \cdot \lambda \cdot 100}{Dv \cdot M \cdot X} \text{ gm/100 ml} \qquad (3)$$

In practice the values of λ, M and X are constant so the two formulae (1) and (3) reduce to:

$$Cc = \frac{Rh}{Dh} \cdot 2.5 \quad g/100 \text{ ml and } \quad Cv = \frac{Rv \cdot 2.5}{Dv} \quad g/100 \text{ ml}$$

DETERMINATION OF DIFFUSIBLE CONTENTS

To determine the amount of material in the cytoplasm and vacuole that existed in a diffusible form, that is, normally retained in the organism by the semi-permeability of the cytoplasmic membranes, as distinct from the structural material, the dry matter concentrations of the cytoplasm and vacuoles were measured with the hyphae mounted in water and again after irrigation with 4% neutral formalin.

Due to the fact that there was a certain amount of movement of the hyphae during the exchange of mounting fluid co-ordinate measurements could not be relied upon for successive measurements of the same vacuoles, so that sketch maps of the surrounding hyphal system were used as an aid to location.

DETERMINATION OF OSMOTIC PRESSURE

Two methods of determining the osmotic pressure of the vacuolar contents were attempted:

(a) The Plasmolytic Method (Bennet-Clark et al, 1936)

This method depends upon irrigating the hyphae with solutions of a range of known osmotic pressures and observing the concentration in which the hyphae show incipicent plasmolysis.

(b) The Plasmometric Method (Hofler, 1920)

Hofler's plasmometric method depends upon the assumption that,

within the experimental limits, the osmotic pressure of the vacuole is related to the volume as if it obeyed the gas laws. The assumption enables the osmotic pressure of the fully turgid cell to be calculated from the change in volume that takes place following transfer from equilibrium with a solution of known osmotic pressure to distilled water. To eliminate the effect of wall pressure it is necessary that the external solution be of such a concentration as to cause a slight plasmolysis.

DETERMINATION OF THE MEAN MOLECULAR WEIGHT OF VACUOLAR CONTENTS

If the dry matter concentration and the osmotic pressure are known the mean molecular weight of the contents of the vacuole can be calculated:

$$\text{Gram Molecular Wt.} = \frac{\text{O.P.} \times \text{Cv}}{2.24}$$

Where O.P. is the osmotic pressure of the vacuole.

In view of the fact that the non-diffusable compounds can be expected to have a high molecular weight and therefore contribute little to the osmotic pressure, this calculation produces a more realistic value if based on the value of the concentration of diffusable material (See Determination of Diffusable Contents).

EXPERIMENTAL RESULTS

EXPERIMENTAL RESULTS

FORM AND STRUCTURE

(a) Vacuolization of Hyphae

Observations of actively growing hyphae of R. solani were made on slide cultures with the positive phase contrast microscope. The apical segment was found to be devoid of vacuoles (Fig 1) and highly refractile. As the hyphae aged vacuoles started to appear, the first being seen as small globose structures attached to the wall when the hyphae were 20-30 minutes old (Fig 2). Increasing age of the hyphae resulted in the further enlargement of the vacuoles and the coalescence of some adjacent vacuoles with a consequent decrease in the number per segment. It was noticed that the vacuoles were not scattered over the wall, but tended to form in longitudinal rows (Figs 3-5). The development of vacuoles up to this stage tended to be similar in all hyphae of the same age, whether they were trunk hyphae or lateral branches. The differences between these became progressively more apparent as the vacuolization proceeded. The cytoplasm in this fungus tends to be withdrawn from the side branches during the maturation of the mycelium and concentrated in the trunk hyphae. This process results in the complete evacuation of cytoplasm from most side branches (Fig 8) and the elimination of vacuoles originally present in the trunk hyphae (Figs 7 and 9). Occasionally, the vacuoles become very extensive just before the cytoplasm is removed as is shown in Figure 6. The appearance of the cytoplasm in

the fully mature resting trunk hyphae is very similar to that in the apices, as can be seen by a comparison of Figures 1 and 9.

(b) The Effect of Cytoplasmic Streaming on Vacuoles

Superimposed upon the changes in volume of the vacuoles described in the previous section are much shorter term changes due to the effect of the cytoplasmic streaming in the hyphae. In actively growing hyphae the vacuoles could be seen constantly changing their shape and position, some would break free from their attachment to the hyphal wall and move with the cytoplasmic stream, while those that remained attached to the wall showed varying degrees of distortion. Coalescence occurred between both attached and free vacuoles. The series of photographs shown in Figure 10 demonstrates the type of change frequently seen. The photographs are of the same section of hypha taken at two minute intervals.

(c) Fine Structure of Vacuoles

In addition to the sections prepared in this study and examined with the electron microscope, the collection of micrographs of fungi held by the electron microscope section of the Division of Biological Sciences has been examined for evidence of vacuolar structure. From this survey and from the references cited in the Literature Review it is clear that vacuoles are seldom well preserved. The tonoplast is most clearly revealed by fixation in unbuffered potassium permanganate as is shown in Figure 11. The outline of this membrane is much less distinct when

osmium fixation is used (Fig 12). It was found that sections of hyphae fixed in osmium very seldom show structures that can be recognised as vacuoles; a more typical appearance is seen in the lower half of Figure 13 where the cytoplasm appears to consist of irregular areas that show a marked difference in electron density. In these sections mitochondria and nuclei are only found embedded in the denser areas, but lipid depsoits are found in both. Fixation in glutaraldehyde solutions preserves the form of the vacuole, but prevents the subsequent uptake of permanganate or osmium by the tonoplast (Figs 14-18) so that the membrane is not distinguishable.

Very few micrographs were seen with vacuoles in a position close to the wall and in each of these cases there appeared to be a layer of cytoplasm between the tonoplast and the plasmalema (Fig 11).

In one preparation of Alternaria tenuis a large central vacuole surrounded by a thin peripheral layer of cytoplasm similar to that found in the higher plant cell (Figs 14-17) was seen. Apart from these no large vacuoles were found in the sections, nor have micrographs of normal mature vacuoles such as those seen in Figures 3-5 been taken in this department or published by other authors.

Micrographs of the hyphae that appear to be empty under the optical microscope confirm that the cytoplasm is completely withdrawn leaving a hyphal tube of wall material only.

A consistent feature of electron micrographs of vacuoles is the appearance of various types of contents which, with the possible

exception of neutral red granules which will be discussed later, are not seen with the optical microscope. The youngest vacuoles are characterized by the presence of irregular flocculent contents and the absence of membrane bound bodies (Fig 11). Older vacuoles frequently contain vesicular structures varying from very small (20-50 mμ) and simple (Fig 12) to relatively large (1-2 μ) and complex with internal structure (Figs 14-17).

From the limited material available there is a suggestion that development of the vesicular contents is accompanied by a corresponding decrease in the flocculent material found in the youngest vacuoles (cf. Figs 11, 12 and 14).

(d) Interferometric Studies

Owing to the regular geometrical form of the hyphae and vacuoles it was possible to use the interferometer microscope to measure directly their solute concentration.

The readings were paired with a value for cytoplasmic concentration being obtained beside each vacuole measured. This was necessary for the calculation of vacuolar concentration, but also enabled the relationship between the two to be demonstrated.

The values for cytoplasmic and vacuolar concentrations are listed in Tables Ia and b and plotted in Fig 19. From these it is evident that there is a double relationship between the two concentration measurements. Below a vacuolar concentration of approximately 5% the correlation is negative, while above this value the correlation is

positive. The range of vacuolar concentration thus increases with increasing cytoplasmic concentration and is most restricted when the cytoplasm is most hydrated.

Table II and Figure 20 show the effect of increasing the nutrient level. The level used, 1/50 N sucrose-peptone, is at least ten times the concentration of nutrient in the plain agar medium used for the previous experiment, but is still a very weak medium. One limitation of the technique is the requirement that the mycelium be very sparse on the slide so as to prevent blocking of the reference beam in the interferometer.

The effect of increasing the nutrient level is seen in an overall increase in both vacuolar and cytoplasmic concentrations, but the relationship between the two remains the same. In both cases the increases in concentration appear to be due primarily to a smaller number of very low values rather than a marked increase in high values.

The results shown in Figures 19 and 20 were obtained from comparatively restricted areas about 10 mm behind the apices, of the mycelium measuring vacuoles in hyphae of all types. Figure 21 shows the effect of measuring the vacuolar concentration in successive vacuoles along a trunk hypha starting at the apex. The most advanced vacuoles measured were not in the apex which is devoid of vacuoles, but are the youngest ones of suitable size and position to be measured. Similarly, not all vacuoles were measured because many were too small

or in an unsuitable position; samples were taken at intervals along the hyphae. Although the variation is very large it is clear that the tendency is for the younger vacuoles to have the lower solute concentration.

In Figure 22 the values of cytoplasmic concentration are plotted in order of position and a reverse trend to that seen in vacuoles is found with the older cytoplasm having a lower concentration. This trend is further confirmed by comparing the concentrations of apices and old hyphae (7-8 days) that have ceased to be vacuolated (Table IV). The apices had a mean concentration of 41% while the old hyphae were 26% dry matter. By measuring the concentration of vacuoles and cytoplasm before and after treatment with 4% formalin an estimate can be made of the concentration in each of small molecules that are free to diffuse out of the hyphae when the semi-permeability of the plasmalemma and tonoplast are lost. Values of concentrations of cytoplasm and vacuoles in hyphae before and after treatment with formalin are given in Table V and plotted in Figure 23. It will be seen that the actual quantities lost are very similar in both the cytoplasm and in the vacuole, but that some sections of hyphae tend to lose more from the vacuoles and others the reverse. This tendency for adjacent vacuoles to be similar is shown by the examples in Figure 24 a-f.

FUNCTION STUDIES ON THE BEHAVIOUR OF VACUOLES

(2) Uptake of Neutral Red

Uptake of neutral red is only visually detectable when it is followed by accumulation of the dye to a higher concentration than the surrounding solution.

When hyphae of R. solani are immersed in a dilute (0.005%) solution of neutral red the chief factor affecting the rate of accumulation is the pH of the solution. For accumulation to take place the pH of the solution must be equal to or more than 7.0.

The first sign of accumulation is the appearance in the hyphae of small intensely stained globular bodies similar to the "neutral red granules" described by other authors. Most of these bodies appear to be inside vacuoles, but some may be free in the cytoplasm (Fig 25a). As the accumulation continues the granules increase in size until they appear to fill the vacuoles completely (Fig 25b). Further exposure to the dye solution results in an increasing accumulation and a reduction in the number of vacuoles. The sequence of micrographs shown in Figure 25 were taken during a period of immersion of 1.1/2 hours. The reduction in a number of the vacuoles is illustrated by the numbers visible in Figures 25b, c and d, which are 40, 35 and 21 respectively. Coalescence is also shown in Figure 28.

If ammonium hydroxide is added to the neutral red solution to give a pH of 9.0 the accumulation is at first very rapid but granules produced at lower pH values do not appear, instead the vacuoles increase in

density uniformly. After a short time (20 mins) the outline of the vacuoles becomes vague and the staining appears to spread into the cytoplasm (Fig 26).

If the hyphae that have accumulated neutral red normally are placed in dilute acid solution (pH $\leq$ 4.0) the dye diffuses out of the vacuoles (Fig 27).

(b) Osmotic Pressure

Attempts to measure the osmotic pressure of the vacuoles were made by both the standard plasmometric and plasmolytic methods.

The plasmometric method was thought to be suitable because of its versatility and the reasonably regular form of the vacuoles, however, when measurements were attempted it was found that the vacuoles were constantly changing their volume through division and coalescence which prevented the method being used.

The plasmolytic method involves the immersion of organism in a series of solutions of varying osmotic pressure and noting the least concentration in which plasmolysis is detectable. This concentration is considered to be equal to that of the vacuole. The two standard solutes used in this method are calcium chloride and sucrose. When the hyphae of R. solani were tested it was found to be extremely difficult to determine whether the contents were plasmolysed or not. In calcium chloride solutions the cytoplasm became granular and the vacuoles started fragmenting at a concentration of 0.06 M

(Fig 29) while in sucrose the concentration had to be raised 1.0 M before the same result was obtained (Fig 30). At no concentrations was plasmolysis similar to that seen in higher plants noticed.

DISCUSSION

DISCUSSION

Vacuoles occur abundantly in the filamentous fungi, but very little cytological study has been concerned with them. They are clearly visible under the phase contrast microscope in Rhizoctonia solani and they appear like empty spaces or indentations attached to the hyphal wall. By the aid of interference microscopy, the study of dye accumulation and electron microscopy, the results obtained from this work reveal that the vacuoles of filamentous hyphae resemble in many respects that in higher plants, but differ from them in their plasmolytic properties.

FORM AND STRUCTURE

The different stages of vacuole development shown in Figures 1-4 indicate an increase in size of vacuoles related to the age of the hyphae. The highest degree of vacuolation occurs in the branch hyphae as the cytoplasm is being withdrawn into the trunk hyphae. The translocation of protoplasm from old hyphae towards the apices and from the branch hyphae to the trunk hyphae was considered by Buller (1933), to be due to vacuolation. He thought that the pressure of the expanding vacuoles caused the streaming of the protoplasm. The highly vacuolated hyphae reach a certain age and become completely empty as the cytoplasm is withdrawn into the older hyphae.

Fusion and change in shape of vacuoles was noted and occurred most actively under the conditions of active streaming. This suggests that the flowing protoplasm may exert a pressure which causes the lateration in shape and coalescence of the vacuoles. Dowding and

Bakerspigel (1954) reported that streaming cytoplasm of Gelasinospora tetrasperma could break vacuoles free from the wall and carry them through the septal pore. However, it was noted repeatedly in this work that vacuoles changed their shape without any streaming being seen.

In R. solani, large numbers of vacuoles are in contact with the cell wall and appear to be empty spaces or indentations in the cytoplasm. However, from electron microscopical study, as shown in Figure 11, the vacuole is seen to be situated close to the hyphal wall with a thin layer of protoplasm lying between them. The vacuole is bounded with an electron-dense tonoplast, and the contents have a flocculent appearance which is characteristic of vacuoles in $KMnO_4$ fixed plant cells (Whaley, 1960). The apparent existence of a layer of cytoplasm between the tonoplast and the plasmalemma could mean the section was cut obliquely, but the existence of content appears to rule out the possibility of the vacuoles being indentations and therefore part of the free space. The electron-density of the tonoplast has been taken by many authors (e.g. Porter and Machada, 1960 and Parsons, 1961) to indicate the existence of phospho-lipoprotein.

It has been suggested that $KMnO_4$ acts as an 'unmasking' agent (Clayton, 1958; Clayton, 1959) enabling the protein to reduce the $KMnO_4$ and increase the electron density; the high density in osmium-fixed material suggests the lipid is highly concentrated.

The osmium-fixed vacuoles of R. solani show infolding of the

tonoplast which suggests some shrinkage of vacuoles took place during the preparative processes. Globular or oval, membrane-bounded organelles occur in the vacuoles of R. solani (Fig 12) and Alternaria solani (Figs 14, 15, 16 and 17). They may be the pre-existing bodies in the vacuoles that have been thought to be the "neutral red granules" and not artifacts of fixative or staining as has been suggested by other authors.

The wrinkled appearance of the tonoplast of A. solani seen in Figure 17 is similar to that described by Goldacre (1962) in rhizoids which were undergoing a rapid increase in cytoplasmic contents.

The interfermometric studies show a complex double correlation to exist between the concentrations of vacuoles and ctyoplasm, indicating a probable dynamic relationship between the two. At least two explanations are possible. First, that there are two sorts of vacuoles present and secondly, that the relationship represents a change that takes place with time. These results were obtained from a limited area of a slide culture and include hyphae of all types, but in Figures 21 and 22 the concentrations are plotted for a single trunk hypha. These suggest that a young vacuole (close to the apex) tends to have a low concentration and be surrounded by high density cytoplasm and as the hypha grows older the concentration of the vacuole tends to rise and the cytoplasm to fall. The explanation is very incomplete because of the large variation

found in all parts of the hypha.

The effect of formalin is to increase the permeability of the cell membranes and allow the diffusable substances to escape (Davies, 1954). If the vacuoles contain most of the ions and small molecules in the cell, which is suggested to account for the osmotic pressure, then the great loss of mass on treatment with formalin should occur from the vacuole. Figure 24 and Table V show that the loss in mass is approximately equal from both vacuoles and cytoplasm.

FUNCTION

As one of the cytoplasmic inclusions, vacuoles have been thought to play a role in accumulation and in regulating the osmotic processes in higher plants. In addition to this the vacuoles of fungi have been thought to exert vacuolar pressure causing the streaming of protoplasm (Buller, 1933), and forcing the cytoplasm and nuclei to the tip of basidium (Corner, 1948, Well, 1964).

The demonstration of neutral red granules in R. solani suggests that the fungus accumulates neutral red by the same process as pancreas acinor cells of mouse (Winfield, 1953 and Lacy, 1954), and Amoeba proteus (Singh, 1937).

It is not possible to say whether the membrane bound bodies seen in the vacuoles in the electron micrographs are the same or develop into the neutral red granules.

The effect of pH on the accumulation of neutral red is consistent

with the idea that the vacuoles are areas of lower pH than the surroundings enclosed in a lipophilic membrane. This suggests that the acid metabolism of the vacuole would be interesting to study.

Levitt has suggested that calcium chloride is an ideal plasmolyte for osmotic experiments because of its poor penetration and low viscosity, but it seems to have a damaging effect on the cytoplasm of R. solani. On the other hand sucrose causes the vacuoles to fragment, and in neither case could plasmolysis like that seen in higher plants be produced. This might be due to the osmotic substances being evenly distributed between the vacuoles and the cytoplasm as shown by the effect of formalin.

Bakerspigel (1954) reported that streaming cytoplasm of Gelasinospora tetrasperma could break vacuoles free from the wall and carry them through the septal pore. However, it was noted repeatedly in this work that vacuoles changed their shape without any streaming being seen.

In R. solani, large numbers of vacuoles are in contact with the cell wall and appear to be empty spaces or indentations in the cytoplasm. However, from electron microscopical study, as shown in Figure 11, the vacuole is seen to be situated close to the hyphal wall with a thin layer of protoplasm lying between them. The vacuole is bounded with an electron-dense tonoplast, and the contents have a flocculent appearance which is characteristic of vacuoles in $KMnO_4$ fixed plant cells (Whaley, 1960). The apparent existence of a layer of cytoplasm between the tonoplast and the plasmalemma could mean the section was cut obliquely, but the existence of content appears to rule out the possibility of the vacuoles being indentations and therefore part of the free space. The electron-density of the tonoplast has been taken by many authors (e.g. Porter and Machada, 1960 and Parsons, 1961) to indicate the existence of phospho-lipoprotein.

It has been suggested that $KMnO_4$ acts as an 'unmasking' agent (Clayton, 1958; Clayton, 1959) enabling the protein to reduce the $KMnO_4$ and increase the electron density; the high density in osmium-fixed material suggests the lipid is highly concentrated.

The osmium-fixed vacuoles of R. solani show infolding of the

SUMMARY

SUMMARY

(1) The development of vacuoles in Rhizoctonia solani Kuhn was shown to be related to the age of the hyphae.

(2) The vacuoles were seen to change their shape and coalesce.

(3) Electron microscopy showed the vacuoles to be surrounded by a tonoplast and to have contents that were flocculent in young vacuoles and vesicular in old vacuoles.

(4) Interferometric studies showed a double correlation between the vacuolar and cytoplasmic contents.

(5) Young vacuoles tended to have lower concentrations and be surrounded by denser cytoplasm than older ones.

(6) Measurements of mass before and after treatment with formalin showed that diffusable substances were equally distributed between the vacuoles and cytoplasm.

(7) Treatment with neutral red solutions showed initial accumulation of the dye in "neutral red granules". Further accumulation of neutral red lead to coalescence of vacuoles.

(8) No clear measurment could be made of the osmotic pressure of the vacuoles.

BIBLIOGRAPHY

BIBLIOGRAPHY

Armstrong, S. H., Budka, M. T. E., Morrison, K. C. and Hassin, M. Preparations and properties of serum and plasma proteins. XII. The refractive properties of the proteins of human plasma and certain purified fractions. J. Am. Chem. Soc. 69: 1747-1753,1947.

Arthur, J. C. The movement of protoplasm in coenocytic hyphae. Ann. Bot. 11: 491-507, 1897.

Baker, J. R. The structure and chemical composition of the Golgi elements. Quart. J. Micr. Sci. 85: 1-71, 1945.

Barer, R. Determination of dry mass, thickness, solid and water concentration in living cells. Nature 172: 1097-1098, 1953.

Barer, R. and Dick, D. A. T. J. Physiology (London). 128: 25 p. 1955.

Barer, R. Phase contrast and interference microscopy in cytology. Physical Techniques in Biological Research 3: Academic Press, New York. 29-90, 1956.

Bath, J. D. and Sponsler, O. L. Molecular structure in protoplasm. The Structure of Protoplasm. 41-79. Ames: Iowa State College Press,1942.

Bennet-Clark, T. A., Greenwood, A. D. and Baker, J. W. Water relations and osmotic pressures of plant cells. New Phytologist 35: 277-291, 1936.

Brown, R. and Cartwright, P. M. The absorption of potassium by cells in the apex of the root. J. Exptl. Bot. 4: 197-221, 1957.

Buller, A. H. R. Researches on the Fungi. Vol.3. Longmans, Green and Co., London, 1933.

Buller, A. H. R. Researches on the Fungi. Vol. 5. Longmans, Green and Co., London, 1935.

Chambers, R. Vital staining with methyl red. Proc. Soc. Exper. Biol. & Med. 27: 809-811, 1930.

Chambers, R. and Chambers, E. C. Explorations into the Nature of the Living Cell. Harvard University Press, Massachusetts, 1961.

Clayton, Blanche-P. Unmasking of sudapophil lipid in the testis of the house-cricket, Aceta domesticus. 99: 453-457, 1958.

Clayton, Blanche-P. The action of fixatives on the unmasking of lipid. Quart. J. Micr. Sci. 100: 269-274, 1959.

Corner, E. J. H. Studies in basidium. I. The ampoule effect, with a note on nomenclature. New Phytologist. 47: 22-31, 1948.

Daniel, C. Pease. Histological Techniques for Electron Microscopy. Academic Pressm New York, 1964.

Davies, H. G. The determination of mass and concentration by microscope interferometry. General Cytological Method. 55-161, Academic Press, New York. Vo.1. 1954.

Davies, H. G. The action of fixatives on the ultra-violet absorbing components of chick fibroblast. Quart. J. Micr. Sci. 95: 423-457, 1954.

Dr Vries, Hugo, Eine Methode zur Analyze der Turgorkraft. Jahr. wiss. Bot. 14: 427-601, 1884.

Dixon, H. H. and Atkins, W. R. G. Osmotic pressures of plants. Sci. Proc. Roy. Dublin Soc. 15: 51-62, 1916.

Dowding, E. S. and Bakerspigel, A. The migrating nucleus. Can. J. Microbiol. 1: 68-78, 1954.

Dyson, J. An Interference Microscope. Proc.Roy. Soc. A204: 170-187, 1950.

Gicklhorn, Beobachtungen uber vitale Farbstoff. Kolloidchem. Beih. 28: 367, 1929.

Goldacre, R. J. Active transport and membrane expansion-contraction cycles. Biological Structures and Function 2. Academic Press, New York, 1962.

Gow, C. M.Sc. thesis. University of Manitoba. 1958.

Hale, A. J. The Interference Microscope in Biological Research. E. and S. Livingstone Ltd., Edinburgh and London, 1958.

Hawker, L. E. Physiology of Fungi. University of London Press Ltd., 1950.

Hawkins, L. A. Growth of parasitic fungi in concentrated solutions. J. Agr.Research 7: 255-260, 1916.

Hober, R. Physical Chemistry of Cells and Tissues. The Blackston Company, 676 p. p.263, 1946.

Hofler, K. Ein Schema fur die osmotische Liestung der Pflanzenzelle. Ber. De Deut. Bot. Gesell. 38: 288-298, 1920.

Isaac, P. K. Cytoplasmic streaming in filamentous fungi. Can. J. Bot. 42: 787-793, 1964.

King, M. K. M.Sc. thesis. University of Manitoba, 1964.

Lacy, D. The origin, identity and function of neutral-red bodies in living fixed frozen-dried Acinous cells of the pancrease of the mouse. J. Roy. Micr. Soc. 75: Series 3. 155-171, 1955.

Levitt, J. Osmotic pressure measuring devices. Instrumental Methods of Experimental Biology. MacMillan Company, New York.

Lilly, V. G. and Bernette, H.C. Physiology of Fungi. McGraw-Hill Book Company, London, 1951.

MacRobbie, E. A. C. J. Gen. Phys. 47: 859-877, 1964.

Manton, I. Observations on stellate vacuoles in the meristem of Anthoceros. J. Exptl. Bot. 13: 161-168, 1962.

Mitchison and Swann. Measurements on sea-urchin eggs with an interference microscope. Quart. J. Micr. Sci. 94: 381-389, 1953.

Parson, D. F. A simple method for obtaining increased contrast in Araldite sections by using postfixation staining of tissues with potassium permanganate. J. of Biophys. Biochem. Cytol. 11: 492-497, 1961.

Porter, K. R. and Machada, R. D. Studies on the endoplasmic reticulum. IV. Its forms and distribution during mitosis in cells of onion root tip. J. Biophys. Biochem. Cytol. 7: 167-186, 1960.

Scarth, G. W. The mechanism of accumulation of dye by living cells. Plant Phys. 1: 215-229, 1926.

Singh, B. N. The cytology of Amoeba proteus 'Y' and the effects of large and small centrifugal forces. Quart. J. Micr. Science. 80: 601-635, 1937.

Taylor, M.and Hayes, C. Amoeba lescherae - its morphology, cytology and life-history. Quart. J. Micr.Sci. 84: 295-328, 1942.

Thatcher, F. S. Osmotic and permeability relations in the nutrition of fungus parasites. Am. J. Bot. 26: 449-458, 1939.

Ursprung, A. and Blum, G. Uber die periodischen Schwankungen des osmotischen Wertes. Ber. Deut. Bot. Gesell. 34: 105-123, 1916.

Weber, F. Hitze-Resistenz funktioniereuder Schliesszellen, Planta 1: 553-557, 1926.

Wells, K. The basidia of Exidia nucleata. I. Ultrastructure. Mycologia, 56: 327-341, 1964.

Whaley, W. G. et al. The ultrastructure of the meristematic cell. Am. J. Bot. 47: 401-449, 1960.

Winfield, S. Morgan. Cytological studies of the Acinar cells of the pancrease of the mouse. Part I. The formation of neutral red granules. Quart. J. Micr.Sci. 94: 141-153, 1953.

Yoshimi Nagatani. Cytological studies on the affinity of the carcinogenic Ago dyes for cytoplasmic components. Int. Rev. Cytol. Vol.10: 243-313, 1960.

Zirkle, C. The plant vacuole. Bot. Rev. 3: 1-30, 1937.

FIGURES AND TABLES

FIGURES 1 - 10. Micrographs of hyphae of *R. solani* growing in slide culture on a plain agar medium. Positive phase contrast. Magnification 1000 x.

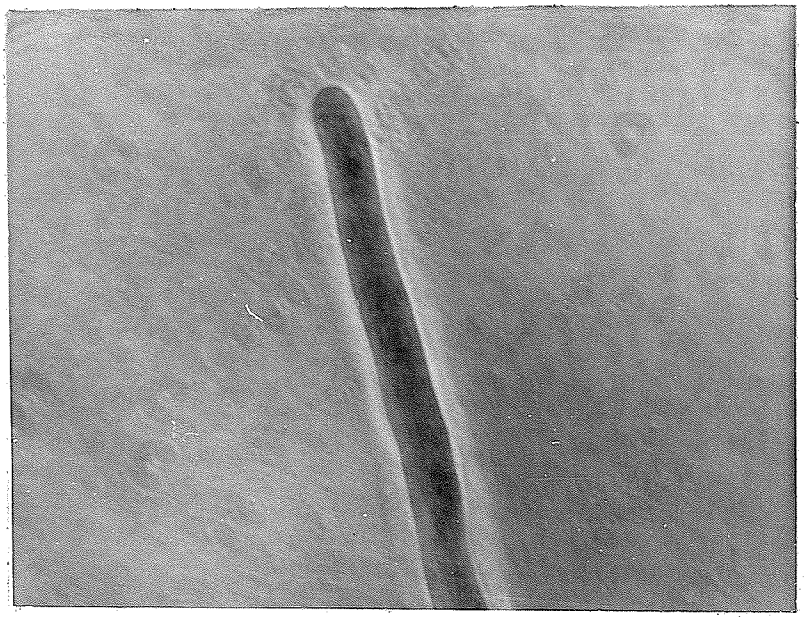


FIGURE 1. Hyphal apex showing dense cytoplasm and absence of vacuoles.

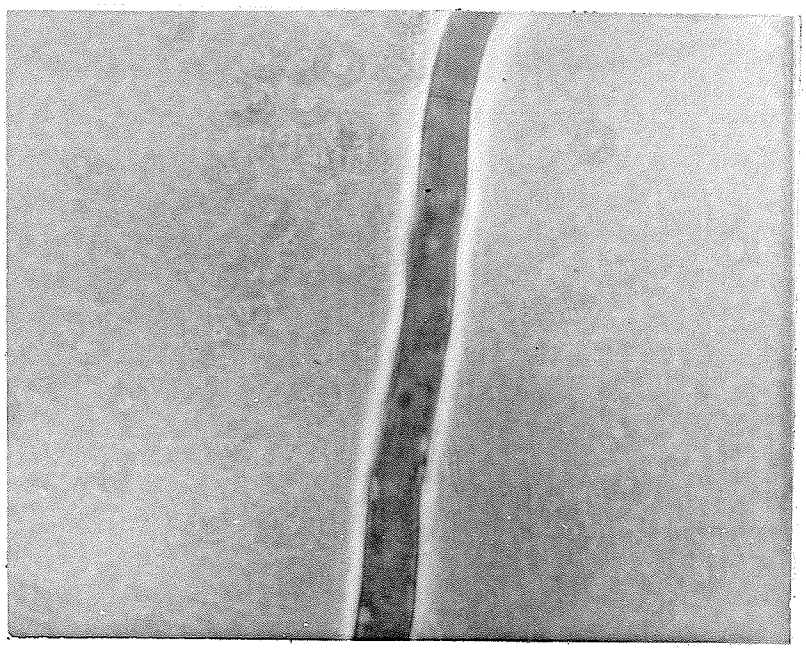


FIGURE 2. Portion of young hypha showing the first appearance of vacuoles.

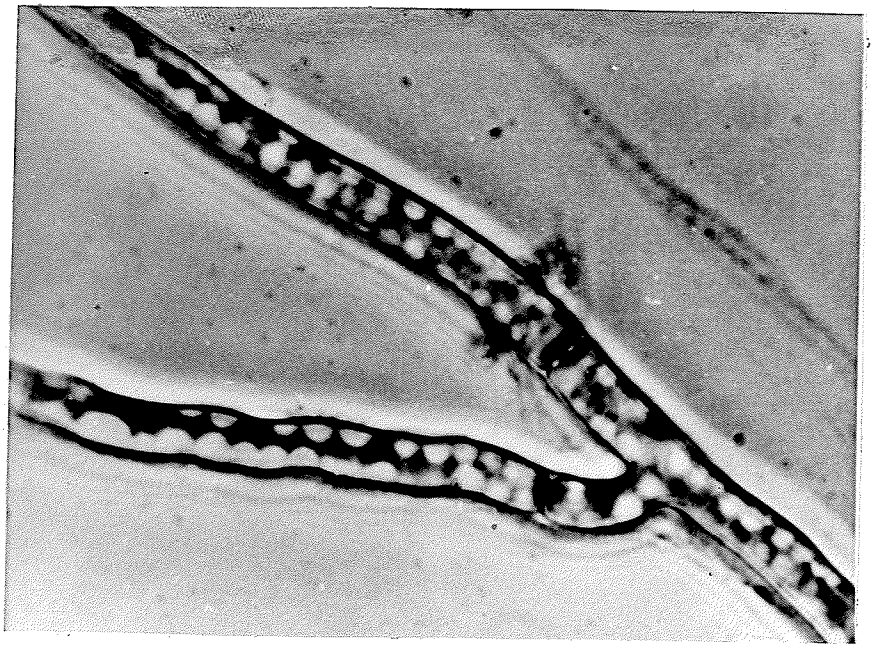


FIGURE 3. Many small vacuoles adhering to the hyphal wall in rows.

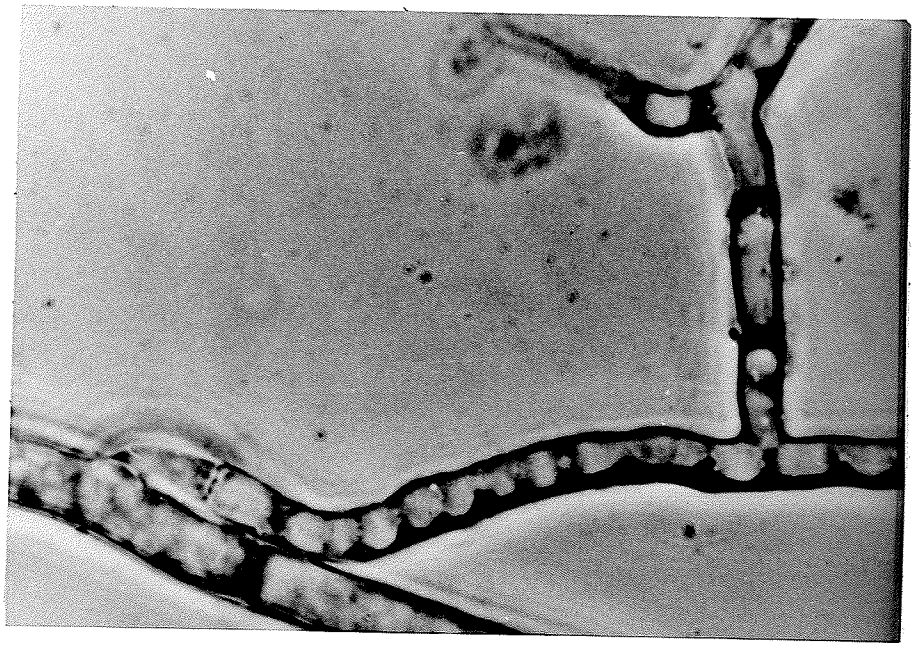


FIGURE 4. An older stage of Figure 3 showing the increase in size and coalescence of adjacent vacuoles.

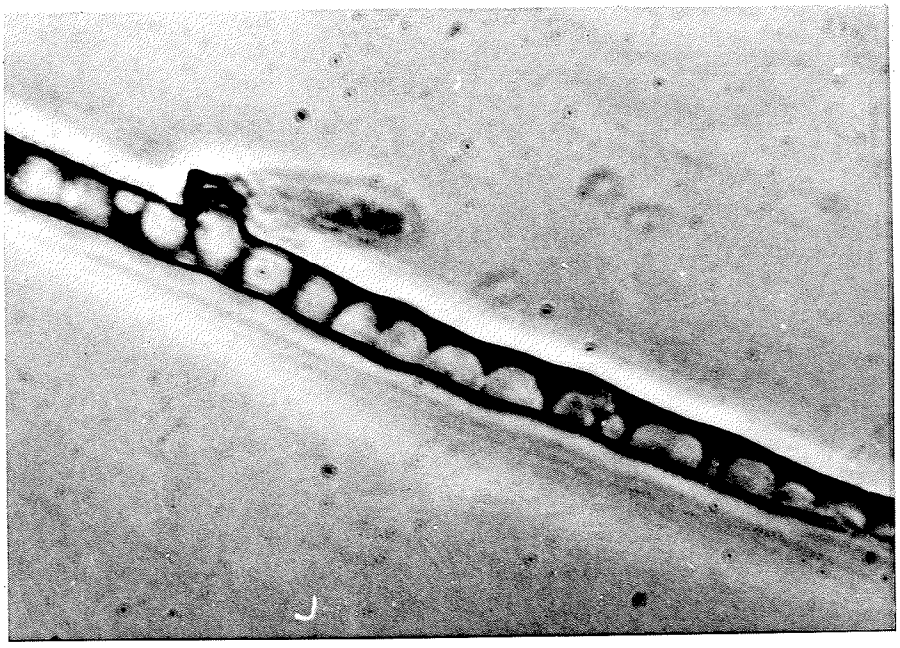


FIGURE 5. A later stage of development than Figure 4.

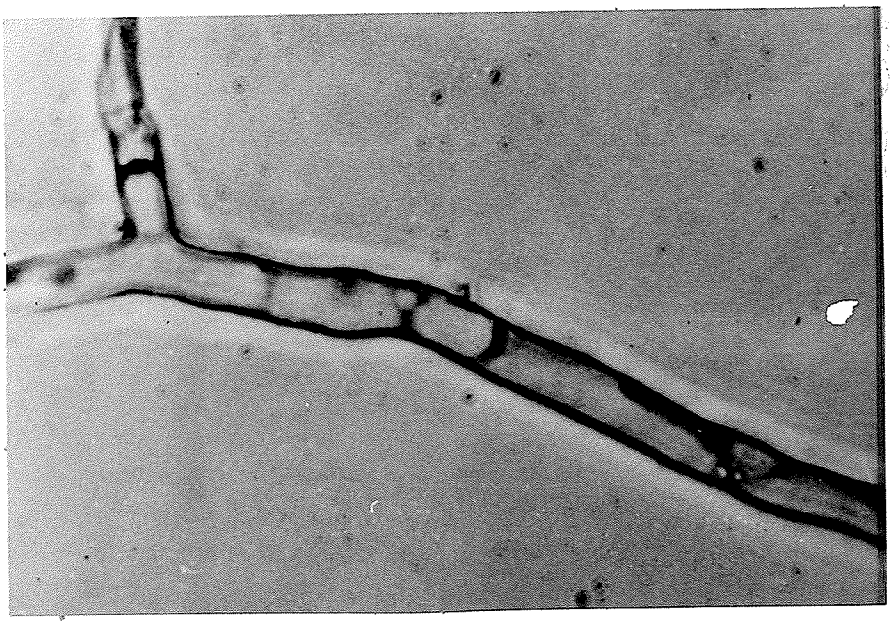


FIGURE 6. A portion of old hyphae (6-7 days) showing attenuation of cytoplasm as occasionally seen prior to evacuation of the hypha.

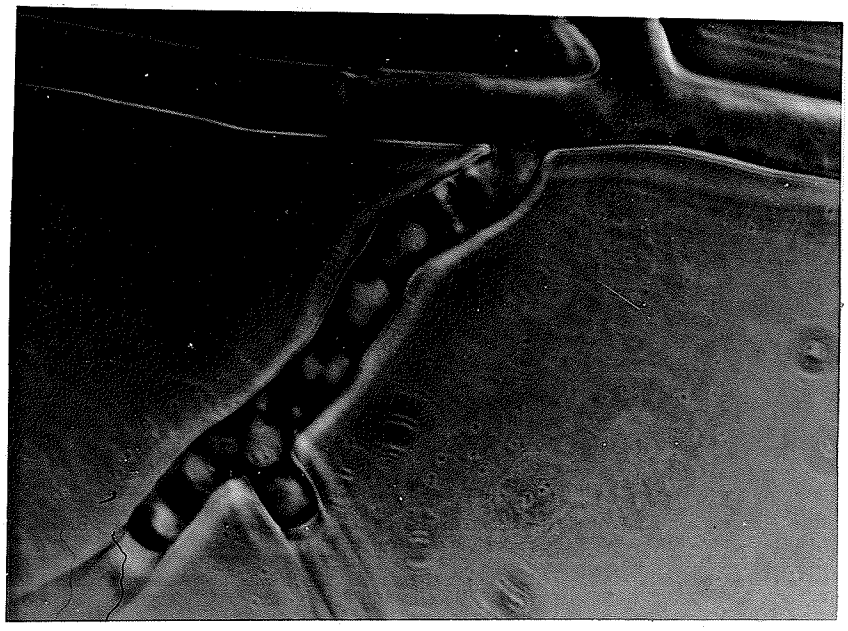


FIGURE 7. Evacuation of cytoplasm of a side branch. The lowest hyphae are empty and the cytoplasm of the vacuolated portion is moving into the full trunk hypha at the top of the micrograph.

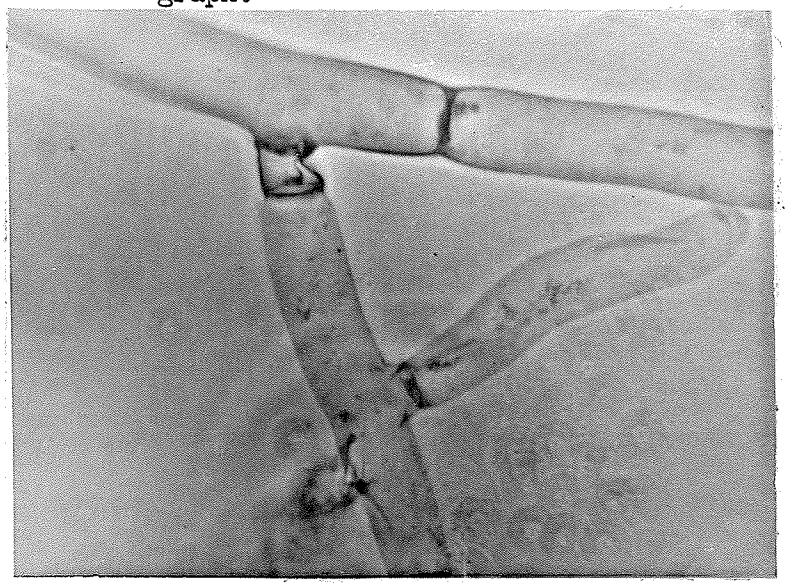


FIGURE 8. Completely evacuated hyphae.

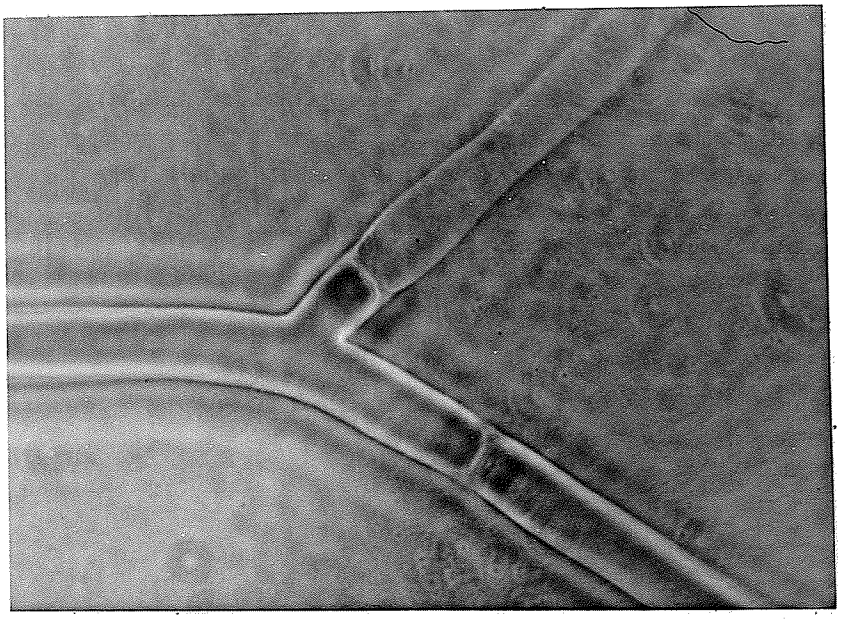


FIGURE 9. A portion of old mycelium (6-7 days) showing an empty branch and full trunk hypha.

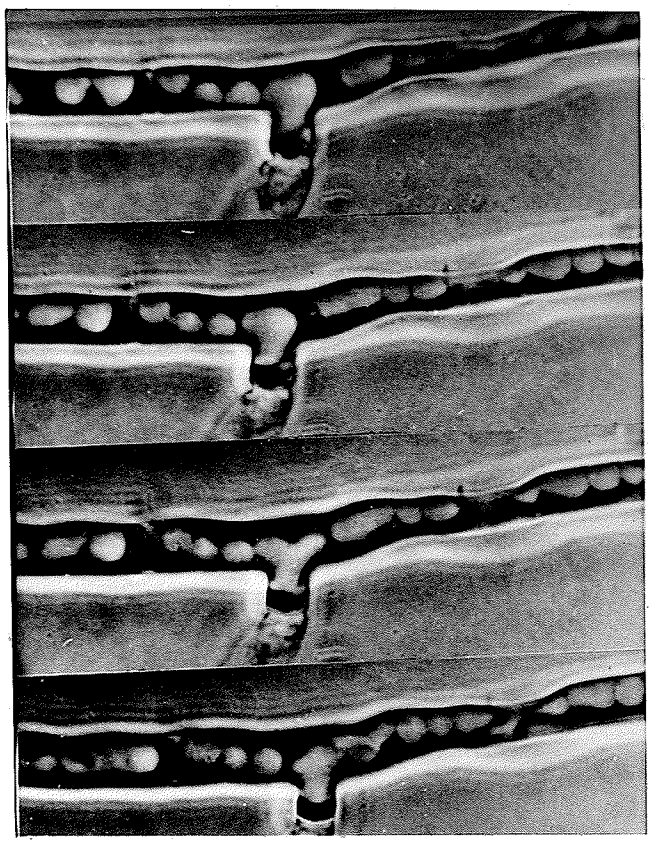


FIGURE 10 (a-d). A series of micrographs of a portion of hyphae showing the change in shape of the vacuoles; 2 minute intervals.

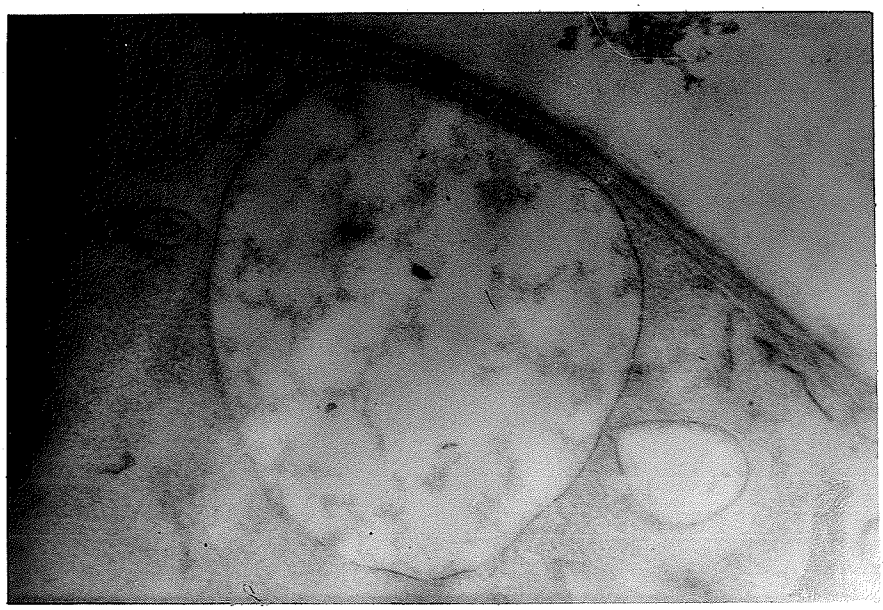


FIGURE 11. Electron micrograph of vacuoles fixed in $KMnO_4$ showing tonoplast, flocculent contents and a layer of cytoplasm between the vacuole and the plasmalema.

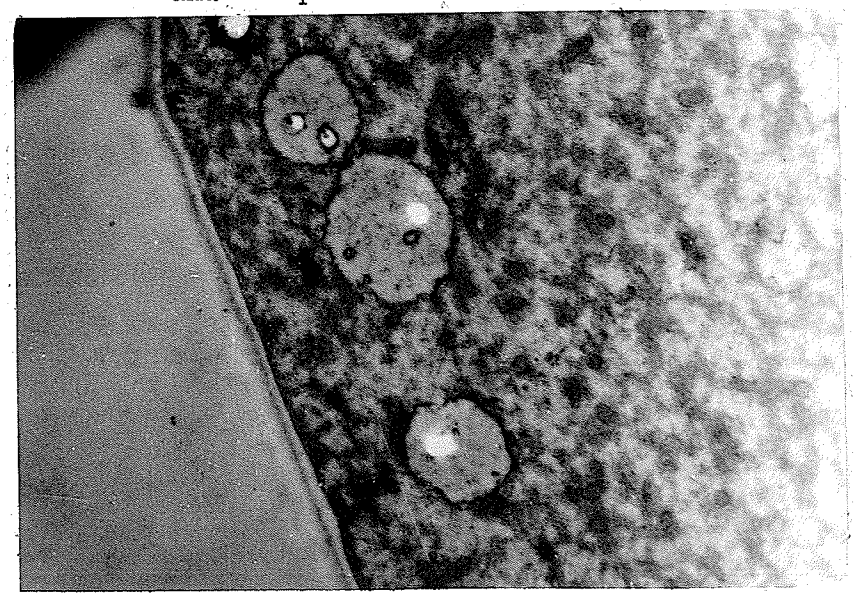


FIGURE 12. Electron micrograph of vacuoles fixed in osmium showing fine vesicular contents.

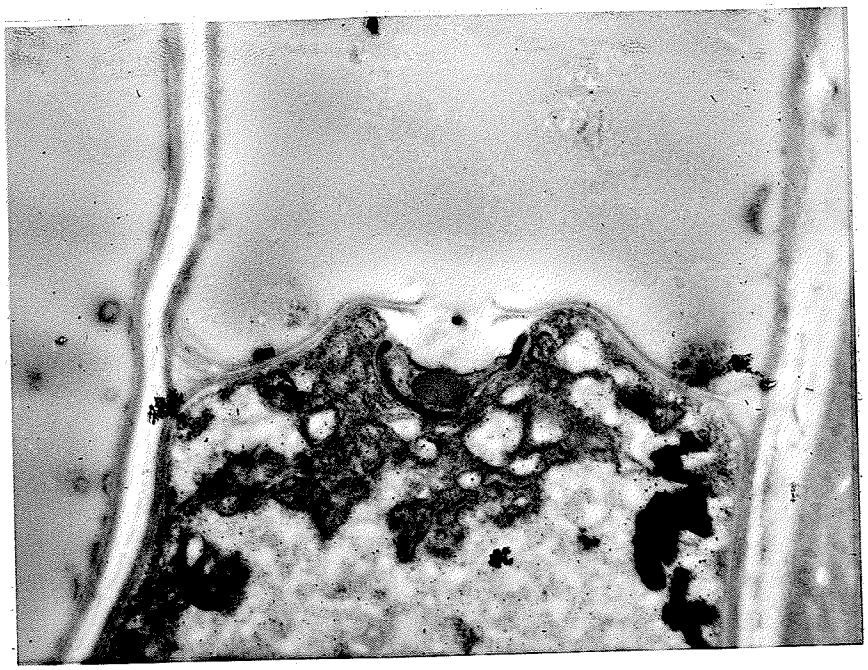


FIGURE 13. Electron micrograph of R. solani fixed in osmium showing a septum between full and empty parts of the hypha.

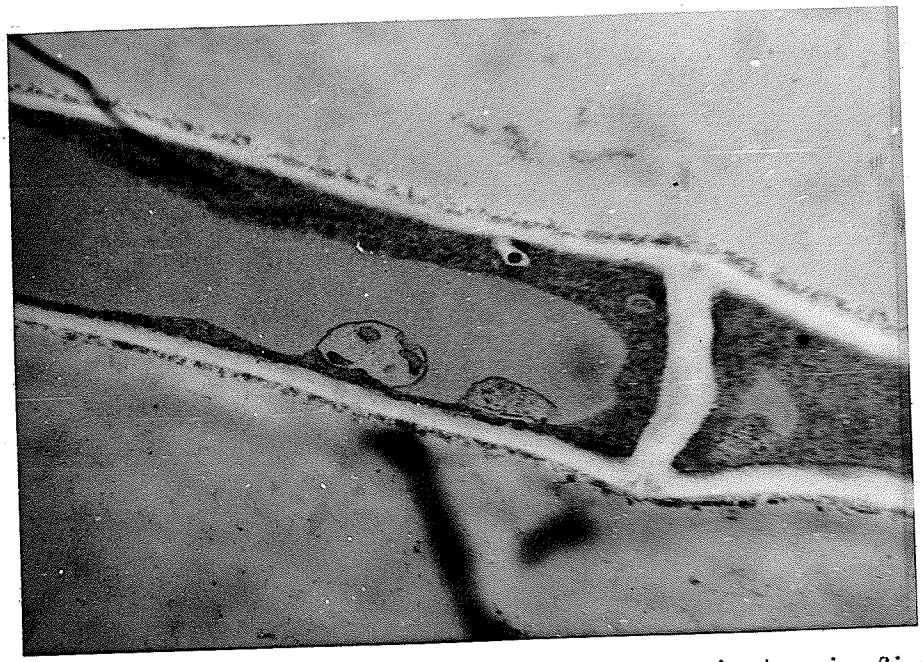


FIGURE 14. Electron micrograph of Alternaria tenuis fixed in glutaraldehyde and $KMnO_4$ showing a large central vacuole containing complex bodies.

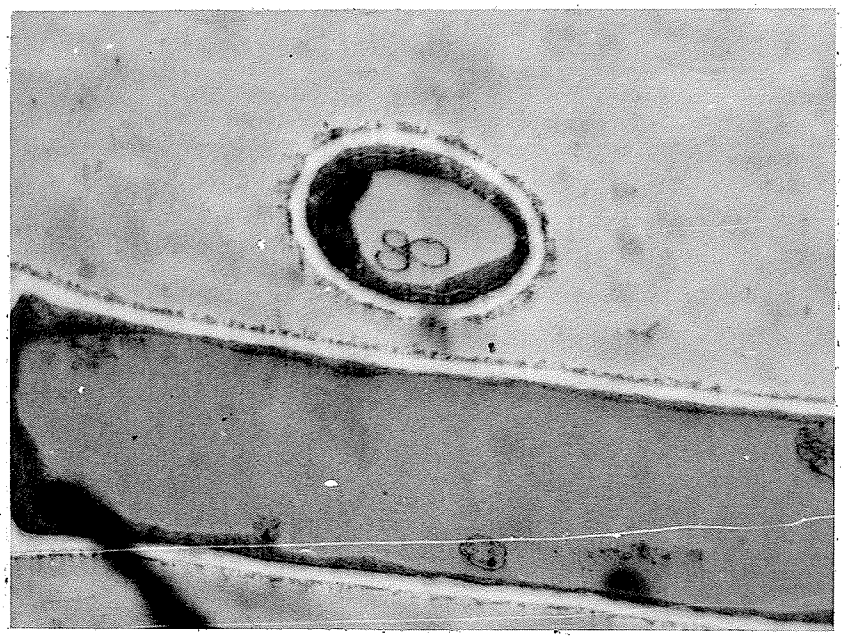


FIGURE 15. Similar to Figure 14 showing membrane bound vesicles.

FIGURE 16. Similar to Figure 15.

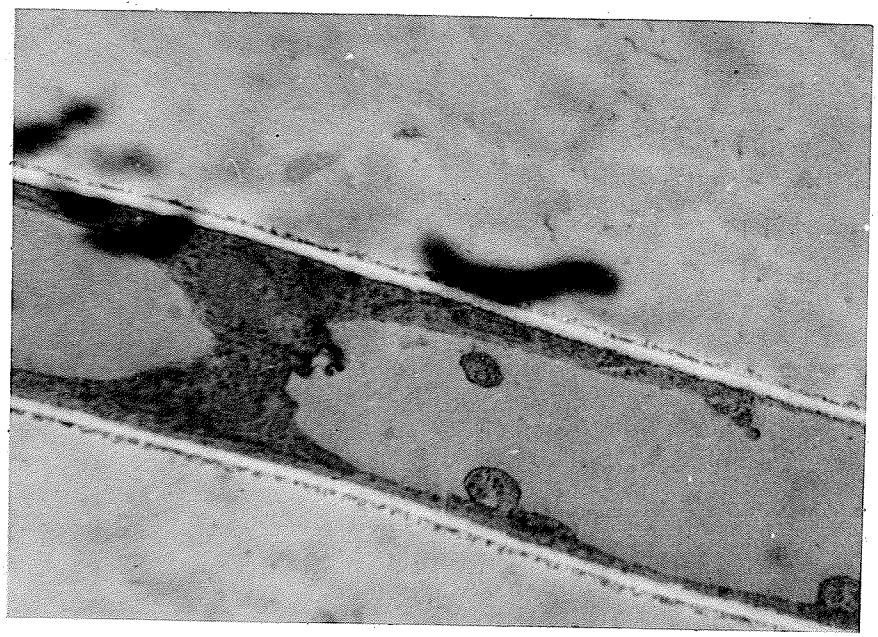


FIGURE 17. Similar to Figure 14.

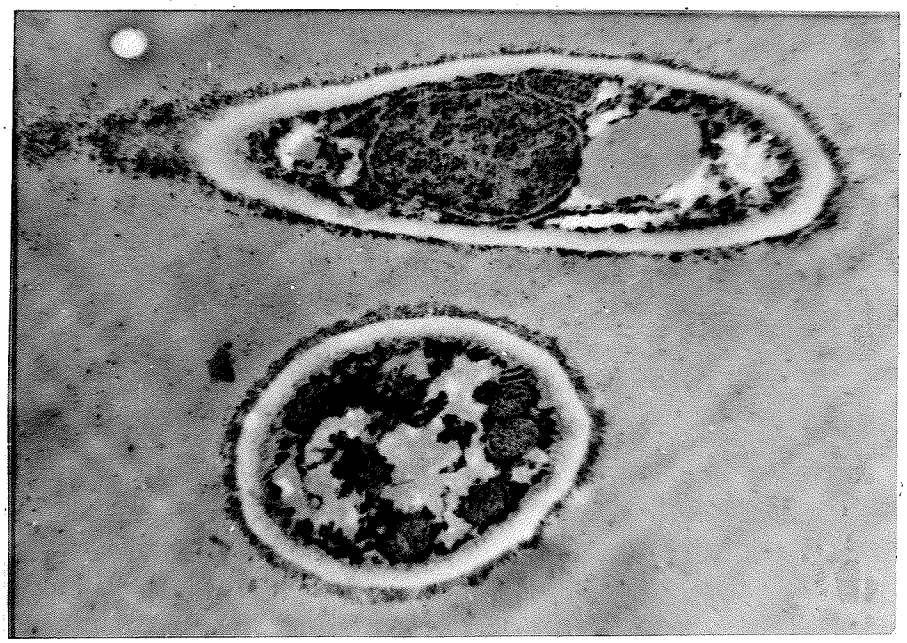


FIGURE 18. Electron micrographs of young hyphae of A. solani fixed in glutaraldehyde and $KMnO_4$ showing the low density of membranes.

FIGURE 19. Diagram showing the relationship between the concentration (g/100 ml) of vacuoles and surrounding cytoplasm. R. solani, slide culture, plain agar.

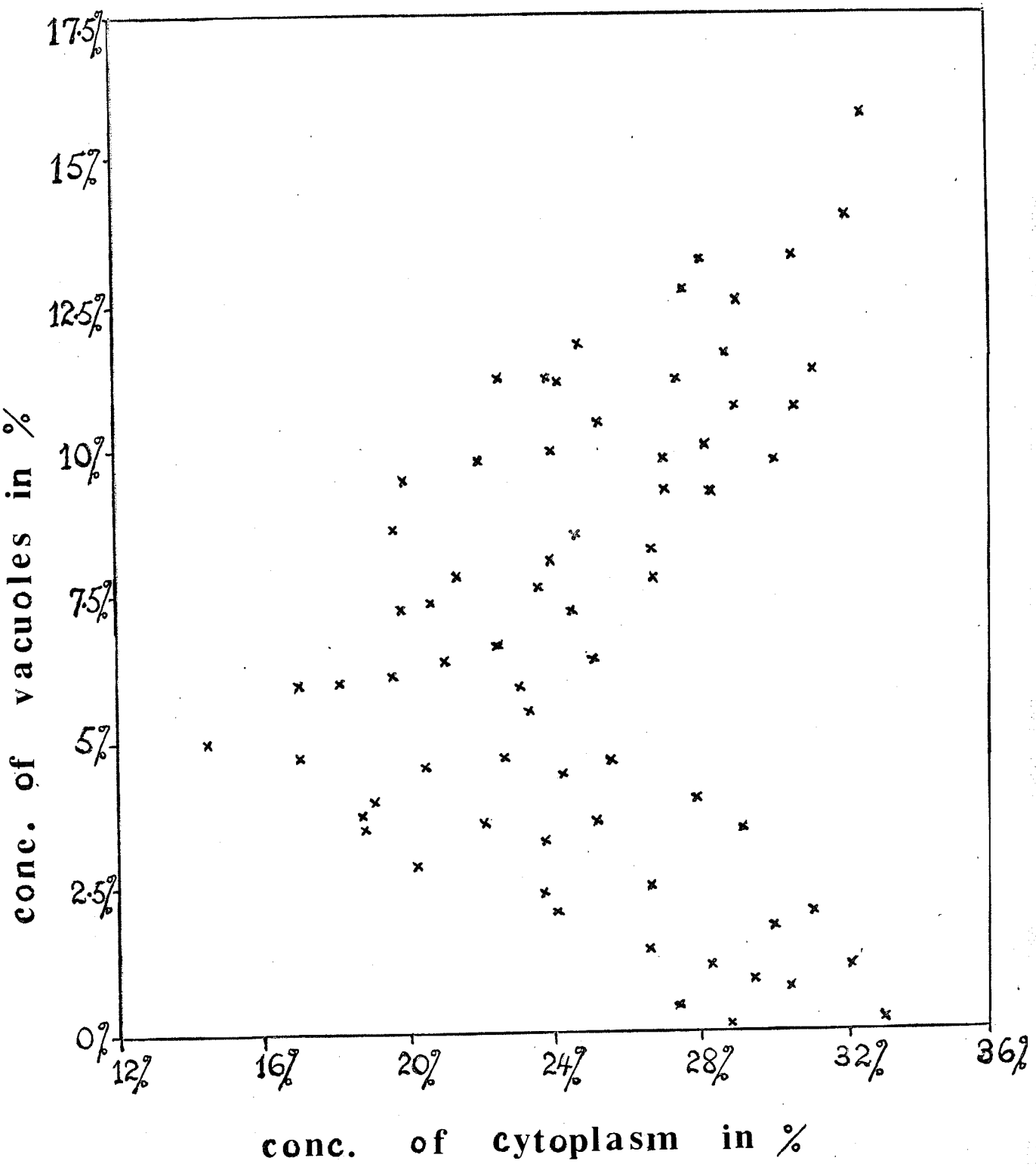

17.5%
15%
12.5%
10%
7.5%
5%
2.5%
0%
12%
16%
20%
24%
28%
32%
36%
conc. of vacuoles in %
conc. of cytoplasm in %

FIGURE 20. As in Figure 19, but culture grown on 1/50 N sucrose-peptone medium.

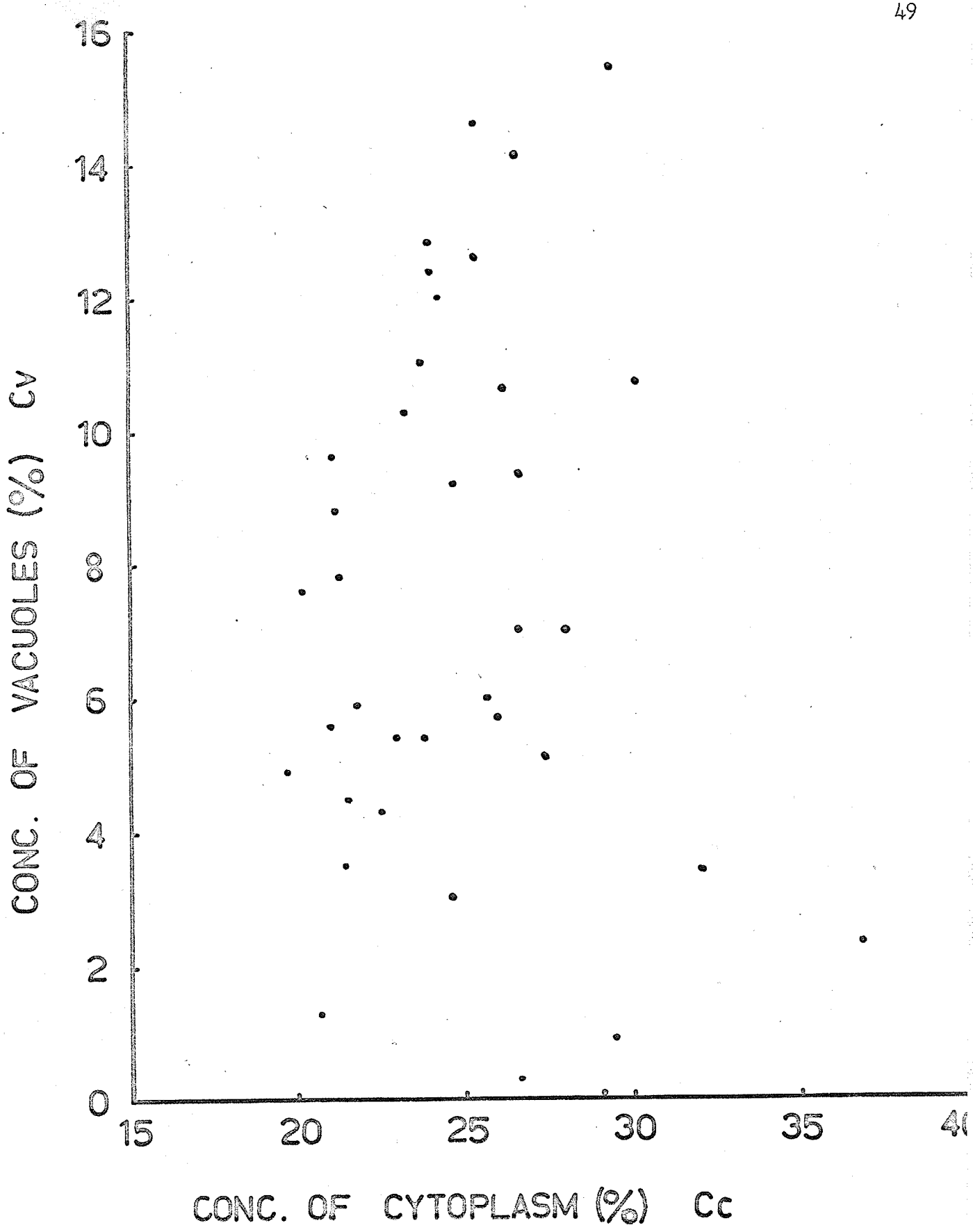

16
14
12
10
8
6
4
2
0
15
20
25
30
35
CONC. OF VACUOLES (%) Cv
CONC. OF CYTOPLASM (%) Cc

FIGURE 21. Diagram of the relationship between vacuolar concentration and distance of vacuole from apex. *R. solani*, slide culture, plain agar.

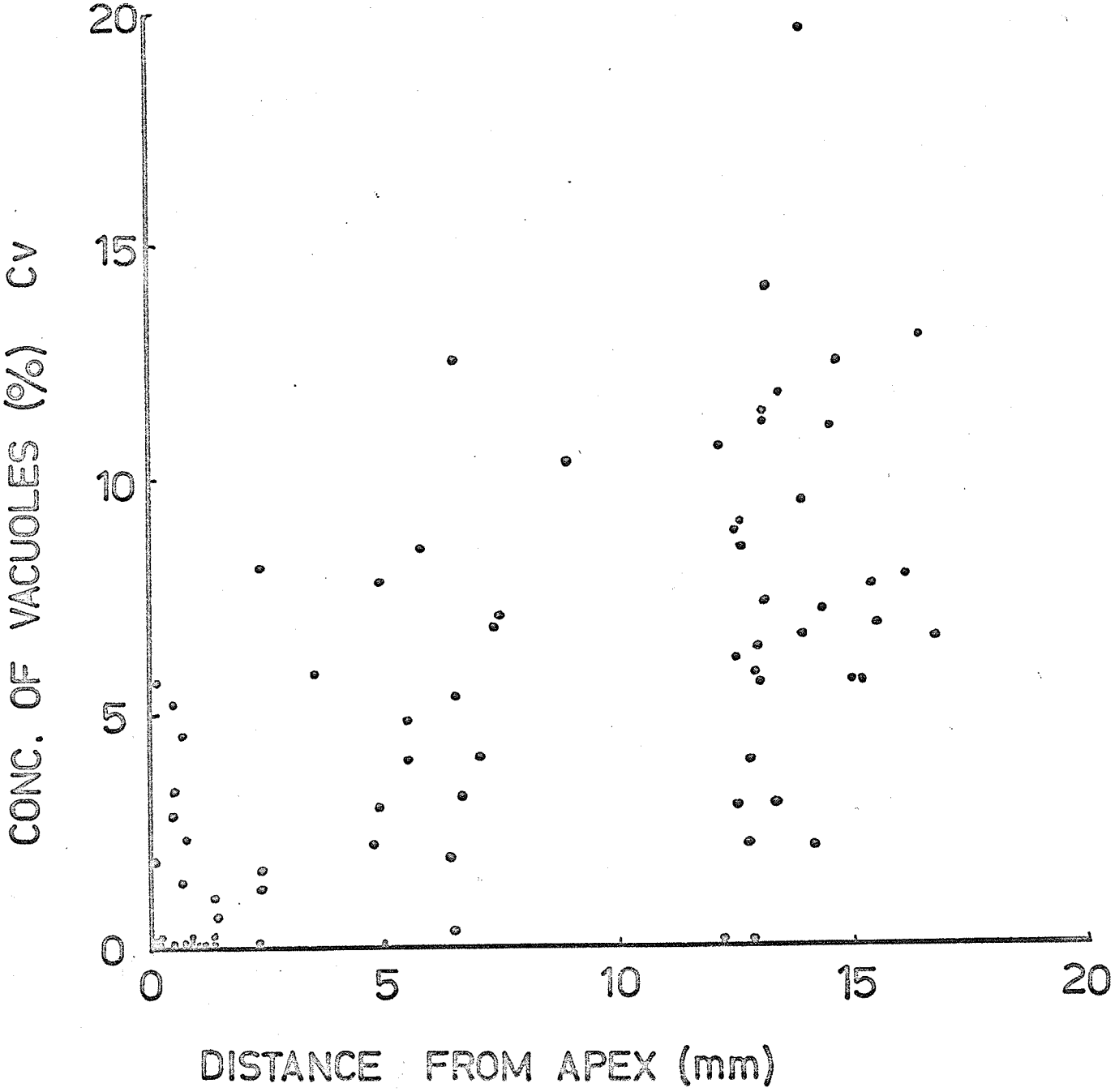

CONC. OF VACUOLES (%) Cv
20
15
10
5
0
0
5
10
15
20
DISTANCE FROM APEX (mm)

FIGURE 22. As in Figure 21, but relationship between cytoplasmic concentration and distance from apex.

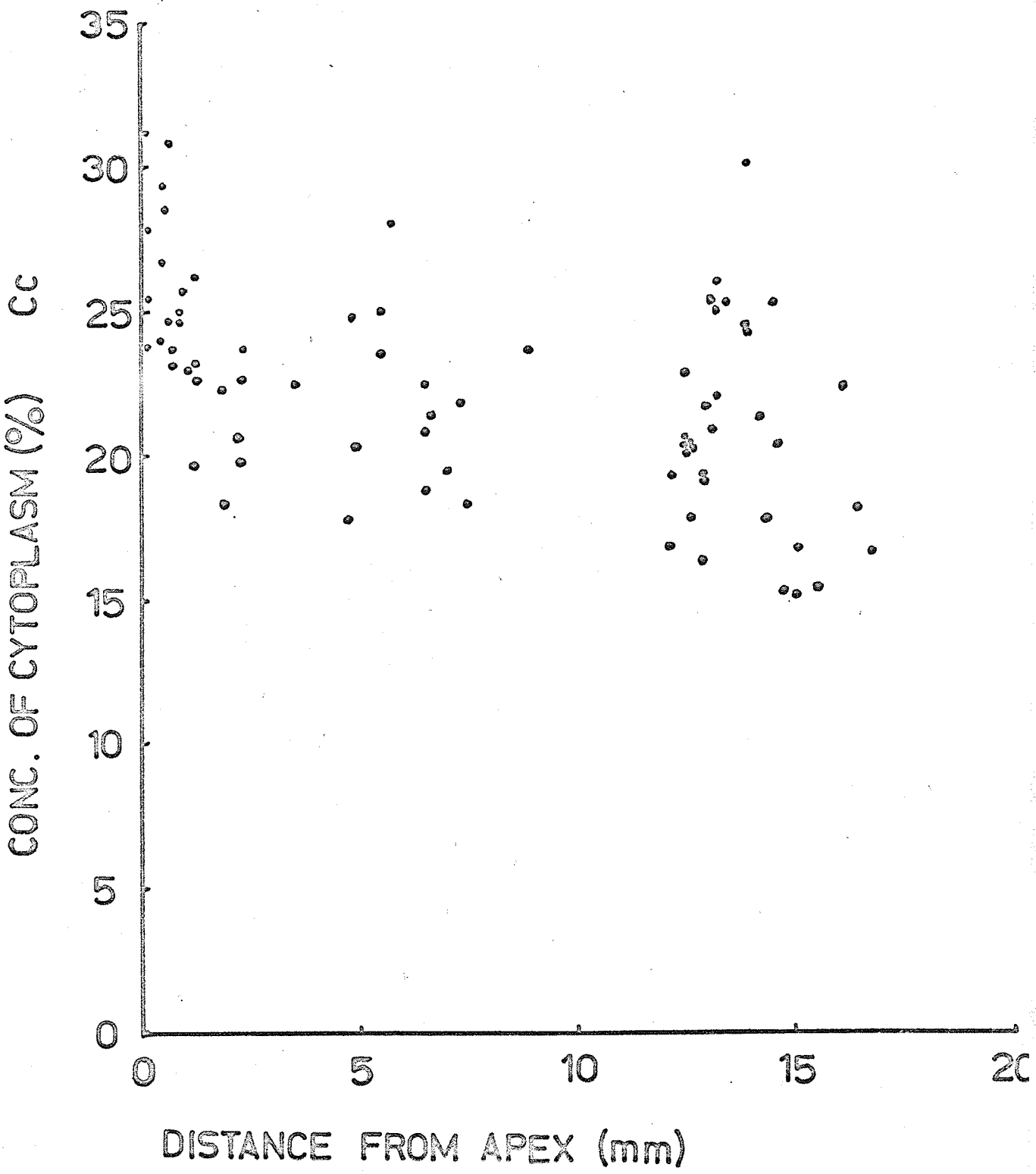

CONC. OF CYTOPLASM (%) Cc
35
30
25
20
15
10
5
0
0
5
10
15
20
DISTANCE FROM APEX (mm)

FIGURE 23. Diagram showing the relationship between vacuolar and cytoplasmic concentrations before and after treatment with 4% formalin. *R. solani*, slide culture, plain agar.

\+ Values obtained in water.

O Values obtained after treatment with formalin.

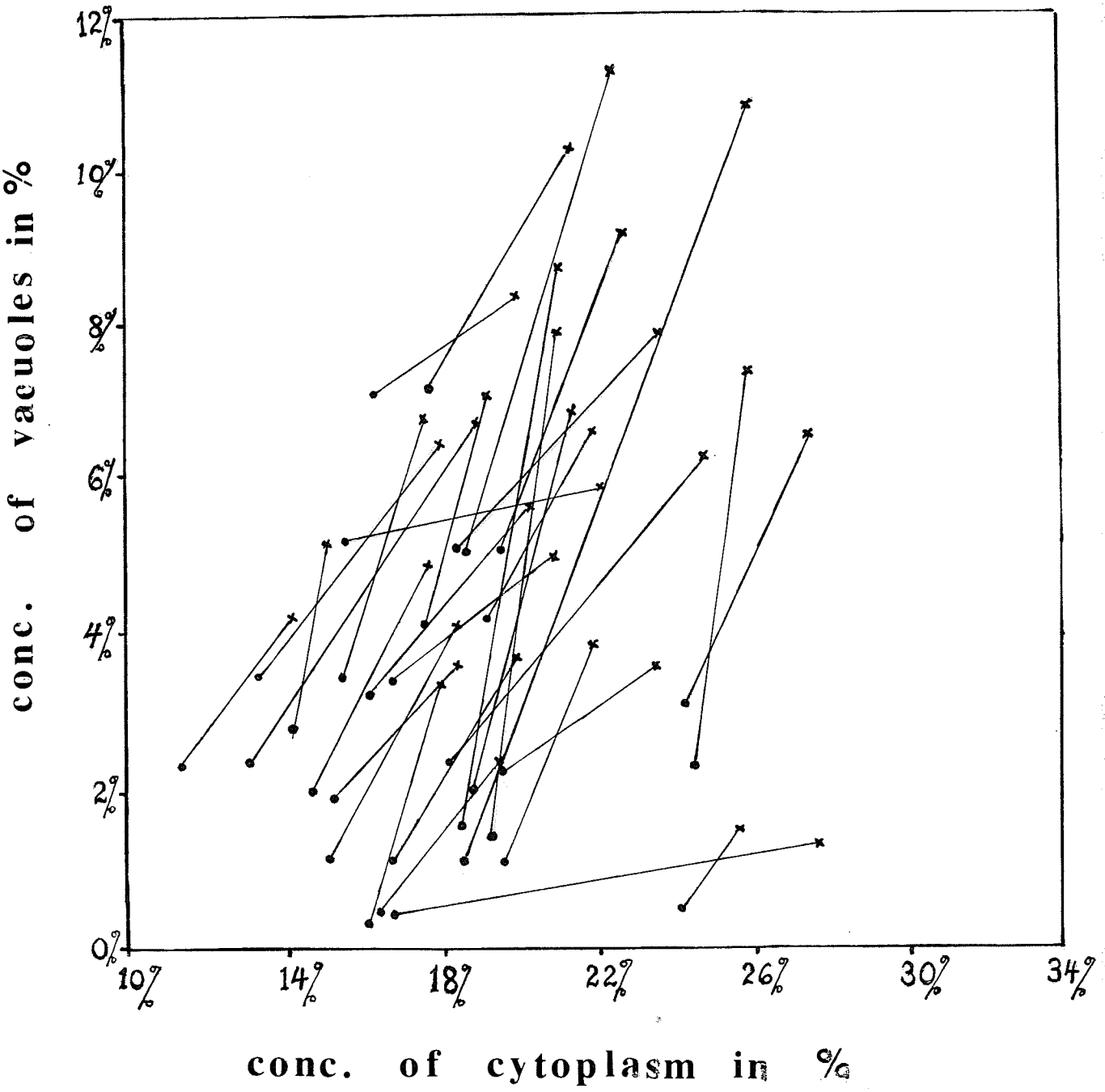

conc. of vacuoles in %
12%
10%
8%
6%
4%
2%
0%
10%
14%
18%
22%
26%
30%
34%
conc. of cytoplasm in %

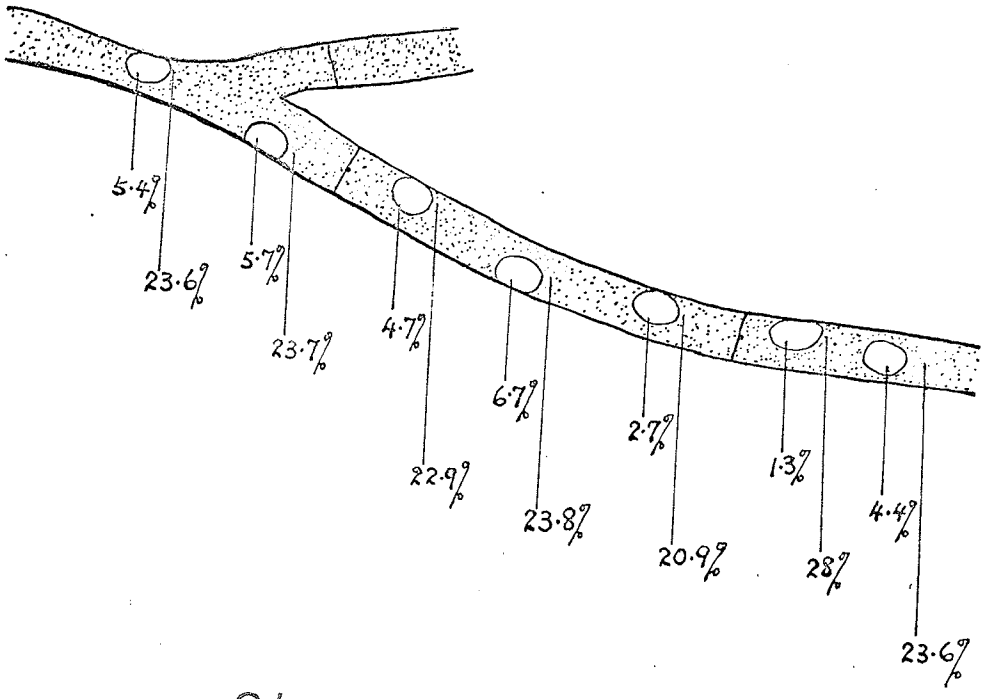
FIGURE 24 (a-f) Drawings of representative portions of hyphae showing differing types of relationship between vacuolar and cytoplasmic concentrations.

(a),(b) and (f): Medium vacuolar concentration
Low cytoplasmic concentration

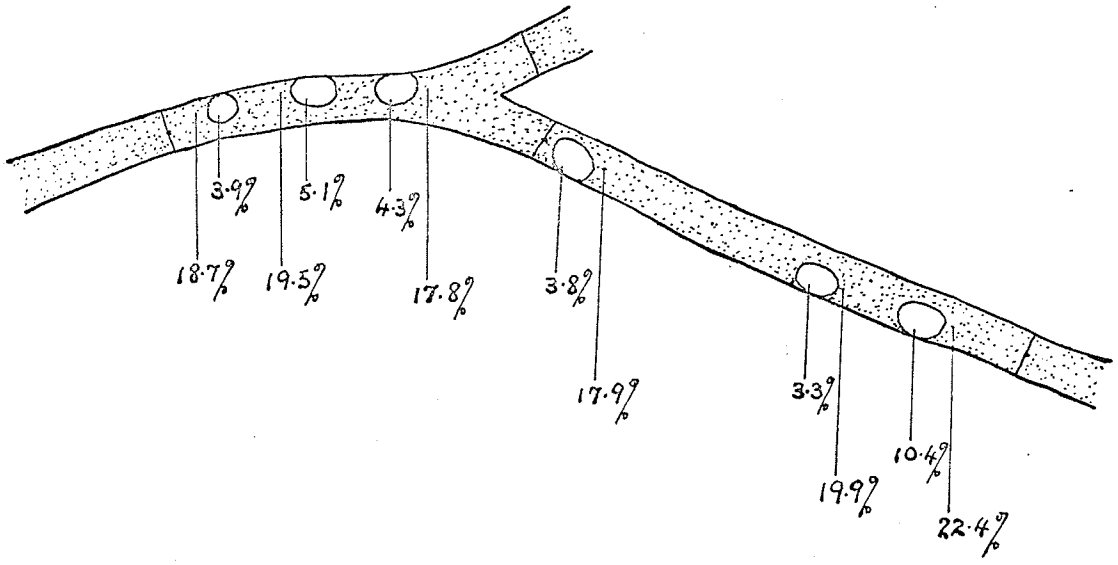
(c) Low vacuolar concentration
High cytoplasmic concentration

(d) High vacuolar concentration
High cytoplasmic concentration

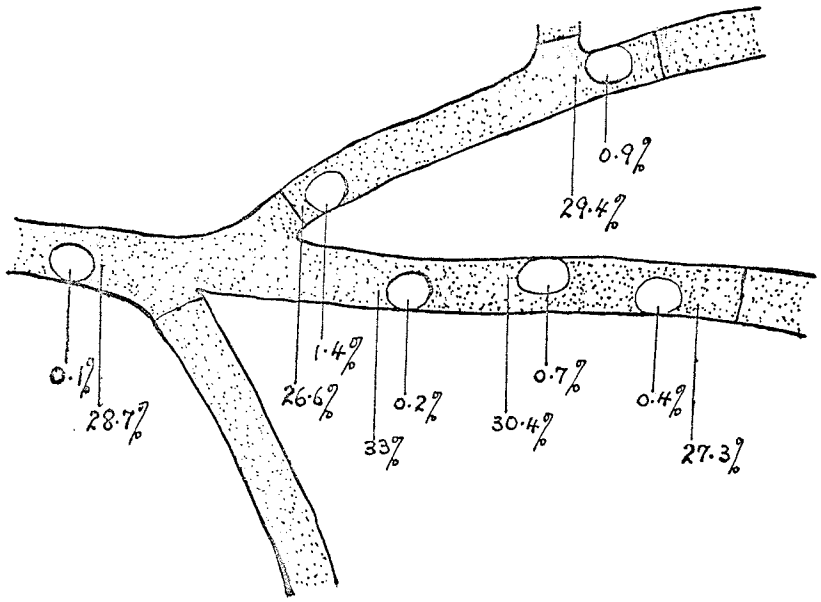
(e) Emptying side branch - low vacuolar concentration.


5·4%
23·6%
5·7%
23·7%
4·7%
22·9%
6·7%
23·8%
2·7%
20·9%
1·3%
28%
4·4%
23·6%

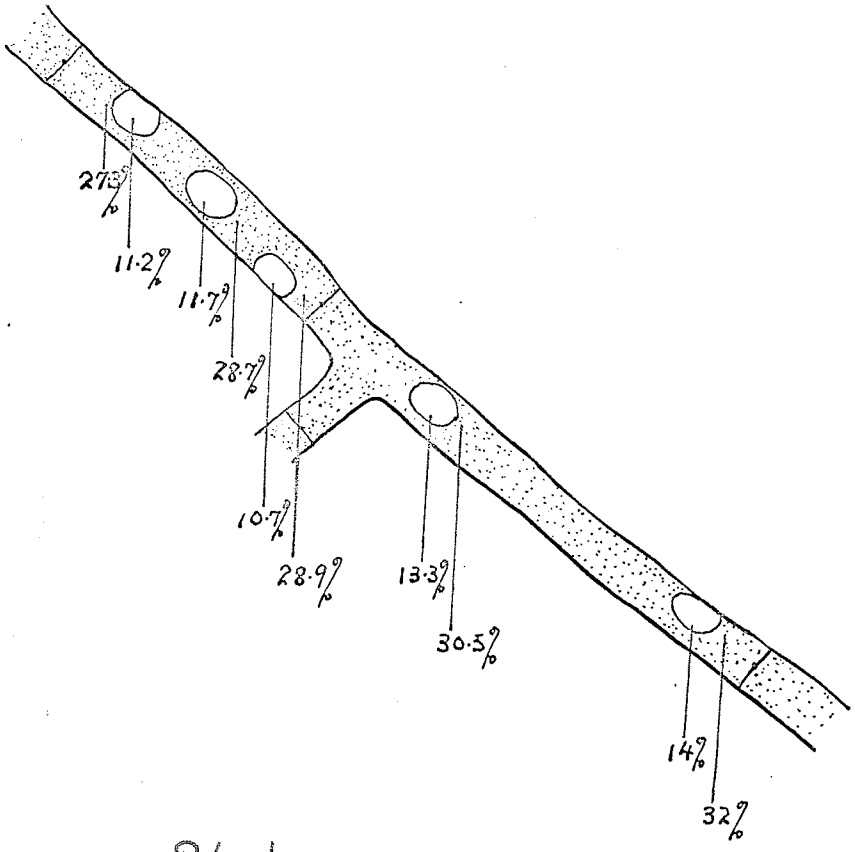
24 a


3·9%
18·7%
5·1%
19·5%
4·3%
17·8%
3·8%
17·9%
3·3%
19·9%
10·4%
22·4%

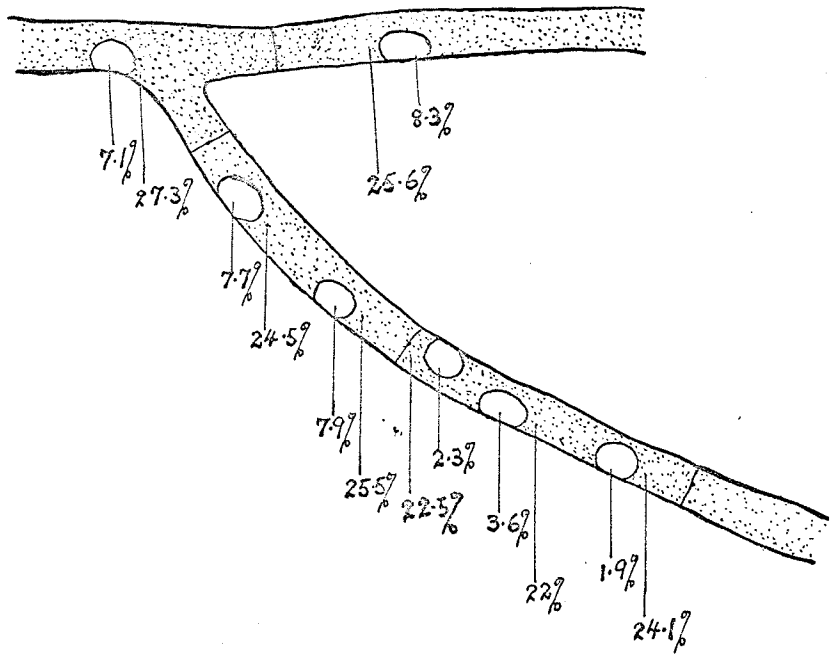
24 b


0.9%
29.4%
0.1%
28.7%
26.6%
1.4%
33%
0.2%
30.4%
0.7%
27.3%
0.4%

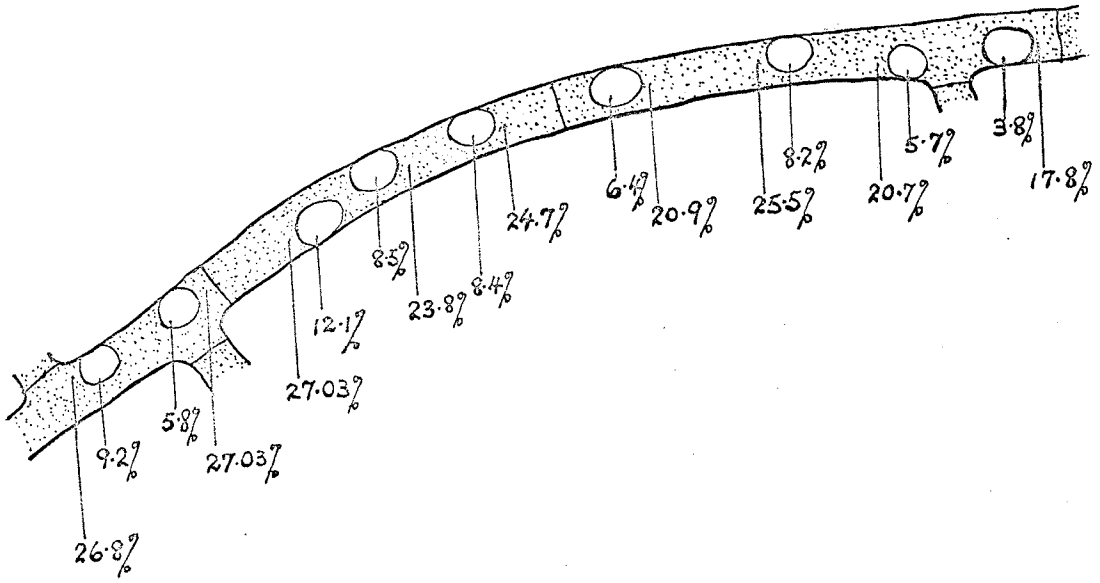
24 c


11.2%
11.7%
28.7%
10.7%
28.9%
13.3%
30.5%
14%
32%

24 d


7·1%
27·3%
7·7%
24·5%
7·9%
25·5%
22·5%
2·3%
3·6%
22%
1·9%
24·1%
8·3%
25·6%

24 e


26·8%
9·2%
5·8%
27·03%
27·03%
12·1%
8·5%
23·8%
8·4%
24·7%
6·4%
20·9%
25·5%
8·2%
20·7%
5·7%
3·8%
17·8%

24 f

FIGURE 25 (a-d) The same portion of hypha of R.solani showing the process of accumulation of neutral red during a period of 1.1/2 hours.

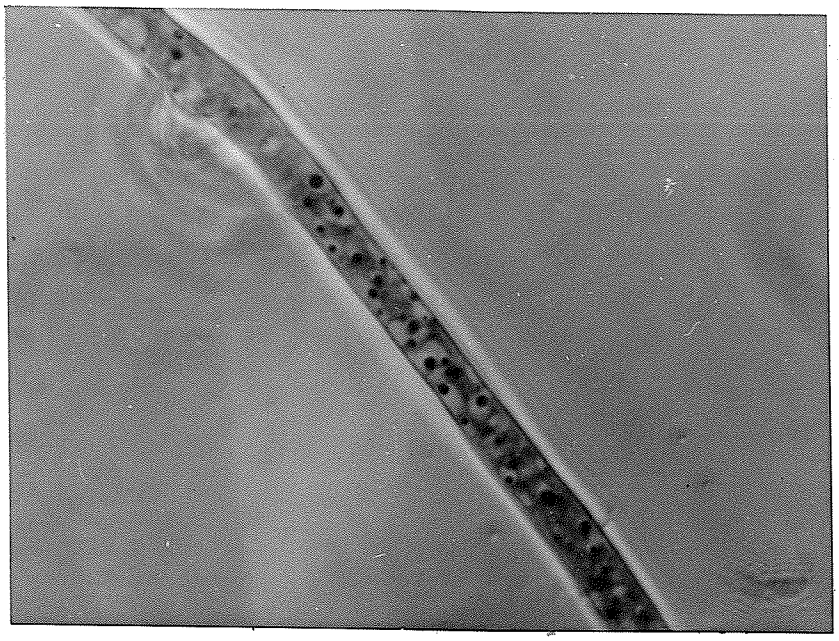


FIGURE 25 (a). The appearance of "neutral red granules".

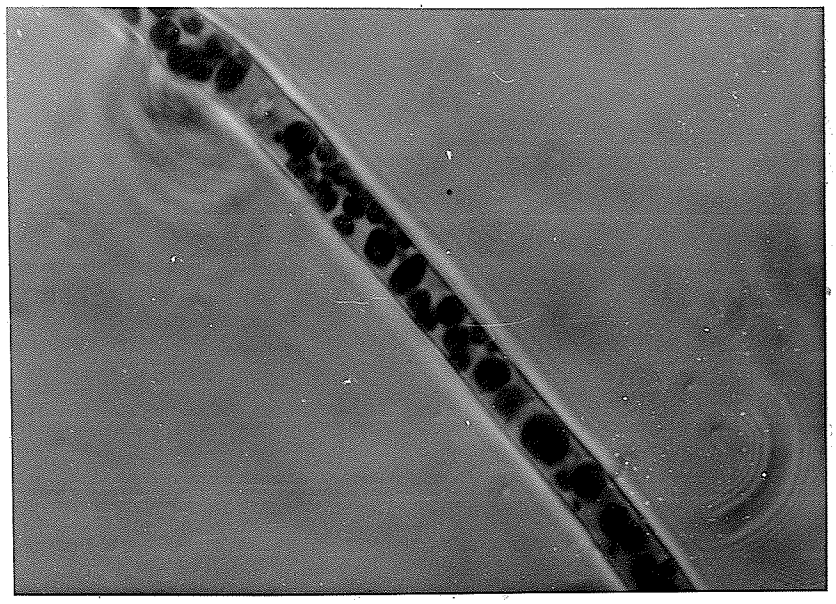


FIGURE 25 (b). A later stage of uptake.

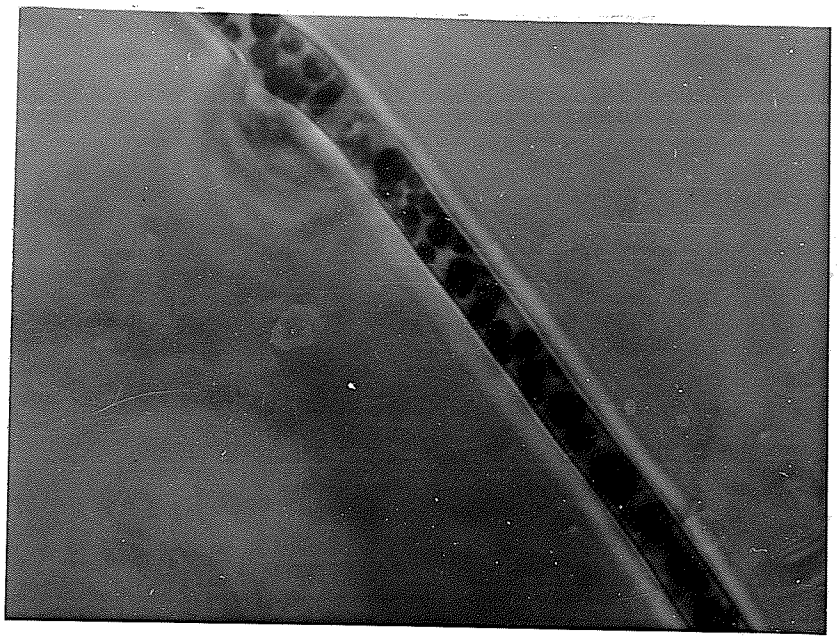


FIGURE 25 (c). As in Figure 25 (b).

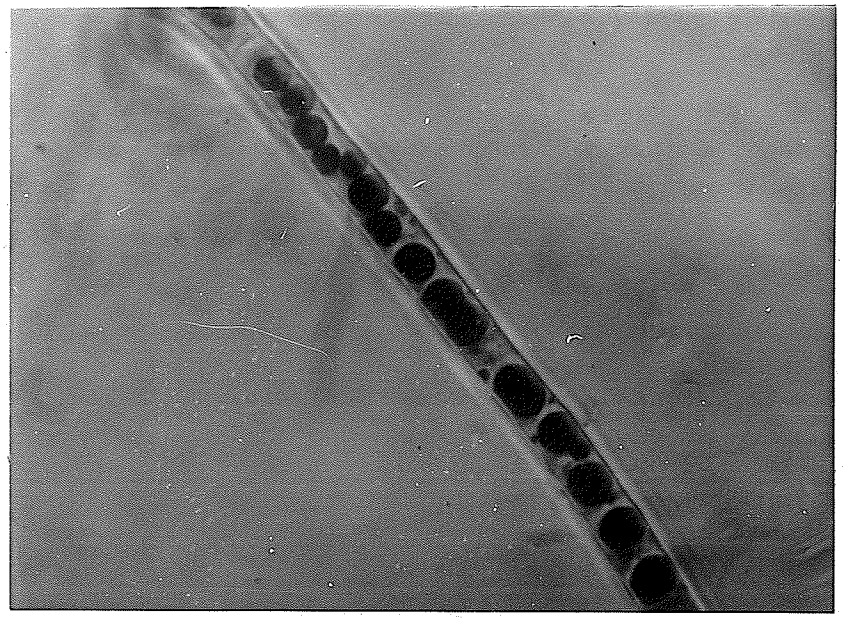


FIGURE 25 (d). As in Figure 25 (c) showing coalescence of vacuoles with increasing dye accumulation.

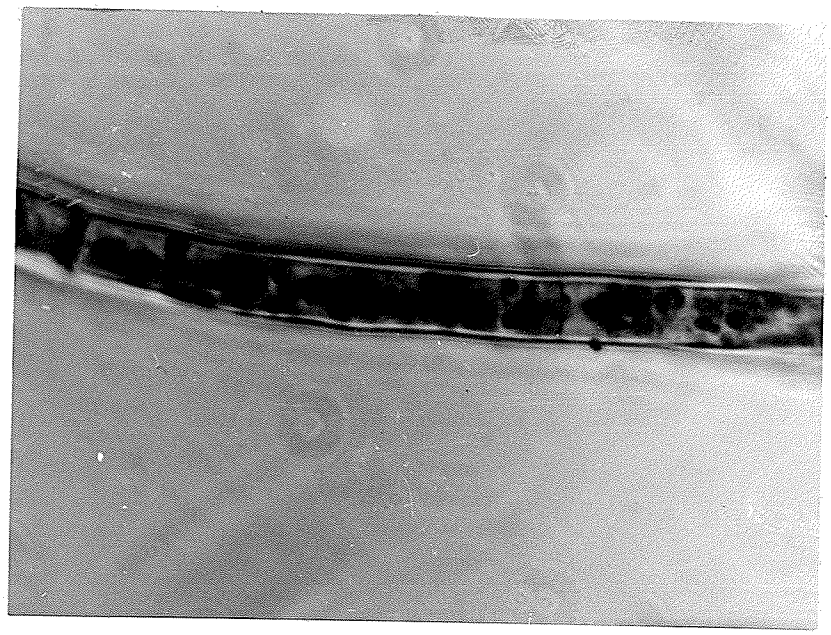


FIGURE 26. Accumulation of neutral red at pH 9.0.

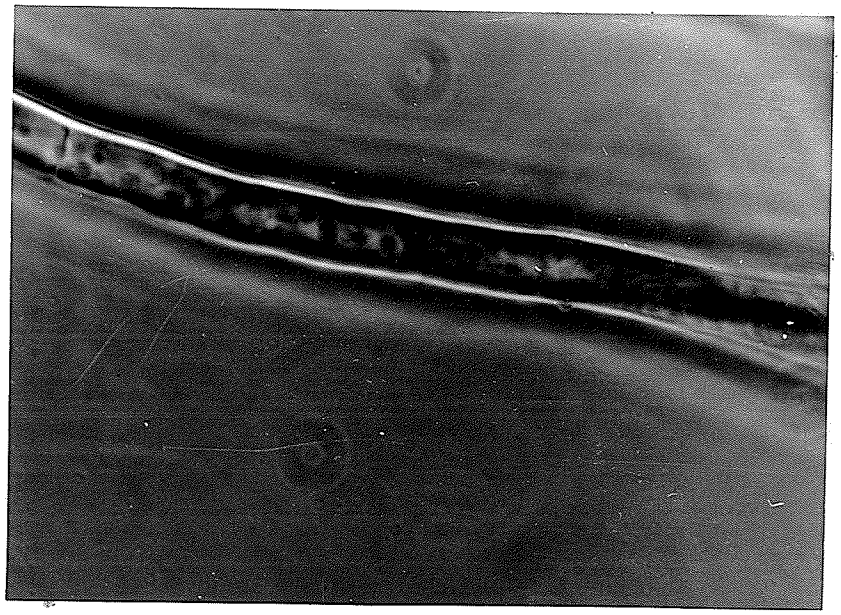


FIGURE 27. Micrograph showing the loss of neutral red after transfer to an acid medium (pH 4.0); compare Figure 25(d).

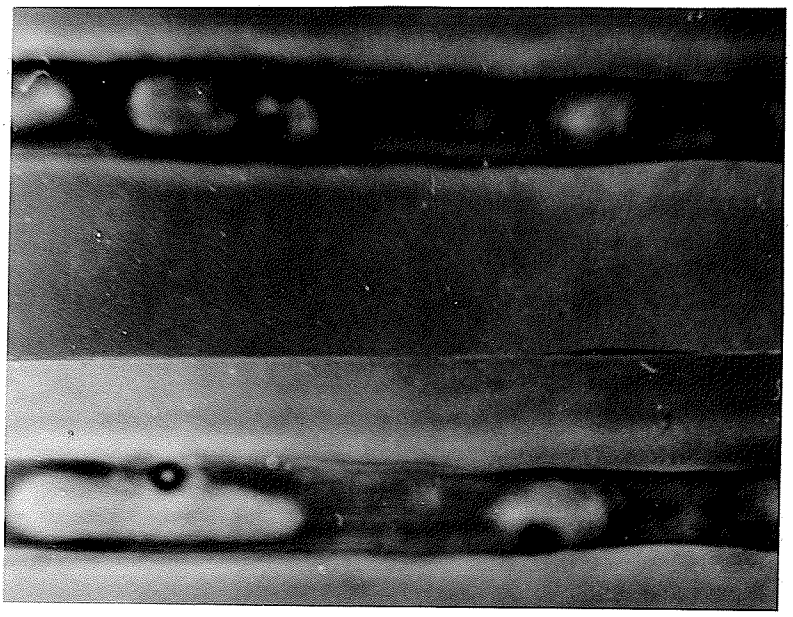


FIGURE 28. Micrograph showing coalescence of vacuoles at an early stage of neutral red accumulation. "Neutral red granule" stage.

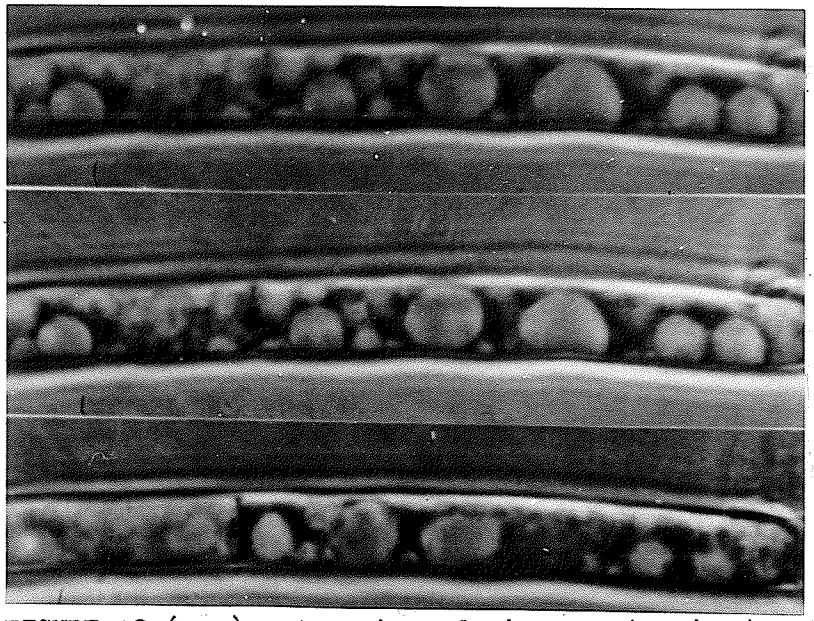


FIGURE 29 (a-c). A series of micrographs showing the effect of $CaCl_2$ solutions of increasing concentration. a) 0.02 M, b) 0.04 M, c) 0.06 M.

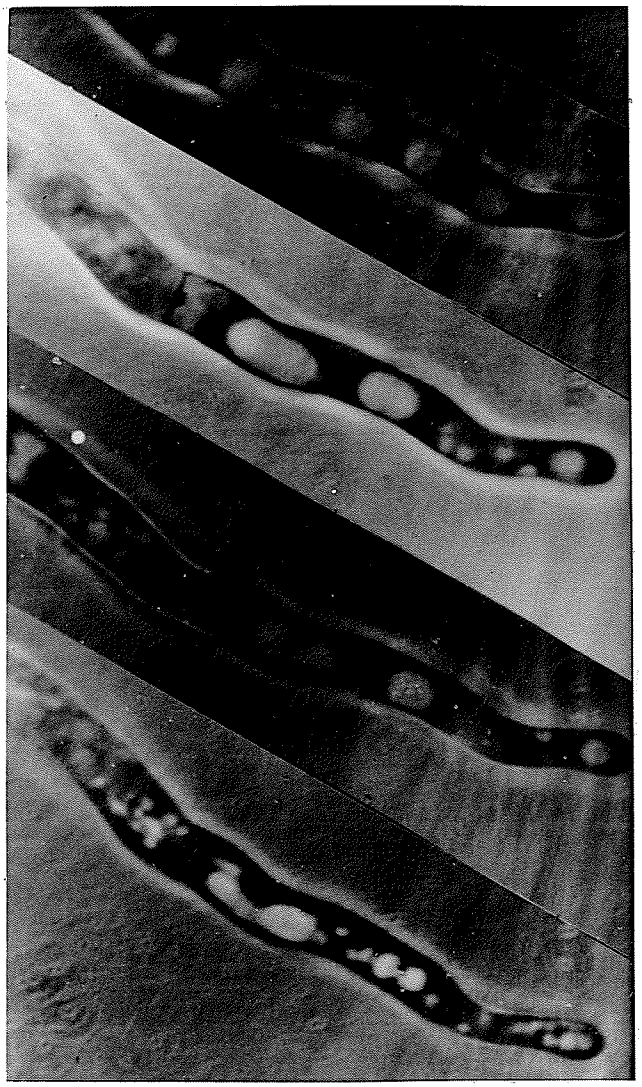


FIGURE 30 (a-d). A series of micrographs showing the effect of sucrose solutions of increasing concentration.
a) water, b) 0.1 M, c) 0.5 M, d) 1.0 M.

TABLE I. Values of diameter (μ) of vacuoles (D_v) and hyphae (D_h) and of concentration (g/100 ml) of vacuoles (C_v) and cytoplasm (C_c) in hyphae of R. solani (plain agar medium - 3 day old slide culture).
(a)

D_v	D_h	C_v	C_c
3.7	6.7	1.1	28.1
4.4	6.3	6.3	25.0
3.5	6.3	2.5	26.6
3.7	6.3	4.1	27.8
3.7	7.4	0.7	30.4
3.7	7.0	0.4	27.3
3.7	7.0	11.2	27.3
4.1	7.4	5.5	23.3
4.1	6.7	7.6	23.6
5.2	7.4	4.8	16.9
4.4	6.3	10.7	30.6
3.7	7.4	9.8	30.0
4.4	7.4	11.7	28.7
3.5	7.4	1.6	29.9
3.7	7.0	9.1	28.4
3.7	7.8	10.7	28.9
3.7	7.8	12.8	27.6
5.6	7.4	3.3	23.6
4.1	7.8	13.3	30.5
4.1	7.0	8.2	26.6
3.7	5.9	15.7	32.5
4.1	5.9	9.3	27.0
3.0	7.4	0.1	28.7
4.4	6.7	7.3	24.4
4.8	6.7	3.7	18.7
5.9	6.3	11.2	23.8
3.7	5.9	7.8	26.6

continued

TABLE I (a) Continued

D_v	D_h	C_v	C_c
2.6	7.0	0.2	33.0
3.5	6.7	10.0	28.1
3.7	7.0	8.3	24.5
4.1	7.0	13.3	28.0
3.7	7.4	9.8	27.0
5.6	5.9	9.8	21.9
4.4	6.7	11.1	22.5
1.9	8.1	0.9	29.4
3.0	7.4	3.5	29.1
5.2	7.8	5.0	14.4
4.8	6.3	9.5	19.8
3.7	7.8	11.1	24.1
3.7	8.5	7.4	20.7
4.8	7.4	4.6	20.3
3.7	8.1	12.5	29.0
3.5	6.7	11.3	31.1
3.0	7.0	1.4	26.6
5.6	6.3	8.1	23.8
5.2	7.0	7.8	21.3
4.1	6.7	3.4	18.7
4.1	7.4	2.3	23.6
5.6	7.0	6.1	19.5
3.5	7.4	11.8	24.6
5.9	6.7	7.2	19.9
3.7	7.0	14.0	32.0
4.1	6.7	1.1	32.0
5.2	6.3	10.4	25.2
4.8	7.0	8.6	10.5
3.7	6.7	6.0	16.9

continued

TABLE I (a) Continued

D_v	D_h	C_v	C_c
3.7	7.0	4.5	24.1
5.2	6.7	6.6	22.5
4.4	7.0	6.4	20.9
4.4	6.3	3.6	25.1
5.2	6.7	4.7	25.5
3.7	6.3	2.1	31.0
4.4	6.3	6.0	23.0
4.8	6.3	6.0	18.0
3.7	7.4	4.0	19.0
3.7	7.4	2.9	20.2
5.2	7.4	3.6	22.0
5.2	7.4	4.8	22.6
5.6	7.4	10.0	24.0
4.8	7.8	2.0	24.0

TABLE I (b). Same as Table I(a), replicate experiment.

D_v	D_h	C_v	C_c
3.5	6.3	3.2	25.8
3.7	6.3	9.1	19.9
2.7	5.9	0	37.8
3.5	6.3	11.1	23.8
3.0	6.7	0	19.9
2.6	5.9	0	29.6
2.2	5.6	4.5	29.3
3.7	6.3	10.1	29.0
3.7	5.9	13.9	27.4
3.7	5.9	8.0	31.7
3.5	5.6	9.6	30.2
3.0	5.9	0.8	21.1
3.5	6.7	0	25.5
3.5	7.0	11.0	19.6
3.5	5.6	6.5	27.0
4.4	5.9	13.2	27.4
3.7	6.7	6.7	22.9
3.7	5.6	13.0	29.3
3.5	6.0	4.0	26.6
3.7	6.7	0	27.4
3.5	6.7	1.9	16.9
4.1	6.7	15.4	24.0
4.8	7.0	11.0	26.7
5.6	6.7	15.1	30.0
5.2	7.0	0.1	21.3
3.5	7.4	2.0	24.0
4.4	6.3	11.5	26.2
3.6	6.3	6.6	26.2

continued

TABLE I (b) Continued

D_v	D_h	C_v	C_c
3.0	7.0	0	22.0
3.7	7.4	3.0	28.0
5.6	6.7	12.2	24.4
3.5	6.3	9.1	24.7
3.5	7.4	3.4	11.2
3.7	7.4	0	23.3
6.3	8.1	8.9	27.2
5.6	7.4	7.5	24.7
4.4	8.1	6.4	26.2
3.5	8.5	1.7	27.9
3.0	6.3	7.2	25.8
5.6	7.8	5.1	28.9
4.8	6.7	9.1	23.6
5.9	6.7	13.8	25.1
3.0	8.9	5.1	28.7
4.4	8.5	9.0	28.5
3.0	7.4	4.7	30.0
3.7	7.4	0	25.4
5.6	7.4	11.3	20.3
3.7	7.8	6.2	24.1
4.4	7.8	0	23.5
4.8	8.5	10.8	25.0
6.7	8.9	8.9	18.3
7.0	8.5	11.0	18.5
5.2	8.5	11.1	23.2
5.9	8.5	8.7	23.5
4.4	8.9	5.2	28.2
4.8	7.4	6.5	23.6
6.3	8.9	4.2	18.9

continued

TABLE I (b) Continued

D_v	D_h	C_v	C_c
4.8	8.5	3.6	25.0
6.7	8.9	7.0	14.9
4.8	5.6	9.5	22.5
4.4	5.2	11.0	24.6
6.7	7.4	6.3	17.6
4.4	7.4	2.4	25.7
3.7	7.8	0	23.5
3.5	7.4	5.6	24.0
7.0	8.1	5.1	13.3
5.9	7.0	12.2	24.9
5.9	7.4	5.7	23.0
5.6	6.7	13.3	28.2
5.9	7.8	9.4	23.8
5.2	7.4	9.1	21.3
4.4	5.9	10.4	27.5
6.3	7.8	13.0	20.6

TABLE II. Values of D_v, D_h, C_v and C_c for a Mycelium of R. solani growing in slide culture on 1/50 N sucrose-peptone agar.

D_v	D_h	C_v	C_c
4.4	5.2	12.6	25.4
3.7	6.3	5.4	23.0
3.0	4.1	2.3	36.8
4.8	5.9	12.8	24.0
5.5	6.3	11.0	23.8
4.4	5.6	14.1	26.5
3.0	7.8	1.3	20.5
4.8	5.9	15.4	29.5
4.4	6.3	14.6	25.4
3.5	5.6	9.2	24.7
4.1	5.9	9.3	26.7
3.7	6.3	3.0	24.6
3.7	5.6	10.6	26.1
4.1	5.9	5.1	27.4
6.3	7.4	12.0	24.3
5.6	7.0	7.8	21.3
4.1	7.0	5.4	23.8
4.4	8.1	4.5	21.5
5.9	8.1	10.3	23.3
5.6	7.0	0.9	29.5
4.1	7.0	0.3	26.6
5.6	7.4	5.7	26.0
5.9	8.5	4.9	19.7
7.4	8.5	7.6	20.2
6.7	8.5	3.5	21.4
7.4	8.9	9.6	21.1
7.8	9.3	5.9	21.8

continued

TABLE II. Continued

D_v	D_h	C_v	C_c
8.4	9.6	12.4	24.1
7.0	9.3	7.0	26.6
7.4	8.5	10.7	30.2
4.4	7.8	4.3	22.5
4.8	7.0	3.4	32.0
3.7	8.1	0	29.1
4.4	6.7	5.6	21.0
5.2	7.4	7.0	28.0
4.8	7.4	6.0	25.7
4.4	7.8	8.8	21.2

TABLE III (a). Values of D_v, D_h, C_v and C_c for a hypha of R. solani at various distances from the apex. (plain agar medium, 3 day old slide culture).

Distance (mm)	D_v	D_h	C_v	C_c
<0.1	3.7	6.7	1.1	28.1
<0.1	4.4	6.3	6.3	25.6
0.1	3.5	6.3	2.5	26.6
0.2	3.0	6.3	4.1	27.8
0.3	3.7	7.4	0.7	30.4
0.3	3.7	7.0	0.4	27.3
0.5	3.7	7.0	11.2	27.3
0.5	4.1	7.4	5.5	23.3
0.5	4.1	6.7	7.6	23.6
0.5	5.2	7.4	4.8	16.9
0.6	4.4	6.3	10.7	30.6
0.7	3.7	7.4	9.8	30.0
0.7	4.4	7.4	11.7	28.7
0.9	3.5	7.8	1.6	29.9
1.0	3.7	7.0	9.1	28.4
1.1	3.7	7.8	10.7	28.9
1.2	3.7	7.8	12.8	27.6
1.2	5.6	7.4	3.3	23.6
1.3	4.1	7.8	13.3	30.5
1.3	4.1	7.0	8.2	26.6
1.5	3.7	5.9	15.7	32.5
1.5	4.1	5.9	9.3	27.0
1.8	3.0	7.4	0.1	28.7
2.0	4.4	6.7	7.3	24.4
2.0	4.8	6.7	3.7	18.7
2.2	5.9	6.3	11.2	23.8

continued

TABLE III (a) Continued

Distance (mm)	D_v	D_h	C_v	C_c
2.2	3.7	5.9	11.6	26.6
2.8	2.6	7.0	0.2	33.0
2.8	3.5	6.7	10.0	28.1
2.8	3.7	7.0	8.3	24.5
3.3	4.1	7.0	13.3	28.0
3.4	3.7	7.4	9.8	27.0
3.4	5.6	5.9	9.8	21.9
4.1	4.4	6.7	11.1	22.5
4.1	1.9	8.1	0.9	29.4
9.6	3.0	7.4	3.5	29.1
9.6	5.2	7.8	5.0	14.4
9.7	4.8	6.3	9.5	19.8
9.7	3.7	7.8	11.1	24.1
10.0	3.7	8.5	7.4	20.7
10.0	4.8	7.4	4.6	20.3
11.0	3.7	8.1	12.5	29.0
11.0	3.5	6.7	11.3	31.1
30.0	3.0	7.0	1.4	26.6
30.0	5.6	6.3	8.1	23.8
30.8	5.2	7.0	7.8	21.3
31.2	3.0	8.9	5.8	25.0
31.2	4.1	6.7	3.4	18.7
31.2	4.1	7.4	2.3	23.6
32.3	4.4	7.4	14.7	24.3
32.5	3.5	7.4	11.8	24.6
32.5	5.9	6.7	7.2	19.9
33.0	3.7	7.0	14.0	32.0
33.1	4.1	6.7	1.1	32.0

continued

TABLE III (a) continued

Distance (mm)	D_v	D_h	C_v	C_c
33.2	5.2	6.3	10.4	25.2
33.3	4.8	7.0	8.6	19.5
33.4	3.7	6.7	6.0	16.9
33.5	3.7	7.0	4.5	24.1
33.7	6.7	5.2	6.6	22.5
33.9	4.4	6.3	3.6	25.1
34.1	5.2	6.7	4.7	25.5
34.1	3.7	6.3	2.1	31.0
34.3	4.4	6.3	6.0	23.0
34.4	4.8	6.3	6.0	18.0
34.7	3.7	7.4	4.0	19.0
34.7	3.7	7.4	2.9	20.2
34.8	5.2	7.4	3.6	22.0
34.9	5.2	7.4	4.8	22.6
35.0	5.6	7.4	10.0	24.0
35.2	4.8	7.8	2.0	24.0

TABLE III (b). Same as Table III (a).
(7 day old culture)

Distance (mm)	D_v	D_h	C_v	C_c
<0.1	3.3	6.7	0	31.2
<0.1	3.3	6.7	0	23.7
0.1	3.7	7.4	0	27.7
0.1	4.1	7.4	5.6	25.4
0.4	4.1	7.4	2.8	24.0
0.5	4.1	7.0	3.3	28.5
0.5	4.4	7.4	5.2	29.4
0.5	3.0	7.8	0	26.7
0.6	3.3	7.4	1.4	24.6
0.6	3.3	6.7	4.5	30.8
0.7	3.0	7.8	2.3	23.2
0.7	3.7	7.4	0	23.7
0.8	4.8	8.1	0	24.6
0.8	3.3	8.5	0	25.0
0.9	3.7	7.8	0	25.7
1.1	3.7	8.1	0	23.0
1.3	3.7	8.5	0	19.7
1.3	4.4	8.5	0	23.2
1.3	3.7	7.8	1.1	26.1
1.4	4.1	8.1	0.6	22.7
1.9	4.1	8.1	0	22.4
1.9	3.7	7.4	6.3	18.4
2.3	3.7	7.8	0	20.6
2.4	4.8	6.3	8.1	22.7
2.4	3.3	8.1	1.3	19.9
2.4	4.1	5.9	1.6	23.2
3.5	5.2	6.7	5.8	22.5
4.7	3.7	8.9	2.2	17.7
4.8	4.8	8.5	7.7	20.3

continued

TABLE III (b) Continued.

Distance (mm)	D_v	D_h	C_v	C_c
4.8	3.0	7.0	3.0	24.8
5.5	3.7	8.5	4.0	23.5
5.5	3.7	8.5	4.8	25.0
5.7	3.3	7.4	8.5	28.0
6.5	4.4	7.8	12.5	20.9
6.5	3.7	8.9	0.4	22.5
6.5	5.2	7.0	5.4	18.8
6.6	4.4	6.7	3.3	21.4
7.0	4.1	6.7	4.1	19.5
7.3	4.4	6.3	6.8	21.9
7.4	4.4	8.1	7.1	18.4
8.9	4.8	8.9	10.4	23.6
12.2	5.2	7.8	12.6	19.3
12.2	4.8	8.1	0	16.9
12.5	4.8	6.7	8.9	22.9
12.5	4.4	7.0	6.2	20.3
12.5	4.1	6.3	3.0	20.3
12.6	4.4	7.0	9.1	20.3
12.6	5.9	7.0	8.5	17.7
12.7	4.4	7.4	2.2	20.2
12.7	4.8	6.3	4.0	20.3
12.8	5.6	8.1	0.1	16.4
12.9	4.1	7.4	5.8	19.3
12.9	6.7	7.4	6.4	19.2
13.0	3.7	7.0	5.6	21.6
18.1	4.4	7.4	11.2	25.3
13.1	7.0	7.8	11.3	20.9
13.2	4.4	7.0	7.4	26.0
13.2	4.8	7.4	14.1	25.0

continued

TABLE III (b) continued

Distance (mm)	D_v	D_h	C_v	C_c
13.3	3.7	8.5	3.1	22.0
13.4	4.1	6.7	11.7	25.2
13.9	3.7	7.4	9.5	24.3
13.9	4.8	6.7	19.6	30.0
13.9	4.4	7.4	6.6	24.2
14.2	4.1	7.0	4.2	21.3
14.3	5.2	7.0	7.2	17.8
14.5	4.4	6.7	11.1	25.2
14.6	5.2	7.8	12.5	20.3
14.7	6.7	7.4	7.7	15.2
15.0	6.3	7.8	5.6	15.1
15.1	5.6	7.4	5.6	16.9
15.5	5.9	8.1	6.9	15.4
16.1	5.2	7.0	7.9	22.4
16.4	4.8	5.9	12.7	18.2
16.7	5.9	7.0	6.6	16.7

TABLE IV. Values of C_c for hyphal apices and portions of non-vacuolate old hyphae (7 days)

Apices	Old Hyphae
42.9	29.4
44.1	22.3
36.6	30.1
41.1	22.3
40.5	28.5
Means and Standard Deviations	
41.04 ± 2.86	26.52 ± 3.89

TABLE V. Values of C_c and C_v in water and 4% formalin. I and II = replicate slides, a-d = adjacent vacuoles.

	Conc. of Cytoplasm in Water	Conc. of Cytoplasm in 4% formalin	$C_{cw}-C_{cf}$	Conc. of Vacuoles in Water	Conc. of Vacuoles in 4% formalin	$C_{vw}-C_{rf}$
I a	19.1	17.6	1.5	7.1	4.1	3.0
	17.6	14.7	2.9	4.9	2.0	2.9
	15.5	14.1	1.4	5.2	2.8	2.4
	14.1	11.3	2.8	4.2	2.3	1.9
	20.9	19.3	1.6	7.9	1.4	6.5
	17.5	15.4	2.1	6.8	3.4	3.4
	19.3	17.1	2.2	4.1	0.1	4.0
II b	18.0	16.1	1.9	3.4	0.3	3.1
	21.3	15.2	6.1	5.7	3.2	2.5
	21.4	18.8	2.6	6.9	1.9	5.0
	22.1	15.6	6.5	5.9	5.2	0.7
	19.9	16.3	3.6	8.4	7.1	1.3
	17.9	13.2	4.7	6.5	3.5	3.0
	23.6	18.5	5.1	7.9	5.1	2.8
	24.7	18.2	6.5	6.3	2.3	5.0

TABLE V continued

	Conc. of Cytoplasm in Water	Conc. of Cytoplasm in 4% formalin	$C_{cw}-C_{cf}$	Conc. of Vacuoles in Water	Conc. of Vacuoles in 4% formalin	$C_{vw}-C_{rf}$
I c	18.9	12.9	6.0	6.7	2.3	4.4
	20.9	16.9	4.0	5.0	3.4	1.6
	23.5	19.5	4.0	3.6	2.2	1.4
	22.7	19.5	3.2	9.2	5.1	4.1
	21.9	19.2	2.7	6.6	4.2	2.4
	18.4	15.3	3.1	3.6	1.9	1.5
	22.3	18.6	3.7	11.3	5.1	6.2
	25.9	18.7	7.2	10.9	1.1	9.8
	21.3	17.7	3.6	10.3	7.2	3.1
I d	19.6	16.4	3.2	2.4	0.4	2.0
	20.9	18.5	2.4	8.8	1.6	7.2
	27.4	24.3	3.1	6.6	3.1	3.5
	21.9	19.6	2.3	3.9	1.0	2.9
	18.4	15.6	2.8	4.1	1.1	3.0
	19.9	16.8	3.1	3.7	1.1	2.6
	25.9	24.5	1.4	7.4	2.3	5.1
	27.6	16.8	10.8	1.3	0.4	0.9
	25.7	24.1	1.6	1.5	0.5	1.0

TABLE V continued

	Conc. of Cytoplasm in Water	Conc. of Cytoplasm in 4% formalin	$C_{cw}-C_{cf}$	Conc. of Vacuoles in Water	Conc. of Vacuoles in 4% formalin	$C_{vw}-C_{rf}$
II a	20.9	19.3	1.6	2.7	0.7	2.0
	23.8	21.3	2.5	6.7	4.2	2.5
	22.9	20.2	2.7	4.7	2.1	2.6
	28.0	17.5	10.5	1.3	0.3	1.0
	23.6	17.5	6.1	4.4	3.0	1.4
	23.7	20.3	3.4	5.7	2.0	3.7
	23.6	20.3	3.3	5.4	1.2	4.2
II b	18.7	13.1	5.6	3.9	0.3	3.6
	19.5	17.4	2.1	5.1	0.4	4.7
	17.8	15.0	2.8	4.3	0.4	3.9
	17.3	14.4	2.9	5.5	3.9	1.6
	17.9	15.8	2.1	3.8	0.1	3.7
	25.2	21.8	3.4	12.8	8.3	4.5
	19.9	16.9	3.0	3.3	0.3	3.0
	22.4	19.5	2.9	10.4	7.5	2.9
II c	24.5	21.6	2.9	7.7	1.9	5.8
	25.5	22.7	2.8	7.9	5.1	2.8
	22.5	16.9	5.6	2.3	0.6	1.7
	22.0	19.9	2.1	3.6	0.9	2.7
	24.1	19.5	4.6	1.9	0.6	1.3
	25.6	18.8	6.8	8.3	1.9	6.4
	27.3	23.1	4.2	7.1	2.9	4.2