A comparative study of growth regulation in fungi

Betool, Zainal Ali

A COMPARATIVE STUDY OF GROWTH REGULATION IN FUNGI

by

BETOOL ZAINAL ALI

B.Sc., M.Sc. (Baghdad University, IRAQ)

A Thesis submitted to the
University of Durham for
the degree of Doctor of Philosophy

Graduate Society,
Durham University,
England

July 1984

Declaration:

The work described in this thesis has been carried out by the undersigned at the Department of Botany of Durham University and has not previously been submitted for any other diploma or degree.

Signed: B. Z. Ali

Date: 17 July 1984

To my sons ADNAN and GHASSAN

ABSTRACT

The effect of two plant growth regulating substances (GA_3 and IAA) has been investigated on growth of *Mucor mucedo* and *Saprolegnia parasitica*. No stimulatory effect was observed at low concentrations of both substances on vegetative growth and macromolecular composition of both fungi. Instead a gradual inhibition was found at high concentrations. The only stimulation with both substances was detected on sporangiospore germination of *M. mucedo* at the range of concentrations between 10 and 100 ppm. Higher concentrations showed a delay of germination.

Macromolecular composition of these fungi as well as *Phycomyces blakesleeanus* was found to be variable. Results were discussed in terms of relation of these figures to growth rate of the test fungi.

Flooding of *M. mucedo* and *P. blakesleeanus* colonies with their liquid culture medium resulted in unexpected responses (bursting and/or swelling of hyphal tips followed by apical branching). Electron microscopy was used as an aid to investigate these phenomena. No observations were obtained directly relating to the bursting of *P. blakesleeanus* hyphal tips. Nevertheless, flooding of *M. mucedo* colonies revealed a high production and accumulation of nearly all cytoplasmic components in the swollen tips which indicated high metabolic activities and thus formation of two or more apical branches.

Studies of regeneration of protoplasts produced from *P. blakesleeanus* and *S. parasitica*, revealed two different

patterns. *S. parasitica* showed unusual regeneration process, protoplast developed an abnormal, very thin hypha(e), sometimes branched with a swollen tip(s), later on a normal hypha developed from the abnormal one and continued to grow as a normal hypha.

Serological methods used to study the relationship between the chitin and cellulose-walled fungi revealed weak antigenic reactivities between representatives of both groups of fungi. Likewise between the members of the chitin-walled fungi, in contrast to the high cross reactivities between the cellulose-walled fungi.

ACKNOWLEDGEMENTS

I want to express my deepest sympathy and appreciation for my former supervisor, Mr. G.H. Banbury. My sincere gratitude and thanks to my supervisor Dr. J.A. Pearson, who took over the responsibility to finish this thesis, together with Dr. M.F. Dewey to whom I owe my deepest gratitude, by correcting the manuscripts and for their endless encouragement and advice after the death of Mr. Banbury.

I am also grateful to Dr. N. Harris for his cooperation and help on electron microscopy work, to Dr. S. Parry (Medical School, Newcastle University) for appreciated effort in injecting and bleeding the rabbits used for preparation of antisera in the serological experiments; to Dr. B. Whitton and Mr. B. Simmon for allowing me to use some of their laboratory equipment and to Dr. N.J. Chaffey for his advice on protein estimation and calculation of the final magnification of the electron microscopy plates.

My thanks are due to Professor D. Boulter for accepting me in his department, to Mr. P. Sidney to his cooperation in preparing the photographs, to Mr. J.H. Whittle (Departmental Superintendent) and to all staff and technicians in Botany Department for their help and support; to the staff of the Science Library and to Mrs. Marion Wilson for typing this thesis.

I wish to express my thanks to the Government of IRAQ and to the Ministry of Higher Education and Scientific Research for the financial support during the period of my study.

I want also to thank my husband's family for their help in looking after my son in my country during all the period of my study.

LIST OF FIGURES

		Page No.
Fig.(1)	Percentage of spore germination of *M. mucedo* in MPG medium containing varying concentrations of GA_3.	25
Fig.(2)	Percentage of spore germination of *M. mucedo* in MPG medium containing varying concentrations of IAA.	26
Fig.(3)	Mean colony diameters of *M. mucedo* grown on MPG medium containing varying concentrations of GA_3 and IAA.	44
Fig.(4)	Mean colony diameters of *Saprolegnia parasitica* grown on PYG medium with varying concentrations of GA_3 and IAA.	45
Fig.(5)	Mean colony diameters of *Saprolegnia terrestris* grown on PYG medium with varying concentrations of GA_3 and IAA.	46
Fig.(6)	Mean colony diameters of *Pythium debaryanum* grown on PYG medium with varying concentrations of GA_3 and IAA.	48
Fig.(7)	Mean colony diameters of *Achlya caroliniana* grown on PYG medium with varying concentrations of GA_3 and IAA.	49
Fig.(8)	Mean colony diameters of *Saprolegnia parasitica* grown on PYG medium with varying concentrations of Kinetin.	50
Fig.(9)	Mean colony diameters of *Saprolegnia parasitica* grown on PYG medium with varying combinations of IAA and Kinetin.	51
Fig.(10)	Dry weight yield of *M. mucedo* grown in MPG medium containing varying concentrations of GA_3.	53
Fig.(11)	Dry weight yield of *M. mucedo* grown in MPG medium containing varying concentrations of IAA.	54
Fig.(12)	Dry weight yield of *S. parasitica* grown in PYG medium containing varying concentrations of GA_3.	56
Fig.(13)	Dry weight yield of *S. parasitica* grown in PYG medium containing varying concentrations of IAA.	57
Fig.(14)	*Mucor mucedo* growing hyphae, from 48 hr. old colony flooded with liquid medium at different intervals.	81 & 82

Page No.

Fig.(15) *Mucor mucedo* colonies showing different types of bursting. 84

Fig.(16) *Phycomyces blakesleeanus*, 48 hr. old colonies flooded with liquid medium at different intervals. 85

Fig.(17) *Achlya beneckii* colonies flooded with liquid medium at different intervals. 87

Fig.(18) *Saprolegnia parasitica* colonies flooded with liquid medium at different intervals. 88

Fig.(19) Protoplasts of *Phycomyces blakesleeanus*. 144

Fig.(20) Protoplasts of *Saprolegnia parasitica*. 145

Fig.(21) Indirect immunofluorescent micrograph of a homologous antigen-antibody reaction of hyphae and conidial heads of *Penicillium waksmanii*. 151

Fig.(22) Indirect immunofluorescent micrograph of a homologous antigen-antibody reaction of a hypha of *Saprolegnia parasitica*. 152

Fig.(23) Indirect immunofluorescent micrograph of a homologous antigen-antibody reaction of a hypha of *Pythium debaryanum*. 152

Fig.(24) Indirect immunofluorescent micrograph of a homologous antigen-antibody reaction of sporangiophores and sporangia of *M. mucedo*. 153

Fig.(25) Indirect immunofluorescent micrograph of a homologous antigen-antibody reaction of sporangiophores and sporangiospores of *Phycomyces blakesleeanus*. 153

Fig.(26) Indirect immunofluorescent micrograph of *Pythium debaryanum* hyphae cross-reacted with *Saprolegnia parasitica* antiserum. 154

Fig.(27) Indirect immunofluorescent micrograph of *Phytophthora parasitica* hyphae cross-reacted with *Saprolegnia parasitica* antiserum. 154

Fig.(28) Indirect immunofluorescent micrograph of *Saprolegnia terrestris* hypha cross-reacted with *Saprolegnia parasitica* antiserum. 155

Fig.(29) Indirect immunofluorescent micrograph of *Achlya beneckii* hyphae cross-reacted with *Pythium debaryanum* antiserum. 155

Fig.(30) Indirect immunofluorescent micrograph of *Apodachlya brachynema* hyphae cross-reacted with *S. parasitica* antiserum. 156

		Page No.
Fig.(31)	Indirect immunofluorescent micrograph of *Sapromyces elongatus* cross-reacted with *S. parasitica* antiserum.	156
Fig.(32)	Indirect immunofluorescent micrograph of *Aphanomyces cladogamus* hyphae cross-reacted with *S. parasitica* antiserum.	157
Fig.(33)	Indirect immunofluorescent micrograph of *Aphanomyces cladogamus* hyphae cross-reacted with *P. debaryanum* antiserum.	157
Fig.(34)	Indirect immunofluorescent micrograph of *Aplanopsis spinosa* hypha cross-reacted with *M. mucedo* antiserum.	158
Fig.(35)	Indirect immunofluorescent micrograph of *Aplanopsis spinosa* hypha cross-reacted with *P. debaryanum* antiserum.	158
Fig.(36)	Indirect immunofluorescent micrograph of *Aphanomyces cladogamus* hyphae cross-reacted with *P. waksmanii* antiserum.	159
Fig.(37)	Indirect immunofluorescent micrograph of *Aphanomyces cladogamus* cross-reacted with *M. mucedo* antiserum.	159
Fig.(38)	Indirect immunofluorescent micrograph of two hyphae of *Pythium debaryanum* cross-reacted with *M. mucedo* antiserum, showing two types of reactions.	160
Fig.(39)	Indirect immunofluorescent of *P. debaryanum* hypha cross-reacted with *M. mucedo* antiserum showing the spotted reaction.	160
Fig.(40)	Yellow-green immunofluorescence of *P. waksmanii* hyphae and conidial heads.	161
Fig.(41)	Yellow-green immunofluorescence of *P. debaryanum* hyphae.	162
Fig.(42)	Blocking of a homologous serological reaction of *P. debaryanum* hyphae by prior treatment with SDS.	163
Fig.(43)	Immunodiffusion pattern of the reactions of undiluted antisera against antigens extracted from mycelia with PBS, SDS, Triton-X100 in addition to culture filtrate.	167 & 168

LIST OF TABLES

		Page No.
Table (1)	Length of hyphal growth units of young mycelia of *M. mucedo* grown on MPG medium, flooded with liquid MPG medium containing varying concentrations of GA_3 and IAA.	29
Table (2)	Length of hyphal growth units of young mycelia of *S. parasitica* grown on PYG medium, flooded with liquid PYG medium containing varying concentrations of GA_3 and IAA.	31
Table (3-12)	Length (μm) and growth rate (μm/min.) of individual hyphae and their branches of *S. parasitica*, flooded 3 hr. with control liquid medium followed by 3 hr. with varying concentration of GA_3 and IAA.	33 - 42
Table (13)	Nucleotides and nucleic acids content of *S. parasitica* (% of fresh weight) grown on PYG medium with varying low concentrations of GA_3 and IAA.	59
Table (14)	Macromolecular composition in percentage of fresh and dry weight of three fungi.	61
Table (15)	Intensity of the immunofluorescence of homologous antigen-antibody reaction by the IMF technique.	147
Table (16)	Intensity of the immunofluorescence of homologous and/or heterologous antigen-antibody reaction by IMF technique.	148
Table (17)	Effect of blocking reagents on the homologous antigen-antibody reaction stained by the IMF technique.	150

LIST OF PLATES

		Page No.
Plate (1)	T.S. through a control hyphal tip of *Phycomyces blakesleeanus*.	90
Plate (2)	T.S. through a subapical region of control hypha of *P. blakesleeanus*.	91
Plate (3)	Higher magnification of part of Plate (2) showing a close association of mvb and plasmamembrane.	92
Plate (4)	Higher magnification through a subapical region of control hypha of *P. blakesleeanus*.	93
Plate (5)	L.S. through a control hypha of *P. blakesleeanus*.	94
Plate (6)	T.S. through a vacuolation zone of control hypha of *P. blakesleeanus*.	96
Plate (7)	L.S. through a burst hyphal tip of *P. blakesleeanus*.	97
Plate (8)	Higher magnification of a burst hyphal tip of *P. blakesleeanus*.	98
Plate (9)	T.S. through a burst hyphal tip of *P. blakesleeanus*.	99
Plate (10)	T.S. through a control hyphal tip of *M. mucedo*.	102
Plate (11)	T.S. through a control hyphal tip of *M. mucedo*.	103
Plate (12)	T.S. through a control hypha of *M. mucedo*.	104
Plate (13)	T.S. through a branch initiation in a control hypha of *M. mucedo*.	105
Plate (14)	T.S. through a hypha of *M. mucedo*, 15 min. after flooding with liquid medium.	106
Plate (15)	T.S. through a branched hypha of *M. mucedo*, 30 min. after flooding with liquid medium.	107
Plate (16)	Higher magnification through (X) region of Plate (15).	108
Plate (17)	Higher magnification of a well developed branch (Y region) of Plate (15).	109
Plate (18)	T.S. through a branch initiation of treated hypha of *M. mucedo*.	110
Plate (19)	T.S. through a vacuolation zone of treated hyphae of *M. mucedo*.	111

LIST OF ABBREVIATIONS

As	:	Antiserum
CW	:	Cell wall
CoA	:	Concanavalin A
dsg	:	Dark staining globule
2,4-D	:	2,4-Dichlorophenoxy acetic acid
D	:	Dictyosome
ER	:	Endoplasmic reticulum
GA_3	:	Gibberellic acid
G	:	Glycogen
IMD	:	Immunodiffusion test
IMF	:	Immunofluorescent technique
IAA	:	Indole-3-acetic acid
K	:	Kinetin
L	:	Lipid droplet
Lo	:	Lomasome
Md	:	Membranous disc
M	:	Mitochondria
Mvb	:	Multivesicular body
NAA	:	Naphthalene acetic acid
Nu	:	Nucleolus
N	:	Nucleus
PCA	:	Perchloric acid
PGRS	:	Plant Growth Regulating Substance
R	:	Ribosome
SDS	:	Sodium Dodecyl Sulphate
2,4,5-T	:	2,4,5-Trichlorophenoxy acetic acid
TCA	:	Trichloroacetic acid
Va	:	Vacuole
V	:	Vesicle

CONTENTS

	Page No.
Abstract	iv
Acknowledgements	vi
List of Figures	viii
List of Tables	xi
List of Plates	xii
List of Abbreviations	xiii
GENERAL INTRODUCTION	1
CHAPTER ONE - PLANT GROWTH REGULATING SUBSTANCES AND GROWTH OF FUNGI	3
1.1 Introduction	4
1.2 Materials and Methods	15
1.2.1 Culture of fungi and growth conditions	15
1.2.1.1 Organisms	15
1.2.1.2 Media	15
1.2.1.3 Preparation of spore suspension	16
1.2.1.4 Maintenance of cultures	16
1.2.1.5 Growth measurements	17
1.2.2 Effect of Plant Growth Regulating Substances (PGRS) on growth of Fungi	18
1.2.2.1 PGRS	18
1.2.2.2 Effect of PGRS on Germination of Sporangio-spores	18
1.2.2.3 Effect of PGRS on Length of Hyphal growth units	19
1.2.2.4 Effect of PGRS on Hyphal Growth Rate	20
1.2.2.5 Effect of PGRS on the Growth of Surface Colonies	20
1.2.2.6 Effect of PGRS on Dry Weight Yield	21
1.2.2.7 Effect of PGRS on Nucleic acids content	21
1.2.3 Macromolecular composition of some fungi	22
1.3 Results	24
1.3.1 Effects of PGRS on Growth	24
1.3.1.1 Effect of PGRS on spore germination	24
1.3.1.2 Effect of PGRS on the length of hyphal growth units	28

Page No.

1.3.1.3 Effect of PGRS on growth rate of primary hyphae 30
1.3.1.4 Effect of PGRS on surface colonies growth 32
1.3.1.5 Effect of PGRS on dry weight yield 52
1.3.1.6 Effect of PGRS on nucleic acids content 58

1.3.2 Macromolecular compositions of some Fungi 60

1.4 Discussion 63

CHAPTER TWO - LIGHT AND ELECTRON MICROSCOPIC STUDIES OF HYPHAL TIP BURSTING 68

2.1 Introduction 69

2.2 Materials and Methods 77

2.2.1 Organisms, culture conditions and light microscopy 77

2.2.2 Electron Microscopy 78

2.2.2.1 Organisms and Sampling 78
2.2.2.2 Fixation, Dehydration and Embedding 79
2.2.2.3 Sectioning, staining and examination 79

2.3 Results 80

2.3.1 Observations by Light Microscopy 80

2.3.2 Observation of electron microscopy 86

2.4 Discussion 112

CHAPTER THREE - PROTOPLASTS REGENERATION USING IMMUNOLOGICAL TECHNIQUES 119

3.1 Introduction 120

3.2 Materials and Methods 128

3.2.1 Organisms 128

3.2.2 Buffers and solutions 128

3.2.3 Protoplasts preparation 130

3.2.4 Antigen preparation 131

3.2.5 Immunization 132

3.2.6 Indirect immunofluorescent antibody technique 133

Page No.

3.2.7 Immunodiffusion test 135

3.2.8 Staining of immunodiffusion gels 136

3.2.9 Agglutination test 137

3.2.10 Concentration and purification of antisera 137

3.2.11 Antigen-Antibody blocking reaction 138

3.2.12 Preparation of pure cell walls 138

3.2.13 Adsorption of antisera 139

3.3 Results 139

3.3.1 Observations on Protoplasts Production 139

3.3.2 Morphological features of Lysis and Protoplasts Production 140

3.3.3 Factors affecting Protoplast Production 141

3.3.3.1 Osmotic stabilizer 141
3.3.3.2 Enzyme concentration 141
3.3.3.3 Age of culture 141

3.3.4 Protoplast isolation and examination 142

3.3.5 Regeneration of Protoplasts 142

3.3.6 Indirect immunofluorescent technique (IMF) 146

3.3.7 Blocking reactions 149

3.3.8 Immunodiffusion test (IMD) 164

3.4 Discussion 169

GENERAL DISCUSSION AND CONCLUSION 176

REFERENCES 178

GENERAL INTRODUCTION

The effects of plant growth regulating substances on cell extension and growth of higher plant cells have been widely studied and reviewed. In contrast most reports of the effect of these growth substances on filamentous fungi, especially, cellulose-walled ones, have been neither conclusive nor extensive. Therefore, it seemed desirable that a new approach to the problem should be undertaken. It was hoped that a comparative study of Gibberellic acid (GA_3) and Indole-3-acetic acid (IAA) effect on growth of numbers of chitin and cellulose-walled fungi would lead to a greater understanding of this subject.

Two fungi, *Mucor mucedo* Burgeff (Zygomycetes) and *Saprolegnia parasitica* (Oomycetes), were used in the first part of this study which focussed on the effect of varying concentrations of GA_3 and IAA on growth of both fungi. The two fungi are well suited because of their rapid normal growth and differences in their cell walls (chitin and cellulose respectively).

During the preliminary studies on growth of *M. mucedo* hyphal tips showed unexpected reactions when the colony grown on solid medium was flooded with the same liquid culture medium. This behaviour was investigated in some other chitin and cellulose-walled fungi (Oomycetes) using light and electron microscopy in an attempt to study the ultrastructural differences which may explain the reasons behind the unexpected responses obtained.

The third part of this study was focussed on protoplast preparation from a representative of each group of fungi using enzymic methods, in an attempt to investigate the wall regeneration process using the immunofluorescent technique.

Serological methods were also used to investigate the antigenic relationship between the two groups of fungi, since these methods were successfully used besides other classical diagnostic procedures of fungi.

CHAPTER ONE

PLANT GROWTH REGULATING SUBSTANCES AND GROWTH OF FUNGI

1.1 Introduction

The fungal cell wall is important because of its rigidity; it maintains the shape of the cell, offers protection by being the outermost cover of cells during development and morphogenesis, and may help to regulate the movement of chemicals between the cell and its environment.

The walls of all fungi consist of a two-phase system, made up of crystalline microfibrils embedded in an amorphous (non-crystalline) matrix. The microfibrils are usually composed of chitin (β1→4) linked polymers of N-acetyl glucosamine), or cellulose (β1→4 linked polymer of glucose), and they are always found in the inner layer of the mature wall and hyphal tips (Hunsley and Burnett, 1970, Gooday and Trinci, 1980). The amorphous matrix, which binds together the different structural components of the wall into the rigid macromolecular complex, is primarily polysaccharide, together with protein and lipids (Bartnicki-Garcia, 1968). Polysaccharides make up about 80-90% of the dry matter of fungal cell wall (Aronson, 1965; Bartnicki-Garcia, 1968; Dick, 1960; Smith and Berry, 1974).

Cell walls of fungi have received much attention because of the generally accepted view that different groups of fungi differ widely in the nature of the polymers that make up their cell walls and because of the realization that morphogenesis of fungal cells is associated with cell wall metabolism. Bartnicki-Garcia (1968) proposed a general classification for fungal walls based on the dual combination of the chief wall polysaccharides. Walls of Zygomycetes are composed of

chitosan-chitin as a polysaccharide, whereas Oomycetes are composed of cellulose-glucan residue. Wessels and Sietsma (1981) proposed a more detailed classification depending on alkali soluble (AS) and alkali insoluble (Al) polymers, in which Zygomycetes contain a glucuronomanno-protein polyphosphate as AS and a polyglucuronic acid, chitosan and chitin as Al. On the other hand Oomycetes contain a β1→3/β1-6-D-glucan, as AS and a β1→3/β1→6-D-glucan and cellulose as Al. Therefore, hyphae of Zygomycetes do not contain any glucan but polysaccharides rich in D-glucuronic acid (Bartnicki-Garcia and Reyes, 1968; Datema *et al*, 1977, a,b; Wessels and Sietsma, 1981).

Oomycete walls possess relatively low amounts of cellulose; Parker *et al* (1963) showed the presence of 10-15% cellulose fibrils in the walls of *Saprolegnia ferax*, Sietsma *et al* (1969) studied the cell wall composition of four genera of Oomycetes and found cellulose to be a minor component (range from 4% dry weight of the cell wall of *Apodachlya* up to 20% in *Pythium* and *Saprolegnia*). However some workers reported higher values around 30-45% for *Pythium butleri*, *Phytophthora heveae* and *Saprolegnia ferax* (Novaes-Ledieu *et al*, 1967). Vaziri-Tehrani (1978) stated that "the major component of the Oomycetes cell walls is not cellulose but R-glucan and chitin can be present". Dietrich (1975) reported the presence of chitin (as evidenced by the identification of glucosamine) in the walls of Saprolegniaceae and Pythiaceae.

Since walls of the Oomycetes are in some ways similar to higher plants in possessing cellulose microfibrils embedded in an amorphous matrix, one might expect to find comparable effects

of plant growth regulating substances (PGRS) on growth and hyphal extension, as observed in higher plants and extensively reviewed by Brian *et al* (1954, 1955), Stowe and Yamaki (1957), Jones (1973).

Auxins and gibberellins have been the most widely studied groups of PGRS having an effect on growth of higher plants. There are differences in the action of gibberellins and auxins as has been reported by Glaston and Purves (1960). There is a possible multiplicity of auxin action and it is difficult to separate the various effects from the elongation process itself. The major effects known to be produced by auxins on elongating cells are as follows:

(i) Decreased protoplasmic viscosity
(ii) Increased rate of protoplasmic streaming
(iii) Altered permeability patterns
(iv) Increased cell wall plasticity
(v) Altered respiratory patterns
(vi) Increased water uptake
(vii) Altered nucleic acid metabolism.

Thus whereas auxins in general are characterized by a multiplicity of physiological responses; gibberellins are characterized by specificity (Brian *et al*, 1955). However, Stowe and Yamaki (1957), Jones (1973) pointed out that all aspects of growth and development of higher plants from seed germination to fruit set can be affected by gibberellins.

The relationship between auxins and fungi has been investigated almost since phytohormones were first demonstrated in higher plants. A growth regulating substance was isolated

from the medium in which the fungus *Rhizopus suinus* was grown. This was called "Rhizopin", and was studied by Nielsen (1930) and Thimann (1935) and shown to be identical in its physiological effects to IAA. Gibberellic acid (GA_3) and other gibberellins have also been isolated in pure form from a strain of *Gibberella fujikuroi* (Saw.) Wr. (conidial state *Fusarium moniliforme* (Sheld.) emend. Snyder and Hansen) (Borrow *et al*, 1955, Stowe and Yamaki, 1957). Since then, it has been shown that auxin production is widespread among fungi belonging to different taxonomic groups (extensively reviewed by Gruen 1959). Studies by various workers have shown that the effects of PGRS on fungi vary to a considerable degree. In chitinous fungi, Banbury (1952) was unable to detect any stimulation of growth or curvature in *Phycomyces blakesleeanus* sporangrophores at low conc of IAA, instead a gradual inhibition at high concentration was seen. Brian *et al* (1954) studied the potential influence of GA_3 on the growth of a number of bacteria and molds, and were unable to detect any effect. Likewise Oravec *et al* (1959), using the potassium salt of gibberellic acid, were unable to detect any effect on *Saccharomyces cerevisiae*. Leelavathy (1969) studied the effect of 4 PGRS, IAA, GA_3, 2,4-D and NAA on the linear growth of fungi isolated from the rhizosphere of some graminaceous plants and concluded that the low concentration used caused no effect on the growth of any of the fungi tested, whereas high concentrations mostly inhibited growth to various extents. Therefore, he drew attention to the fact that these results explain partially why foliar sprays of the same PGRS failed to produce any significant change in the

rhizosphere fungal population of the graminaceous plants. Leonian and Lilly (1937) tested about a hundred fungi (cellulose and chitin-walled fungi) with regard to the effect of IAA, and came to the conclusion that the higher concentration of this substance proved toxic and the lower ones failed to induce any stimulation. Murdia (1939) investigated the effect of IAA and Phenyl acetic acid (PAA) on the growth of cellulose-walled fungi in the family Saprolegniaceae: *Achlya dubia*, *Aphanomyces campostylus*, *A. cladogamus*, *Pythiopsis intermedia* and *Thraustotheca clavata* where low concentrations induced no acceleration of growth but high concentrations caused a gradual inhibition. The same negative results were obtained with *Achlya sp*, *Isoachlya anisopora* var. *indica*, *Saprolegnia monoica* and *Brevilegnia gracilis* (Bhargava, 1946). Mehrotra (1951) studied the effect of 2,4-D, 1-NAA, IAA and r-3-indolyl butyric acid on growth and reproduction of four species of the genus *Phytophthora*. No acceleration of growth or induction of sexual structures were seen at low concentrations, however high concentrations showed considerable retardation in the growth.

Despite these reports on the inhibitory action of PGRS, there are numerous reports of the stimulation of growth of fungi. Richards (1949) reported stimulation in growth of some chitin-walled fungi to various extents in the presence of 1-NAA, 2,4-D, 2,4,5-T and IAA. Nysterakis (1954) in his studies on *Neurospora tetrasperma* found that as much as 100mg/L IAA gave a 60% to 70% promotion of growth. Gruen (1959) listed only a few reports of stimulation of growth of fungi induced by various auxins. Klein (1962) found that the rates

of growth of inositol-less colonial mutant (319) and a lysine-less mutant (4545A) of *Neurospora crassa* were markedly stimulated by K and GA_3, while wild type strains of *N. crassa* and *N. tetrasperma* were not so affected. Auxins were shown to elongate cells of yeast *Saccharomyces ellipsoideus* only in a respiration-deficient mutant under special cultural condition (Yanagishima, 1963, a,b,c). However, Gruen (1959) sums up the work done on the effect of auxins on all groups of fungi, the general observation was that high concentrations of all the regulators were toxic, while low concentrations of some were either ineffective or promoted growth.

Effect of IAA on spore germination of fungi was first studied by Windisch (1937), no overall effect was seen, except the usual inhibition at high concentrations. Richards (1949) found promotion of germination of *Aspergillus candidus* conidia with NAA, but not with IAA, 2,4-D or 2,4,5-T. According to Guttenberg and Strutz (1952) IAA caused up to 75% germination in *Ustilago zeae* spores, while the controls in water did not germinate. Van Sumere *et al* (1957) observed strong promotion of germination in *Puccinia graminis* uredospores treated with IAA and other compounds. The percentage promotion by IAA depended on the spore concentration, since an increase in spore concentration led to a decrease in germination of the controls. With high spore concentration IAA (200 mg/L) increased the germination but with low concentration the same amount of IAA was actually inhibitory. It was suggested that IAA might reverse a self-inhibition of spores. Nakamura *et al* (1978) reported that IAA or 2,4-D enhances germination of *N. crassa* conidia at the density of $2x10^6$ conidia/cm^3 (shortening the

time for completion of germination by approximately 1 hr.). Nakamura *et al* (1982) further investigated the role of IAA on conidial germination of *N. crassa* and found that the conidial density effect at $2x10^4$ and $2x10^5$ conidia/cm^3 was eliminated by the addition of auxin IAA or 2,4-D: the optimum concentration of IAA and 2,4-D being around 10^{-6}M. They found that this effect of auxin was mediated by the active substance(s) for conidial germination which was related to the conidial density effect. The effect of this active substance(s) which was detected in the cell-free filtrate of the germination medium, was enhanced in concentration of 10^{-6}M IAA.

Much work has been done on the influence of auxin on nucleic acids and protein synthesis in higher plants. Auxin induced-growth is concomitant with increase in RNA and protein synthesis, and this is prevented by inhibitors of RNA and protein synthesis and by an anti-auxin (Key and Hason, 1961; Datko and Maclachlan, 1968; Masuda, 1968). Additionally Tanimoto and Masuda (1968) reported that auxin causes the cell wall loosening by promoting the hemicellulase activity. One of the hemicellulases responsible for cell wall loosening may be β-1,3-glucanase which, in fact, induces a marked elongation. This was also confirmed later by Shimoda and Yanagishima (1971) in their studies on the auxin-induced growth of yeast cells. Masuda (1978) extensively reviewed auxin induction of cell wall loosening in higher plants.

Likewise, GA_3 was also reported to influence nucleic acid and protein metabolism in higher plants, directly (Giles and Myers, 1966; Broughton, 1968) or indirectly by enhancing

the *de novo* synthesis of α-amylase (Yomo, 1960; Paleg (1960). Other enzymes which were also found to be enhance as a result of GA_3 treatment were protease (Jacobsen and Varner 1967), β-glucanase and ribonuclease (Bennett and Chrispeels 1972, see also Jones and Jacobsen 1978) in aleurone tissue of barley. Additionally, inhibitors of protein synthesis, such as puromycin and cycloheximide, were also found to inhibit the GA_3-induced formation of α-amylase, and the incorporation of labelled amino acids into protein (Varner 1964, Varner *et al* 1965, Filner and Varner 1967). In addition to the postulated control of RNA metabolism, GAs were also found to stimulate cell division (Lang 1966) and DNA synthesis (Lang 1967).

Effect of GA_3 on the synthesis of DNA, RNA and protein in the yeast *Hansenula wingei* was reported by Makarem and Aldridge (1969); total contents of nucleic acids and protein/cm^3 of culture were highest in cultures supplemented with $10\mu g/cm^3$ GA_3 and this was concomitant with the increase in number of cells and buds.

Progress has been made on site of action of auxins in higher plants. Generally, auxins do not act directly on the wall but rather within the cytoplasm or at the plasma membrane. Since this action is partially separated from the cell wall, there should be some form of communication most likely a chemical messenger of some kind, between the cytoplasm and the wall. Many workers, Hager *et al* (1971), Rayle (1973), Rayle and Cleland (1972), Cleland (1980), have suggested that the wall loosening factor was simply hydrogen ions. The "Acid growth theory" then developed and in its present form

states that: Plant cells, upon exposure to auxin, excrete protons outwardly into the cell wall with the result that the pH of the wall solution decreases, allowing loosening and extension to occur. Acidic solutions (pH less than 5) have long been known to enhance the rate of cell elongation in shoot tissues (Bonner, 1934, Rayle and Cleland, 1970). Models or ideas suggested to explain how auxin induces H^+-excretion were reviewed in detail, (Cleland and Rayle, 1978, Vorobiev and Manusadzianas, 1983).

No such promoting effects of acidic acids on growth of fungi has been reported. Although within the last few years it has become apparent that the plasma membrane of probably all eukaryotic cells is equipped with H^+- translocating ATPases functioning as electrogenic proton pumps under physiological conditions. This has been shown in detail with *Saccharomyces cerevisiae* (Serrano, 1978; Peeters and Borstpauwels, 1979), *Neurospora crassa* (Bowman and Slayman, 1977; Bowman *et al*, 1981, a,b), *Schizosaccharomyces pombe* (Dofour and Goffeau, 1980). The generation by these enzymes of a proton gradient (alkaline inside) is the active step within a rapid circulation of protons across fungal cytoplasmic membranes, (Roos, 1981; Slayman, 1982): the H^+-influx being coupled with the uptake of sugars, amino acids and other nutrients. A direct coupling of proton extrusion with the uptake of cations has been demonstrated in *Saccharomyces cerevisiae* (K^+-H^+ exchange, Dofour and Goffeau, 1980). Acidification of the growth medium observed in cultures of *Penicillium cyclopium*, from an initial pH value 5.6 to below 3, was mostly due to the extrusion of protons into the medium (Roos and Luckner, 1984). In the absence of NH_4^+, protons

were extruded together with citrate. This finding suggests that the extrusion of H^+ is coupled with the influx of NH_4^+. With the exception of *N. crassa* this aspect of energy coupling of NH_4^+ transport has not yet been investigated in fungi (Roos and Luckner, 1984).

Since plant pathogens (other than viruses) have been shown to produce growth regulating substances in culture, the idea has grown that pathogens induce many symptoms of disordered growth as a result of producing an alien growth substance or by contributing in excess the same growth substance as is found in the host endogenous pool (Sequiera, 1973 and Pegg, 1976). Pegg (1981) has listed examples of increased levels of auxins in a variety of plant diseases. Much work has been done on hyper auxiny in diseases caused by biotrophic pathogens and in particular by rusts. Most increases in IAA or IAA equivalents in diseased plants have been of the order of five-fold, but Shaw and Hawkins (1958) reported increases of 24-fold in the susceptible wheat infected with *Puccinia graminis*. Unlike most diseases where IAA is increased, rust-infected cereals do not manifest abnormal cellular growth.

The work on the use of PGRS in the control of fungal diseases, reviewed by Leelavathy (1969) has shown that their effect on fungi is highly variable. Some workers recorded inhibitory action by spraying PGRS on fungi causing various plant diseases, whilst others reported no effect in controlling some fungal plant diseases. Hale *et al* (1962) showed that PGRS caused an increase in size and number of leaf spots

in some varieties of corn inoculated with *Helmithosporium carborium*. Sullia (1966) reported an increase in fungal population in the rhizosphere of cultivated and wild leguminous plants as a result of the foliar spray of different PGRS. The work of Leelavathy (1966) showed that there was no significant change in the rhizosphere fungal population of graminaceous plants as a result of foliar sprays of GA_3 and IAA. Several investigators (Mitchell and Marth, 1946; Nutman *et al* (1945) have suggested that the rates of disappearance of certain PGRS used as herbicides and the loss of their toxicity in the soil may be a result of the ability of certain micro-organisms either to utilize or to decompose these substances.

In this part of the present investigation, a comparative study of growth and macromolecular composition of members of chitin (*Mucor mucedo*) and cellulose-walled fungi (*Saprolegnia parasitica*), subjected to various concentrations of IAA and GA_3 was investigated in an attempt to study the response effect of these two groups of fungi especially the cellulose-walled ones to these growth substances.

1.2 Materials and Methods

1.2.1 Culture of fungi and growth conditions

1.2.1.1 Organisms: The test organisms used in this study were: *Mucor mucedo* Burgeff minus strain obtained from Centraal Bureau vor Schimmelcultures - Baarn, Holland and *Saprolegnia parasitica* (212B). Other Oomycetes were also used in the preliminary work on the growth of surface colonies, these were: *Saprolegnia terrestris* (212C) *Achlya caroliniana* (152C) and *Pythium debaryanum* (401C). All Oomycetes were supplied from the culture collection in the Botany Department at the University of Reading, England by Dr. M. Dick.

1.2.1.2 Media:

A - Malt - Peptone - Glucose (MPG).

Malt extract (Oxoid)	20g
Peptone	5g
Glucose	20g
Agar	15g
Dist. water	1L

B - Peptone - Yeast extract - Glucose (PYG).

Peptone	1.25g
Yeast extract	1.25g
Glucose	3g
Agar	15g
Dist. water	1L

C - Yeast - starch (YS).

Yeast extract	4g
Starch (soluble)	15g
KH_2PO_4	1g
$MgSO_4 . 6H_2O$	0.5g
Agar	15g
Dist. water	1L

All media were autoclaved at 15 p.s.i. for 15 min. Chemcals used in this work were from BDH unless otherwise stated.

1.2.1.3 Preparation of spore suspension:

Mucor mucedo spore suspension was prepared from 1-3 weeks old slant cultures on MPG medium, harvested by covering the culture with sterile distilled water and brushing gently with an inoculation loop. The spore suspension was filtered through 6 layers of sterile muslin to remove hyphae or sporangiophores, and then washed by centrifugation for 10 min. at 2000xg. The spores were suspended in sterile distilled water and counted in a haemocytometer. The concentration of spores was adjusted to 12.5×10^6 spores/cm^3 prior to inoculation.

For *S. parasitica*, an inoculum of spore cysts was prepared according to the idea that mycelia of aquatic fungi which are well nourished in rich nutrients form sporangia and discharge spores when transferred to a nutrient poor environment such as water. Therefore, 10-12 discs inoculum (6mm diameter from 24 hr. old culture on PYG medium at $24^{\circ}C$), were transferred into $100cm^3$ flask containing $25cm^3$ of sterile distilled water. Flasks were left at room temperature ($20^{\circ}C \pm 2$) on a rotary shaker at 160rev/min. Water was changed 5-6 times. After 24 hr. in water, spore cysts were collected by decantation, counted in a haemocytometer and the concentration of spores was adjusted to 4.4×10^6 spores/cm^3.

1.2.1.4 Maintenance of cultures: Stock cultures of *M. mucedo* were maintained on MPG agar slants (Gooday, 1968) in test tubes at $4^{\circ}C$. Colonies were grown aseptically on the same medium in petri dishes at $24^{\circ}C$ for 4-5 days.

For *S. parasitica* and other aquatic fungi, Griffins and Breuker's culturing method (1969) was used, stock cultures were maintained on solid Emerson's YS medium (Emerson 1958) in Petri dishes at 4°C and were transferred every 2 to 4 months. Vegetative colonies were grown aseptically on PYG agar (Cantino and Lovett 1960) in Petri dishes at 24°C for 4 days. Stock cultures were also maintained in water at 4°C in screw-top universal bottles containing 10 cm^3 of distilled water and 2-3 hemp seeds, which had been autoclaved at 15 p.s.i. for 15 min.

1.2.1.5 Growth measurements: In most physiological and bioassay experiments on fungi, measurement of colony diameter and radial growth rate are frequently used to measure fungal growth. However, the rate of growth of a fungus can be defined most precisely in terms of its specific growth rate or doubling time in submerged culture (Trinci and Gull 1970). Likewise Trinci (1973) has suggested that growth ofamycelium may also be considered in terms of the duplication of a hypothetical "growth unit" which consists of a tip associated with a strain specific length of hypha (Trinci 1979). This growth unit simply represents the ratio between total hyphal length and number of tips, and could be used as a parameter for branching density (Trinci 1973). Therefore, the experimental work on the effect of various concentrations of PGRS on growth was studied in five cases: spore germination, length of hyphal growth units and growth rate of young mycelia, colony diameters on solid media, mycelial dry weight and nucleic acids content.

1.2.2 Effect of Plant Growth Regulating Substances (PGRS) on growth of Fungi

1.2.2.1 PGRS: The PGRS used in this study were: Indole-3-Acetic Acid (IAA), Gibberellic acid (GA_3) and Kinetin (K).

Concentrations ranging from 0.1 ppm. to 400 ppm. were prepared by dissolving differing amounts of these substances in a known small volume of acetone in separate tubes prior to make up in culture media. When acetone - PGRS solutions were added to the other ingredients, the solvent constituted 1% of the final medium. Therefore 1% acetone was used in all control experiments.

In order not to subject the acid growth substances to prolonged sterilization by heat and to overcome the problem of acid hydrolysis of the agar, it appeared desirable to add the PGRS after sterilization of the media had been completed.

400 ppm. concentration of IAA and GA_3 was kept as the maximum concentration because it was shown by many investigators working on IAA that this concentration, or slightly lower, showed complete inhibition of growth of many fungi (Richards 1949, Leelavathy 1969).

1.2.2.2 Effect of PGRS on Germination of Sporangio spores: This method was performed on *Mucor mucedo* sporangiospores only. Germination experiments were done by using $25cm^3$ of MPG liquid medium containing various concentrations of PGRS in $250cm^3$ Erlenmyer flasks inoculated with $1cm^3$ of spore suspension which were incubated on a Gallenkamp Rotary Incubator at 160rev/min. at

16°C. Samples of 0.1cm^3 from the flasks were examined microscopically every hour to assess germination. Spores were recorded as having germinated when an emerging germ-tube was perceptible. Germination counts were calculated from observations of 200 spores. The diameters of at least 25 spores were recorded at the beginning (Zero time) and just prior to emergence of the germ-tubes.

1.2.2.3 Effect of PGRS on Length of Hyphal growth units: Petri dishes containing about 20cm^3 of solid medium were overlaid with uncoated cellophane (supplied by W.E. Cannings, Avonmouth, Bristol), which had been previously sterilized by boiling in water. 0.1cm^3 of spore suspension was dispersed over the surface with a sterile glass spreader and overlaid with another layer of sterilized cellophane. The cellophane prevented growth into the medium and thus ensured that the mycelium grew in a single plane. Inoculated plates were left overnight at room temperature (20°C±2). Cultures were examined from 11.5-13 hrs. after inoculation, 100-150µm total hyphal length for *M. mucedo* and 300-500µm total hyphal length for *S. parasitica*. To observe the growth the following method of Bartnicki-Garcia and Lippman (1972a) was used. Petri dishes were placed on the stage of a microscope equipped with a camera. The microscope was also fitted with a plastic Petri dish cover (15cm diameter) attached to the low power (10X) objective. Cultures were subjected to a flow system (drop by drop) of liquid medium containing varying concentrations of GA_3 and IAA in a biurett. The medium was pumped onto the upper surface of the upper layer

of cellophane at a rate of 50cm^3/hr. Excess medium was removed by suction at the opposite side of the colony to permit a rapid stream of medium to travel across the fungal colony. Total hyphal lengths and number of tips were recorded on unfixed mycelia after 6 hr. treatment by measurements made on enlarged prints or tracing of the negatives by the microprojector. The photography records were supplemented by a rapid direct observation using an occular micrometer.

1.2.2.4 Effect of PGRS on Hyphal Growth Rate: Effect of PGRS on growth rate of very young mycelia of *S. parasitica* was also determined using the same culture conditions and method described in the last section (Bartnicki-Garcia and Lippman 1972a), except that mycelia were subjected to a flow system of control liquid medium for 3 hrs., followed by another 3 hr. treatment with varying concentrations of GA_3 and IAA dissolved in liquid medium. Lengths of primary hyphae and their first branches of the first order were recorded at 30 min. intervals using an occular micrometer converted to give a record of growth rate in terms of μm/min.

1.2.2.5 Effect of PGRS on the Growth of Surface Colonies: Petri dishes (9 cm. diameter) containing approximately 20cm^3 of medium, were inoculated centrally with a 6mm. diameter plug cut from a slightly submarginal area of a mature colony growing on solid medium. *M. mucedo* was grown on MPG medium. All Oomycetes were cultured on PYG medium. Growth was recorded by measuring the colony diameter at 24 hr. intervals in two directions at

approximately right angles. For each treatment there were 4 replicates.

1.2.2.6 Effect of PGRS on Dry Weight Yield: Submerged cultures were grown in 100cm^3 conical flasks containing 25cm^3 of MPG or PYG media with varying concentrations of PGRS. Each flask was inoculated with 1cm^3 of spore suspension. Cultures of *M. mucedo* were incubated on a Gallenkamp Rotary incubator at 16^{o}C, whilst cultures of *S. parasitica* were grown under stationary conditions at 24^{o}C. Mycelial dry weights were determined at 24 hr. intervals for 6 and 4 days, for *M. mucedo* and *S. parasitica* respectively, by suction filtration through previously weighed Whatman No.1 filter papers. The mycelium was washed twice with 25cm^3 of distilled water, dried to constant weight at 80^{o}C, cooled in a desiccator and reweighed. Dry weight was expressed in terms of grams/50cm^3 medium.

1.2.2.7 Effect of PGRS on Nucleic acids content: Cultures of *S. parasitica*, grown on PYG liquid medium with varying concentrations of GA_3 and IAA at 24^{o}C for 4 days, were used for the estimation of nucleic acids after harvesting the mycelium on filter paper and washing several times with distilled water. Methods described by Pearson (1969), Storck and Morril (1977) and Saadi (1980) were used. Mycelia, 0.2-0.5g, were frozen with liquid air and ground with a mortar and pestle. 5cm^3 of 5% Trichloroacetic acid (TCA) was added to the ground mycelium and a full homogenization was carried out. The mixture was centrifuged at full speed (2000xg) in a MSE bench centrifuge for 10 min. at room temperature. This was

repeated twice and the supernatants containing nucleotides were pooled and retained in a 25cm^3 volumetric flask, which was subsequently used for the quantitative estimation of nucleotides. 5cm^3 of ethanol/ether/chloroform (3:2:1) was added to remove lipids from the pellet. The mixture was shaken vigorously and then centrifuged as above. The supernatant was discarded, and 5cm^3 of 0.3N KOH was added to the pellet, and the mixture digested at 32^{o}C overnight (18hr.). The digested mixture was centrifuged as above, and the supernatant was retained in a 50cm^3 volumetric flask. Another 4cm^3 of 0.3N KOH was added to the pellet and centrifuged. The supernatant was retained and pooled together with the first fraction; these were used for the quantitative estimation of RNA. The remaining pellet was used for DNA extraction by digesting with 2cm^3 of 5% (V/V) Perchloric acid (PCA) at 75^{o}C for 10 min. This should precipitate protein and hydrolyze DNA. After centrifugation as above, the supernatant was retained and made up to 5cm^3 in a volumetric flask. Nucleotides, RNA and DNA were estimated by a direct absorbance at 260nm. wavelength using a spectrophotometer (Perkin-Elmer 402). Concentrations were calculated using the formula:

$$22OD\ 260\text{-}290 = 1mg/cm^3.$$

1.2.3 Macromolecular composition of some fungi

Nucleotides and nucleic acids were estimated by the methods described previously, from fresh mycelia (after filtration and several washings with distilled water) and dried mycelia (at 80^{o}C) of three fungi: *Mucor mucedo*, *Phycomyces*

blakesleeanus, Burgeff (used by Ramadani, 1978 and Saadi, 1980, originally from Baarn culture collections) and *Saprolegnia parasitica*. The three fungi were grown on PYG liquid medium under static conditions. The same method was used for preparation of sporangiospore suspension from *M. mucedo* (described earlier) was used for *P. blakesleeanus*. Spores were activated before inoculation by a heat shock in a water bath at 45^{O}C for 10 min.

Protein was also estimated from fresh and dry mycelia of these three fungi by a modification of Lowry *et al*, (1951) after freezing the mycelia with liquid air and grinding with a mortar and pestle. Ground mycelia were suspended in 3-5cm^3 1N NaOH, boiled in a water bath for 5 min., then centrifuged as above. 0.03-0.1cm^3 supernatant was taken and made up to 1cm^3 volume with distilled water in a test tube. 0.9 cm^3 of reagent "A" was added (Potassium, sodium tartrate 2g, sodium carbonate (anhydrous) 100g, dissolved in 500cm^3 IN NaOH, made up to one litre with distilled water), mixed well and allowed to stand for 10 min. at room temperature. 0.1cm^3 of reagent "B" (Potassium, sodium tartrate 2g, cupric sulphate (anhydrous) 1g, dissolved in a solution containing 90cm^3 distilled water, plus 10cm^3 IN NaOH) was added and mixed well, allowed to stand for 10-15 min. 3cm^3 of Folin-Ciocalteu phenol reagent (2N SIGMA) diluted 1cm^3 in 15cm^3 with distilled water were added very rapidly and mixed within a second or two. After 10 min. or longer, the samples were read in the spectrophotometer used earlier. Protein was calculated from a standard curve prepared from lysozyme at a range of concentrations of 10-350µg/cm^3. Results of nucleic acids and protein were calculated in terms of percentage fresh and dry weight.

1.3 Results

1.3.1 Effects of PGRS on Growth

1.3.1.1 Effect of PGRS on spore germination:

Under normal conditions, the ungerminated spore of *M. mucedo* was elliptical or subcylindrical, about twice as long as broad, variable in sizes, 6.9-10μm long by 3-6μm wide.

In most fungi particularly in Mucorales (Ekundayo and Carlile, 1964, Mandels, 1953) germ-tube emergence is preceded by swelling of the spore brought about by a water uptake following a change in the permeability of the spore wall. One to two hours after inoculation in the control medium and in the medium containing various concentrations of GA_3 and IAA, the spores had begun to swell. The initial swelling was greater along the equator than longitudinally, so that just before germ-tube emergence the spores were spherical with a diameter of 17-20μm.

In the control medium the emergence of the germ-tube was first observed after 8 hrs. of incubation and completed during incubation for 15 hrs.

GA_3 and IAA (Figures 1 and 2) showed no acceleration of germination over the control at the whole range of concentrations of both substances, *i.e.* no difference was observed in the time required for the emergence of the first germ-tube. A possible exception was 100 ppm. GA_3, the germination was accelerated by an hour before the control. On the other hand the results showed a gradual shortening of time required for the completion of germination with the increasing concentrations of GA_3 from 10 up to 100 ppm., in which the highest percentage

Fig. 1: Percentage of spore germination of *M. mucedo* in MPG medium containing varying concentrations of GA_3.

Incubation at 16 °C

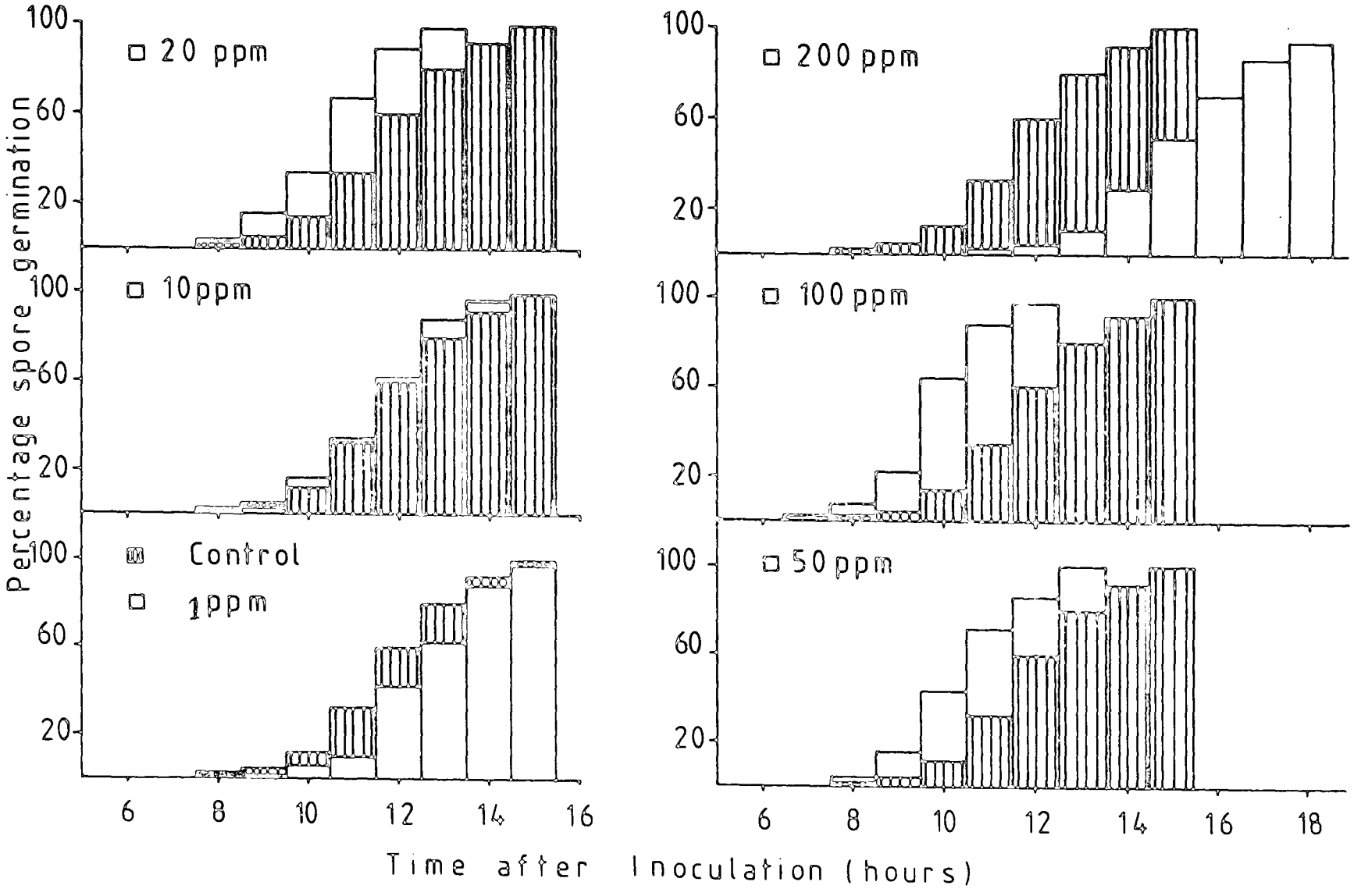
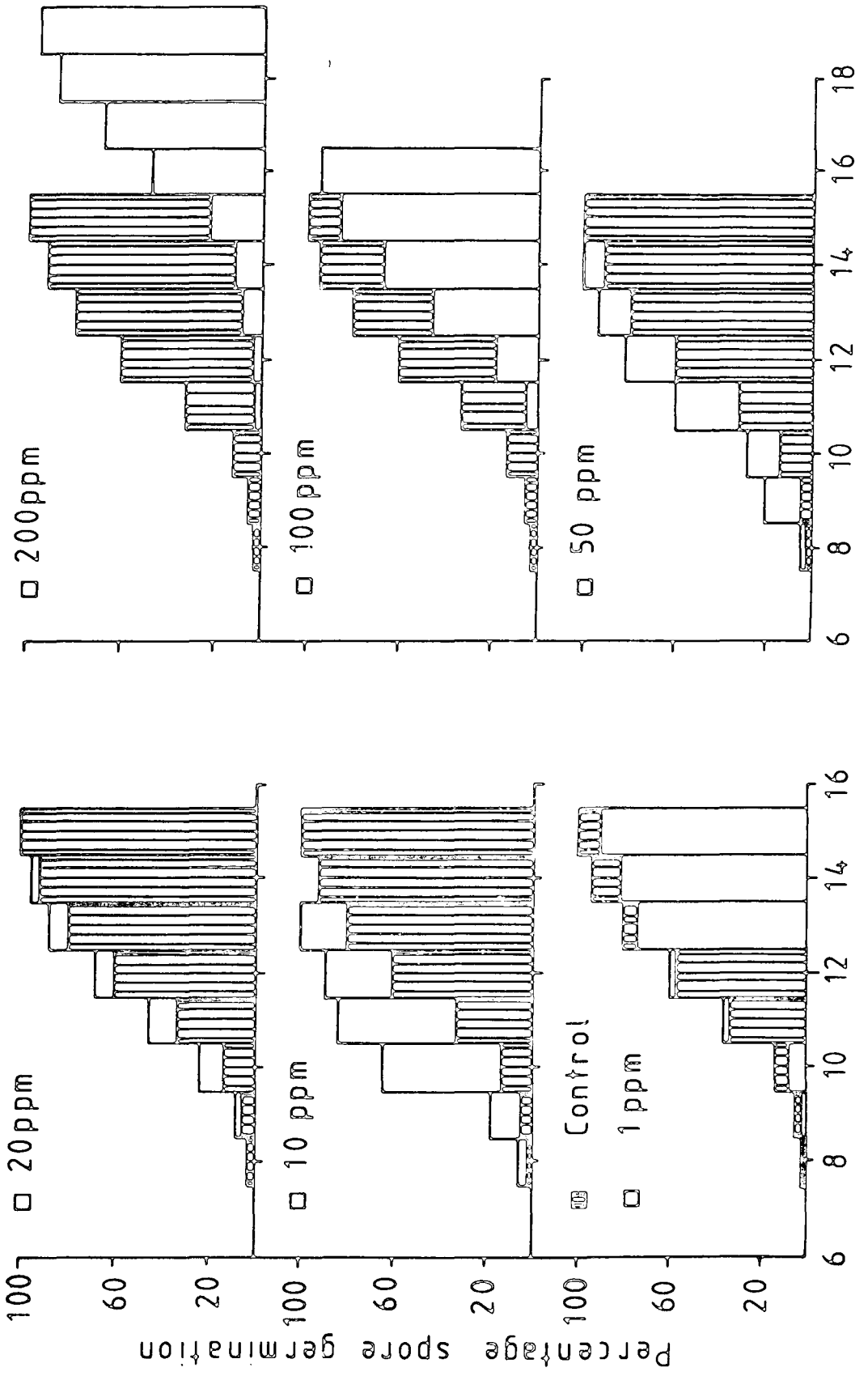

Percentage spore germination
100
60
20
20 ppm
10ppm
Control
1ppm
200 ppm
100 ppm
50 ppm
6
8
10
12
14
16
18
Time after Inoculation (hours)

Fig. 2: Percentage of spore germination of *M. mucedo* in MPG medium containing varying concentrations of IAA.

Incubation at 16 °C


Percentage spore germination
100
60
20
□ 20ppm
□ 10 ppm
Control
□ 1ppm
□ 200ppm
□ 100ppm
□ 50 ppm
6
8
10
12
14
16
18
Time after Inoculation (hours)

of germination was accomplished within 12 hrs. of incubation. While on the high concentration of both substances, 200 and 400 ppm. the first germ-tube was delayed by 3 hrs. after the control, and the highest percentage of germination was accomplished within 18-19 hrs. after inoculation. The only concentration of IAA that induced higher percentage of germination and shortened the time required for completion of germination (13 hrs.) was 10 ppm. This acceleration was reduced with increasing concentrations (20 and 50 ppm.) up to 100 ppm. in which this concentration showed some inhibition of spores germination and a delay in the initial germination.

The addition of GA_3 and IAA had almost no effect on swelling or permeability of spores.

1.3.1.2 Effect of PGRS on the length of hyphal growth units: Measurements of colony radius do not give any information about the earliest growth of the colony during the first few hours after germination. Therefore, the following experiment was set up to investigate any variation in length of the hyphal growth unit of a developing germ-tube or young mycelia. The initial length of the germ-tubes before treatment (11.5-13 hrs. after inoculation) were 100-150µm. After 6 hrs. treatment with particular concentrations of GA_3 and IAA, the average length of hyphal growth units of 10-15 randomly selected young mycelia of *M. mucedo* (Table 1), were between 106.67 and 180.46µm. These figures did not reveal any clear stimulation or inhibition by either substances because of the inconstant variation in the length of hyphal growth unit between high and low values with the increasing concentration of PGRS from 0.1 ppm. up to

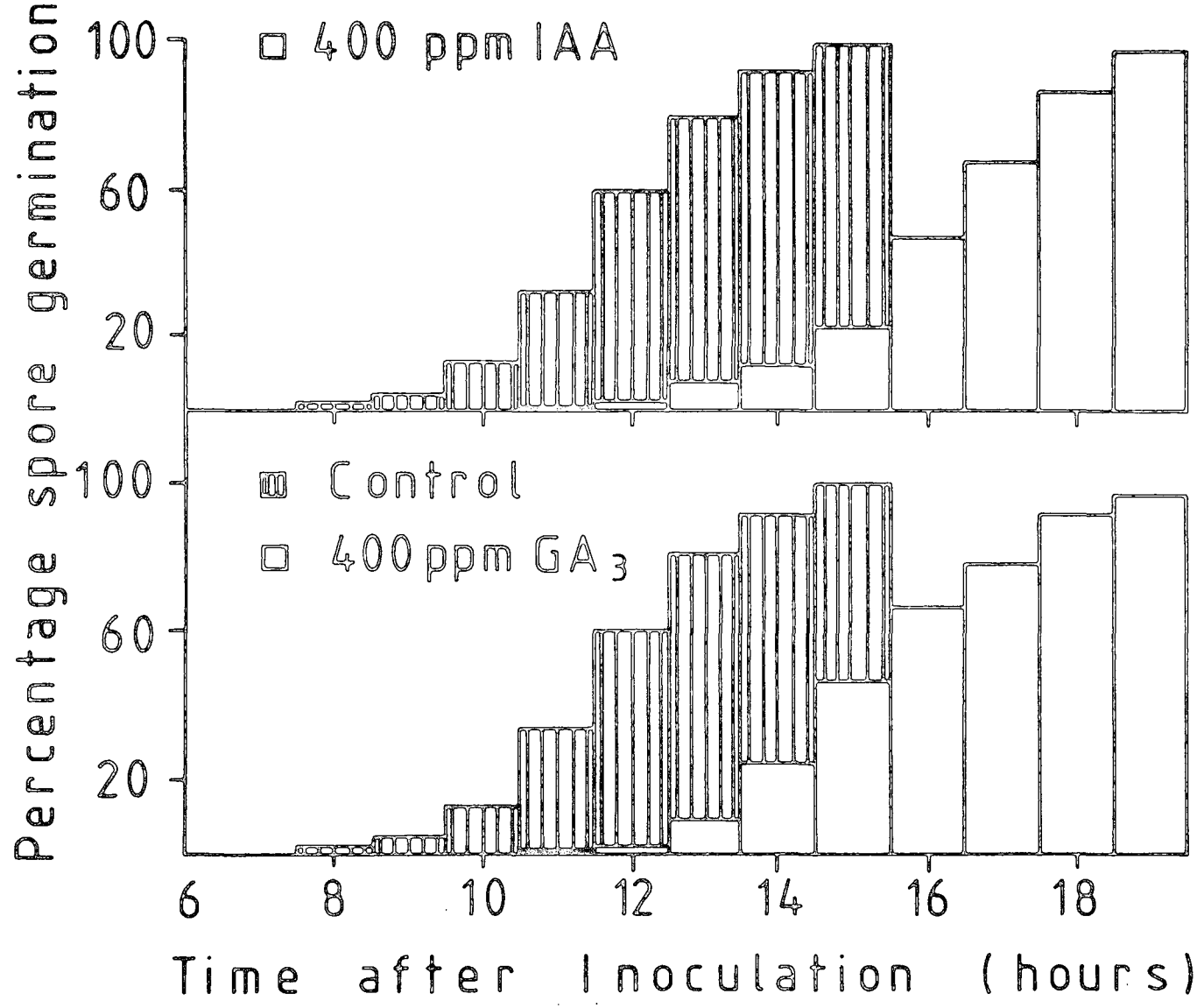

Percentage spore germination
100
60
20
400 ppm IAA
100
60
20
Control
400 ppm GA3
6
8
10
12
14
16
18
Time after Inoculation (hours)

Table (1) Length of hyphal growth units of young mycelia of *M. mucedo* grown on MPG medium, flooded with liquid MPG medium containing varying concentration of GA_3 and IAA.

Experiment was carried out at room temperature (20 °C + 2)

continue Figure 1 and 2. Spore germination of *M. mucedo* with 400 p.p.m. GA_3 and IAA.

Treatment	Length of hyphal growth units (µm)																			AVERAGE LENGTH OF HYPHAL GROWTH UNIT
	Number of hyphal tips																			
	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	
Control		189·75	171·05	114·87	94·28	107·25	104·5			124·2		119·4								128·16
0·1 GA_3		136·12	125·4	91·6	86·42	108·2	131·38		94		80·3									106·67
0·2 GA_3		151·25		88·9		199	210·8	156·7	218·5	174·43										171·37
0·5 GA_3			127·6	120	107·86		134·4	109·15	115·75	75·6										112·9
1·0 GA_3	121	119·6	172·7	91·6	139·3	109·28	100·8	127·6	92·5											119·37
5·0 GA_3		220·68	181·5	148·5		153·31														175·99
10·0 GA_3			149·6	206·7	209·73		146·35	155·9		151·47										169·96
20·0 GA_3			169·4	200·27	123·06	101·75	103·8		125·25	136·12	148·9									138·56
0·1 IAA				165	136·7	110·63	129·11	79·2		89·8			104·13						119·69	116·78
0·2 IAA			136·4	208·08	132·16		163·7		155·5	130·6	101·1		166·1							149·2
0·5 IAA					168·25	169·4	182·1	163·7			115·5									159·71
1·0 IAA			198	147·5	206·6	173·9		155·26	201·5											180·46
5·0 IAA	214·5		152·35	142·08	171·07			121·5	143											157·41
10·0 IAA	218·15		194·15	132	146·4	137·5	110													156·36
20·0 IAA		303·87			167·33	163·61	69·6	147·4	109·5	79·75		182·28				139·94				151·47

20 ppm. Likewise, there were also large variations or irregularities between the replications of mycelia possessing different number of tips within each treatment individually and within mycelia containing the same number of tips at the whole range of concentrations of both substances. With *S. parasitica* (Table 2), the results showed a more or less regular increase in the average length of hyphal growth units with the increasing concentrations of GA_3 from 0.1 ppm. up to 10 ppm.; at this concentration the difference between the control and the treated mycelia was at its highest value. With IAA treatment, results showed irregular inhibition with increasing concentration from 0.2 ppm. up to 20 ppm., especially at concentration 5, 10 and 20 ppm. These results also showed variation between mycelial replications within each treatment and between mycelia containing the same number of tips over the whole range of concentrations.

1.3.1.3 Effect of PGRS on growth rate of primary hyphae: The results of the effect of GA_3 and IAA at low concentrations on the growth rate of young mycelia grown for 3 hrs. with control medium followed by three hours treatment with these substances were shown by Tables (3-11) and summarized in Table (12). The growth rate of individual hyphae was studied by measuring the increase in length of hyphae and their branches. The spores sown on the agar plates germinated at different times and when random measurements were taken the hyphae found were already of different lengths. Although the hyphae on the 3 hr. control treatment (with liquid medium only), were all under the same conditions, they were growing at different rates. Further, none

Table (2) Length of hyphal growth units of young mycelia of *S. parasitica* grown on PYG medium, flooded with liquid PYG medium containing varying concentrations of GA_3 and IAA.

Experiment was carried out at room temperature (20 ℃ + 2)

Treatments	Length of hyphal growth units (μm)												AVERAGE LENGTH OF HYPHAL GROWTH UNIT
	Number of hyphal tips												
	2	3	4	5	6	7	8	9	10	11	12	13	
Control	606·83	524·3	409·75		350·16	325·28							443·26
0·1 GA_3	682	556·54	670·06	485·1	806·6								640·06
0·2 GA_3	431·7		551·37	581·46	633·84		566·5						552·97
0·5 GA_3	404·8	581·82	398·75	389·95									443·83
1·0 GA_3		931·3		811·61	608·65								783·85
5·0 GA_3	671	819·45	770·08	562·1	495		570·62						648·04
10·0 GA_3	982·66	691·12	675·12		891	732·28							794·43
20·0 GA_3				700·7	700·93	645·85	572·68	626·99		637			647·35
0·1 IAA	503·25	344·6	609·12	506	412·3	306·42	525·25	490·72	551·1	534·19			478·29
0·2 IAA				357·5	324·5	266·3	325·64		337·97		247·04		309·82
0·5 IAA			401·5		533·11	551·92	532·1	521·8					508·08
1·0 IAA		238·3	185·6		425·33	456·66	317·1			270			315·49
5·0 IAA		248·41	190·56	158·4	145·54	154·7							179·52
10·0 IAA	264	191·55	243·81	208·26	187		193·87	182·1				188·6	207·39
20·0 IAA		163·16		227·7	227·77	232·93	206·93			133·5			198·66

of the hyphae taken by themselves maintained its own steady growth rate, but each went through a series of changes. At first when the hypha was very short, the elongation was very slow, but the rate gradually increased. As in the parent hyphae, the branches usually started slowly and increased in rate as the time passed. They show fluctuations in rates similar to those shown by primary hyphae. Comparison of the results of Tables (3-11), could be properly made between the hyphae attending more or less the same initial length, by dividing the initial lengths of the hyphae into 9 groups (7.7-15.4μ range of difference in lengths in each group tested), as shown in Table (12). As a result most of the variations remained within all the groups and no marked stimulation or inhibition of growth could be detected after the 3 hr. treatment with the whole range of concentrations of GA_3 and IAA. Further, no effect could be observed on the rate of elongation of parent hyphae as a result of branch formation.

1.3.1.4 Effect of PGRS on surface colonies growth: Colonies of *M. mucedo*, on control MPG medium were characterized by a very compact growth with a thin and dense advancing peripheral ring of the vegetative hyphae. These colonies were not quite regular in outline, and had a very dense cluster of sporangiophores, which characteristically grew upwards to the lid of the Petri dish. The under surface of the colonies, particularly the centres, were usually coloured yellow having a rich carotene content.

Table (3) Length (μm) and growth rate (μm/min.) of individual hyphae and their branches of *S. parasitica*, 6 hr. flooded with control liquid medium.

Experiment was carried out at room temperature (20 °C + 2)

H_1 , H_2 : Hyphae examined in this mycelium

Time (hours)	Mycelium 1										Mycelium 2							
	H_1			H_2							H_1				H_2			
	No. of tips			No. of tips							No. of tips				No. of tips			
	1	2	3	1	2	3	4	5	6	7	1	2	3	4	1	2	3	4
0·0	84·7 (-)			123·2 (-)							154 (-)				15·4 (-)			
0·5	107·8 (0·77)			154 (1·02)							177·1 (0 77)				38·5 (0·77)			
1·0	138·6 (1·02)			200·2 (1·54)							231 (1 79)				61·6 (0·77)			
1·5	200·2 (2·05)			277·2 (2·5)							300·3 (2·3)				115·5 (1·79)			
2·0	261·8 (2·05)			385 (3·5)							354·2 (1·79)				200·2 (2·8)			
2·5	315·7 (1·79)			508·2 (4·1)							446·6 (3·08)				277·2 (2·5)			
3·0	361·9 (1·54)			600·6 (3·08)							492·8 (1 54)				292 6 (0 5)	15·4 (-)		
4·0	462 (1·66)	138·6 (-)		908·6 (5·1)	69·3 (-)	100·1 (-)	100·1 (-)				616 (2·05)	77 (-)	77 (-)		369·6 (1·28)	215·6 (3·3)		
4·5	508·2 (1·54)	215·6 (2·5)		1001 (3 08)	154 (2·8)	154 (1·7)	154 (1·7)				677·6 (2 05)	184·8 (3·5)	215 6 (4 6)		400·4 (1·02)	315·7 (3·3)	38·5 (-)	
5·0	569·8 (2·05)	354·2 (4 6)		1155 (5 1)	169·4 (0·5)	246 4 (3·08)	215·6 (2·08)				754·6 (2 5)	292·6 (3 5)	354·2 (4·6)		492 8 (3·08)	446·6 (4 3)	115·5 (2·5)	
5·5	631·4 (2·05)	462 (3·5)	38·5 (-)	1262 8 (3·5)	261·8 (3·08)	385 (4·6)	346·5 (4·3)				823·9 (2·3)	369·6 (2·5)	469 7 (3·8)		569 8 (2·5)	569·8 (4·1)	223·3 (3·5)	
6·0	723 8 (3 08)	569 8 (3 5)	107 8 (2·31)	1386 (4 1)	408·1 (4·8)	539 (5·1)	492·8 (4·8)	15·4 (-)	23 1 (-)	30 8 (-)	985·6 (5·3)	531·3 (5 3)	616 (4 8)	15 4 (-)	785 4 (7 1)	800 8 (7·7)	408·1 (6 16)	15 4 (-)

Table (4) Length (μm) and growth rate (μm/min.) of individual hyphae and their branches of *S. parasitica*, flooded 3 hr. with control liquid medium followed by 3 hr. with medium containing 0.5 p.p.m. GA_3.

Experiment was carried out at room temperature (20°C + 2)

H_1 , H_2 : Hyphae examined in this mycelium

Time (hours)	Mycelium 1				Mycelium 2						Mycelium 3					
	H_1		H_2		H_1		H_2				H_1	H_2				
	No. of tips		No. of tips		No. of tips		No. of tips				N.O.T	No. of tips				
	1	2	1	2	1	2	1	2	3	4	1	1	2	3	4	
0·0	115·5 (-)		53·9 (-)		77 (-)		92·4 (-)				46·2 (-)	77 (-)				3h Control
0·5	138·6 (0·77)		92·4 (1·28)		100·1 (0·77)		138·6 (1·54)				46·2 (0)	130·9 (1·79)	46·2 (-)			
1·0	207·9 (2·3)		130·9 (1·28)		138·6 (1·2)		169·4 (1·02)				46·2 (0)	169·4 (1·28)	77 (1·02)			
1·5	269·5 (2·05)		184·8 (1·79)	15·4 (-)	231 (3·08)		246·4 (2·5)	30·8 (-)			46·2 (0)	215·6 (1·53)	115·5 (1·28)			
2·0	338·8 (2·3)		231 (1·54)	77 (2·05)	346·5 (3·8)		346·5 (3·3)	107·8 (2·5)			100·1 (1·79)	292·6 (2·56)	184·8 (2·3)			
2·5	400·4 (2·05)		323·4 (3·08)	184·8 (3·5)	369·6 (0·77)		408·1 (2·05)	177·1 (2·3)			138·6 (1·28)	385 (3·07)	277·2 (3·07)			
3·0	431·2 (1·02)		338·8 (0·5)	231 (1·54)	385 (0·5)		462 (1·79)	200·2 (0·76)			184·8 (1·53)	431·2 (1·53)	308 (1·02)			
4·0	477·4 (0·77)	69·3 (-)	492·8 (2·5)	400·4 (2·8)	508·2 (2·05)	46·2 (-)	677·6 (3·5)	338·8 (2·3)	77 (-)		385 (3·3)	508·2 (1·28)	500·5 (3·2)			3h with 0·5ppm GA_3
4·5	477·4 (0)	154 (2·8)	492·8 (0)	523·6 (4·1)	600·6 (3·08)	77 (1·02)	716·1 (1·28)	369·6 (1·02)	107·8 (1·02)		508·2 (4·1)	539 (1·02)	585·2 (2·8)			
5·0	554·4 (2·5)	200·2 (1·54)	616 (4·1)	608·3 (2·8)	693 (3·08)	161·7 (2·8)	847 (4·3)	492·8 (4·1)	231 (4·1)		569·8 (2·05)	677·6 (4·6)	723·8 (4·6)	23·1 (-)		
5·5	669·9 (3·8)	323·4 (4·1)	785·4 (5·6)	708·4 (3·3)	847 (5·1)	277·2 (3·8)	985·6 (4·6)	623·7 (4·3)	354·2 (4·1)		723·8 (5·13)	816·2 (4·6)	847 (4·1)	77 (1·79)	15·4 (-)	
6·0	800·8 (4·3)	446·6 (4·1)	908·6 (4·1)	862·4 (5·13)	1061·4 (5·6)	431·2 (5·1)	1139·6 (5·1)	777·7 (5·1)	523·6 (5·6)	30·8 (-)	1031·8 (10·26)	1001 (6·1)	1031·8 (6·1)	169·4 (3·07)	92·4 (2·5)	

Table (5) Length (µm) and growth rate (µm/min.) of individual hyphae and their branches of *S. parasitica*, flooded 3 hr. with control liquid medium followed by 3 hr. with medium containing 1 p.p.m. GA_3.

Experiment was carried out at room temperature (20 °C + 2)

H_1 , H_2 : Hyphae examined in this mycelium

Time (hours)	Mycelium 1					Mycelium 2						Mycelium 3						
	H_1	H_2				H_1			H_2			H_1			H_2			
	N.O.T.	No. of tips				No. of tips			No. of tips			No. of tips			No. of tips			
	1	1	2	3	4	1	2	3	1	2	3	1	2	3	1	2	3	
0·0	11·5 (-)	123·2 (-)				123·2 (-)			7·7 (-)			46·2 (-)			138·6 (-)			3h Control
0·5	15·4 (0·12)	154 (1·02)				154 (1·02)			15·4 (0·25)			77 (1·02)			169·4 (1·02)			
1·0	46·2 (1·02)	200·2 (1·5)				184·8 (1·02)			23·1 (0·25)			84·7 (0·25)	15·4 (-)		215·6 (1·5)			
1·5	123·2 (2·56)	261·8 (2·05)	30·8 (-)	30·8 (-)	23·1 (-)	246·4 (2·05)	15·4 (-)		77 (1·79)	15·4 (-)		123·2 (1·28)	69·3 (1·79)		308 (3·07)			
2·0	138·6 (0·51)	277·2 (0·5)	38·5 (0·25)	46·2 (0·5)	46·2 (0·76)	261·8 (0·5)	30·8 (0·5)	38·5 (-)	138·6 (2·05)	23·1 (0·25)		169·4 (1·5)	107·8 (1·28)		369·6 (2·05)			
2·5	177·1 (1·28)	331·1 (1·79)	46·2 (0·25)	46·2 (0)	77 (1·02)	315·7 (1·79)	53·9 (0·76)	53·9 (0·5)	184·8 (1·5)	23·1 (0)		200·2 (1·02)	177·1 (2·3)		438·9 (2·3)			
3·0	215·6 (1·28)	369·6 (1·28)	53·9 (0·25)	46·2 (0)	107·8 (1·02)	338·8 (0·76)	92·4 (1·28)	92·4 (1·28)	231 (1·5)	30·8 (0·25)		215·6 (0·5)	231 (1·79)		508·2 (2·3)			
4·0	308 (1·54)	492·8 (2·05)	123·2 (1·15)	107·8 (1·02)	261·8 (2·5)	431·2 (1·5)	200·2 (1·7)	323·4 (3·8)	338·8 (1·79)	77 (0·76)	30·8 (-)	331·1 (1·9)	431·2 (3·3)	30·8 (-)	546·7 (0·64)	107·8 (-)	38·5 (-)	3h with 1ppm GA_3
4·5	385 (2·5)	585·2 (3·07)	231 (3·5)	200·2 (3·01)	385 (4·1)	485·1 (1·79)	308 (3·5)	462 (4·6)	415·8 (2·5)	138·6 (2·05)	69·3 (1·28)	385 (1·79)	600·6 (5·6)	107·8 (2·5)	69·3 (4·8)	169·4 (2·05)	84·7 (1·5)	
5·0	454·3 (2·3)	654·5 (2·3)	338·8 (3·5)	261·8 (2·05)	462 (2·5)	531·3 (1·5)	400·4 (3·07)	554·4 (3·07)	485·1 (2·3)	154 (0·5)	138·6 (2·3)	446·6 (2·05)	693 (3·07)	161·7 (1·79)	785·4 (3·07)	238·7 (2·3)	138·6 (1·79)	
5·5	539 (2·8)	739·2 (2·8)	462 (4·1)	369·6 (3·5)	539 (2·5)	600·6 (2·3)	523·6 (4·1)	770 (7·1)	616 (4·3)	246·4 (3·07)	246·2 (3·5)	508·2 (2·05)	739·2 (1·5)	200·2 (1·28)	862·4 (2·5)	277·2 (1·28)	200·2 (2·05)	
6·0	616 (2·5)	816·2 (2·5)	577·4 (3·8)	462 (3·07)	616 (2·5)	646·8 (1·5)	616 (3·07)	877·8 (3·5)	654·5 (1·28)	284·9 (1·28)	323·4 (2·5)							

Table (6) Length (μm) and growth rate (μm/min.) of individual hyphae and their branches of *S. parasitica*, flooded 3 hr. with control liquid medium followed by 3 hr. with medium containing 5 p.p.m. GA_3.

Experiment was carried out at room temperature (20 °C + 2)

H_1 , H_2 : Hyphae examined in this mycelium

Time (hours)	Mycelium 1						Mycelium 2						Mycelium 3									
	H1		H2				H1						H2			H1					H2		
	No. of tips		No. of tips				No. of tips						No. of tips			No. of tips					No. of tips		
	1	2	1	2	3	4	1	2	3	4	5	6	1	2	3	1	2	3	4	5	1	2	
0·0	38·5 (-)		123·2 (-)				61·6 (-)						123·2 (-)			154 (-)							3h Control
0·5	46·2 (0·25)		138·6 (0·5)				73·15 (0·38)						146·3 (0·76)			192·5 (1·28)							
1·0	77 (1·02)		177·1 (1·28)				92·4 (0·64)	7·7 (-)					177·1 (1·02)			215·6 (0·76)					3·8 (-)		
1·5	115·5 (1·28)		261·8 (2·8)				130·9 (1·28)	23·1 (0·5)					246·4 (2·3)			292·6 (2·5)					3·8 (0)		
2·0	138·6 (0·76)		323·4 (2·05)	23·1 (-)			138·6 (0·25)	61·6 (1·28)					323·4 (2·5)			323·4 (1·02)					84·7 (2·69)		
2·5	154 (0·51)		369·6 (1·79)	61·6 (1·28)			138·6 (0)	115·5 (1·79)					408·1 (2·8)			392·7 (2·3)	19·25 (-)	3·85 (-)			119·3 (1·1)		
3·0	200·2 (1·5)		454·3 (2·5)	92·4 (1·02)			138·6 (0)	169·4 (1·79)					492·8 (2·8)			446·6 (1·79)	23·1 (0·12)	15·4 (0·38)			154 (1·1)		
4·0	392·7 (3·2)		646·8 (3·2)	238·7 (2·4)	7·7 (-)		138·6 (0)	400·4 (3·8)					646·8 (2·5)	46·2 (-)		616 (2·8)	177·1 (2·5)	115·5 (1·6)	23·1 (-)	23·1 (-)	292·6 (2·3)		3h with 5ppm GA3
4·5	415·8 (0·76)	7·7 (-)	739·2 (3·07)	277·2 (1·28)	11·55 (0·12)		138·6 (0)	523·6 (4·1)					716·1 (2·3)	53·9 (0·25)		631·4 (0·5)	231 (1·79)	146·3 (1·02)	30·8 (0·25)	30·8 (0·25)	308 (0·5)		
5·0	539 (4·1)	11·55 (0·12)	862·4 (4·1)	369·6 (3·07)	19·25 (0·25)		146·3 (0·25)	639·1 (3·8)	30·8 (-)				800·8 (2·8)	92·4 (1·2)	15·4 (-)	693 (2·05)	338·8 (3·5)	223·3 (2·5)	77 (1·5)	30·8 (0)	338·8 (1·02)		
5·5	693 (5·1)	19·25 (0·25)	1031·8 (5·6)	492·8 (4·1)	61·6 (1·4)	61·6 (-)	146·3 (0)	800·8 (5·3)	138·6 (3·5)	46·2 (-)	30·8 (-)	15·4 (-)	939·4 (4·6)	215·6 (4·1)	30·8 (0·5)	708·4 (0·5)	492·8 (5·1)	354·2 (4·3)	184·8 (3·5)	84·7 (1·79)	431·2 (3·1)	15·4 (-)	
6·0	816·2 (4·1)	77 (1·9)	1185·8 (5·1)	631·4 (4·6)	115·5 (1·7)	169·4 (3·5)	146·3 (0)	985·6 (6·1	261·8 (4·1)	154 (3·5)	69·3 (1·28)	15·4 (0)	1093·4 (5·1)	369·6 (5·1)	46·2 (0·5)	754·6 (1·5)	646·8 (5·1)	423·5 (2·3)	338·8 (5·1)	161·7 (2·5)	577·5 (4·8)	69·3 (1·79)	

Table (7) Length (μm) and growth rate (μm/min.) of individual hyphae and their branches of *S. parasitica*, flooded 3 hr. with control liquid medium followed by 3 hr. with medium containing 10 p.p.m. GA_3.

Experiment was carried out at room temperature (20 °C + 2)

H_1 , H_2 : Hyphae examined in this mycelium

Time (hours)	Mycelium 1					Mycelium 2					Mycelium 3									
	H 1			H 2		H 1			H 2		H 1					H 2				
	No. of tips			No. of tips		No. of tips			No. of tips		No. of tips					No. of tips				
	1	2	3	1	2	1	2	3	1	2	1	2	3	4	5	1	2	3	4	
0·0	7 7 (-)			30·8 (-)		100·1 (-)					46·2 (-)	15·4 (-)				107·8 (-)				3h Control
0·5	107·8 (1·02)			30·8 (0)		123·2 (0·76)					46·2 (0)	30·8 (0·5)				146·3 (0·76)				
1·0	138·6 (1·02)			30·8 (0)		130·9 (0·25)					46·2 (0)	46·2 (0·5)				169·4 (0·76)				
1·5	154 (0·5)			61·6 (1·02)		169·4 (1·2)	7·7 (-)				69·3 (0·76)	84·7 (0·76)				207·9 (1·28)				
2·0	169·4 (0·5)			107·8 (1·5)		200·2 (1·02)	15·4 (0·25)		7·7 (-)		107·8 (1·28)	107·8 (0·76)				261·8 (1·79)				
2·5	207·9 (1·28)			130·9 (0·76)		215·6 (0·5)	30·8 (0·5)		23·1 (0·5)		123·2 (0·5)	146·3 (0·76)				308 (1·5)				
3·0	246·4 (1·28)			184·8 (1·79)		246·4 (1·02)	46·2 (0·5)		38·5 (0·5)		154 (1·02)	184·8 (1·28)				331·1 (0·76)	46·2 (-)			
4·0	315·7 (1·15)	115·5 (-)		323·4 (2·3)		300·3 (0·89)	92·4 (0·7)		92·4 (1·79)		254·1 (1·66)	277·2 (1·5)				485·1 (2·5)	207·9 (2·6)	7·7 (-)		3h with 10ppm GA_3
4·5	315·7 (0)	169·4 (1·79)		392·7 (2·3)		338·8 (1·28)	138·6 (1·5)		123·2 (0·52)		277·2 (0·76)	308 (1·02)				539 (1·79)	277·2 (2·3)	23·1 (0·5)		
5·0	338·8 (0·76)	231 (2·05)	15·4 (-)	462 (2·3)		361·9 (0·76)	169·4 (1·02)	15·4 (-)	169·4 (1·5)		308 (1·02)	308 (0)				569·8 (1·02)	338·8 (2·05)	61·6 (1·28)		
5·5	369·6 (1·02)	323·4 (3·07)	61·6 (1·5)	546·7 (2·8)		400·4 (1·28)	246·4 (2·5)	77 (2·05)	246·4 (2·5)		369·6 (2·05)	392·7 (2·8)	15·4 (-)	15·4 (-)		631·4 (2·05)	415·8 (2·5)	154 (3·07)		
6·0	400·4 (1·02)	415·8 (3·07)	107·8 (1·5)	623·7 (2·5)	30·8 (-)	423·5 (0·76)	277·2 (1·02)	154 (2·5)	338·8 (3·07)	23·1 (-	446·6 (2·5)	492·8 (3·3)	77 (2·05)	61·6 (1·5)	46·2 (-)	693 (2·05)	515·9 (3·3)	269·5 (3·8)	77 (-)	

Table (8) Length (μm) and growth rate (μm/min.) of individual hyphae and their branches of *S. parasitica*, flooded 3 hr. with control liquid medium followed by 3 hr. with medium containing 0.5 p.p.m. IAA.

Experiment was carried out at room temperature (20 °C + 2)

H_1 , H_2 : Hyphae examined in this mycelium

Time hours	Mycelium1			Mycelium 2				Mycelium 3											
	H_1	H_2		H_1		H_2		H_1						H_2					
	N.O.T.	No. of tips		No. of tips		No. of tips		No. of tips						No. of tips					
	1	1	2	1	2	1	2	1	2	3	4	5	6	1	2	3	4		
0·0	38·5 (-)	92·4 (-)		100·1 (-)		46·2 (-)		61·6 (-)	92·4 (-)					92·4 (-)	3·85 (-)			3h Control	
0·5	46·2 (0·25)	92·4 (0)		115·5 (0·5)		61·6 (0·5)		69·3 (0·25)	107·8 (0·5)					107·8 (0·5)	46·2 (1·4)				
1·0	46·2 (0)	107·8 (0·5)		123·2 (0·25)		77 (0·5)		77 (0·25)	107·8 (0)					138·6 (1·02)	92·4 (1·5)				
1·5	46·2 (0)	107·8 (0)	23·1 (-)	123·2 (0)	23·1 (-)	92·4 (0·5)		115·5 (1·28)	154 (1·5)					169·4 (1·02)	123·2 (1·02)				
2·0	46·2 (0)	123·2 (0·5)	30·8 (0·25)	123·2 (0)	46·2 (0·76)	130·9 (1·28)		184·8 (2·3)	207·9 (1·79)					231 (2·05)	192·5 (1·79)				
2·5	53·9 (0·25)	138·6 (0·5)	46·2 (0·5)	154 (1·02)	46·2 (0)	169·4 (1·28)		215·6 (1·02)	215·6 (0·25)	23·1 (-)				254·1 (0·76)	231 (1·28)				
3·0	53·9 (0)	146·3 (0·25)	69·3 (0·76)	169·4 (0·5)	77 (1·02)	215·6 (1·5)		231 (0·5)	246·4 (1·02)	38·5 (0·5)				277·2 (0·76)	269·5 (1·28)				
4·0	53·9 (0)	215·6 (1·15)	107·8 (0·64)	231 (1·02)	138·6 (1·02)	308 (1·5)		261·8 (0·5)	308 (1·02)	123·2 (1·4)	7·7 (-)			323·4 (0·75)	354·2 (1·4)	15·4 (-)	23·1 (-)	3h with 0·5ppm IAA	
4·5	77 (0·76)	215·6 (0)	130·9 (0·76)	261·8 (1·02)	169·4 (1·02)	331·1 (0·76)	15·4 (-)	261·8 (0)	323·4 (0·5)	138·6 (0·5)	15·4 (0·25)	15·4 (-)		346·5 (0·76)	385 (1·02)	15·4 (0)	61·6 (1·28)		
5·0	115·5 (1·28)	231 (0·5)	154 (0·76)	292·6 (1·02)	184·8 (0·5)	338·8 (0·28)	46·2 (1·02)	277·2 (0·5)	331·1 (0·25)	184·8 (1·5)	61·6 (1·5)	30·8 (0·5)	7·7 (-)	354·2 (0·25)	415·8 (1·02)	15·4 (0)	92·4 (1·02)		
5·5	138·6 (0·76)	261·8 (1·02)	169·4 (0·5)	331·1 (1·28)	215·6 (1·02)	369·6 (1·02)	77 (1·02)	292·6 (0·5)	385 (1·79)	246·4 (2·05)	123·2 (2·05)	53·9 (0·76)	7·7 (0)	400·4 (1·5)	446·6 (1·02)	15·4 (0)	184·8 (3·07)		
6·0	200·2 (2·05)	338·8 (2·5)	215·6 (1·5)	385 (1·79)	261·8 (1·5)	415·8 (1·5)	123·2 (1·5)	323·4 (1·02)	446·6 (2·05)	338·8 (3·07)	215·6 (3·07)	107·8 (1·79)	7·7 (0)	454·3 (1·28)	508·2 (2·05)	23·1 (2·05)	315·7 (3·8)		

Table (9) Length (µm) and growth rate (µm/min.) of individual hyphae and their branches of *S. parasitica*, flooded 3 hr. with control liquid medium followed by 3 hr. with medium containing 1 p.p.m. IAA.

Experiment was carried out at room temperature (20 °C + 2)

H_1 , H_2 : Hyphae examined in this mycelium

Time (hours)	Mycelium 1					Mycelium 2					Mycelium 3				
	H_1			H_2		H_1		H_2			H_1		H_2		
	No. of tips			No. of tips		No. of tips		No. of tips			No. of tips		No. of tips		
	1	2	3	1	2	1	2	1	2	3	1	2	1	2	
0 · 0	107·8 (-)			—		57·75 (-)		30·8 (-)			92·4 (-)		23·1 (-)		3h Control
0 · 5	107·8 (0)			3·85 (-)		57·75 (0)		30·8 (0)			92·4 (0)		30·8 (0·25)		
1 · 0	107·8 (0)			15·4 (0·38)		7·7 (0·64)		30·8 (0)			92·4 (0)		38·5 (0·25)		
1 · 5	107·8 (0)			46·2 (1·02)		7·7 (0)		46·2 (0·5)			107·8 (0·5)		38·5 (0)		
2 · 0	107·8 (0)			7·7 (1·02)		92·4 (0·5)		61·6 (0·5)			107·8 (0)	7·7 (-)	38·5 (0)		
2 · 5	130·9 (0·76)			107·8 (1·02)		123·2 (1·02)		7·7 (0·5)			15·4 (1·5)	30·8 (0·76)	38·5 (0)		
3 · 0	169·4 (1·28)			15·4 (1·5)		15·4 (1·02)		123·2 (1·5)			161·7 (0·25)	61·6 (1·02)	38·5 (0)		
4 · 0	292·6 (2·05)			30·8 (2·5)		277·2 (2·05)		223·3 (1·6)			261·8 (1·6)	138·6 (1·28)	38·5 (0)		3h with 1ppm IAA
4 · 5	315·7 (0·76)			354·2 (1·5)		292·6 (0·5)		246·4 (0·76)			292·6 (1·02)	184·8 (1·5)	38·5 (0)		
5 · 0	361·9 (1·5)	7·7 (-)	7·7 (-)	415·8 (2·05)	15·4 (-)	323·4 (1·5)		30·8 (2·05)	7·7 (-)		338·8 (1·5)	254·1 (2·3)	38·5 (0)	38·5 (-)	
5 · 5	415·8 (1·7)	38·5 (1·02)	30·8 (0·76)	508·2 (3·07)	53·9 (1·2)	346·5 (0·76)	23·1 (-)	346·5 (1·28)	38·5 (1·02)	30·5 (-)	415·8 (2·5)	323·4 (2·3)	38·5 (0)	92·4 (1·79)	
6 · 0	462 (1·5)	53·9 (0·5)	46·2 (0·5)	554·4 (1·5)	84·7 (1·02)	369·6 (0·76)	46·2 (0·76)	354·2 (0·25)	7·7 (1·28)	53·9 (0·76)	477·4 (2·05)	369·6 (1·5)	38·5 (0)	138·6 (1·5)	

Table (10) Length (μm) and growth rate (μm/min.) of individual hyphae and their branches of *S. parasitica*, flooded 3 hr. with control liquid medium followed by 3 hr. with medium containing 5 p.p.m. IAA.

Experiment was carried out at room temperature (20 °C + 2)

H_1 , H_2 : Hyphae examined in this mycelium

Time (hours)	Mycelium 1						Mycelium 2					
	H_1				H_2		H_1	H_2				
	No. of tips				No. of tips		N.O.T.	No. of tips				
	1	2	3	4	1	2	1	1	2	3	4	
0·0	177·1 (-)				53·9 (-)		77 (-)	115·5 (-)	46·2 (-)			3h Control
0·5	192·5 (0·5)				77 (0·76)		92·4 (0·5)	138·6 (0·76)	53·9 (0·25)			
1·0	254·1 (2·05)				123·2 (1·5)		123·2 (1·02)	192·5 (1·79)	77 (0·76)			
1·5	346·5 (3·07)	15·4 (-)			215·6 (3·07)		200·2 (2·5)	261·8 (2·3)	123·2 (1·5)			
2·0	492·8 (4·8)	77 (2·05)			346·5 (4·3)		238·7 (1·28)	308 (1·5)	177·1 (1·79)			
2·5	646·8 (5·13)	107·8 (1·02)			408·1 (2·05)		277·2 (1·28)	354·2 (1·5)	246·4 (2·3)			
3·0	785·4 (4·1)	154 (1·5)			446·6 (1·28)		277·2 (0)	431·2 (2·5)	308 (2·05)			
4·0	1047·2 (4·6)	492·8 (5·6)	46·2 (-)	38·5 (-)	646·8 (3·3)	46·2 (-)	431·2 (2·5)	646·8 (3·5)	616 (5·13)			3h with 5ppm IAA
4·5	1232 (6·1)	677·6 (6·1)	77 (1·02)	107·8 (2·3)	708·4 (2·05)	84·7 (1·28)	508·2 (2·5)	754·6 (3·5)	754·6 (4·6)	30·8 (-)	30·8 (-)	
5·0	1383·3 (5·04)	810·9 (4·4)	143·1 (2·2)	238·5 (4·3)	763·2 (1·8)	143·1 (1·9)	592·9 (2·8)	877·8 (4·1)	877·8 (4·1)	61·6 (1·02)	61·6 (1·02)	
5·5	1550·25 (5·5)	1001·7 (6·36)	310·05 (5·5)	375·7 (3·9)	930·1 (5·5)	286·2 (4·7)	715·5 (4·08)	1025·5 (4·9)	1025·5 (4·9)	190·8 (4·3)	190·8 (4·3)	
6·0	17649 (7·1)	1192·5 (6·36)	477 (5·5)	548·5 (6·36)	1001·7 (2·3)	429·3 (4·7)	810·9 (1·02)	1144·8 (3·9)	1144·8 (3·9)	238·5 (3·9)	262·3 (1·59)	

Table (11) Length (μm) and growth rate (μm/min.) of individual hyphae and their branches of *S. parasitica*, flooded 3 hr. with control liquid medium followed by 3 hr. with medium containing 10 p.p.m. IAA.

Experiment was carried out at room temperature (20°C + 2)

H_1 , H_2 : Hyphae examined in this mycelium

Time (hours)	Mycelium 1				Mycelium 2									Mycelium 3													
	H 1				H 1				H 2					H 1										H 2			
	No. of tips				No. of tips				No. of tips					No. of tips										No. of tips			
	1	2	3	4	1	2	3	4	1	2	3	4	5	1	2	3	4	5	6	7	8	9	10	1	2	3	4
0·0	92·4 (-)				61·6 (-)				107·8 (-)					231 (-)										15·4 (-)			
0·5	107·8 (0·5)				92·4 (1·02)				130·9 (0·76)					277·2 (1·5)										30·8 (0·5)			
1·0	154 (1·5)				130·9 (1·28)				161·7 (1·02)					338·8 (2·05)										61·6 (1·02)			
1·5	184·8 (1·02)				161·7 (1·02)				184·8 (0·76)					400·4 (2·05)										77 (0·5)			
2·0	231 (1·5)				215·6 (1·79)				207·9 (0·76)					485·1 (2·8)										107·8 (1·02)			
2·5	292·6 (2·05)	7·7 (-)			277·2 (2·05)				246·4 (1·28)					539 (1·79)	7·7 (-)									154 (1·5)			
3·0	361·9 (2·3)	53·9 (1·5)			338·8 (2·05)				323·4 (2·5)					708·4 (5·6)	77 (2·3)									254·1 (3·3)			
4·0	454·3 (1·5)	184·8 (2·1)			415·8 (1·2)				431·2 (1·7)	23·1 (-)				92·4 (3·5)	169·4 (1·5)									338·8 (1·4)			
4·5	531·3 (2·5)	261·8 (2·5)			446·6 (1·02)	15·4 (-)	30·2 (-)	46·2 (-)	508·2 (2·5)	123·2 (3·3)	46·2 (-)	30·8 (-)		985·6 (2·05)	231 (2·05)	30·8 (-)	30·8 (-)	15·4 (-)	23·1 (-)					508·2 (5·6)	38·5 (-)	46·2 (-)	
5·0	577·5 (1·5)	308 (1·5)	30·8 (-)		462 (0·5)	107·8 (3·07)	115·5 (2·8)	130·9 (2·8)	585·2 (2·5)	215·6 (3·07)	138·6 (3·07)	123·2 (3·07)	15·4 (-)	1001 (0·5)	277·2 (1·5)	69·3 (1·28)	107·8 (2·5)	69·3 (1·79)	92·4 (2·3)	15·4 (-)				539 (1·02)	92·4 (1·79)	92·4 (1·5)	15·4 (-)
5·5	608·3 (1·02)	323·4 (0·5)	77 (1·5)	30·8 (-)	492·8 (1·02)	146·3 (1·28)	154 (1·28)	169·4 (1·28)	662·2 (2·5)	231 (0·5)	169·4 (1·02)	169·4 (1·5)	84·7 (2·3)	1031·8 (1·02)	292·6 (0·5)	92·4 (0·76)	154 (1·5)	107·8 (1·28)	115·5 (0·76)	30·8 (0·5)	15·4 (-)			539 (0)	123·2 (1·02)	107·8 (0·5)	30·8 (0·5)
6·0	623·7 (0·5)	338·8 (0·5)	92·4 (0·5)	46·2 (0·5)	492·8 (0)	169·4 (0·76)	169·4 (0·5)	200·2 (1·02)	693 (1·02)	246·4 (0·5)	200·2 (1·02)	200·2 (1·02)	107·8 (0·76)	1047·2 (0·5)	308 (0·5)	107·8 (0·5)	184·8 (1·02)	123·2 (0·5)	138·6 (0·76)	46·2 (0·5)	30·8 (0·5)	15·4 (-)	15·4 (-)				

3h Control

3h with 10ppm IAA

Table (12) Growth rates of primary hyphae with their number of tips of *S. parasitica*, flooded for 3 hr. with control medium followed by 3 hr. treatment with varying concentrations of GA_3 and IAA.

A: Control medium

B: Control medium + GA3 or IAA

( ):Number of branches developed on the primary hypha

Treatment	Groups of initial hyphal length (μm)																	
	7·7 - 23·1		30·8 - 46·2		53·9 - 61·6		77 - 84·7		92·4 - 107·8		115·5 - 123·2		138·6 - 154		177·1		231	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Control	1·54 (1)	2·7 (3)					1·54 (0)	2·01 (2)			2·65 (0)	4·36 (6)						
0·5 GA_3					1·58 (1)	3·16 (1)	1·07 (0) 1·96 (1)	3·75 (1) 3·16 (3)	2·05 (1)	3·76 (3)	1·75 (0)	2·05 (1)						
1·0 GA_3	1·24 (1) 1·13 (0)	2·35 (2) 2·2 (0)									1·36 (3) 1·19 (2)	2·48 (3) 1·71 (2)						
5·0 GA_3			0·89 (0)	3·4 (1)	0·42 (1)	0·04 (5)					1·83 (1) 2·05 (0)	4·06 (3) 3·3 (2)	1·62 (2)	1·71 (4)				
10·0 GA_3	0·94 (1)	1·7 (4)	0·59 (1) 0·85 (0)	1·62 (4) 2·4 (1)			0·94 (0)	0·85 (2)	0·81 (1) 1·24 (1)	0·98 (2) 2·01 (3)								
0·5 IAA			0·08 (0) 0·94 (0)	0·81 (0) 1·11 (1)	0·94 (2)	0·51 (5)			0·38 (1) 0·29 (1) 1·02 (1)	1·19 (1) 1·06 (3) 0·98 (1)								
1·0 IAA	0·08 (0)	0 (1)	0·51 (0)	1·28 (2)	0·53 (0)	1·19 (1)			0·38 (1) 0·34 (0)	1·75 (1) 1·62 (1)								
5·0 IAA					2·18 (0)	3·08 (1)	1·11 (0)	2·96 (0)			1·75 (1)	3·96 (3)			3·37 (1)	5·44 (3)		
10·0 IAA					1·54 (0)	0·85 (3)			1·19 (0) 1·49 (1)	2·05 (4) 1·45 (3)							2·65 (1)	1·88 (9)

The vegetative growth of *S. parasitica* colonies was dense and compact, particularly in the centre as compared with the peripheral region, and cottony in appearance.

The results of the effect of various concentrations of GA_3 and IAA on *M. mucedo* surface growth (colony diameter) as given in Figure (3), failed to show any stimulation of growth over the whole range of low concentrations; on the other hand higher concentrations showed a considerable reduction in the growth. With 400 ppm. GA_3, 200 and 400 ppm. IAA inhibition was noticeable after the first day of incubation.

The effect of these substances on *S. parasitica* (Fig.4) was quite similar to the effect on *M. mucedo*, over the whole range of GA_3 concentrations, except at the very low concentrations, 0.1, 0.2 and 0.3 ppm., which showed a very slight increase in colony diameter over that of the control (6-8%) after the first day of incubation. The inhibitory effect with 400 ppm. concentration of GA_3 was stronger with *M. mucedo* than with *Saprolegnia parasitica*. There was no stimulation in surface growth yield with the whole range of low concentrations of IAA, instead a gradual inhibition of growth with the increasing concentrations from 10 ppm. up to 200 ppm. 400 ppm. showed a lethal effect which permitted no growth of the mycelium.

As a preliminary study with the Oomycetes, different fungi were used to test their response to GA_3 and IAA. The effect of GA_3 on *Saprolegnia terrestris* (Fig.5) was neither stimulation nor inhibition, except perhaps a very slight inhibition on 400 ppm. Whereas which IAA the effect was similar

Fig. 3: Mean colony diameters of *M. mucedo* grown on MPG medium containing varying concentrations of GA_3 and IAA.

Incubation at 24 °C

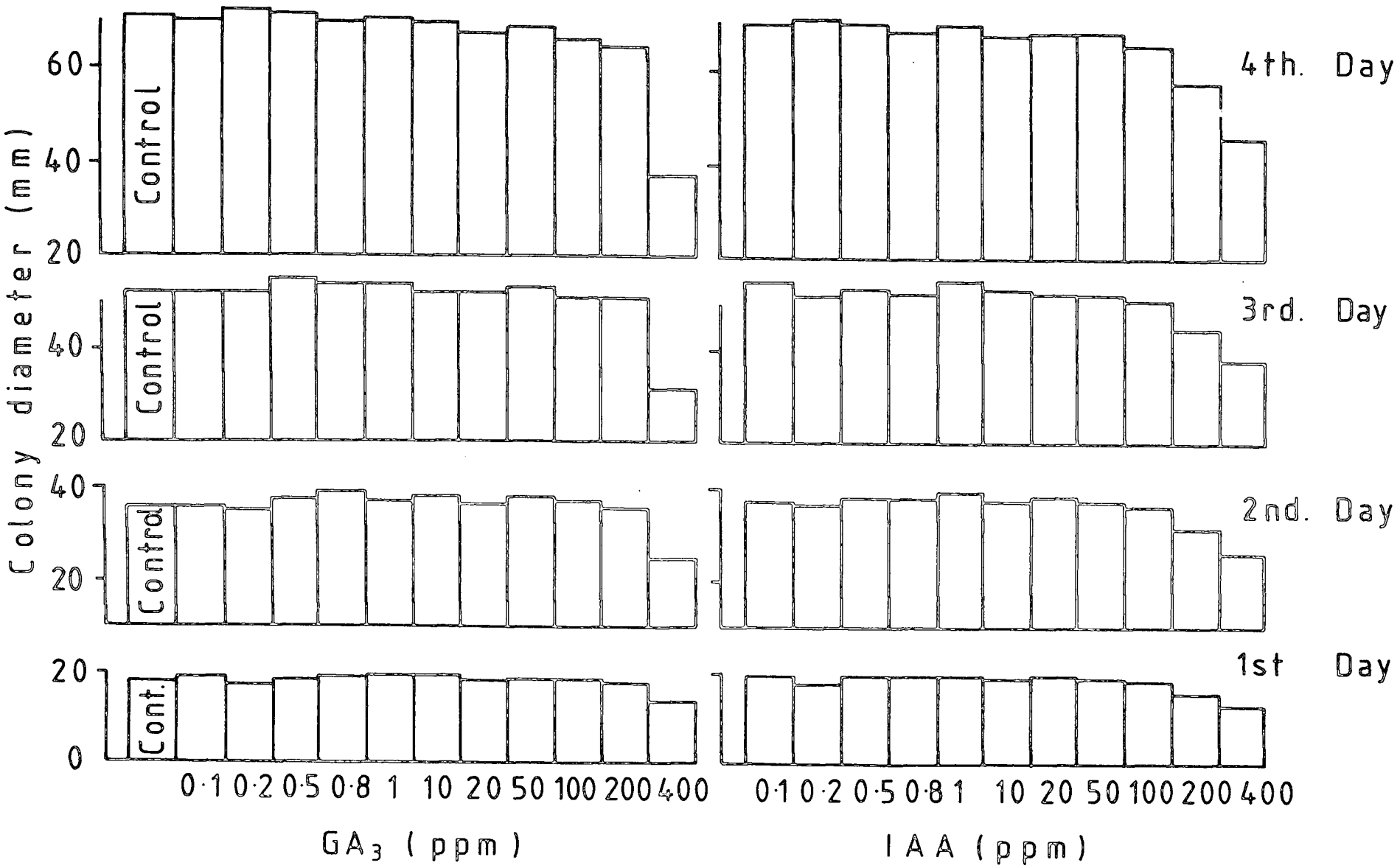

Colony diameter (mm)
60
40
20
Control
4th. Day
40
20
Control
3rd. Day
40
20
Control
2nd. Day
20
0
Cont.
1st Day
0·1 0·2 0·5 0·8 1 10 20 50 100 200 400
GA3 (ppm)
0·1 0·2 0·5 0·8 1 10 20 50 100 200 400
IAA (ppm)

Fig. 4: Mean colony diameters of *Saprolegnia parasitica* grown on PYG medium with varying concentrations of GA_3 and IAA.

Incubation at 24 °C

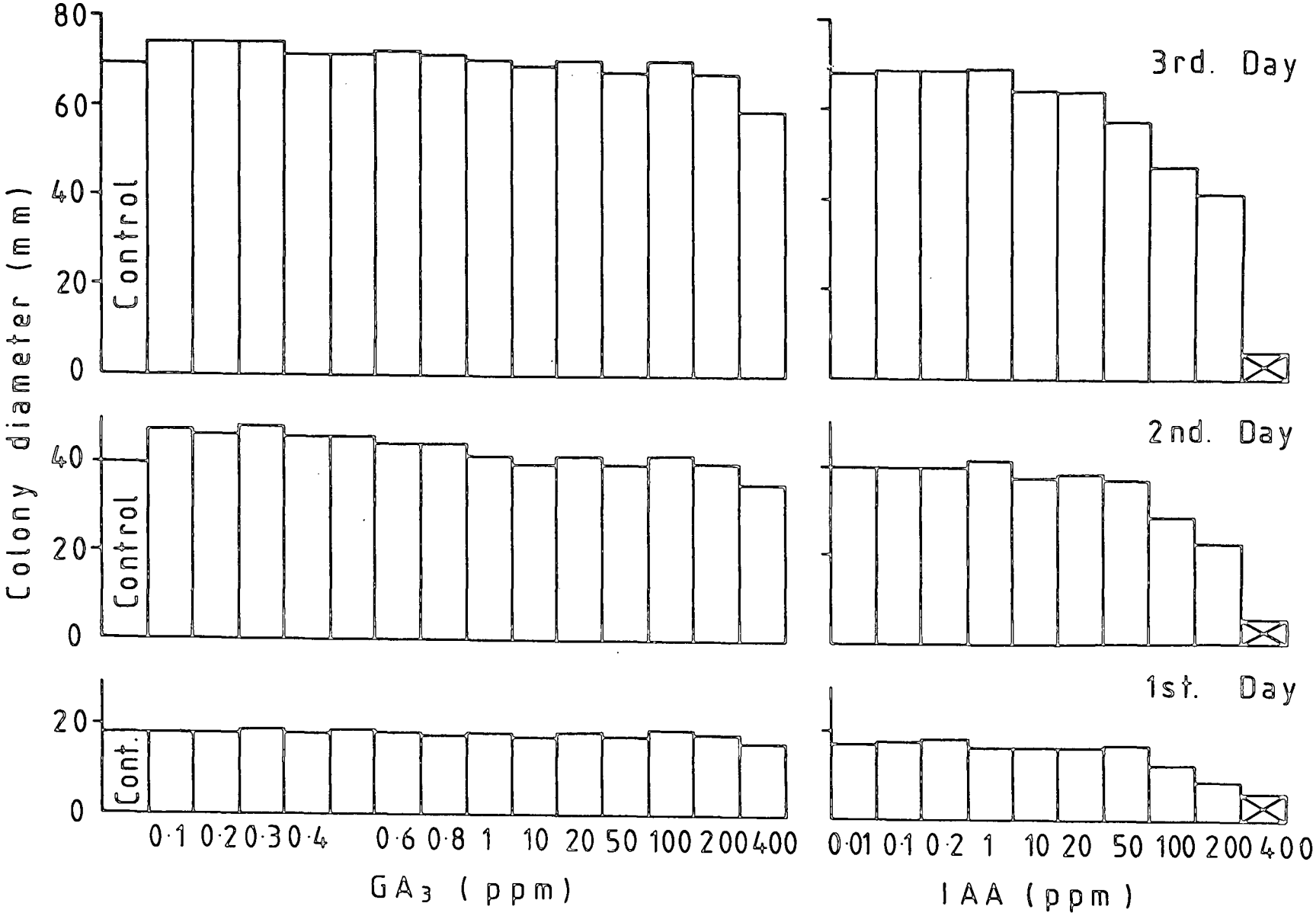
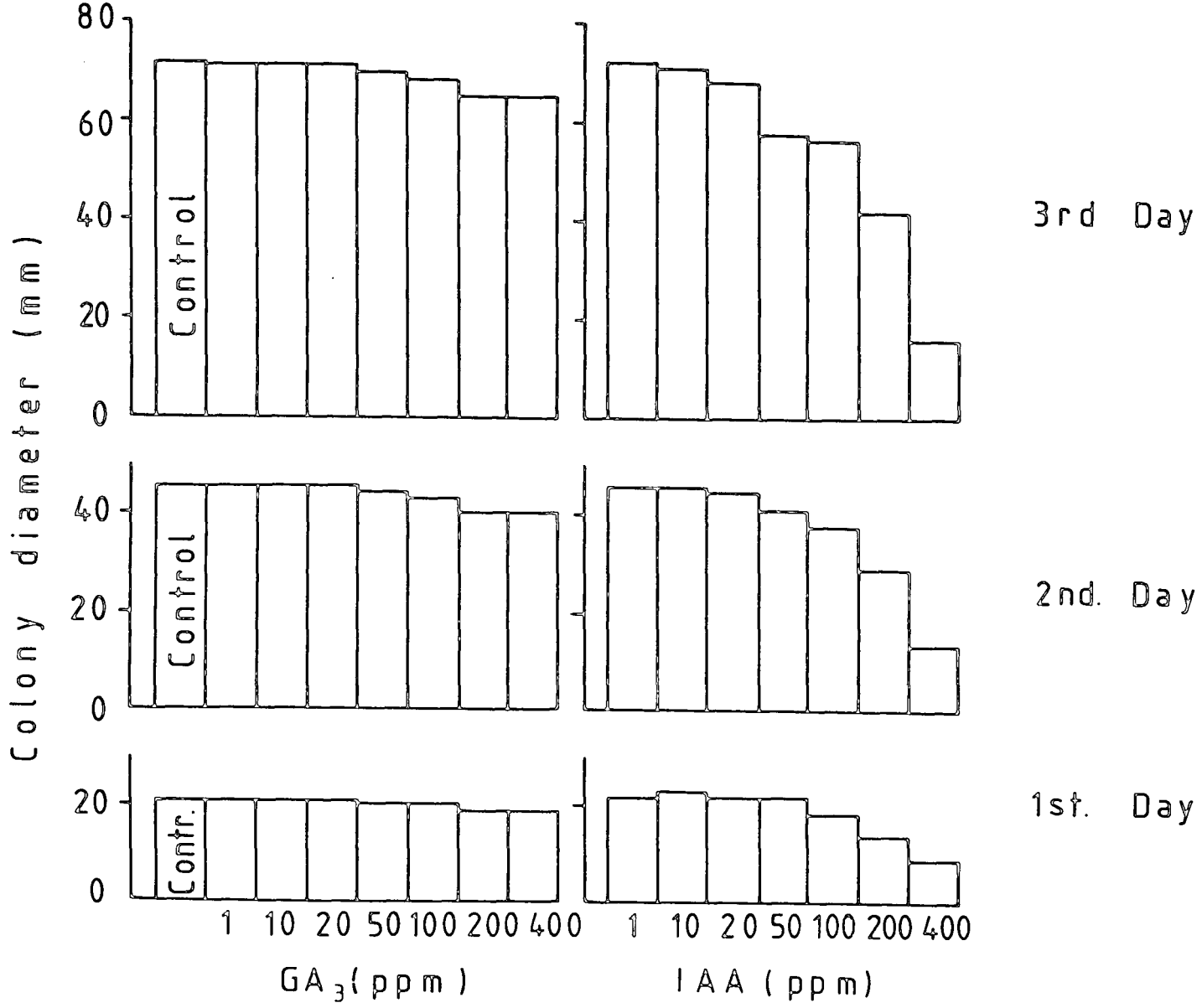

Colony diameter (mm)
80
60
40
20
0
Control
3rd. Day
40
20
0
Control
2nd. Day
20
0
Cont.
1st. Day
0·1 0·2 0·3 0·4 0·6 0·8 1 10 20 50 100 200 400
GA3 (ppm)
0·01 0·1 0·2 1 10 20 50 100 200 400
IAA (ppm)

Fig. 5: Mean colony diameters of *Saprolegnia terrestris* grown on PYG medium with varying concentrations of GA_3 and IAA.

Incubation at 24 °C


Colony diameter (mm)
80
60
40
20
0
Control
3rd Day
40
20
0
Control
2nd. Day
20
0
Contr.
1st. Day
1 10 20 50 100 200 400
GA3(ppm)
1 10 20 50 100 200 400
IAA (ppm)

to that with *S. parasitica*, the gradual inhibition began at 20 ppm. until 400 ppm. in which the growth yield was less than a quarter of the control growth.

Pythium debaryanum (Figure 6) showed a very fast growth on this medium, so the intervals between readings were reduced to two days. With this fungus also, the effect of GA_3 was found to be similar to the effect on the fungi described previously, whilst with IAA the results were found to be similar to those with *S. parasitica*. Inhibition was stronger and there was no growth at 400 ppm.

Achlya caroliniana (Figure 7) showed neither stimulation nor inhibition up to the first day of incubation with the whole range of GA_3 concentrations. After the first day, the inhibition was quite noticeable until the fifth day of incubation with the range of concentration 50 to 400 ppm. The effect was about the same with IAA concentration with this fungus, gradual inhibition which increased as the fungus grew older at the same range of GA_3 concentrations, 50-400 ppm.

Since some enhancing of auxin effects have been noticed in higher plants on addition of Kinetin (Adamson, 1962; Miller, 1956), a similar response at the same combination of concentration noticed to stimulate plant cells was looked for in the fungi tested. No acceleration of growth of *S. parasitica* surface colonies was found either with various concentrations of Kinetin (Figure 8) or with a range of combination of low concentrations of IAA and Kinetin (Figure 9).

However, Kinetin at a concentration of 20 ppm. showed a slight decrease in the colony diameter.

Fig. 6: Mean colony diameters of *Pythium debaryanum* grown on PYG medium with varying concentrations of GA_3 and IAA.

Incubation at 24°C

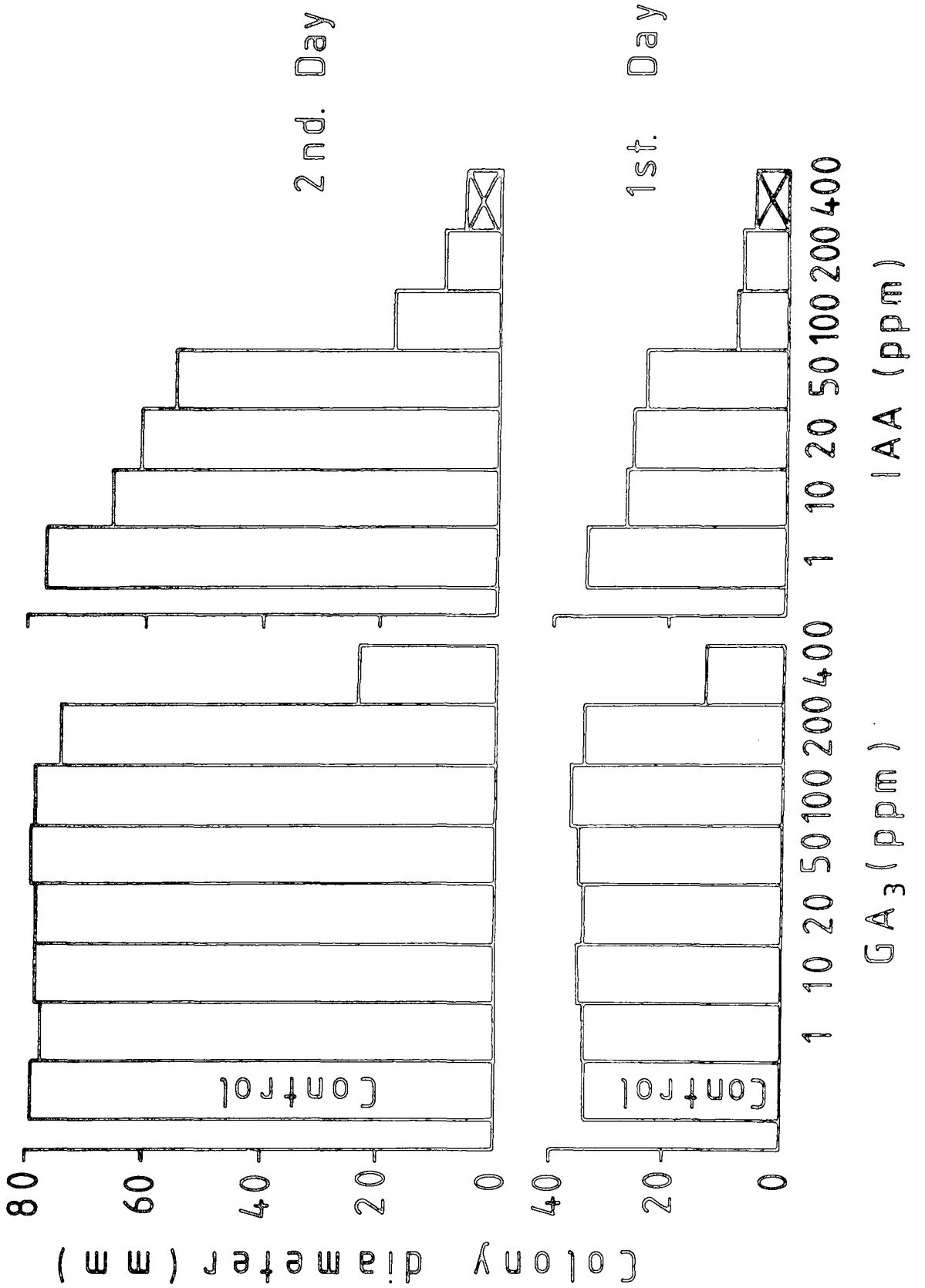

2nd. Day
1st. Day
Control
Control
colony diameter (mm)
80
60
40
20
0
40
20
0
1 10 20 50 100 200 400
1 10 20 50 100 200 400
GA3 (ppm)
IAA (ppm)

Fig. 7: Mean colony diameters of *Achlya caroliniana* grown on PYG medium with varying concentrations of GA_3 and IAA.

Incubation at 24°C

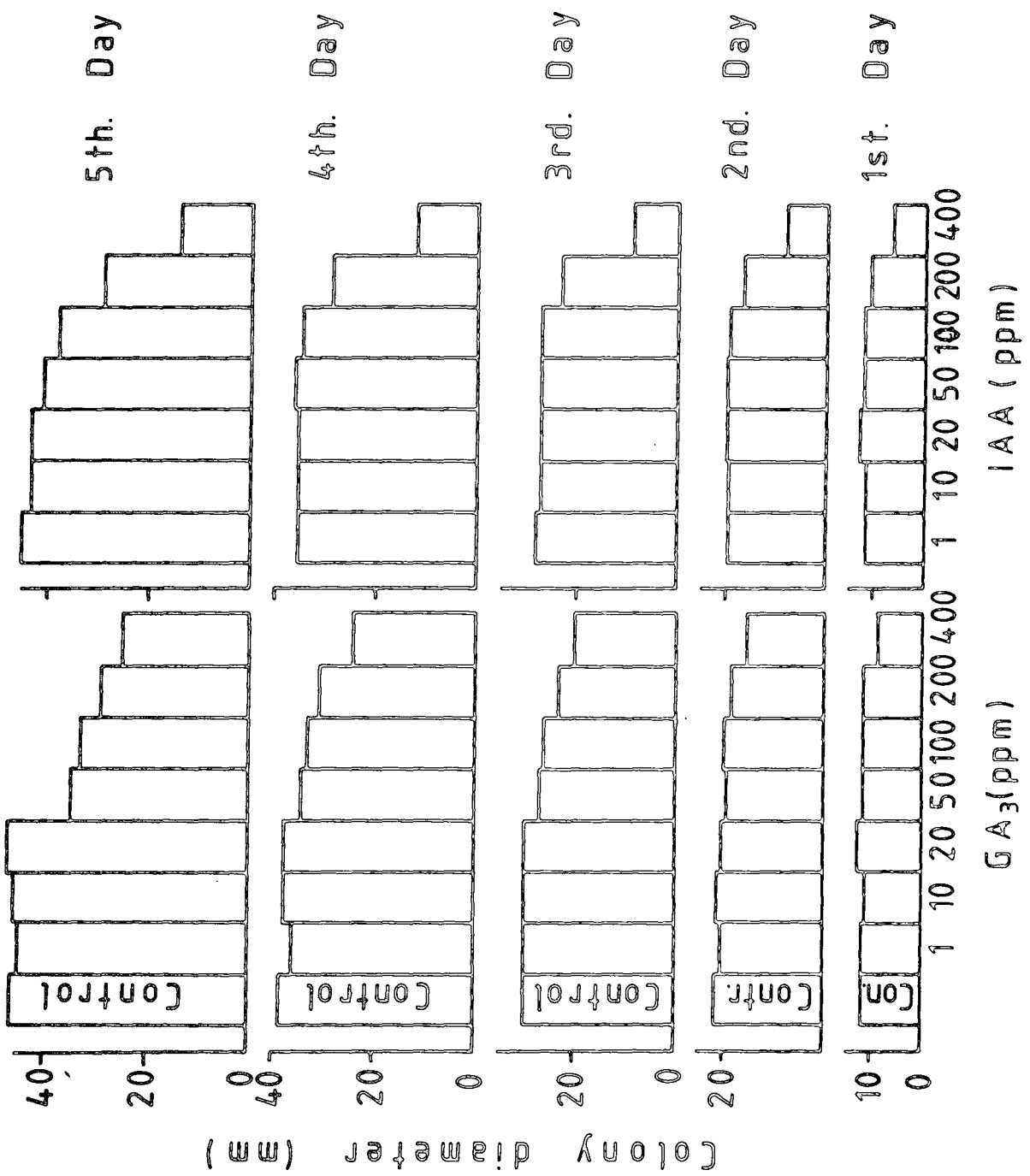

5th. Day
4th. Day
3rd. Day
2nd. Day
1st. Day
Control
Contr.
Con.
Colony diameter (mm)
0
10
20
40
1 10 20 50 100 200 400
GA3(ppm)
1 10 20 50 100 200 400
IAA (ppm)

Fig. 8: Mean colony diameters of *S. parasitica* grown on PYG medium with varying concentrations of Kinetin.

Incubation at 24 °C

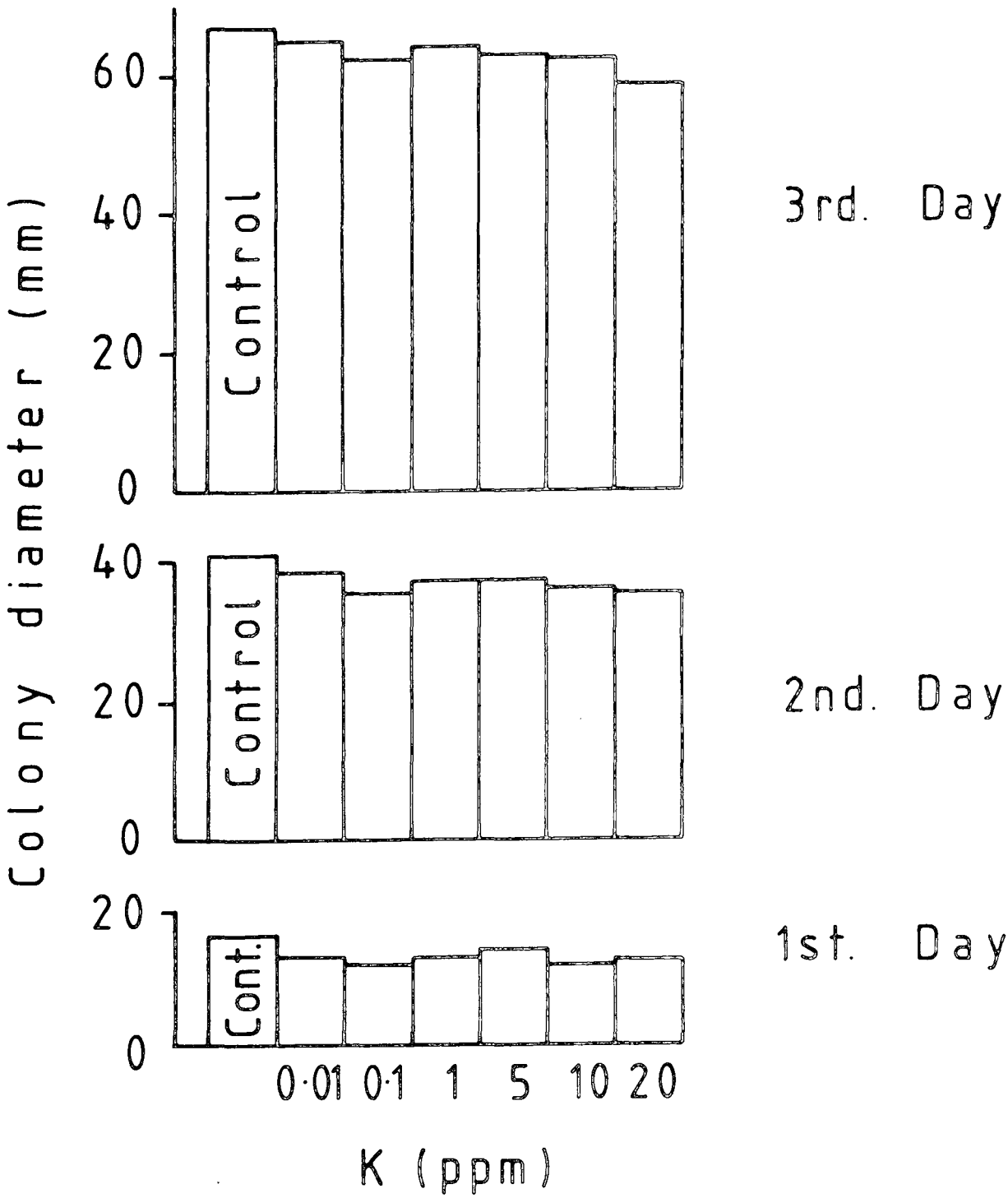
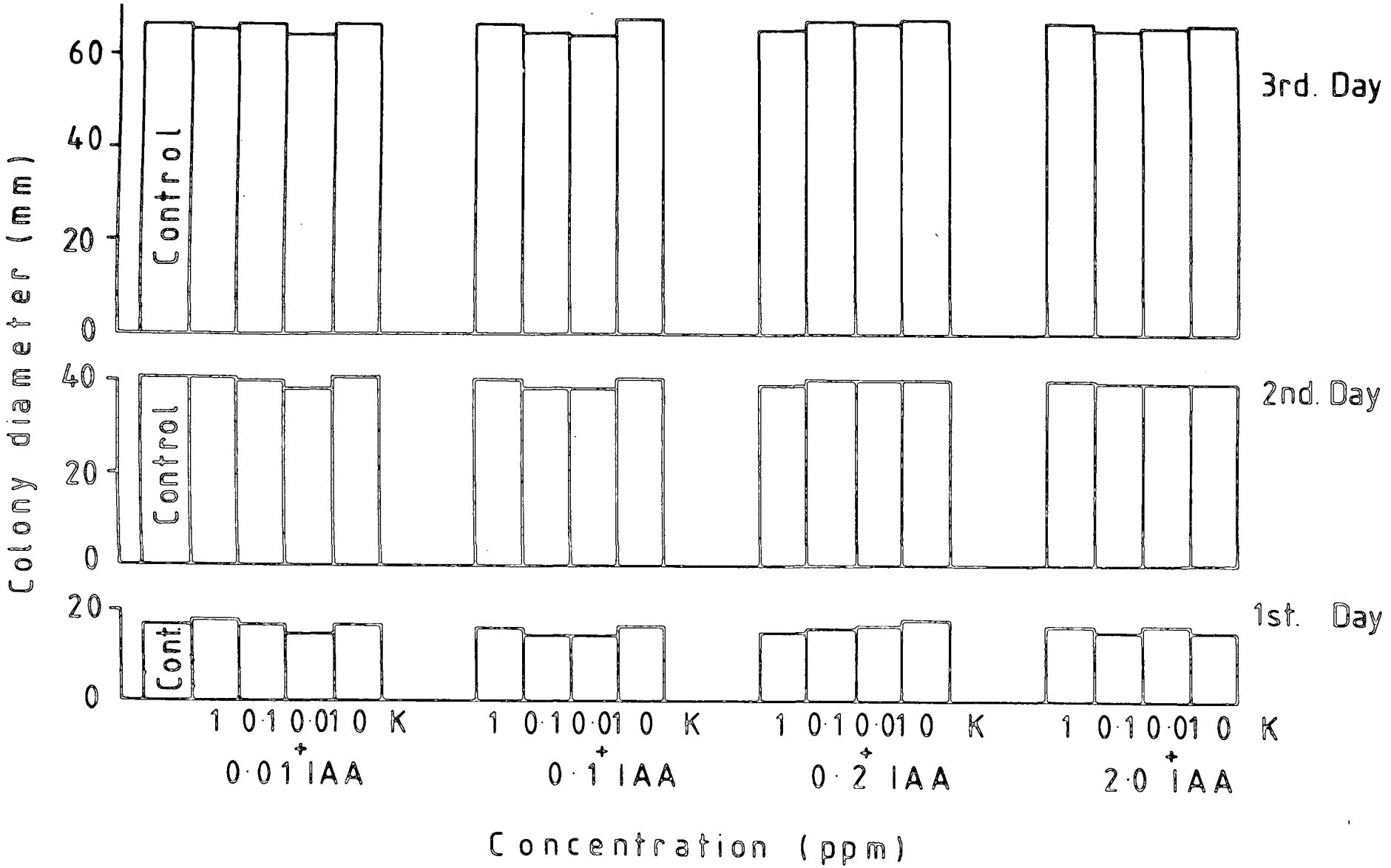

Colony diameter (mm)
60
40
20
0
Control
3rd. Day
40
20
0
Control
2nd. Day
20
0
Cont.
1st. Day
0·01 0·1 1 5 10 20
K (ppm)

Fig. 9: Mean colony diameters of *S. parasitica* grown on PYG medium with varying combinations of IAA and Kinetin.

Incubation at 24 °C


Colony diameter (mm)
60
40
20
0
Control
3rd. Day
40
20
0
Control
2nd. Day
20
0
Cont
1st. Day
1 0·1 0·01 0 K
0·01 + IAA
1 0·1 0·01 0 K
0·1 + IAA
1 0·1 0·01 0 K
0·2 + IAA
1 0·1 0·01 0 K
2·0 + IAA
Concentration (ppm)

There was no observable difference in the appearance of the vegetative mycelium over the whole range, except on *M. mucedo* at 400 ppm. GA_3 and IAA, the sporangiophores with sporangial heads were slightly reduced in number.

1.3.1.5 Effect of PGRS on dry weight yield:

Growth of *M. mucedo* in MPG and PYG liquid media (which was used in other parts of this work as a growth medium as well), was not in a filamentous manner, the young mycelia originated from the inoculated sporangiospores aggregated to form a number of small mycelial masses, spherical pellets were never formed even under static conditions.

Figures (10) and (11) demonstrate the effects of different concentrations of GA_3 and IAA on the growth of *M. mucedo*, the only concentration of both substances which showed very slight increase in dry weight matter over the control was 1 ppm, higher concentrations than that reduced the yield to different extents, the amount of reduction was proportional to the increase in concentration. Curves with GA_3 and IAA concentration appear to be more or less regular and demonstrate an inverse relationship between yield and concentration during all the intervals of recording.

It is obvious from these figures also that after a day of incubation, there was no clear inhibition. However, later on a sharp decrease in growth was noticeable especially after the second day, compared to the growth of the control and at 1 ppm. concentration. Figure (11) also showed that no inhibition occurred at 10 or 20 ppm. two days after incubation, whilst after that time the inhibition was very obvious. Higher

Fig. 10: Dry weight yield of *M. mucedo* grown in MPG medium containing varying concentrations of GA_3.

Incubation at 16 °C

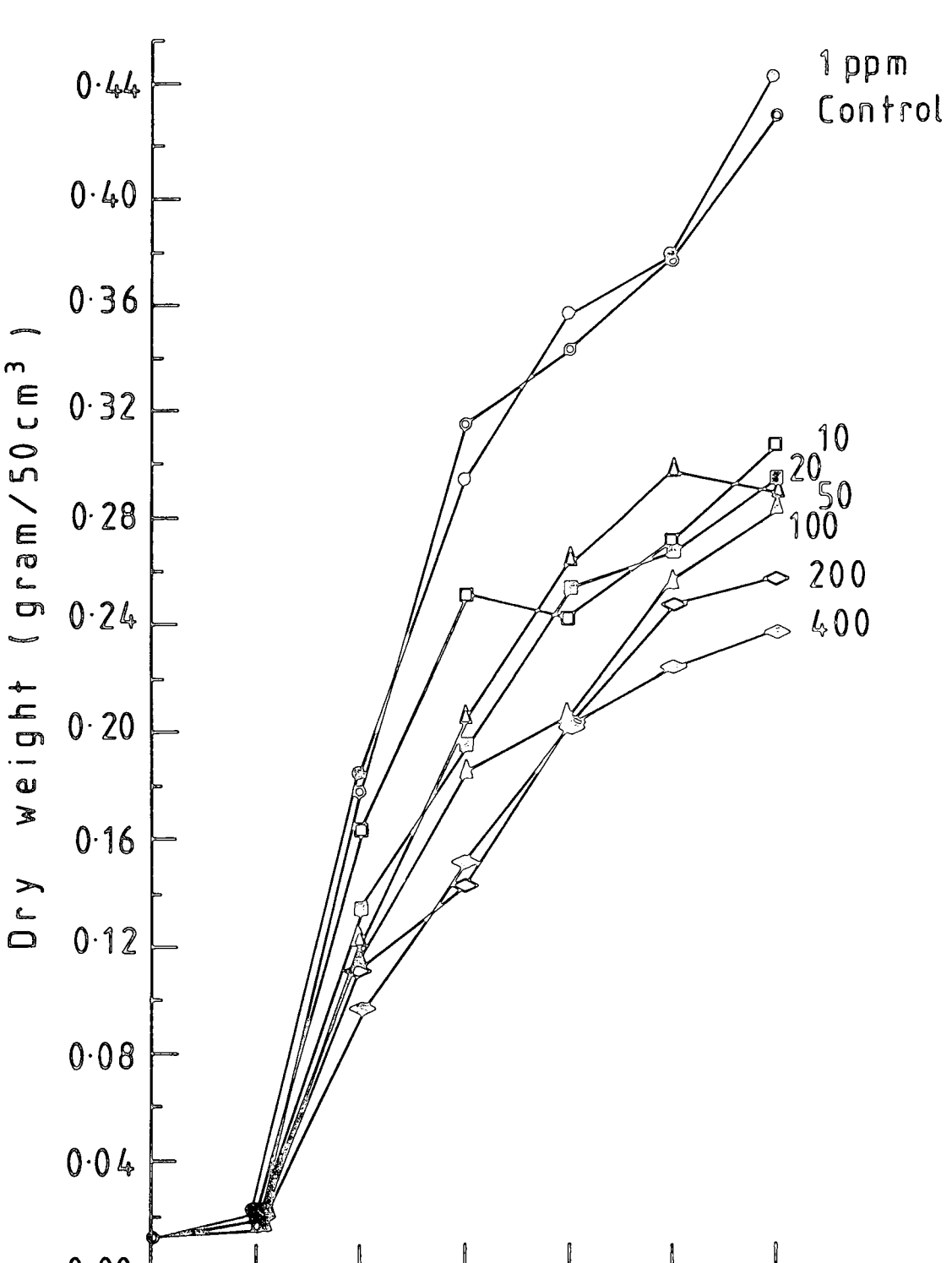
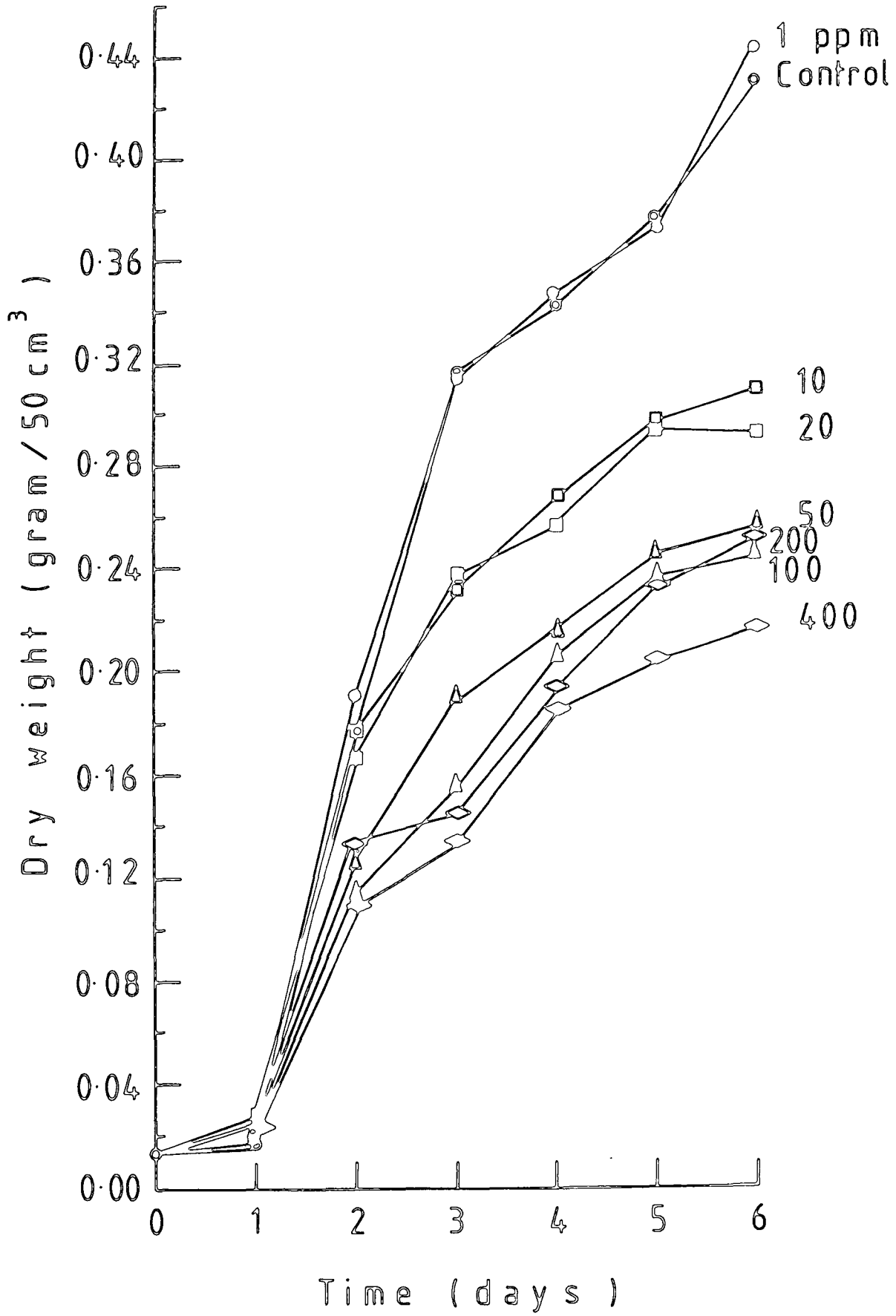

0·44
0·40
0·36
0·32
0·28
0·24
0·20
0·16
0·12
0·08
0·04
0·00
Dry weight (gram/50cm³)
0
1
2
3
4
5
6
Time (days)
1 ppm
Control
10
20
50
100
200
400

Fig. 11: Dry weight yield of *M. mucedo* grown in MPG medium containing varying concentrations of IAA.

Incubation at 16 °C


1 ppm
Control
10
20
50
200
100
400
0·44
0·40
0·36
0·32
0·28
0·24
0·20
0·16
0·12
0·08
0·04
0·00
Dry weight (gram/50 cm³)
0
1
2
3
4
5
6
Time (days)

concentrations than that showed a marked difference after the first day of incubation.

These reactions may be due to the effect of these acid growth regulating substances on the pH of the medium as the fungus grows older, or due to the accumulation of metabolic products, or a direct effect on the mycelium. The initial pH of the medium before autoclaving and addition of the PGRS was 6. No attempts were made to adjust the pH value of the medium after the addition of the PGRS. Although Richards (1949) reported that the process of sterilization and the addition of acid growth regulating substances at various concentrations lowered the pH of the buffered medium, this did not appear to be a significant factor in either stimulation or inhibition of many fungi tested. Richards also found that the final pH values for a given concentration of any of the PGRS used did not vary more than 0.2 to 0.4 of a pH unit.

Preliminary work on the effect of IAA concentration on the dry weight yield of *S. parasitica* showed a very toxic effect at $\geqslant$ 20 ppm, therefore concentrations of $\leqslant$ 20 ppm. were used, since a stimulatory effect of the growth substances was to investigate.

Figures (12) and (13) demonstrate the effect of GA_3 and IAA at low concentration on the growth yield of *S. parasitica*. No marked stimulation could be observed from the effect of GA_3, instead there was an irregular decrease or slight inhibition with the increasing concentrations up to 20 ppm. With IAA, no stimulation of growth on the very low concentrations could be detected. Higher concentrations caused a

Fig. 12: Dry weight yield of *S. parasitica* grown in PYG medium containing varying concentrations of GA_3.

Incubation at 24 °C

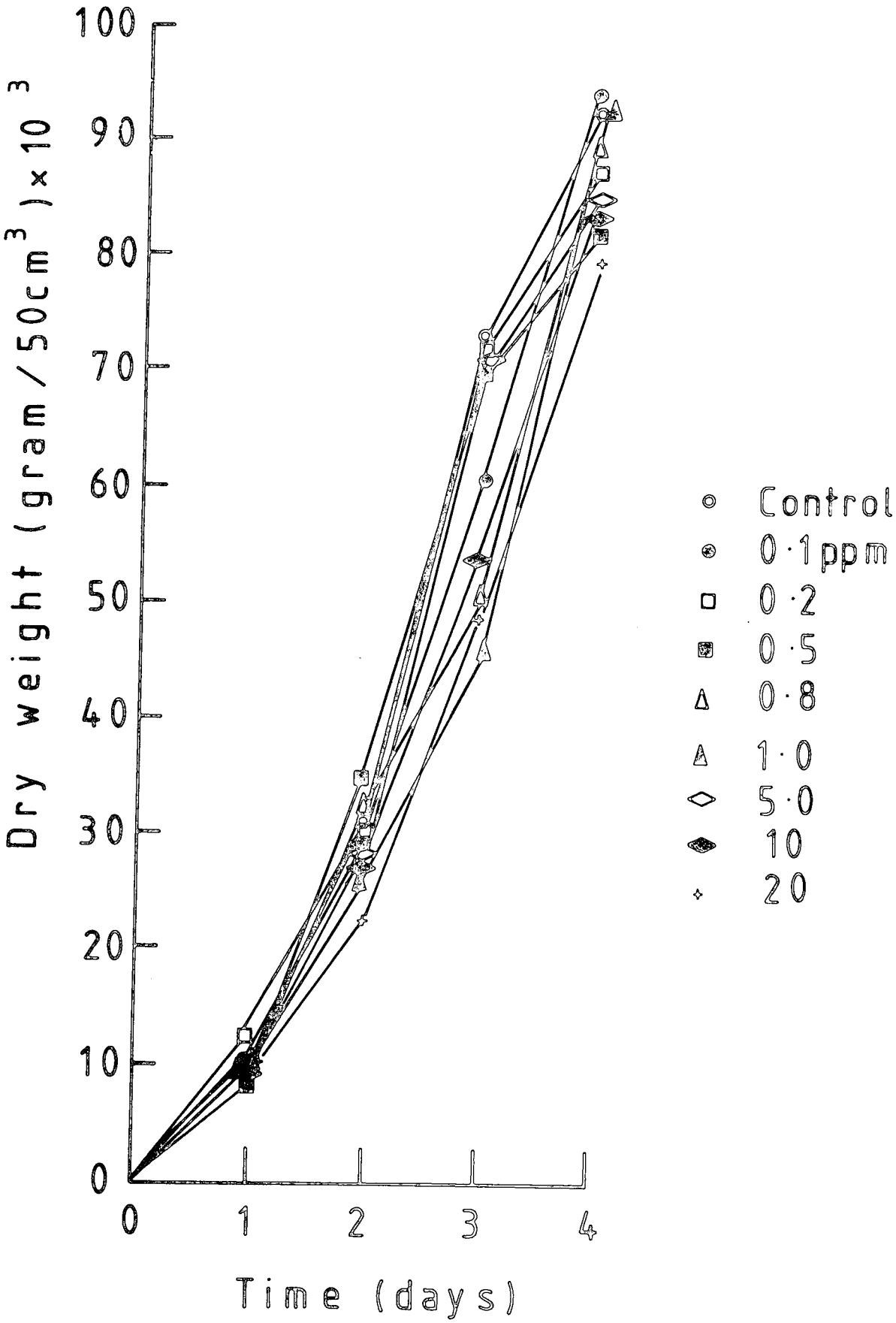
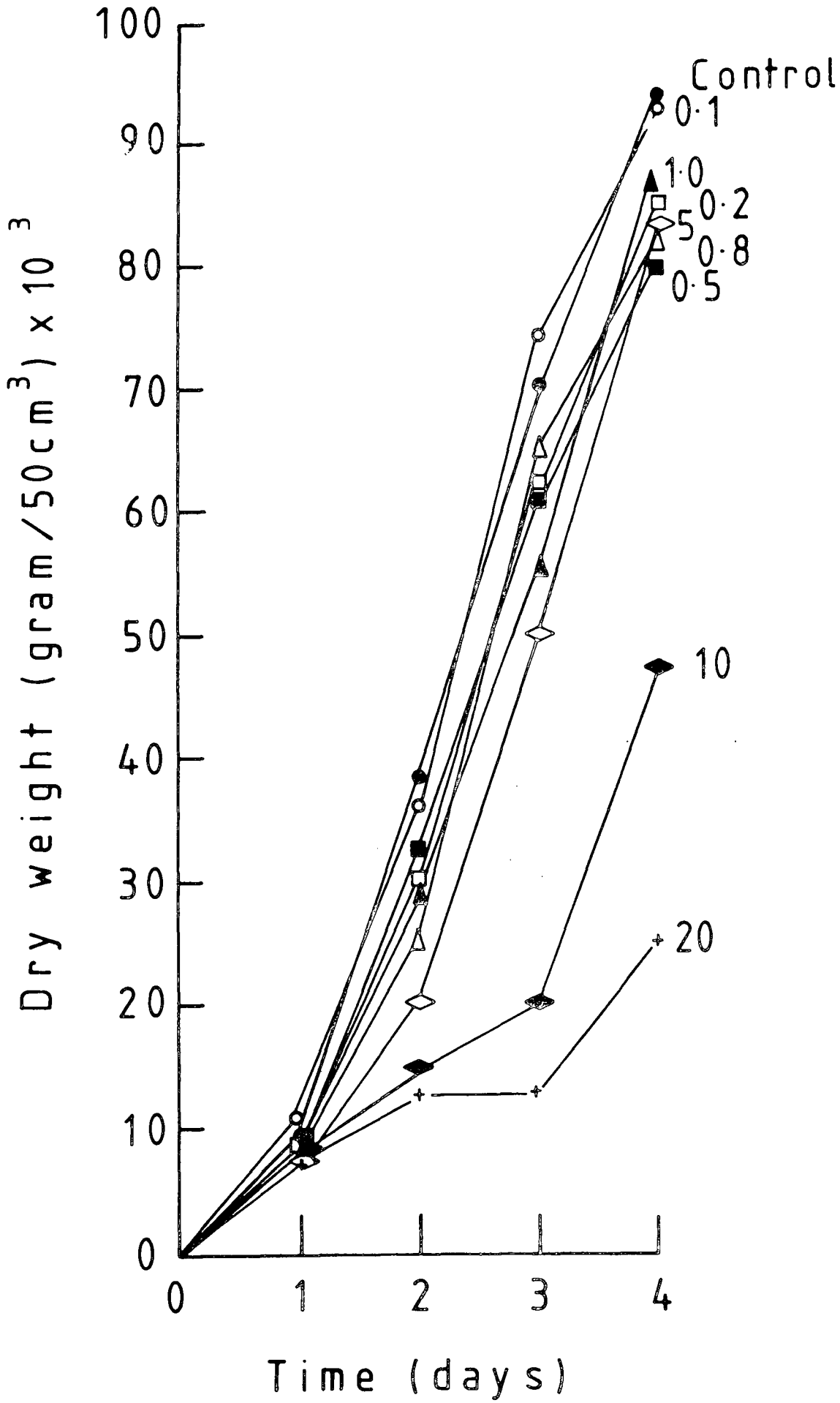

Dry weight (gram/50cm³)×10³
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
Time (days)
Control
0·1ppm
0·2
0·5
0·8
1·0
5·0
10
20

Fig. 13: Dry weight yield of *S. parasitica* grown in PYG medium containing varying concentrations of IAA.

Incubation at 24 °C


Dry weight (gram/50cm^3) x 10^3
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
Time (days)
Control
0·1
1·0
0·2
5
0·8
0·5
10
20

very sharp decrease in growth especially at 20 ppm, in which some inoculated flasks showed a complete inhibition of growth. Because of the irregularity they were repeated and it was consistently showed no growth.

1.3.1.6 Effect of PGRS on nucleic acids content: Many technical difficulties arose when methods for protein determination were applied. The procedure of Lowry *et al* (1951) performed on cold and hot 5% NaOH or hot 5% TCA or Phosphate-buffered saline pH 7.2, yielded a turbidity which interfered with the colorimetric measurements. However, the interference was overcome by an extraction with 1N NaOH. Hot IN NaOH extracted more protein than cold IN NaOH.

The present study reports the effect of varying low concentrations of GA_3 and IAA on the synthesis of nucleotides, DNA and RNA in *S. parasitica*. It is apparent from the data of Table (13), that there is no marked increase or decrease in the percentage of total amounts of nucleotide, DNA and RNA over the whole range of both substances compared with the control.

These results coincide with the results obtained from the vegetative growth of the colonies treated at the same concentration except at 10 ppm. IAA which showed a marked decrease in the dry weight matter. This may indicate that the inhibitory effect of these substances prevented the growth of the whole mycelium and not on a particular component, since same original weight was used for the determination.

TABLE 13 Nucleotides and nucleic acids content of *S. parasitica* (% of fresh weight) grown on PYG medium with varying low concentrations of GA_3 and IAA

Treatments	Nucleotides	RNA	DNA
Control	0.414	0.646	0.016
0.1 ppm. GA_3	0.469	0.661	0.016
0.2 " "	0.426	0.787	0.0095
0.5 " "	0.431	0.626	0.0095
0.8 " "	0.386	0.777	0.0134
1 " "	0.401	0.727	0.0095
5 " "	0.318	0.606	0.0075
10 " "	0.325	0.606	0.0065
20 " "	0.335	0.616	0.0128
0.1 ppm. IAA	0.446	0.595	0.0095
0.2 " "	0.464	0.565	0.0078
0.5 " "	0.401	0.666	0.0073
0.8 " "	0.431	0.636	0.0065
1 " "	0.315	0.636	0.0126
5 " "	0.358	0.717	0.0131
10 " "	0.45	0.626	0.011

1.3.2 Macromolecular compositions of some Fungi

Macromolecular compositions of fresh and dry mycelia of *M. mucedo*, *P. blakesleeanus* and *S. parasitica* were estimated. The ratio of fresh weight to dry weight of these fungi were: 1:0.473, 1:0.312 and 1:0.29 respectively. On the basis of fresh and dry weight results, Table (14) shows that both the chitin-walled fungi had more or less similar amounts of DNA which was higher than that of *S. parasitica*. These two chitin-walled fungi were also contained nearly similar amounts of protein extracted from dry mycelia. In contrast, fresh mycelia of *M. mucedo* showed higher protein content than *P. blakesleeanus* (23.7 and 14% respectively) which was also higher than *S. parasitica*. Additionally, on both fresh and dry weight matter, *M. mucedo* contained higher nucleotides and RNA than the other two fungi tested.

TABLE 14 Macromolecular composition in percentage of fresh and dry weight of three Fungi

FUNGI	% fresh weight				% dry weight			
	Nucleo-tides	RNA	DNA	Protein	Nucleo-tides	RNA	DNA	Protein
Mucor mucedo	0.97	3.17	0.04	23.73	3.3	6.12	0.094	39.74
Phycomyes blakesleeanus	0.49	1.05	0.05	14	2.036	2.104	0.0914	38.88
Saprolegnia parasitica	0.414	0.646	0.016	6.1	2.59	2.79	0.0273	29.77

1.4 Discussion

The lack of effect of both PGRS at low concentrations, and a gradual inhibition at high levels on the vegetative growth of fungi tested is in agreement with the findings of many investigators dealing with the responses of a number of filamentous fungi to PGRS (see Introduction for references), *i.e.* instances of stimulation in growth are rare while inhibition at high concentrations is more common.

IAA showed more toxic or inhibitory effects than GA_3 at the same high concentrations, especially with *S. parasitica* and *P. debaryanum* in which 400 ppm. IAA showed a complete inhibition on solid medium. In liquid cultures (dry weight yield), both substances showed a high toxicity or inhibition which began at 10 ppm. especially with *S. parasitica*. The high toxic effect on this fungus in particular may be due to insufficient oxygen because of the static condition in which the fungus was grown. Although Richards (1949) reported no relationship between the PGRS added and the pH of the medium which could affect the growth yield. Lien *et al* (1971) observed a pH-dependent inhibition with IAA concentration larger than 6×10^{-5} M on *Chlorella fusca*. They ascribed this pH dependency to the uptake of IAA which is known to involve the undissociated molecule rather than the ion.

The lack of effect of the low concentrations (0.1-20 ppm.) of both PGRS on the macromolecular composition of *S. parasitica* agrees with the lack of effect on the vegetative growth at the same concentrations. The only stimulatory effect of

both substances over the control was detected in sparangiospore germination of *Mucor mucedo* at the range of concentrations between 10 and 100 ppm. Higher concentrations showed a delay of germination behind the control. No effect was observed on the swelling of spores even on the high suppressing concentrations which delayed the germination. This finding agrees with Richards (1949) on *Aspergillus nidulans* conidia, in which no effect of PGRS was observed on swelling even at a complete inhibitory concentration. The positive effect of these substances on sporangiospore germination at the concentration mentioned above compared to the lack of such a response in vegetative hyphae could be attributed to major differences in cell wall composition between spores and hyphae. Bartnicki-Garcia (1968) and Van Laere *et al* (1977) had observed such differences in *Mucor rouxii* and *P. blakesleeanus* respectively, where glucosamines (chitin or chitosan) were the major cell wall carbohydrates in hyphae and sporangiophores, whereas glucose was predominant in spore walls. However, Nakamura *et al* (1978, 1982) reported that auxin effect as a regulator in conidial germination of *Neurospora crassa* was mediated by the active substance(s). Some substances found in the germination medium of *N. crassa* conidia appeared to be essential for conidial germination. These factors were identified as hexapeptides, a group of iron-transport compound (Harowitz *et al*, 1976). No clear effect could be detected from the work on the hyphal growth unit. This is due to the oscillations which may be ascribed to the young age of the mycelia. Also mycelia were selected randomly from originally unsyn-

chronized germinating spores which meant that the samples were of different lengths. It has been observed that after spore germination the hyphal growth unit of a germ tube increases in length until it produces the first branch, at this point the hyphal growth unit is halved (Plomely, 1959; Trinci, 1973). The amplitude of oscillations in the undifferentiated mycelia increases in size; eventually the hyphal growth unit attains a more or less constant value. Trinci (1973) found that the length of the hyphal growth unit of mycelia of *N. crassa* grown on solid media was constant for a given set of environmental conditions. However, Caldwell and Trinci (1973) observed that the mean length of the growth unit of selected mycelial fragments of *Geotrichum candidum* increased with the number of tips possessed by the hypha.

With respect to hyphal growth rate, the results were also quite variable and no clear effect could be detected. This may be attributed to minor changes in environmental conditions. This is in contrast with other reported work which it has been detected that on germination of the spore, the mycelium (unbranched hypha) grows at a constant rate for a time, but later the growth rate increases until the colony is growing exponentially (Smith, 1924; Plomely, 1959). These two authors also drew attention to differences in rate of growth between hyphae and in the same hypha at different times.

Nucleic acids and protein content of the fungi tested are more or less in agreement with the findings of others using different fungi, such as *Rhizopus nigricans* (Wegener and Romano, 1963), *Penicillium griseofulvum* (Bent and Morton, 1964), *Phycomyces blakesleeanus* (Saadi, 1980).

The level of nucleic acids and in particular of RNA was reported to be related to the rate of growth (Alberghina *et al* 1979). Therefore, these results may be a reflection of a higher growth rate in *M. mucedo* compared to the other two fungi tested. However, the growth of the vegetative colonies on solid medium showed the reverse, *i.e. S. parasitica* grew faster than *M. mucedo*. But it must be taken into account that *M. mucedo* produces more branches than *S. parasitica* and hence thicker and denser colonies were produced from *M. mucedo* on solid medium. Additionally, in liquid culture, as was mentioned earlier, *M. mucedo* did not grow as spherical pellets even under static conditions, in contrast it showed aggregation of small mycelial masses. This may explain the high content of RNA in these small masses, which reflect younger colonies than the single large spherical pellet of *S. parasitica* at the same age. It has been reported that nucleic acids and protein content decline in older starved mycelium (Bent and Morton, 1964) and this may explain the observation seen in this study.

Although some promotive effects upon the fungal growth have been recorded in the literature (Gruen, 1959), it is clear that the striking growth-promotion effect of PGRS is largely limited to higher plant cells. These cells are best distinguished from all others by the possession of a unique complex of chemical components in the cell wall. These include cellulose, hemicellulose, non-cellulosic polysaccharides, pectic substances, and lignin (Glaston and Purves, 1960). Therefore, there is something about this complex of substances (or one of them) in the cell wall that is essential for PGRS

reaction which absent or not produced in fungal walls. This lack of responses was observed despite the fact that some cellulose and non-cellulosic polysaccharides are present in Oomycetes cell walls. Lack of responses may also be due to the adaptation or loss of responses of these fungi to the low concentration of PGRS, since it has been shown that auxins and gibberellins production is widespread among fungi belonging to different taxonomic groups.

CHAPTER TWO

LIGHT AND ELECTRON MICROSCOPIC STUDIES OF HYPHAL TIP BURSTING

2.1 Introduction

In filamentous fungi, growth occurs as a prolongation of hyphal tips, *i.e.* restricted to the apical region of the filament (Smith, 1923; Zalokar, 1959; Trinci and Banbury, 1967; Bartnicki-Garcia, 1973). The tapered region of the tip involved in this growth is called the extension zone. Additionally, this growth is highly polarized in that although percursors of wall polymers are synthesized in a large volume of cytoplasm, they are only incorporated into the primary wall at the hyphal tip.

The phenomenon of hyphal tip growth has been repeatedly described (Reinhardt, 1892; Smith, 1923; Castle, 1958; Zalokar, 1959; Robertson, 1965, 1968; Carlile, 1966; Bracker, 1967). Likewise theoretical considerations (Green, 1974; Trinci and Saunder, 1977; Koch, 1982) have made it clear that wall expansion is maximal at the extreme tip and declines towards zero at the base of the extension zone, according to a predictable gradient dependent on the shape of the apical dome. The correlation between wall synthesis and wall plasticity has been a matter of much speculation. One model, advanced by Bartnicki-Garcia (1973) considers that the apex is unable to expand until loosened by hydrolytic enzymes. Such lysins would cause breaks in existing polysaccharide chains, producing loci at which insertion of new chains occurs by the action of biosynthetic enzymes. Another earlier model is based on the assumption that the new wall material laid down at the apex is initially plastic but is gradually turned into a more rigid material (Robertson, 1968). Likewise, Wessels *et al* (1983) presented evidence and model which indicated that

complex formation between chitin and β-glucan, which starts just under the extreme tip and continues subapically, accounts for the change from plasticity toward rigidity of the wall during morphogenesis of *Schizophyllum commune* whilst as long as the two components occur in the wall as separate polymers (usually present in the very tip) the wall may be relatively plastic.

Although the osmotic pressure in hyphal tips is considerably higher than that of the surrounding medium (Adebayo, *et al* 1971), and normal extension growth depends on this differential (Park and Robinson, 1966a), gross osmotic potential differences do not exist between the apical and basal cells of *Neurospora crassa* hyphae. However slight differences in water potential probably exist such that there is a net turgor pressure difference between base and tip (Robertson and Rizvi, 1968). This is thought to play an important role in wall extension at the tip (Robertson, 1968; Trinci, 1978).

The problem of the apical extension growth of fungal hyphae has been approached by investigating the bursting of hyphal tips in response to various experimental treatments. Earlier work (Robertson, 1958, 1959) showed that immersion of colonies of *Fusarium oxysporum* in solutions of differing osmotic potential led to changes in the morphology of the hyphal apex. Hypertonic solutions caused withdrawal of water from the apex, hypotonic solutions allowed swelling of the apex, the effect of both was to bring about a temporary arresting of apical extension and this was followed by subapical branching.

An indication of one of chitin's functions in the wall has been obtained from a mutant of *Aspergillus nidulans*, which cannot synthesize glucosamine and therefore chitin at high temperature (Katz and Rosenberger, 1971, a). This mutant grows at the non-permissive temperature provided an osmotic stabiliser is added to the medium and produces mycelium low in chitin; with no osmotic stabiliser the cells die, and swollen and empty hyphae are seen (Cohen *et al*, 1969). Chitin thus appears to be required to maintain the chemical strength of the wall. Katz and Rosenberger (1971, b) found that both hyphae of *A. nidulans* low in wall chitin and those with a normal chitin content distort and lyse only in growth medium and not in water or buffer. Thus walls low in chitin are not mechanically weak but become so, as do normal walls, during growth in the absence of chitin synthesis. Therefore, at least in the early stages of wall extension the presence of all normal components appear to be necessary to provide stability (Katz and Rosenberger, 1971, b). The importance of chitin in the growth of hyphal tips was also supported by the finding that application of Polyoxin D, an inhibitor of chitin synthetase, or of lysozyme (with chitinase activity), both caused bursting of hyphal tips of *Mucor rouxii* (Bartnicki-Garcia and Lippman, 1972, a,b); due to continuing lysis after arrestment of synthesis of chitin. In addition, sorbose, an inhibitor of wall glucan synthetase (Mishra and Tatum, 1972), caused apical disintegration in hyphae of *Neurospora crassa* (Rizvi and Robertson, 1965).

A wide diversity of treatments caused rapid and extensive bursting of hyphal tips of *Mucor rouxii*. Most hyphal tips

from colonies grown on full-strength agar medium burst readily when flooded with distilled water. In contrast, hyphal tips from colonies grown on diluted medium survived flooding with distilled water but succumbed to dilute aqueous solutions (particularly acids, but also neutral salts, EDTA, alcohols, acetone and detergents). Apex bursting was generally inhibited by alkaline solutions but took place at certain concentrations of ethanolamine or NH_4OH. Flooding with divalent metal ions (Mg^{2+}, Mn^{2+}, Ca^{2+}) caused pronounced swelling of the hyphal apex followed by an occasional burst (Bartnicki-Garcia and Lippman, 1972, a). Dabbagh *et al* (1974) examined cell wall and protoplast growth in saprophytic species of the *Cryptococci* which grow normally at 27°C but lyse when shifted to 37°C. With osmotic stabiliser lysis does not occur at 37°C and protoplasmic growth continues.

It is considered that apical or subapical branching is not a normal feature of hyphal marphogenesis (Butler, 1966). It may occasionally occur spontaneously (Robertson, 1965, Park and Robinson, 1966, b), or be induced experimentally, *e.g.* by flooding hyphae with water (Robertson, 1958, 1959), griseofulvin solution (Robertson, 1965), succinic acid or extracts from culture filtrates (Park and Robinson, 1966, b). However, apical branching was observed as a normal feature of hyphal morphogenesis at the margins of colonies of *Aspergillus nidulans* and *Geotrichum lactis* (Trinci, 1970).

Circumstantial evidence from such experiments has been used to support the view that the proposed plasticization may be due to localized enzymic lysis of existing wall polymers (Bartnicki-Garcia and Lippman, 1972, a), which increased by these treatments. Those lytic enzymes are likely to be carried

in vesicles along with synthetic enzymes and wall precursors (Girbardt, 1969; Grove *et al*, 1970; Cortat *et al*, 1973).

Therefore during normal tip growth a delicate balance must exist between deposition of new material, synthetic activity, lytic activity and turgor pressure which provides the force for elongation.

Hyphal tips have been found to possess cytochemical and structural properties which make them distinct from the rest of the thallus (Brunswick, 1924; Fultz and Sussman, 1966; McClure *et al*, 1968; Grove and Bracker, 1970; Gull and Trinci, 1974). On the basis of cytoplastic components, fungal hyphae are distinctly organized into an apical zone and a subapical zone. The characteristic feature of the apical cytoplasm is an accumulation of vesicles, and the lack of most other cytoplasmic components. The subapical zone contains the organelles and inclusions in addition to vesicles, in lower frequency, with greatest concentration around dictyosomes. Some distance from the hyphal apex, the subapical zone merges into zone of vacuolation, the degree of vacuolation increases with distance from the apex and is paralleled by an increase in lipid content (McClure, *et al*, 1968; Grove and Bracker, 1970; Grove *et al*, 1970; Grove, 1978; Trinci, 1978). The transition between apical and subapical zone is gradual over a distance of several microns, and mitochondiia are typically clustered in this transitional region (Grove *et al*, 1970).

In *Saprolegnia* the apical region, 10-40μm long is found to be devoid of mitochondria and nuclei (Heath *et al*, 1971). In *Pythium ultimum*, the growing apex 3-5μm deep zone contained

vesicles; mitochondiia and other large components were lacking in the apex. The subapical region extended up to several hundred microns basipetally, nuclei were absent from the anterior 15-20μm of the subapical zone but were distributed throughout the rest of the hypha. Vacuolation began 40-100μm behind the apex and increased progressively with increasing distance from the tip (Grove *et al*, 1970). About 80% of the apical 1μm of a hypha of *Neurospora crassa* is occupied by vesicles and this value drops sharply to about 5% at the base of the extension zone (Collinge and Trinci,1974).

Although concentrations of vesicles have been found in the tips of growing hyphae of fungi representing all major groups, the septate fungi, Zygomycetes, Oomycetes and Chytridiomycetes, can be delineated on the basis of vesicle distribution patterns (Grove and Bracker, 1970; Roose and Turian, 1977).

Indirect evidence derived mostly from ultrastructural studies suggests that cytoplasmic vesicles play a decisive role in wall growth by transporting wall precursors and/or the necessary enzymes to the apices and branching sites (Grove and Bracker, 1970; Bartnicki-Garcia, 1973; Mullins and Ellis, 1974; Trinci, 1978; Aronson, 1981).

Electron microscopic studies suggest that these cytoplasmic vesicles do not transport wall fibrillar component, furthermore it is now almost certain that the microfibrillar skeleton of the wall is synthesized *in situ* either on the outer surface of the plasmamembrane or within the wall fabric, while the amorphous matrix material is probably synthesized in the cytoplasm (Smith *et al*, 1981).

Although vesicles have not been isolated from moulds (Trinci, 1978), cytochemical methods for polysaccharide localization have been applied to a few species and have provided evidence that apical vesicles contain materials which could serve as wall precursors (McClure *et al*, 1968; Heath *et al* 1971; see also Grove, 1978). Likewise the Ascomycete *Hypomyces chlorinus* (Dargent and Touzé-Soulet, 1976) in which the apical wall, plasmalemma and apical vesicles show similar cytochemical reactivity.

The possibility that the apical vesicles of a growing hypha release lytic enzymes at the apex is high. Matile *et al* (1971), described the occurrence of the wall lytic β-1,3-glucanase in lysomal vesicles concentrated at the site of bud formation in *Saccharomyces cerevisiae*. The secretory budding vesicles contain glucanase, mannan and mannan synthase (Cortat, 1971; Cortat *et al*, 1973). Nolan and Bal (1974) reported the localization of cellulase activity in vesicles in hyphae of *Achlya ambisexualis* responding to the sex hormone, antheridiol, by antheridial branching. In Oomycetes also Févre (1978, 1979), and Mullins and Ellis (1974), described localization of vesicles containing cellulase and glucanases in hyphae of *Saprolegnia monoica* and *Achlya ambisexualis*, respectively at sites of wall formation, hyphal apices and branches.

Apical vesicles are formed in some fungi from dictyosomes. Grove *et al* (1970) reported that in *Pythium ultimum*, vesicles budded from the smooth endoplasmic reticulum, fused to form new dictyosome cisternae and as the cisterna passed through

the dictyosomes, its membrane was gradually transformed until it assumed the chracteristics of the protoplasmic membrane. Similarly Févre (1979) postulated that dictyosomes rich in morphogen enzymes and β(1-4)-linked polysaccharides produce the apical vesicles which secret their contents into the walls by fusing with the plasmalemma. In some other fungi vesicles were reported to form from smooth endoplasmic reticulum (Girbardt, 1969).

The mechanism of migration of the apical vesicles to the direction of the tip is controversial though several hypotheses are being considered. Slayman and Slayman (1962) described in *Neurospora crassa* a decrease in the membrane potential from the old part of a hypha to the tip. Bartnicki-Garcia (1973) suggested that the potential gradient established might drive the vesicles to the apical area by electrophoresis. However, Jennings (1973, 1979) proposed that an osmotic flow caused by a decline in Potassium pumps (ATPase) toward the tip plays a role. The involvement of microtubules and/or microfilaments in the transport or orientation of vesicles is also a speculative subject. A ring of filamentous structures associated with the plasma membrane of budding cells of *S. cerevisiae* has been described by Byers and Goetsch (1976). It has been suggested that this ring is involved in a control mechanism which limits vesicle fusion to very determined places. Microfilaments have been described in filamentous fungi (Gull, 1975), but evidence for their participation in the transport of secretory vesicles is still fragmentary.

Preliminary studies using the flooding method of Bartnicki-Garcia

and Lippman (1972, a) (Materials and Methods 1) on *M. mucedo* showed an unexpected phenomena (Bursting and/or swelling of hyphal tips followed by apical branching). Therefore, in the present study, some other chitin and cellulose-walled fungi were tested following flooding with their liquid culture medium, using the light microscope. In addition electron microscopy was also used to study the ultrastructural changes of two chitin-walled fungi, *M. mucedo* and *P. blakesleeanus* which reacted strongly with the flooding in an attempt to see whether the apical vesicles which transport enzymes and wall precursors to the hyphal tips are involved in these reactions; by recording the percentage of accumulation and fusion of these vesicles in the treated hyphal tips compared to the control.

2.2 Materials and Methods

2.2.1 Organisms, culture conditions and light microscopy

Mucor mucedo was grown on 1% Malt extract medium. Using the method of Bartnicki-Garcia and Lippman (1972, a) (described under methods 1, excluding underlying and overlying the grown colony with cellophane) to study the effect of flooding with liquid culture medium (1% Malt extract) on colonies grown in Petri dishes (containing approximately 20cm^3 solid medium) or on a very thin layer of medium on a slide. The inoculum was a block of agar with growing mycelium, incubated at room temperature (20^{o}C±2). In the case of slide cultures, inoculated slides were incubated in sterilized Petri dishes containing moist filter paper.

Several fungi were tested including *M. mucedo*, *Phycomyces blakesleeanus* Burgeff and *Allomyces arbuscula* as chitin-walled fungi; *Achlya ambisexualis* (163a), *A. beneckii* (128a), *Dictyuchus sterile* (341c), *Phytophthora parasitica* (240a), *Pythium debaryanum* (401a), *P. middletonii* (HCC11), *Saprolegnia parasitica* (211b) *and S. terrestris* (212c) as cellulose-walled fungi. References to wall composition of fungi, *i.e.* chitin or cellulose are: Aronson and Machlis (1959), Crook and Johnston (1962), Parker *et al* (1963), Bartnicki-Garcia (1966); Cooper and Aronson (1967); Dietrich (1975).

All aquatic or cellulose wall fungi (obtained from the culture collection of University of Reading) were grown on PYG solid medium (materials 1) and subjected to a flow system of PYG liquid medium. Fungi were studied at different culture ages, 18, 24, 42, 48, and 72 hrs. after inoculation by taking photographs at different intervals using a camera attached to the microscope (materials 1).

2.2.2 Electron Microscopy

2.2.2.1 Organisms and Sampling: The ultrastructure of the two chitinous fungi, *M. mucedo* and *P. blakesleeanus*, in which their hyphal apices showed different responses upon flooding with liquid medium were examined. Twenty-four hour old colonies, grown on agar medium were used, after flooding and responses had occurred in hyphal tips. The colonies were covered by a very thin layer of 0.5% agar in distilled water to prevent the mycelium and the cell contents from washing away in the subsequent preparations for electron microscopy.

The materials used were small pieces of agar containing hyphae, cut with a razor blade from the edge of the colony at intervals: zero time (before flooding), 15 min. and 45 min. after flooding, (*M. mucedo*).

2.2.2.2 Fixation, Dehydration and Embedding: The preparation schedule was as follows:

1 - Fix 2.5% Glutaraldehyde and 1% Formaldehyde in 0.05M cacodylate buffer at pH 7.0 for 1 hr.
2 - Wash with distilled water for 2x5 min.
3 - Post fix with 1% (w/v) osmium tetroxide in distilled water for 1 hr.
4 - Dehydrate in 25% ethanol (v/v water) for 2x10 min.
5 - Dehydrate in 50% ethanol (v/v water) for 2x10 min.
6 - " " 75% " " " " "
7 - " " 95% " " " " "
8 - " " dry absolute alcohol " " " "
9 - Embed in 50% epoxy resin/absolute alcohol for 1 hr.
10 - Embed, transfer to 100% epoxy resin for 1 hr, and then polymerized at 70°C for 24-48 hrs.

All preparations were made at laboratory temperature. The resin used was epoxy resin, ERL - 4206 (Spurr 1969).

2.2.2.3 Sectioning, staining and examination: Mycelia and treated hyphae were selected in the polymerized blocks using a 10X light microscope objective, trimmed under a stereomicroscope with razor blades and the sections were

cut on LKB ultrotome using glass knives. Sections were collected on formvar coated copper grids, stained for 5 min. on a drop of a saturated aqueous uranyl acetate (UAC), rinsed in distilled water, stained for another 5 min. on a drop of lead citrate (Pb cit), rinsed in distilled water, and dried. Sections were examined in a Philips EM400 electron microscope.

2.3 Results

2.3.1 Observations by Light Microscopy

As a result of flooding the colonies grown on 1% malt extract agar plates with liquid 1% malt extract at room temperature there was a striking effect on the morphology of the apices of the main growing hyphae. With *M. mucedo*, the extension of the apices immediately ceased, thereafter (1.5-2 min.), almost all growing tips developed swellings which persisted for 5-7 min. before regrowth took place. On prolonged incubation the regrowth generally took the form of two or more hyphal tubes emerging from the shoulder of the swollen apex, which grew as a normal hyphae (Fig.14 (A→D). This behaviour resembled that described by Robertson (1958), Bartnicki-Garcia and Lippman (1972,a). Very few apices produced only one such protuberance and the hypha grew on with an obvious kink to mark the position of arrestment. Also very few tips burst. No difference was detected between the behaviour of the apices with different ages of the colonies tested. However, the response was varied from experiment to experiment. In a few cases there was extensive bursting of

Fig. 14: *Mucor mucedo* growing hyphae, from 48 hr. old colony flooded with liquid medium at : X 143

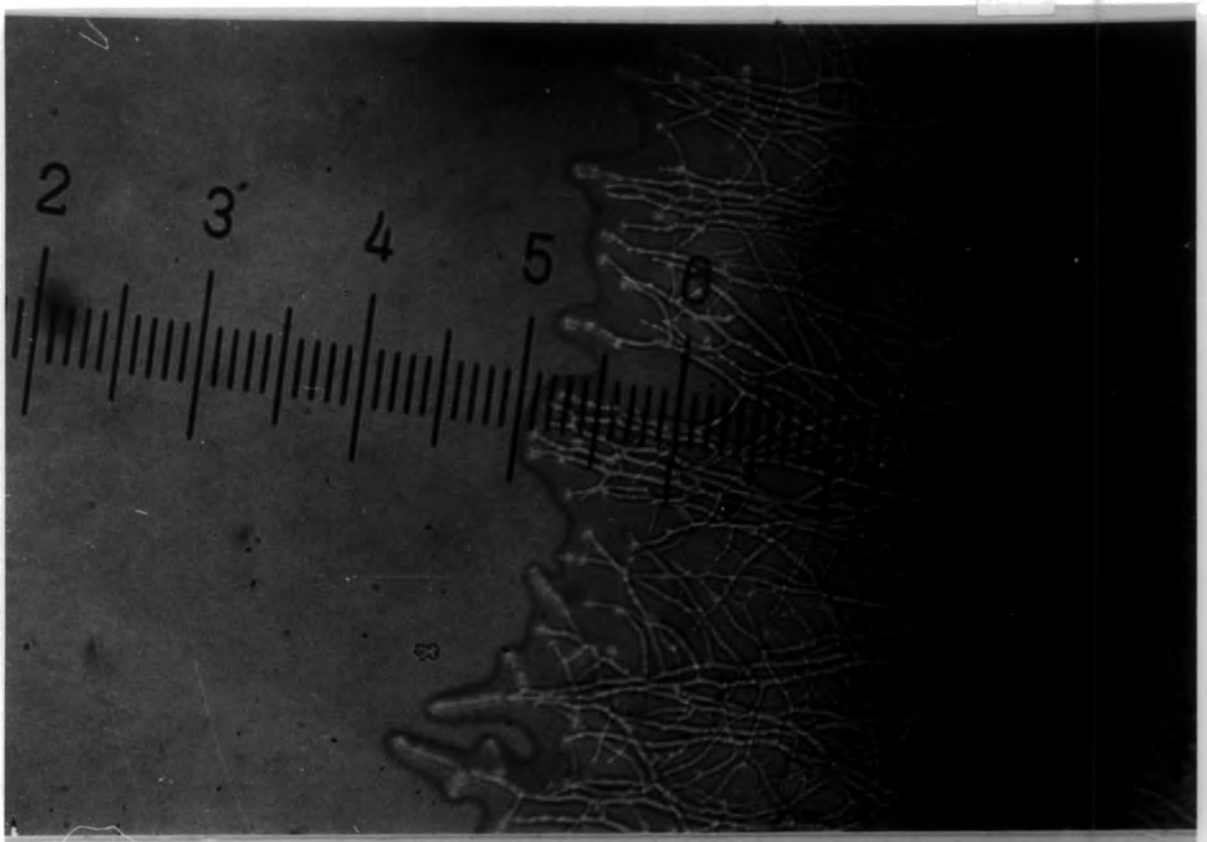
A - zero time (before addition of liquid medium

B - 10 min. after flooding, swelling of hyphal tips

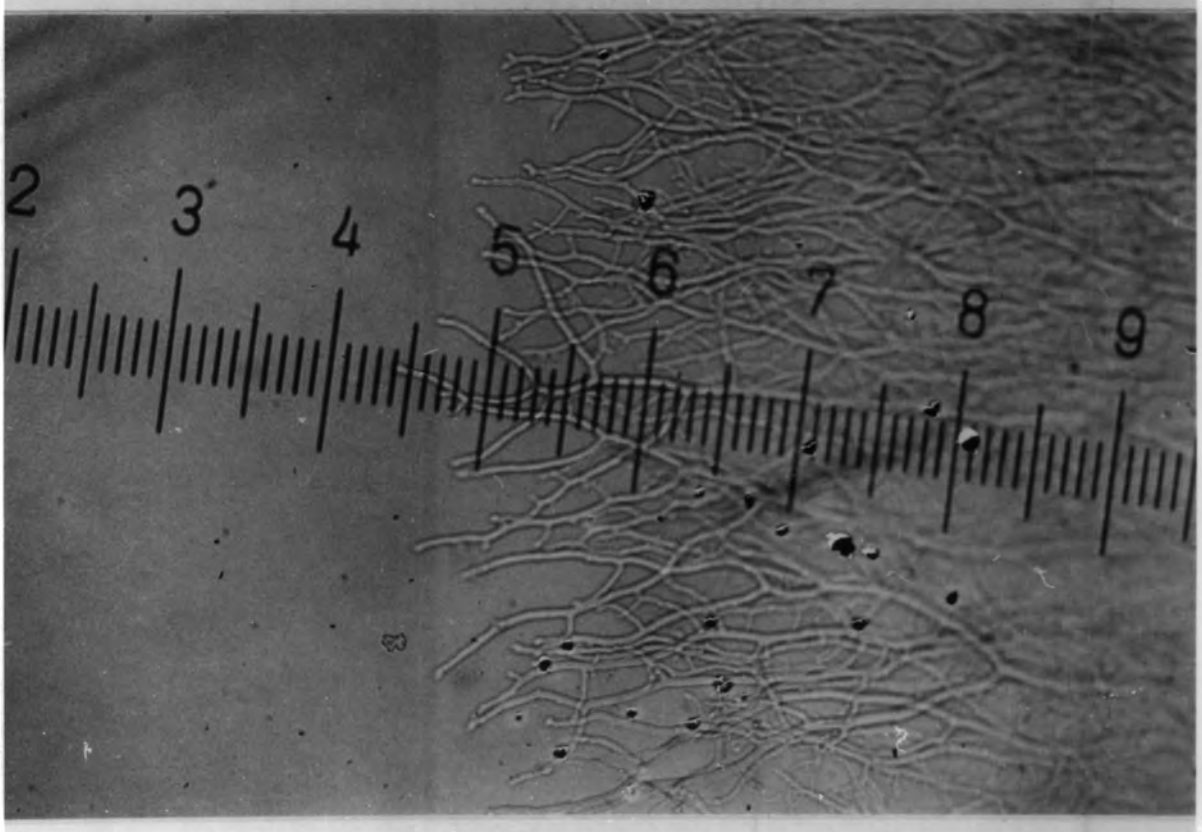
C - apical branching of *M. mucedo*, 45 min. after flooding

D - apical branching of *M. mucedo*, 60 min. after flooding.

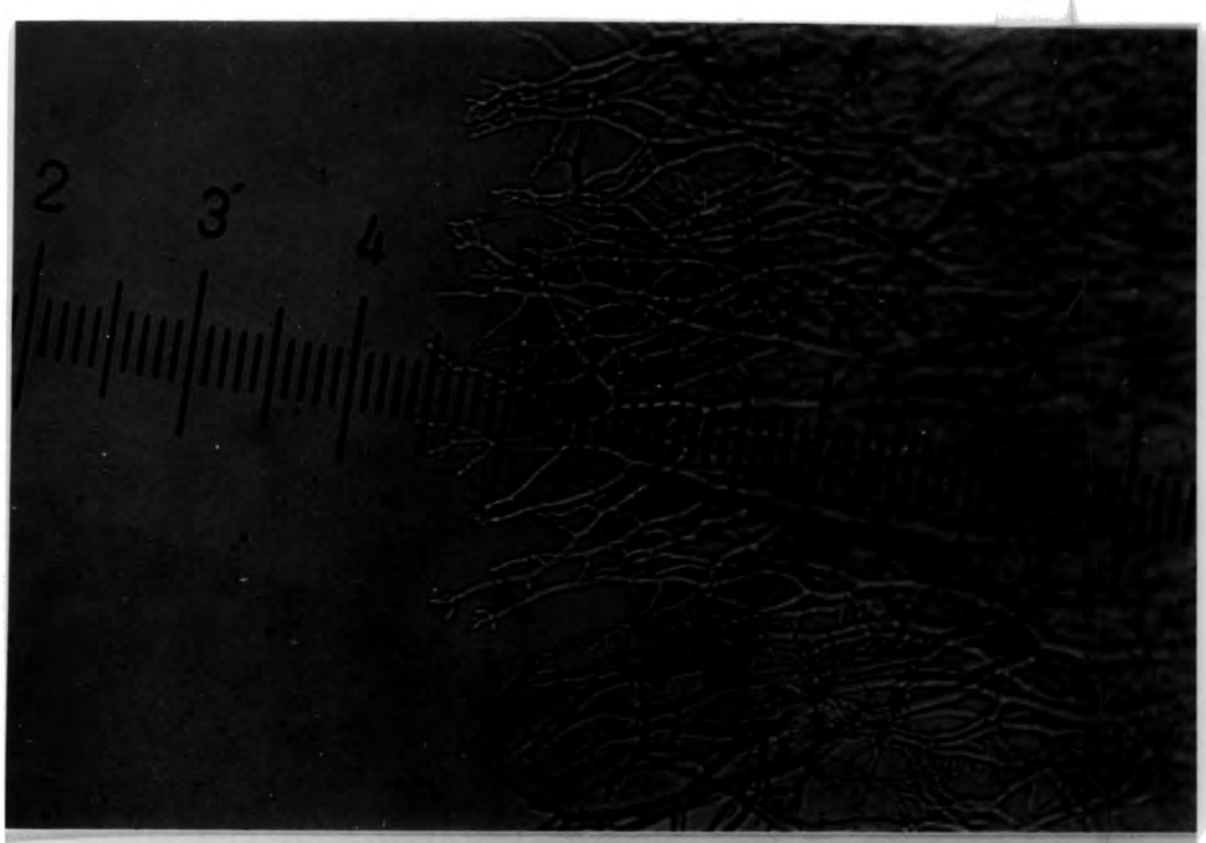
N. B. : A was not the same culture as B, C, D



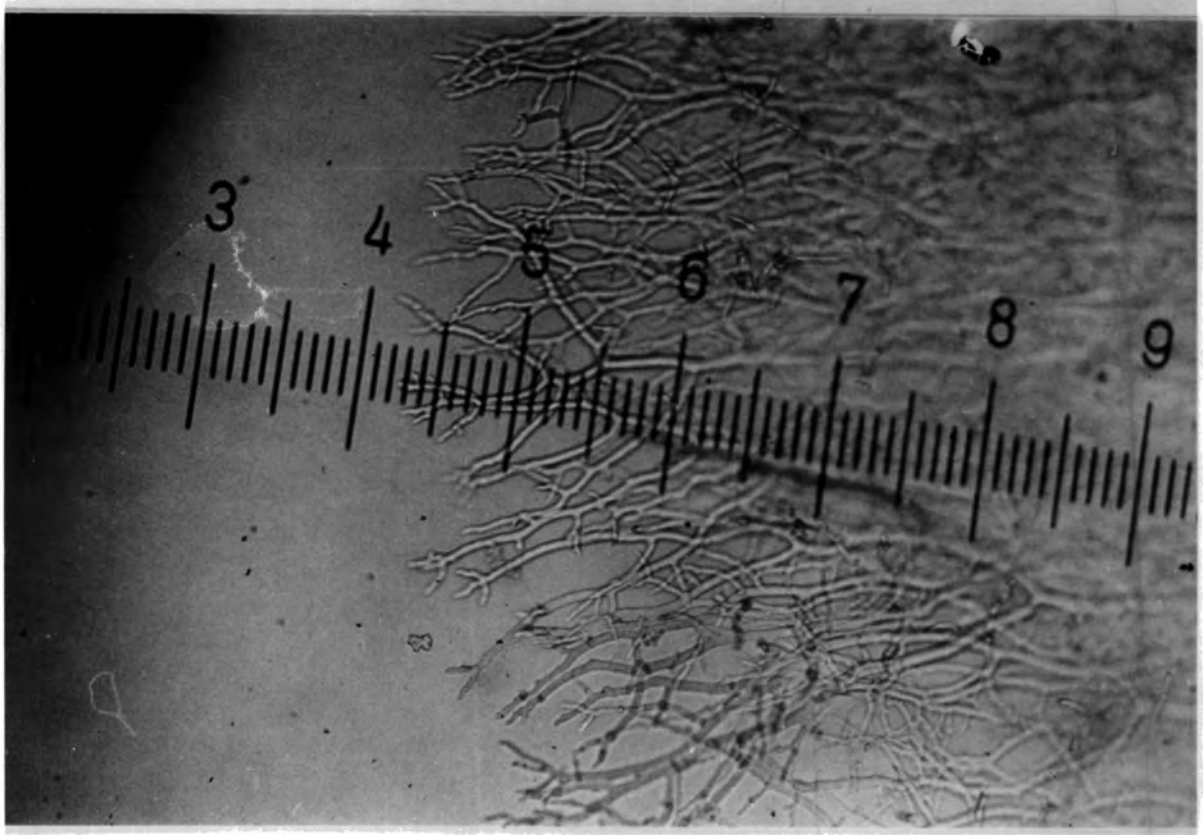
A



B



C



D

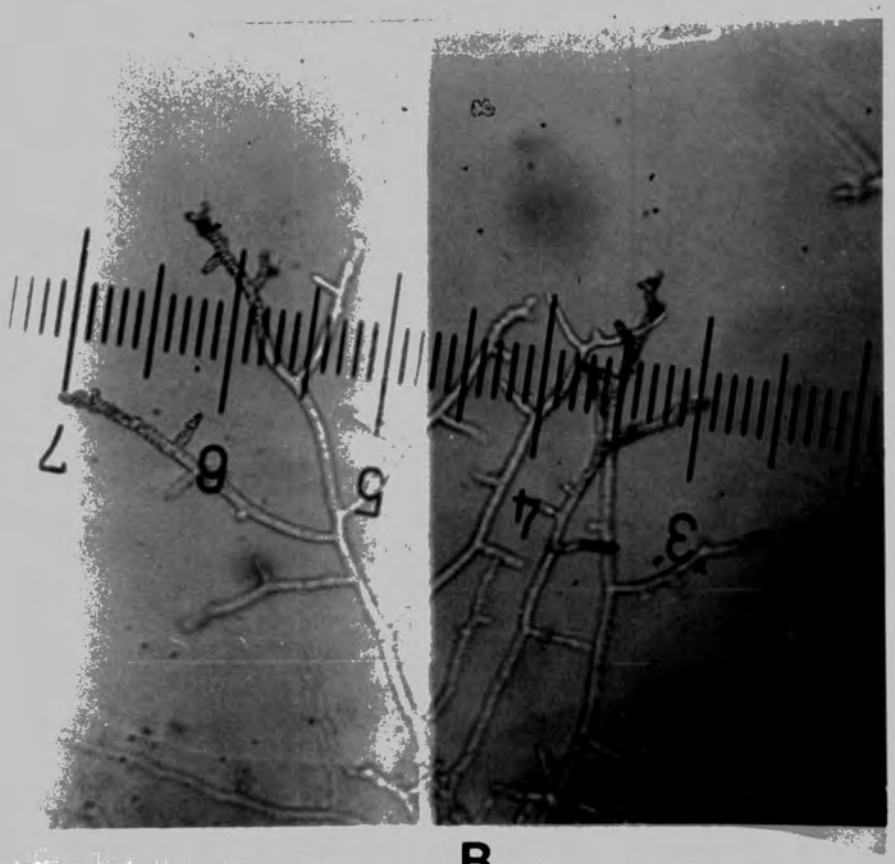
hyphal apices after a few seconds of flooding with liquid medium (Fig. 15(A). In a few others there was a marked disintegration of the previously swollen branched apices after 30-40 min. of flooding, which can easily be detected since the cytoplasm became coarsely granulated (Fig.15(B): this variation probably reflecting minor changes in environmental conditions.

When a colony of *Phycomyces blakesleeanus* grown on 1% malt extract agar was flooded with liquid medium, there was a very fast and extensive bursting of the hyphal tips around the entire colony margin just a few seconds after flooding (3-5 sec.), Fig. 16 (B). In most cases the cytoplasm was violently discharged as a globule through a small opening at the apical dome; sometimes extruded as a long narrow twisting mass. In most cases bursting occurred mainly through the apical dome and rarely behind it (through the lateral walls (Fig. 16(C). Since the mycelial hyphae are branched and non-septate, presumably a single burst within connected tips would release the turgor pressure from a leading hypha or a side branch, but rarely both Fig.16 (C). This bursting pattern is similar to that observed by Bartnicki-Garcia and Lippman (1972,a), and Dow and Rubery (1975). Also there were marked internal changes within the bursting hyphae such as the cytoplasm becoming coarsely granulated. In the remaining unburst hyphae, the response was similar to that observed with *Mucor mucedo*; there was a short delay in growth after flooding (1.5-2 min.) thereafter a slight swelling of hyphal apices was followed which prolonged for 2-3 min. before regrowth took place in the form of mostly one protuberance on

Fig. 15: A - Hyphal tip bursting of *M. mucedo*, 48 hr. old colony, 10 min. after flooding with liquid medium, X143.

B - Disintegration of apical branched hyphae of *M. mucedo*, 48 hr. old colony, 40 min. after flooding with liquid medium, X143.

A



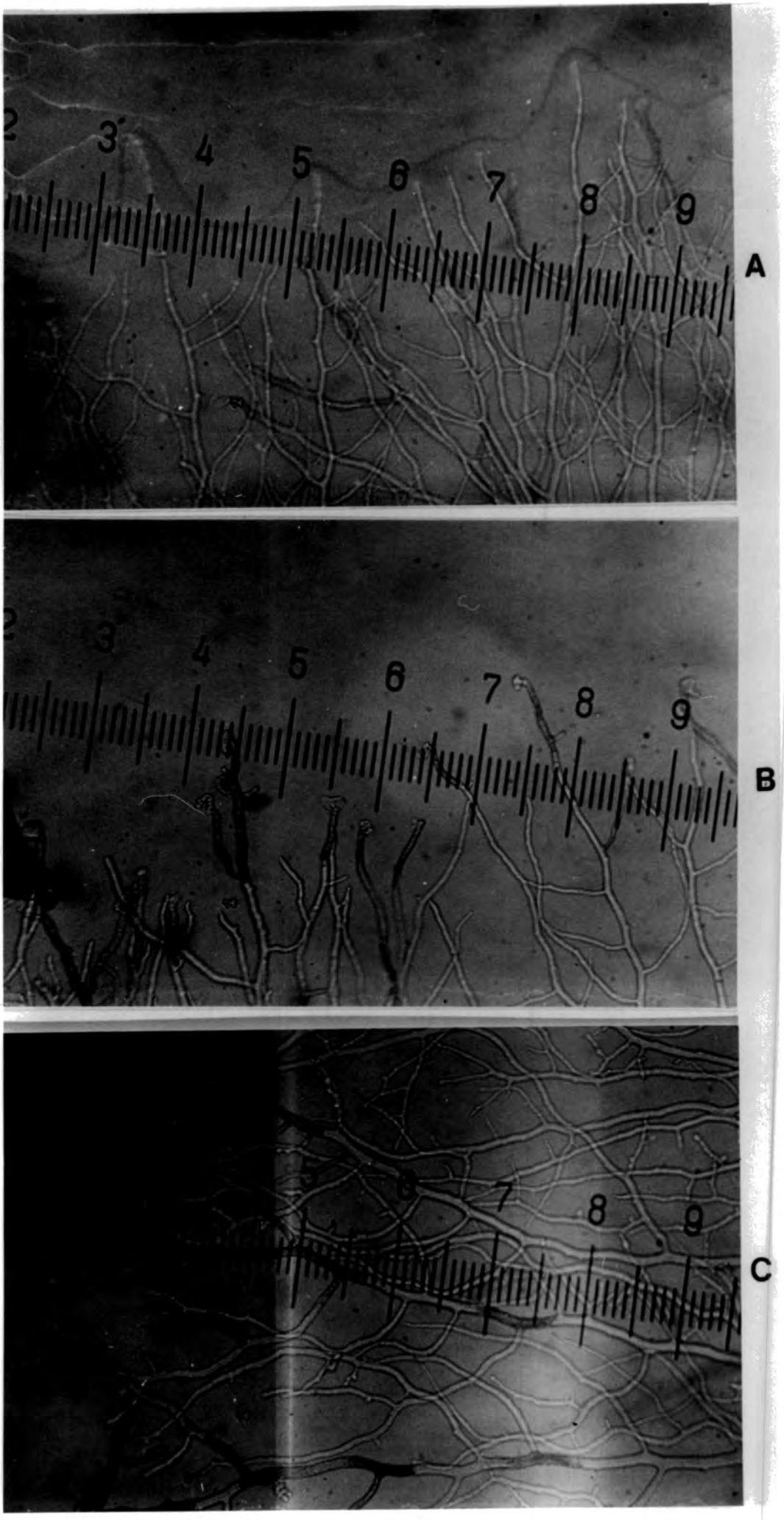
B

Fig. 16: *Phycomyces blakesleeanus*, 48 hr. old colony flooded with liquid medium at: X143

A - zero time (before addition of liquid)

B - 5-10 seconds after flooding showing apical bursting

C - apical bursting and lateral disintegration of hyphae.


2
3
4
5
6
7
8
9
A
2
3
4
5
6
7
8
9
B
6
7
8
9
C

the shoulder of the swollen apex, which grew on with an obvious kink to mark the position of arrestment. With *P. blakesleeanus* also no relationship was found between the age of the colony and the responses to flooding.

An occasional burst hyphal tip was seen on the margin of a colony of *Phycomyces blakesleeanus* which had not been subjected to any exogenous disturbance during the normal course of colony development.

The following aquatic fungi: *Achlya ambisexualis*, *A. beneckii*, *Allomyces arbuscula*, *Dictyuchus sterile*, *Phytophthora parasitica*, *Pythium debaryanum*, *P. middletonii*, *Saprolegnia parasitica*, and *S. terrestris*, grown on PYG solid medium flooded with liquid PYG medium, were tested. None of them was found to produce apical bursting on either swollen tips (Figs. 17 and 18), even the *A. arbuscula* which is known to contain chitin microfibrils in its wall.

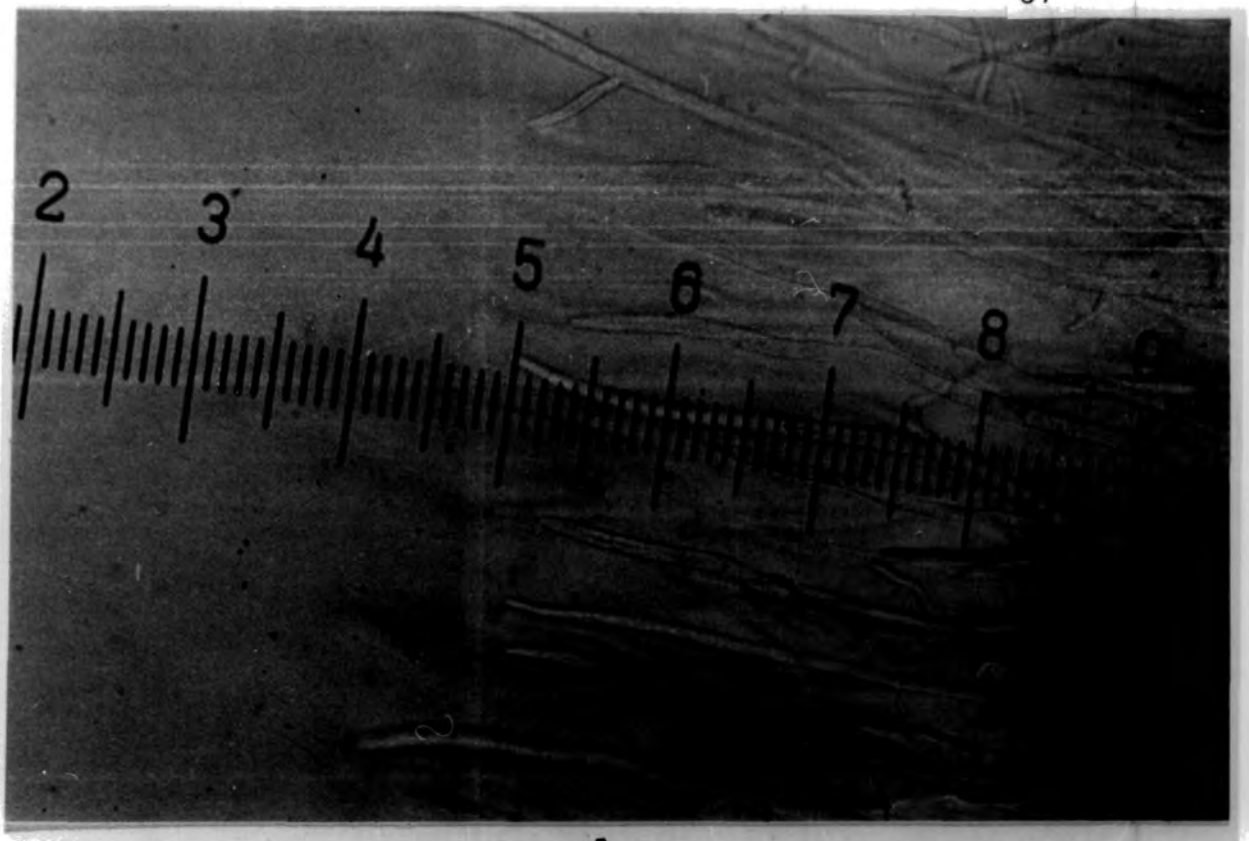
2.3.2 Observation of electron microscopy

Numerous attempts to obtain a longitudinal sections through a normal growing hyphal tip were made with a low degree of success. Nevertheless, sections of vegetative hyphae of *Phycomyces blakesleeanus* obtained in this study represent the three differentiated zones of the growing hypha. Plate (1) shows a section through a hyphal tip which was characterized by the significant accumulation of cytoplasmic vesicles and a lower frequency of other cytoplasmic components. It appears also that all apical vesicles were more or less the same in size and appearance, *i.e.* containing very dark-staining globules. Sections below this region contained less apical

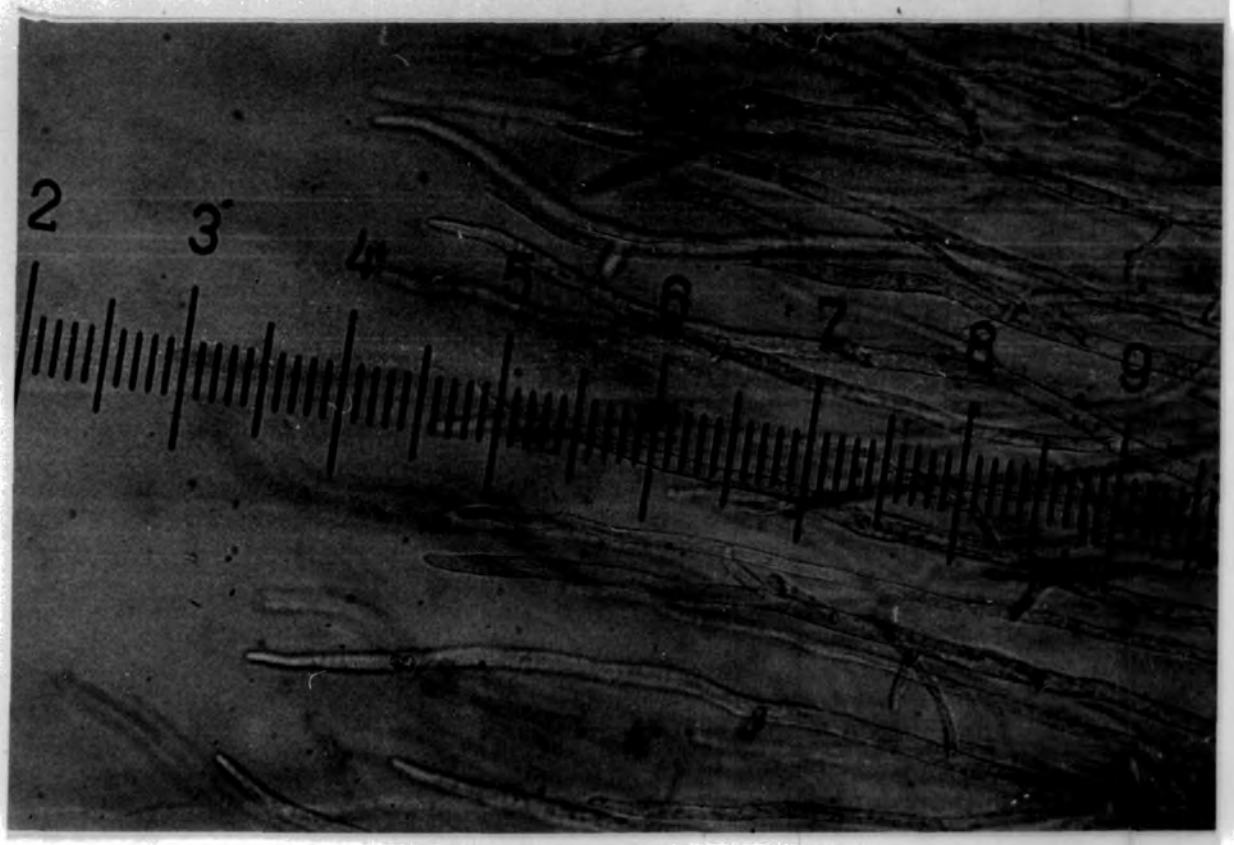
Fig. 17: *Achlya beneckii*, 48 hr. old colony: X143

A - zero time (before flooding)

B - 30 min. after flooding with liquid medium.



A



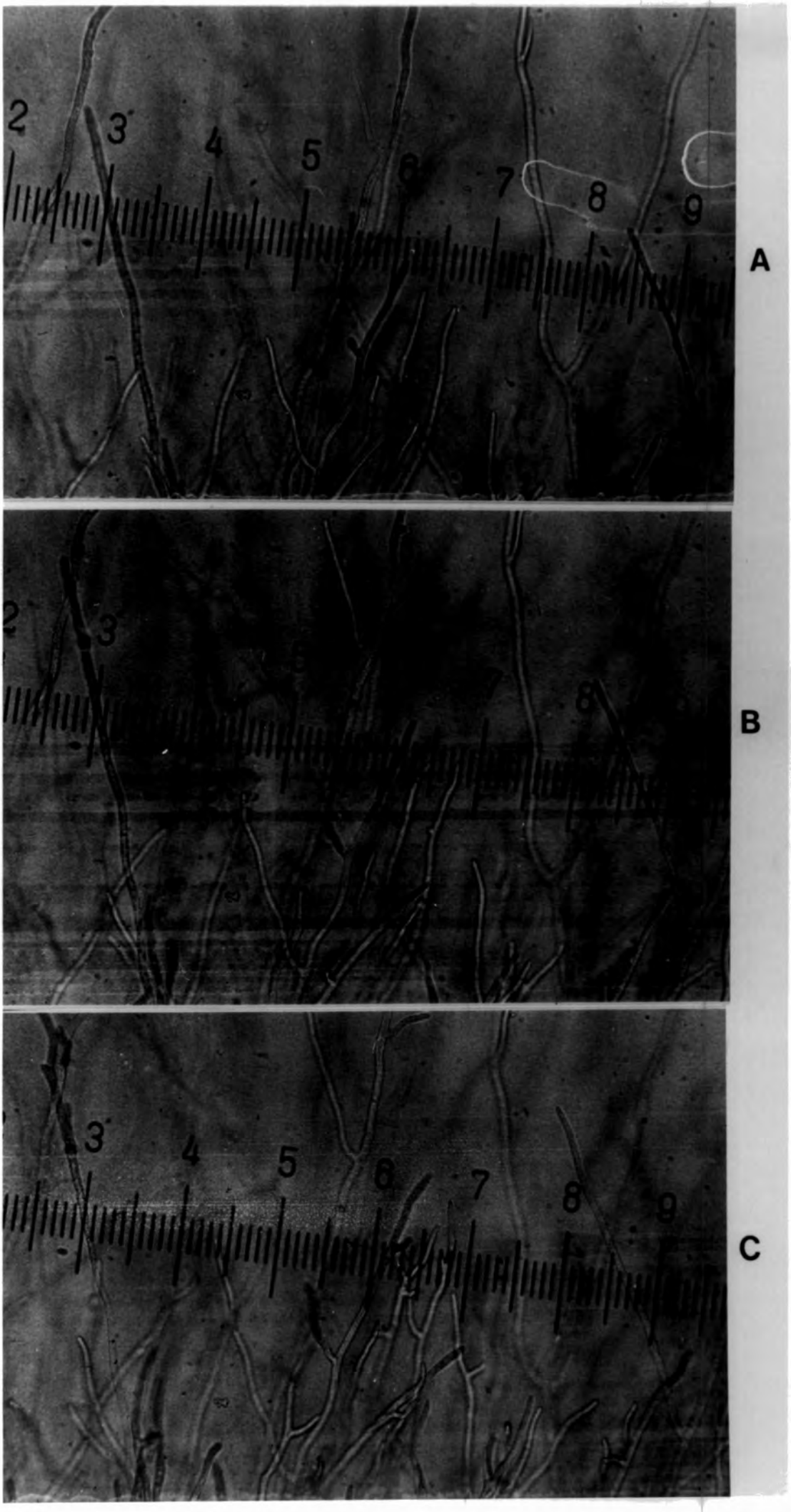
B

Fig. 18: *Saprolegnia parasitica*, 48 hr. old colony, X143

A - zero time

B - 15 min. after flooding

C - 30 min. after flooding.


2
3
4
5
6
7
8
9
A
2
3
8
B
3
4
5
6
7
8
9
C

vesicles and more cytoplasmic components (Plates, 2,4). Vesicles of various sizes and shapes, similar in appearance to those observed in the fungal tips of many fungi (Grove, 1978; Ramadani, 1978), were found throughout the sections; these constituted of large vesicles with mostly dark-staining globules (as above) and small vesicles with a medium to dark granular content, plus others with very opaque granules (Plates, 3,4).

Mitochondria appeared to have extremely different sizes and shapes. In transverse sections (Plates, 2,4), they appeared rather uniform, mostly spherical, but some were elongated with irregular profiles. Sometimes a discontinuity could be seen in the outer membrane (Plate 4). In longitudinal section (Plate 5), they looked more elongated, irregular, some were rectangular in shape with cristae inserted at the edges and a granular content in the middle. Dictyosomes occurred as stacks of short tubules or vesicles of irregular profile (Plates 2,3). Endoplasmic reticulum was very sparse, occurring as sheets of double membrane, smooth or rough surfaced, *i.e.* lined with ribosomes (Plate 4), which were observed to be densely packed in the cytoplasm and around all organelles (Plates 4,5). Free multivesicular bodies (mvb) were also found in almost all sections scattered in subapical and vacuolation zones (Plates 2,3). In subapical regions the mvb were found in a close association with the plasmalemma (arrows, Plates 2,3), producing pockets of vesicles identical in appearance to lomasomes.

With increasing distance from the apex, the vacuolation zone developed, which was characterized by the presence

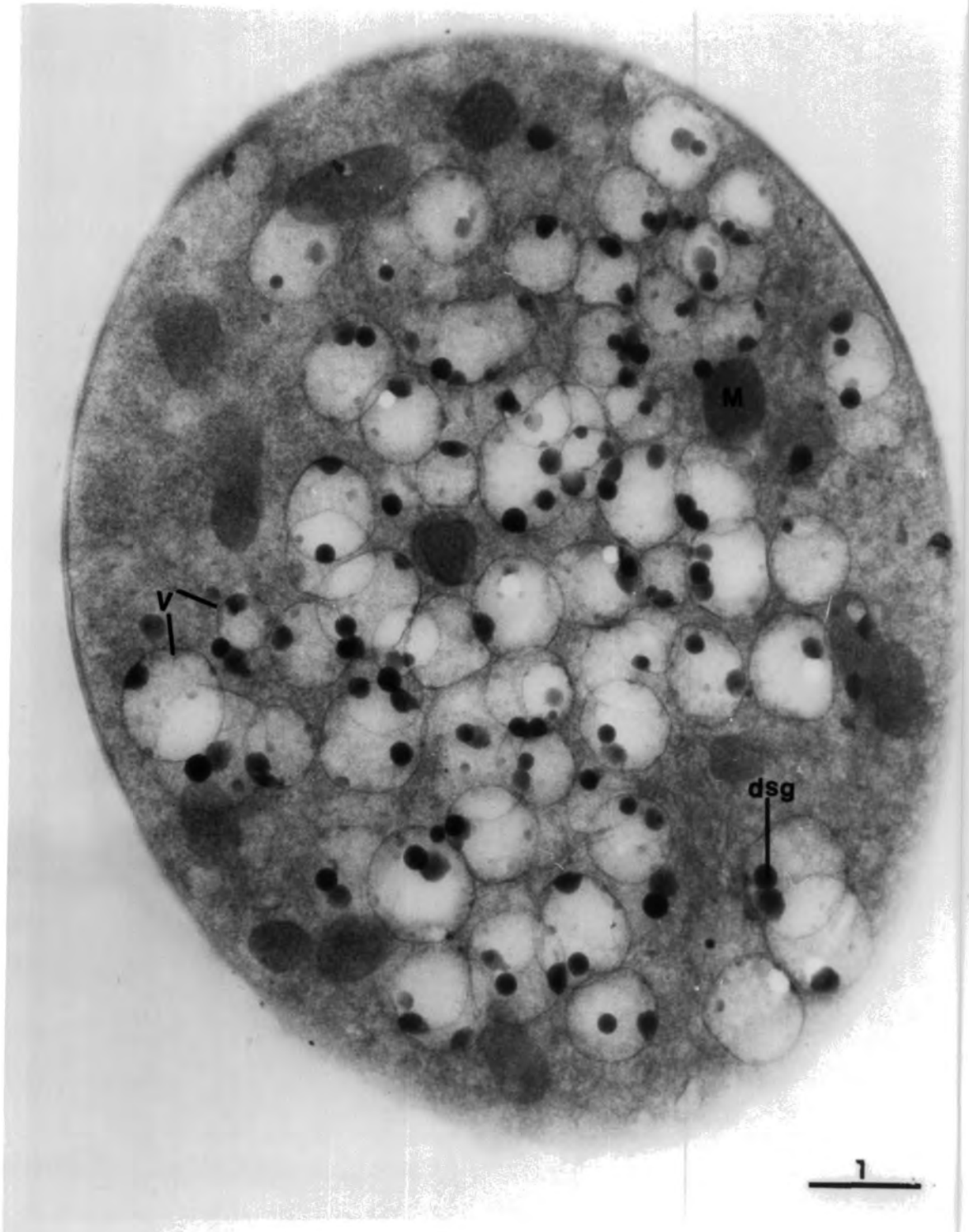


Plate 1 T.S. through a control hyphal tip of Phycomyces blakesleeanus

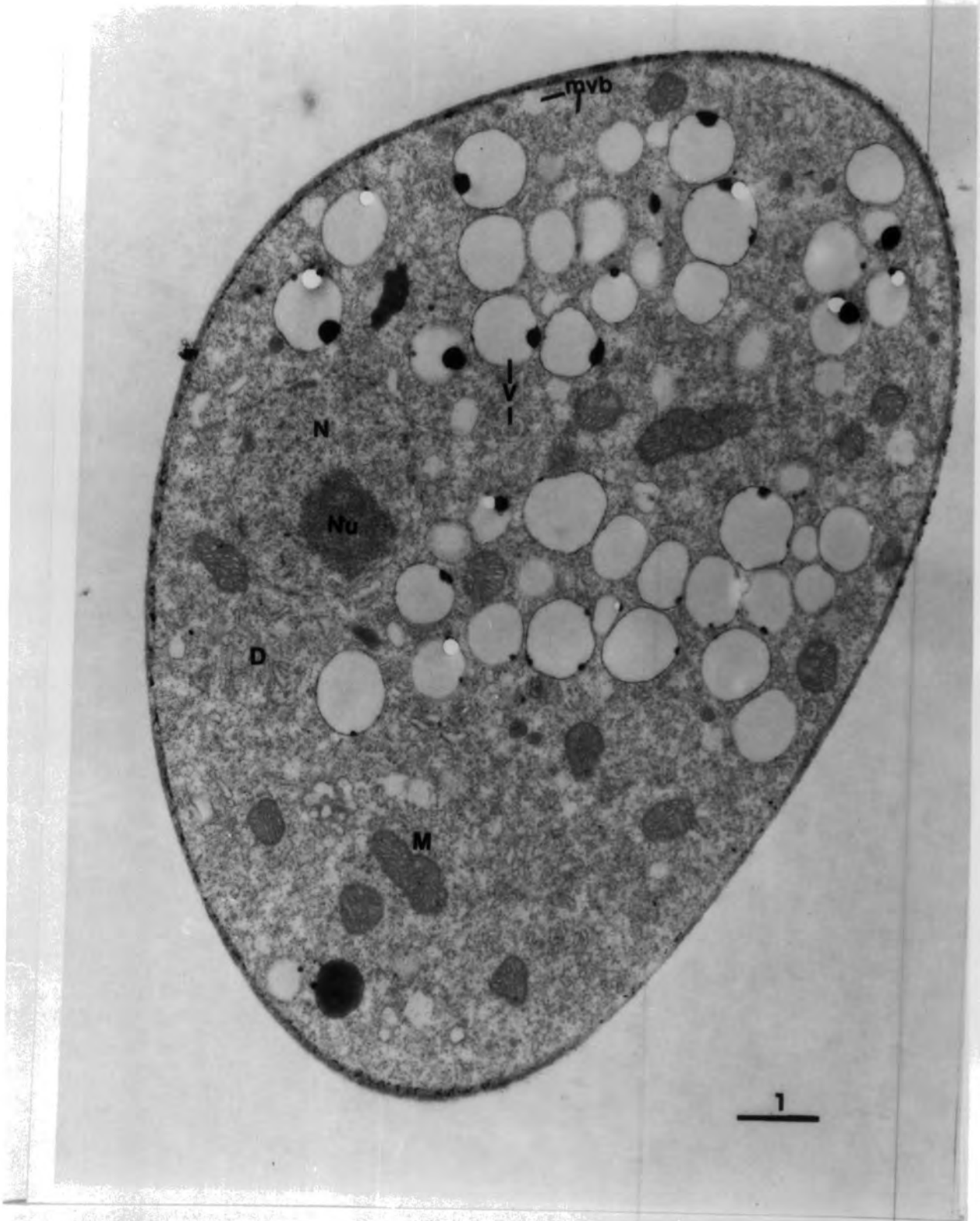


Plate 2 T.S. through a sub apical region of control hypha of Phycomyces blakesleeanus

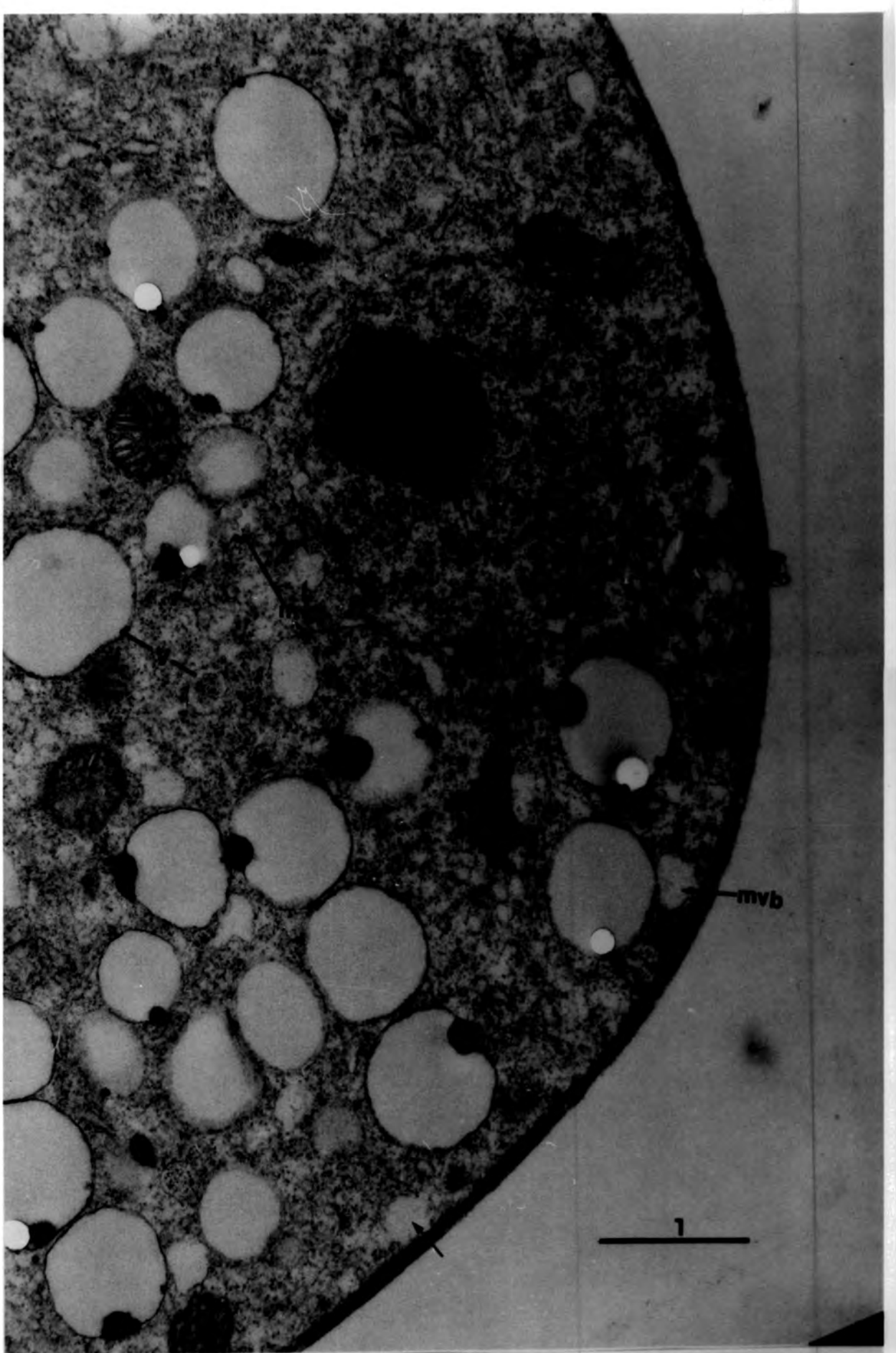


Plate 3 Higher magnification of part of Plate 2 showing a close association of mvb and Plasmamembrane

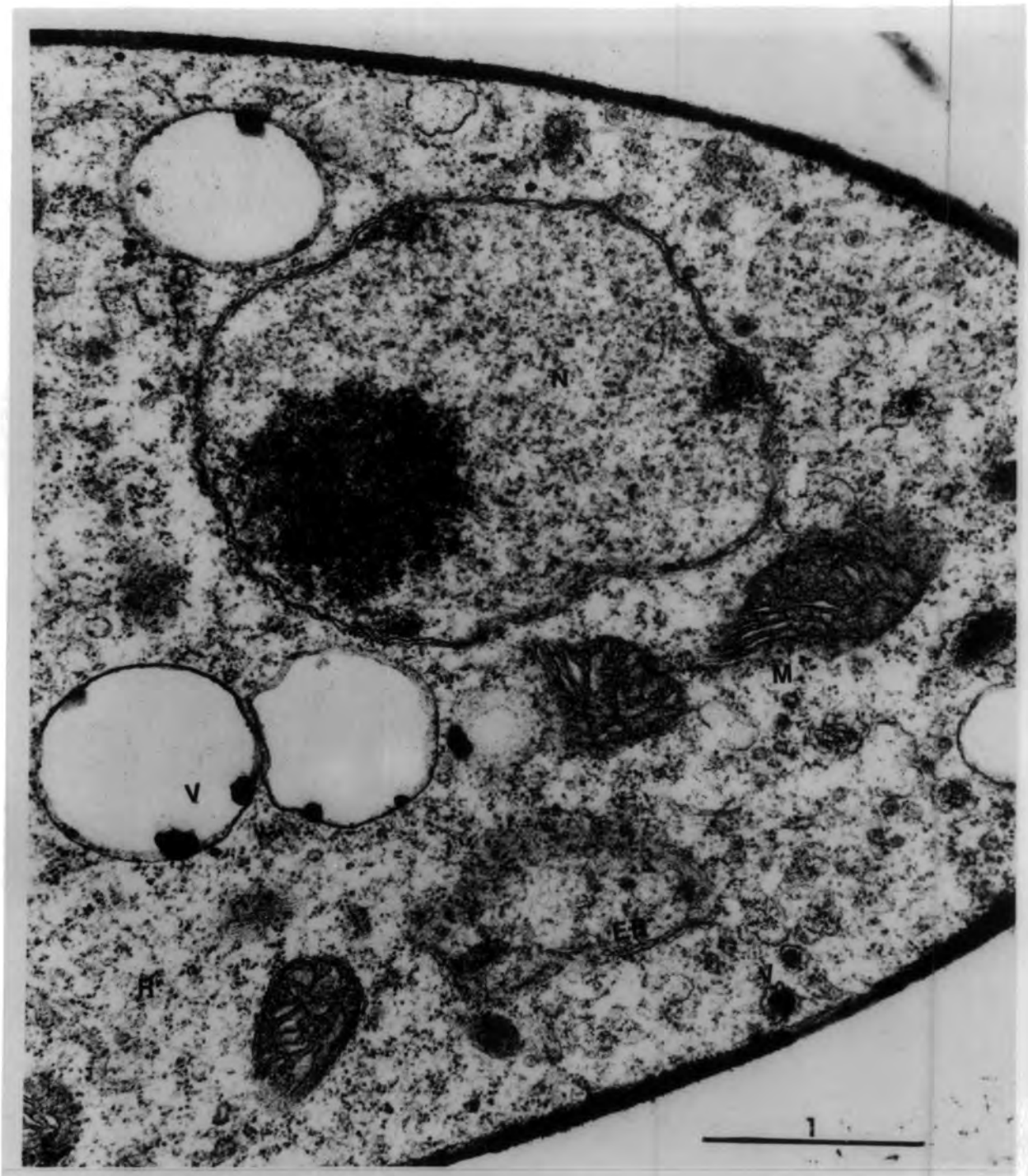


Plate 4 Higher magnification through a sub apical region of control hypha of Phycomyces blakesleeanus

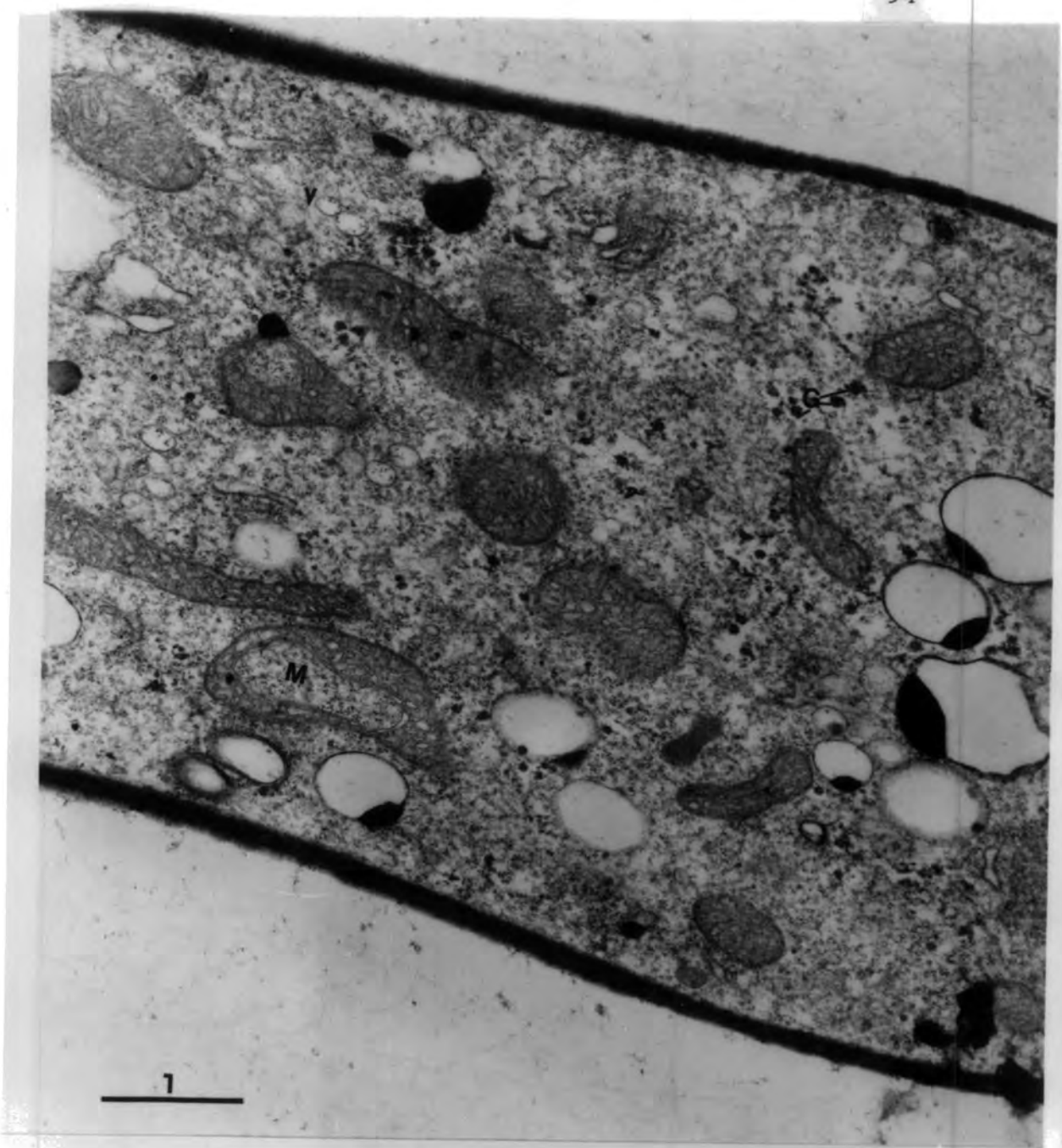


Plate 5 L.S. through a control hypha of Phycomyces blakesleeanus

of large vacuoles, less cytoplasmic components, absence of vesicles except mvb, and highly electron dense aggregates designated as food reserves because of their resemblance to glycogen aggregates (Plate 6). In this region also there seems to be an extension of the plasmalemma into the cytoplasm which appeared as an aggregation somewhat like the membranous discs. Similar membranous structures have been observed by Ramadani (1978) in hyphae and germinating spores of *P. blakesleeanus*.

The presence of low levels of glycogen granules in sections throughout the subapical area (Plate 5), compared to high glycogen content in vacuolation area, proved to be a useful marker for the location of sections along the hyphae, *i.e.* low glycogen content represent young region and high glycogen content represent old regions (Zalokar, 1959).

Since bursting of hyphal tips occurred within a few seconds of flooding the growing hyphae with liquid medium, therefore it was difficult to observe any differences of effect of flooding on hyphal tips before bursting. Plates 7,8 represent a longitudinal sectionsthrough burst tips which showed only a mass or aggregates of cytoplasm containing most of the cytoplasmic components (as seen in the control hypha), at the hyphal tip. It is also indicated in Plate 8 that the wall of the hyphal tip is not disintegrated or lysed but seems to be weakened in one point, pushed off and folded along the side of the hypha.

Transverse sections of a control hypha of *Mucor mucedo* showed quite a difference in shape and appearance of

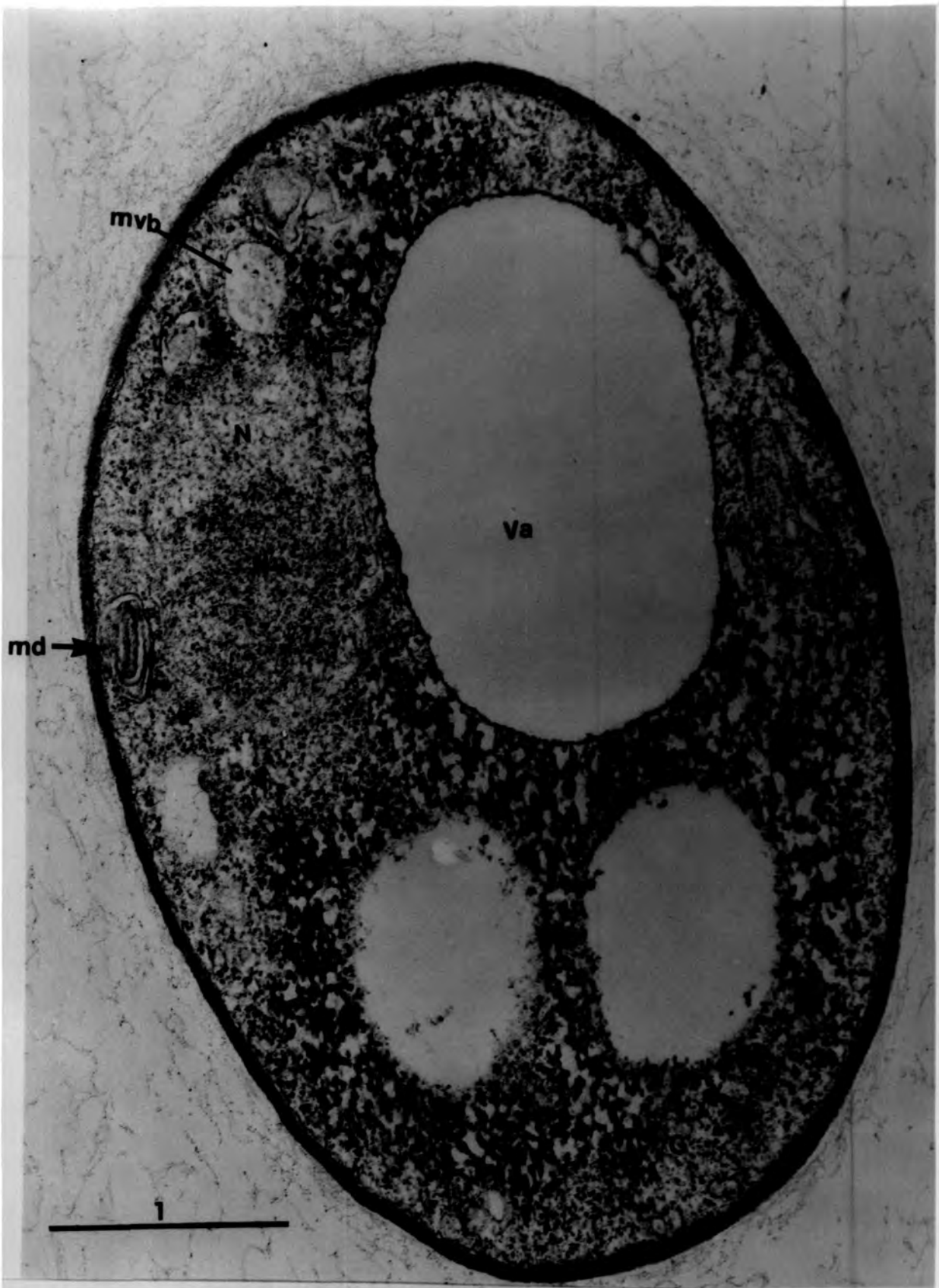


Plate 6 T.S. through a vacuolation zone of control hypha of Phycomyces blakescleeanus

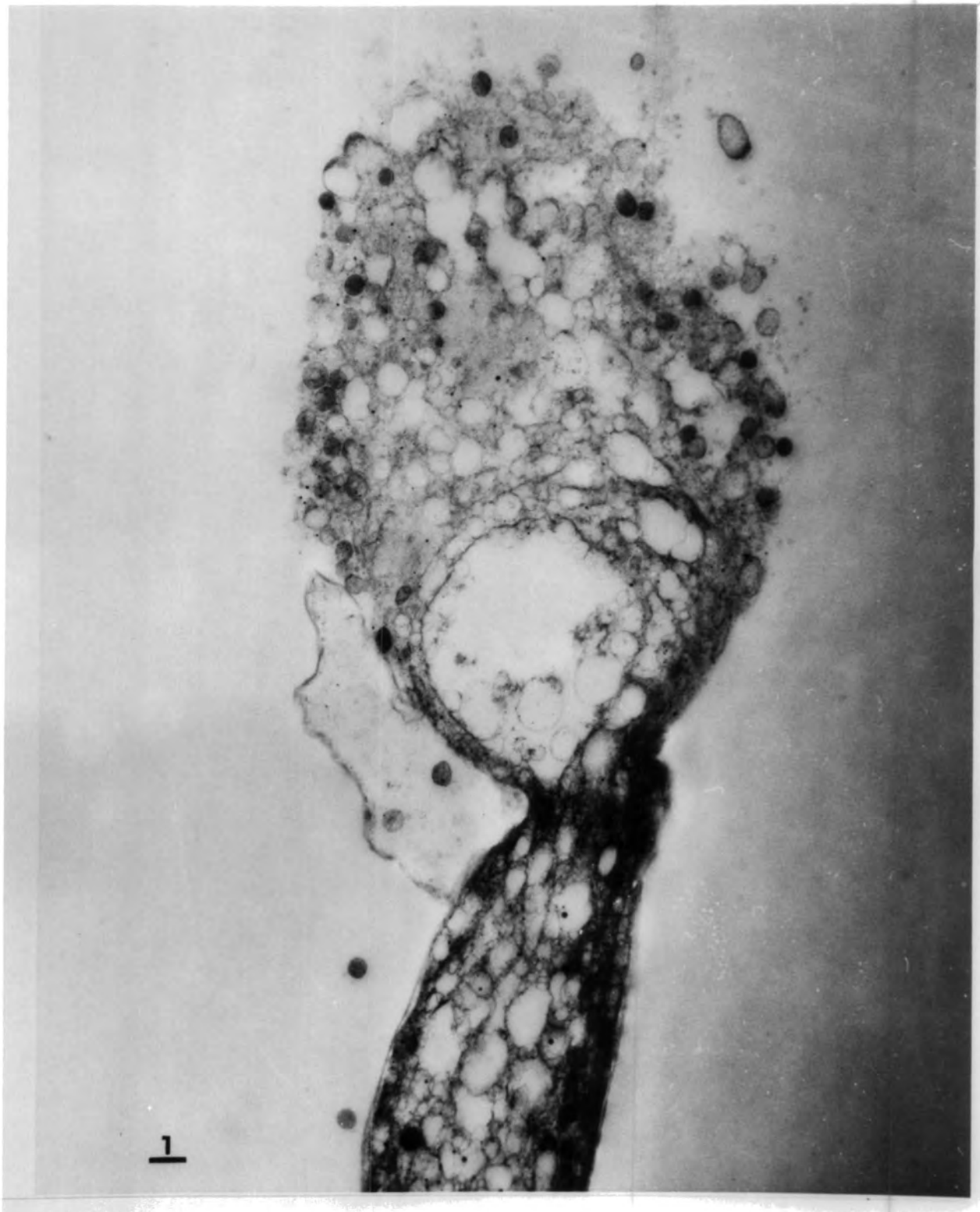


Plate 7 L.S. through a burst hyphal tip of Phycomyces blakesleeanus

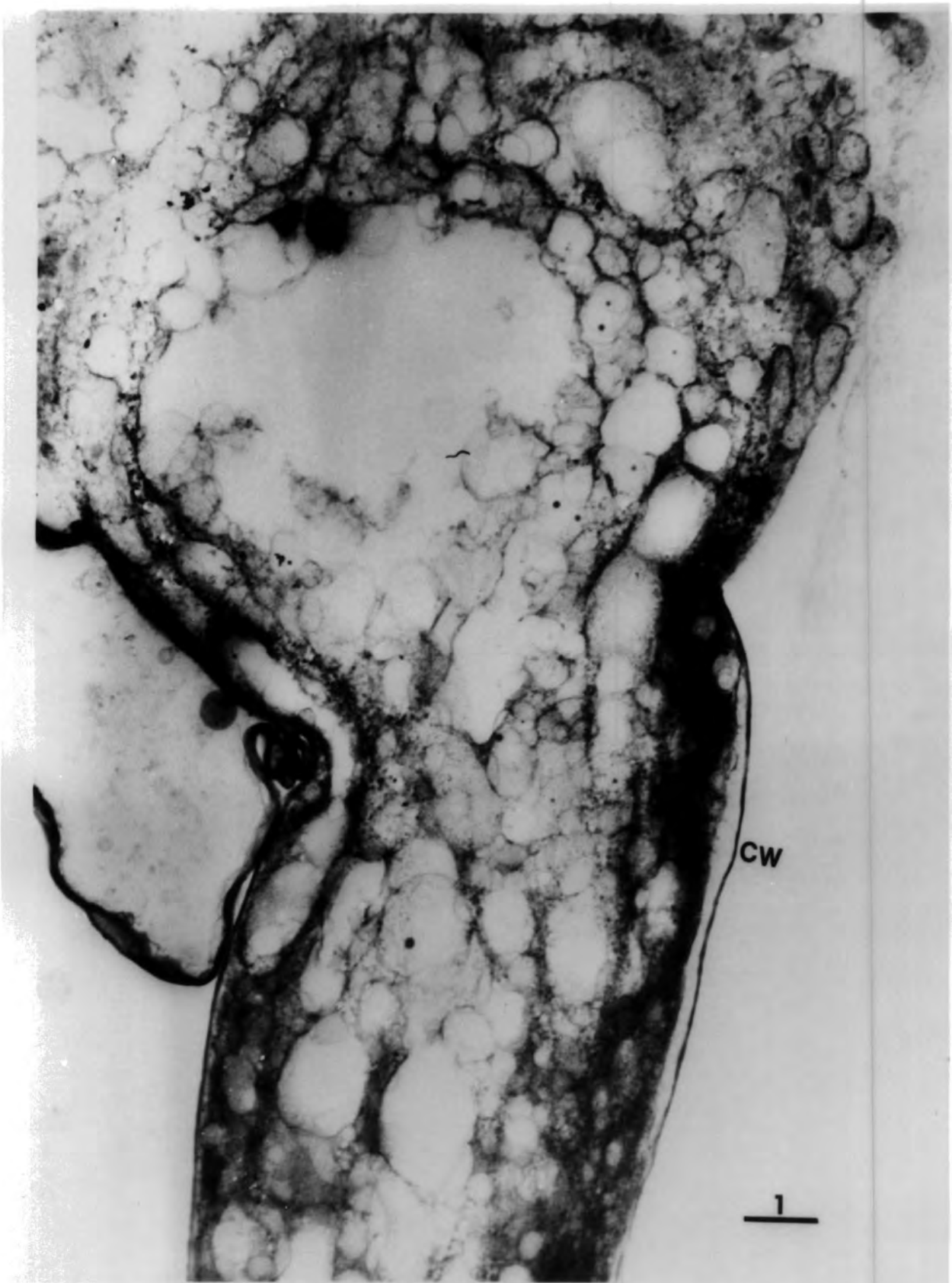


Plate 8 Higher magnification of a burst hyphal tip of Phycomyces blackesleeanus

Plate 9 T.S. through a burst hyphal tip of Phycomyces blakesleeanus

cytoplasmic vesicles. Plate (3) showed large vesicles with mostly irregular profiles, lacking dark staining globules (found in *Phycomyces*). Some vesicles showed a medium staining content in the form of amorphous or fibrous inclusions. Small vesicles were more or less similar to those observed in *Phycomyces blakesleeanus, i.e.* with medium to dark granular contents. Others appeared with opaque granules (Plates 10,11).

A third type of vesicle-like structures were also observed. These were fairly large, possessed a double membrane with membranous and amorphous inclusions; some of these vesicles showed a close association or fusion with the plasmalemma (Plate 11 arrow). Continuity between cytoplasmic vesicles and the plasma membrane was also seen (Plate 10 arrows) as has been observed by other investigators on the curved portion of the hyphal apex (Grove, 1978; Ramadani, 1978). Mitochondiia showed more uniformity, mostly spherical and possessing organized cristae. A few showed discontinuities or distorted profiles (Plate 10) on cristae (Plate 11). All other cytoplasmic components appearing in these sections resembled the ones seen earlier in the subapical region of *Phycomyces blakesleeanus*, except that no multivesicular bodies were detected in this region of *Mucor mucedo*. However, mvb were quite often seen in regions below the above ones or in older regions in connection with the plasma-membrane (Plate 12). Lomasome-like structures also occurred in this region (arrows).

Sections through a normal lateral branch formation showed a significant accumulation of large and small vesicles in addition to lomasome-like structures (Plate 13). Also, quite a considerable number of mvb were observed at the apex

and at potential sites of branch initiation where the wall must extend. This was also observed in *Fusarium culmorum* hyphae (Marchant and Smith, 1968). The most striking feature of the earlier reaction of *Mucor mucedo* hyphal tips upon flooding with liquid medium was a high overall increase in almost all cytoplasmic components (Plate 14). Numerous vesicles of various sizes, different in shape and appearance from those observed in the control hypha were concentrated near the periphery in particular site(s) of the apex. The presence of these vesicles in such particular sites suggests that they might be involved in some processes related to the cell wall and may also indicate the site of branch initiation. Ribosomes were very conspicuous and abundant. The numbers of mitochondiia (variable in size and shape) and nuclei were also greatly increased as compared with the control hypha, prolonged flooding of these hyphae with liquid medium led to the development of two or more apical branches (Plate 15), which showed the same organization of cytoplasmic constituents as those of main hyphae. Occasional aggregates of glycogen-like materials and lipid droplets were also observed in apices of these branches (Plate 18). No mvb and lomasome-like structures were observed, except in the older parts of the hyphae and branches (Plate 19), where these structures were found to be quite abundant.

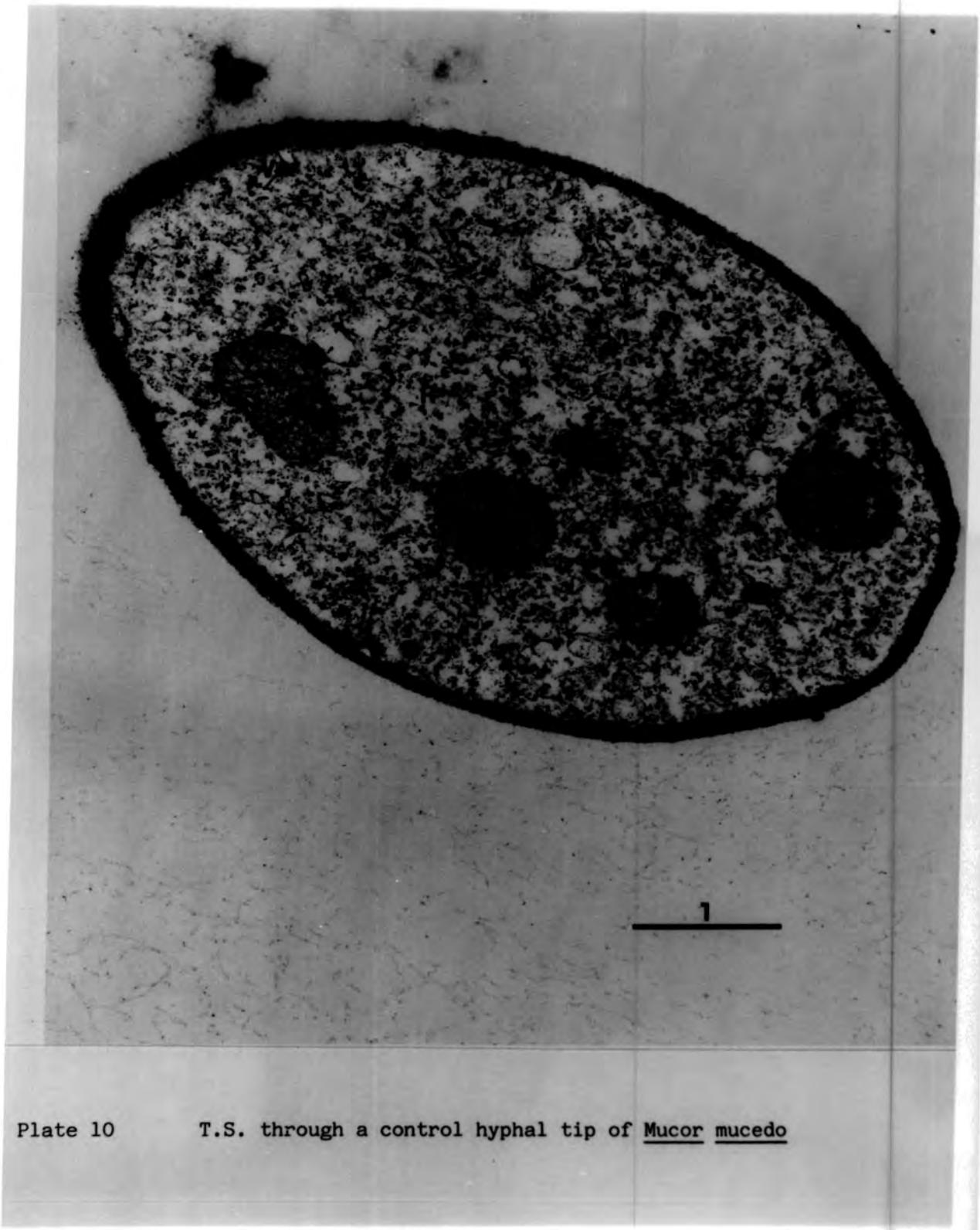


Plate 10 T.S. through a control hyphal tip of Mucor mucedo

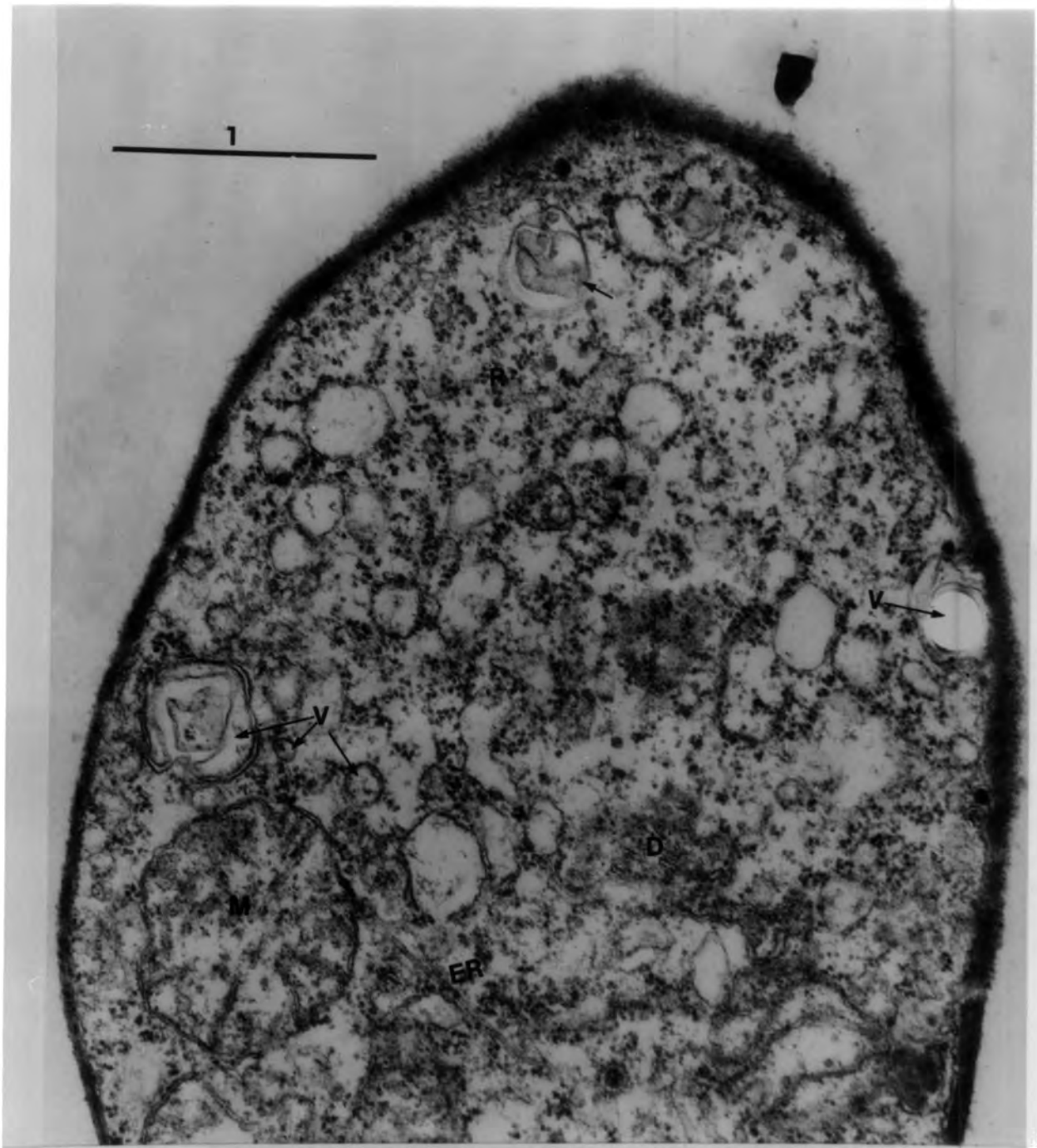


Plate 11 T.S. through a control hyphal tip of Mucor mucedo

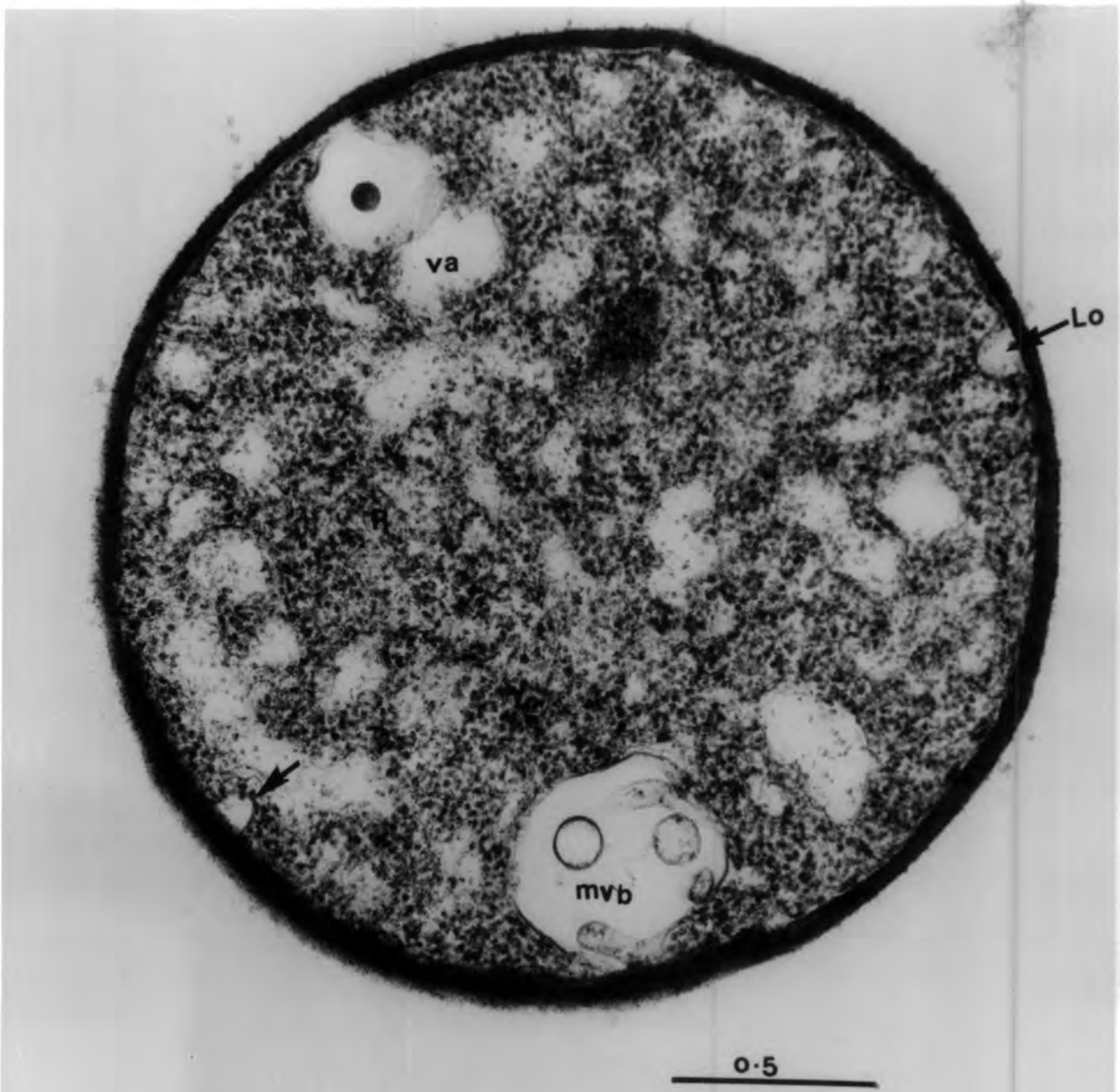


Plate 12 T.S. through a control hypha of Mucor mucedo

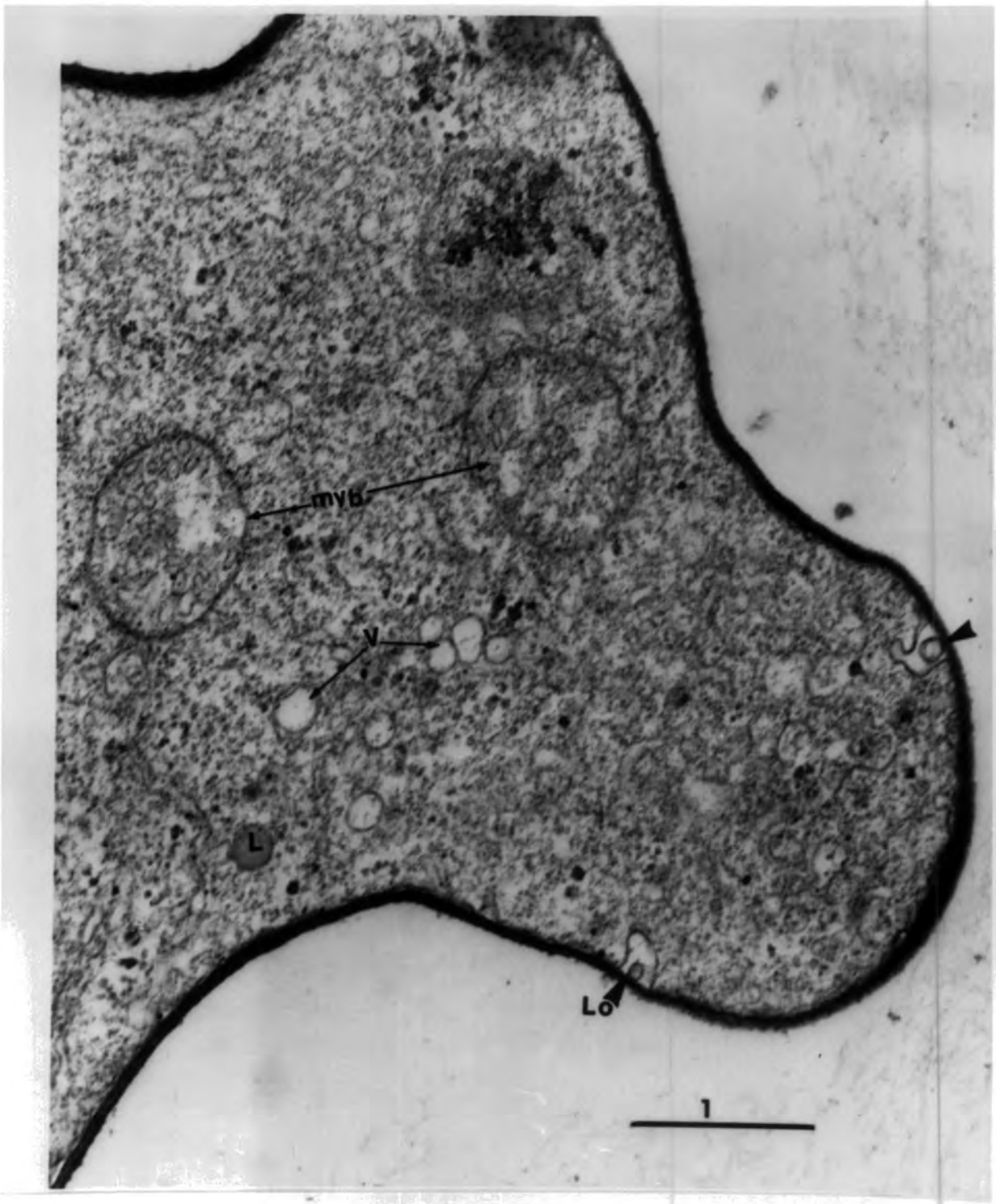


Plate 13 T.S. through a branch initiation in a control hypha of Mucor mucedo

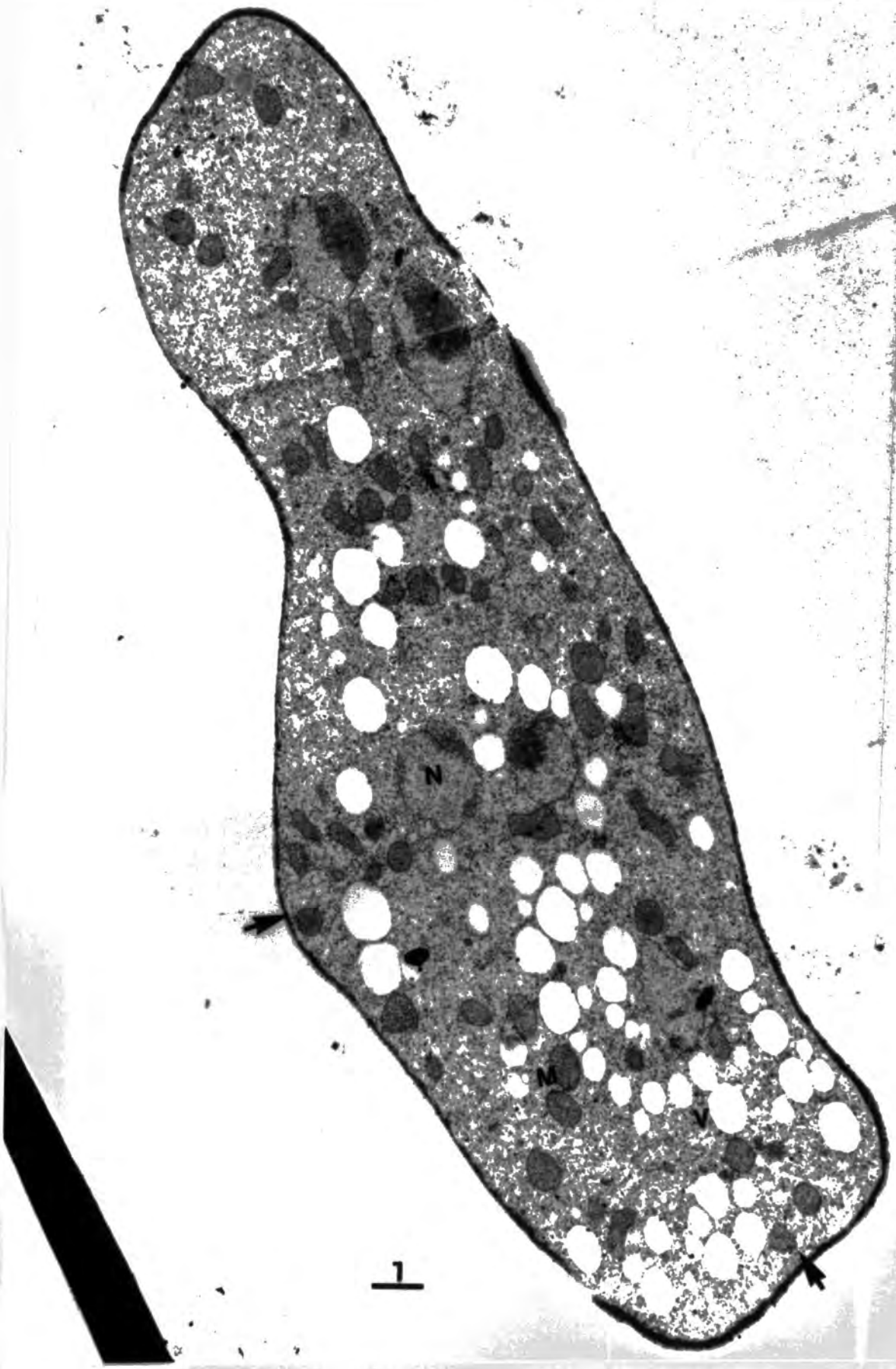
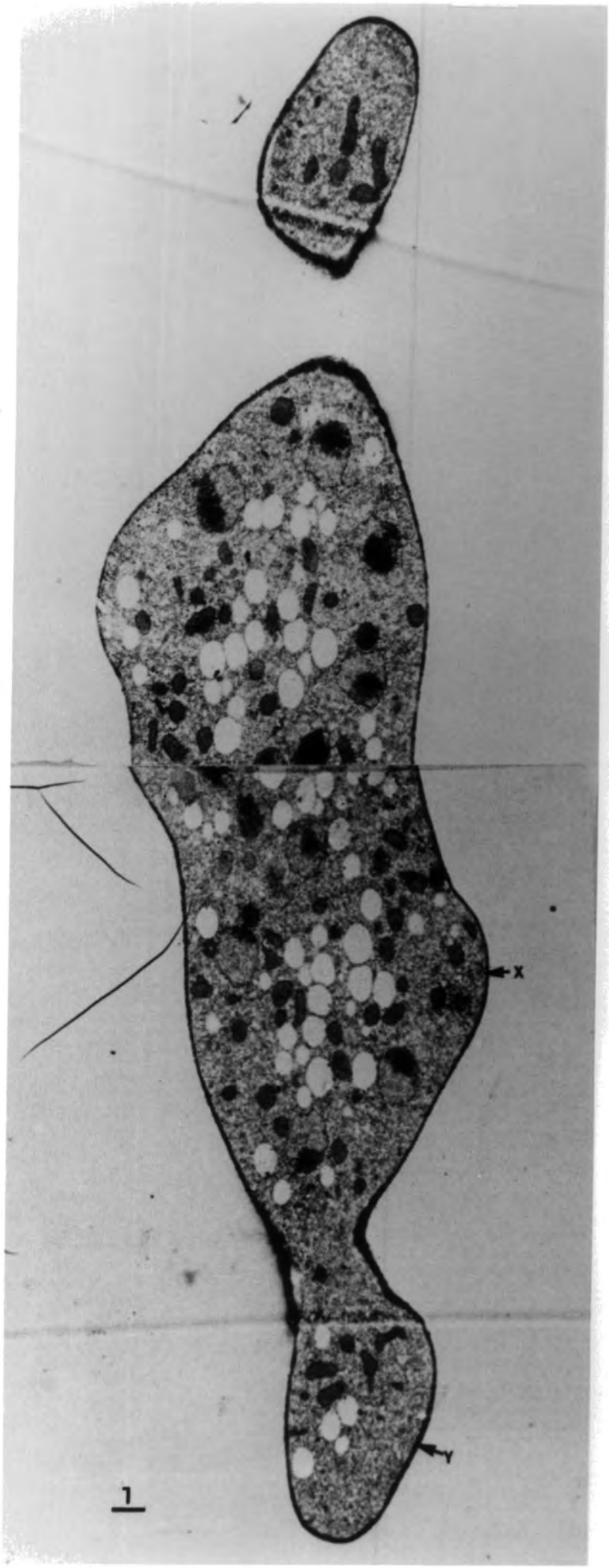


Plate 14 T.S. through a hypha of Mucor mucedo, 15 min after flooding with liquid medium

Plate 15: T.S. through a branched hypha of *M. mucedo*, 30 min. after flooding with liquid medium.


X
Y
1

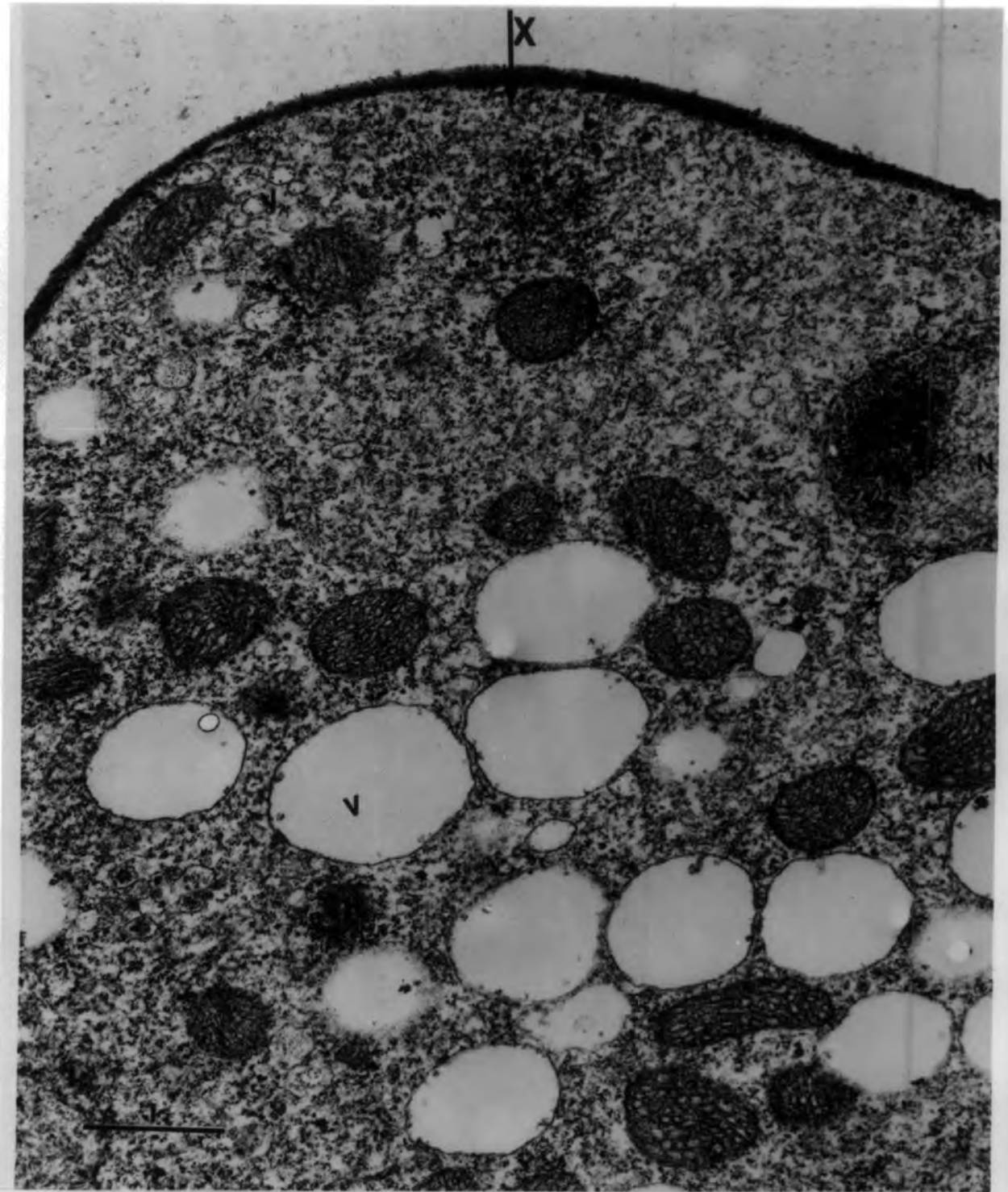


Plate 16 Higher magnification through (x) region of Plate 15

Plate 17 Higher magnification of a well developed branch (Y region) of plate 15

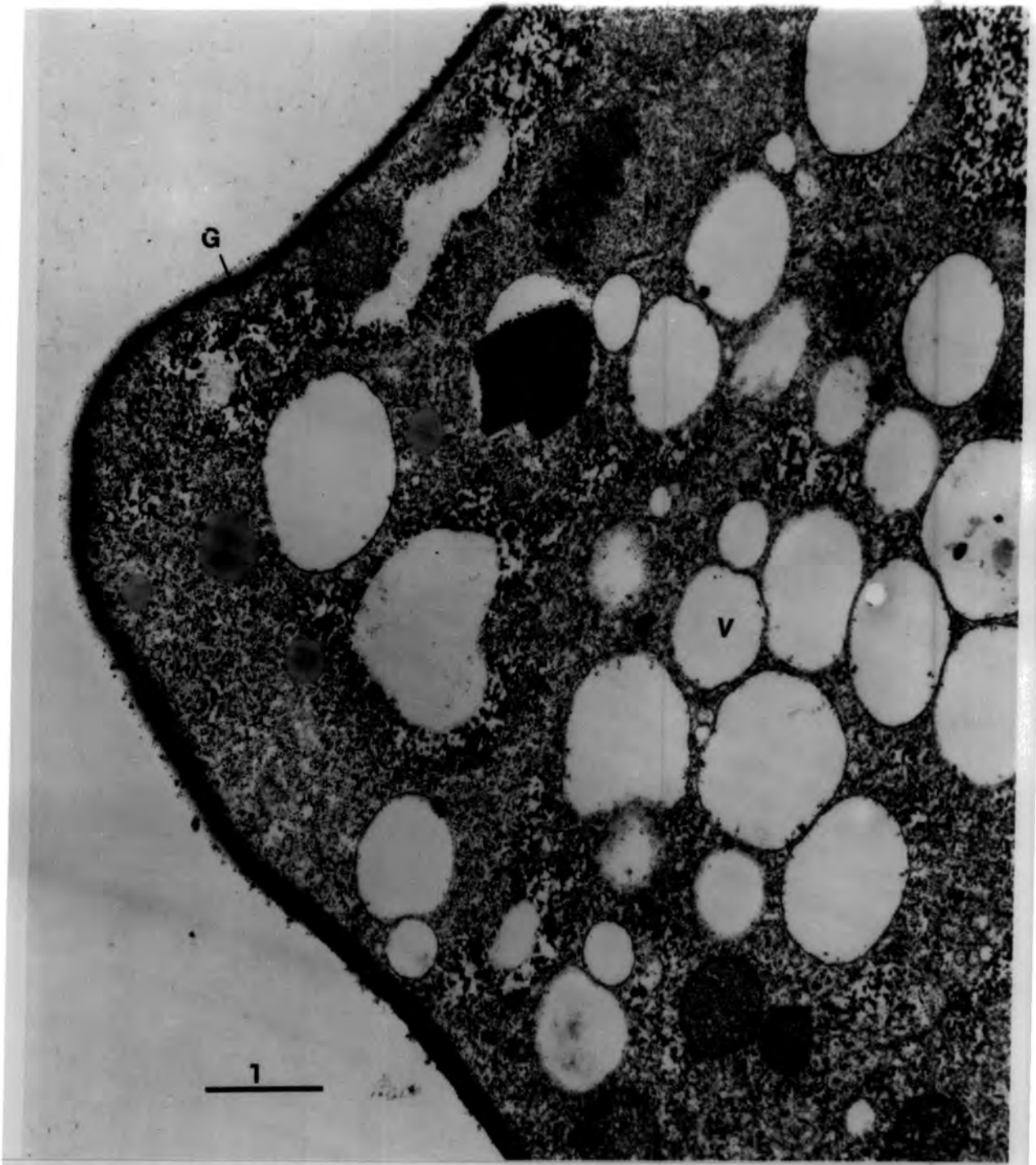


Plate 18 T.S. through a branch initiation of treated hypha of Mucor mucedo

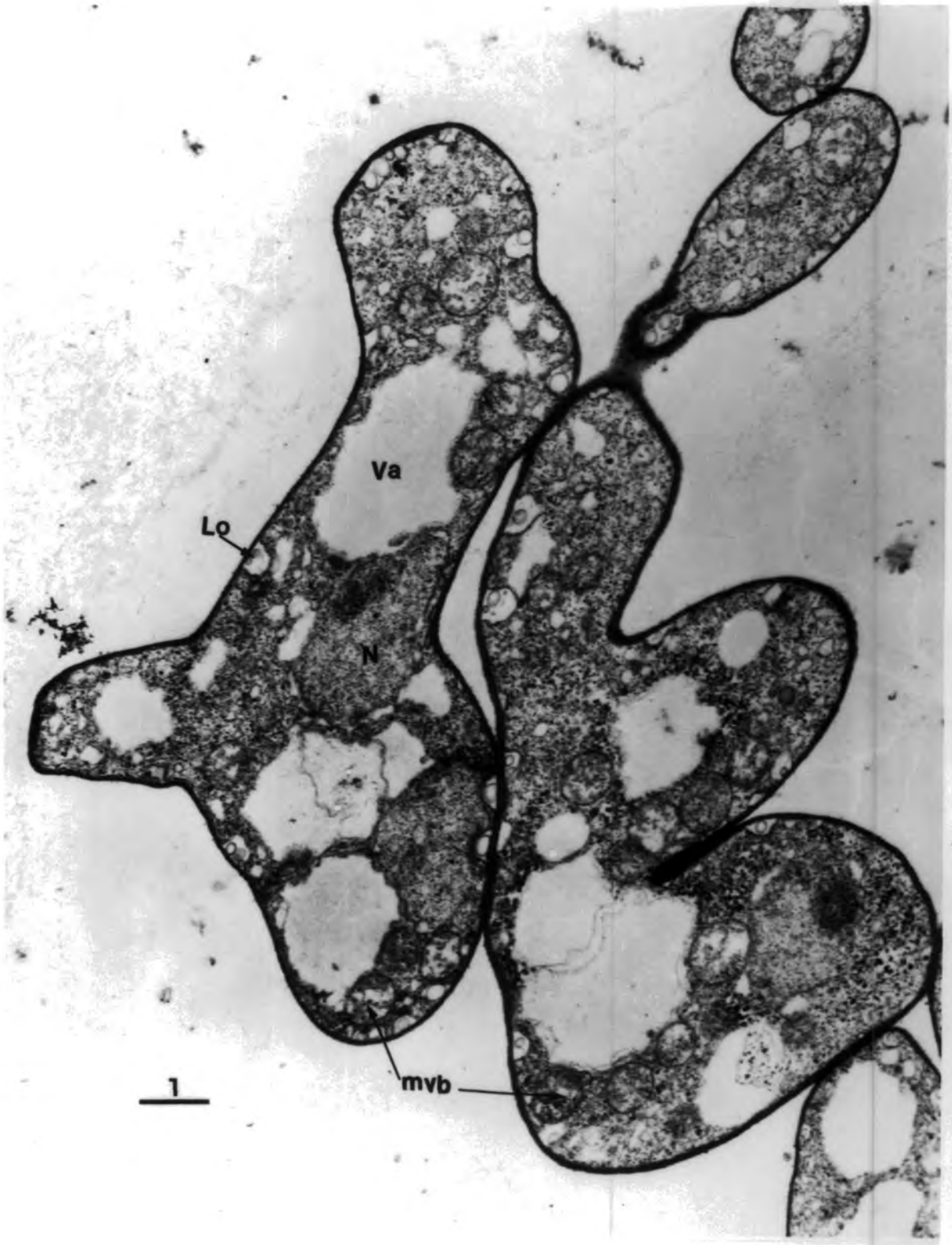


Plate 19 T.S. through a vacuolation zone of treated hypha of Mucor mucedo

2.4 Discussion

Ultrastructural studies of both species of fungi, belonging to the same class, represented the typical features of the cytoplasmic constituents described for other species of fungi (Grove, 1978). Sections showed some similarities in their major types of protoplasmic components and organization. However, some variations were also detected, particularly in shape, size and staining property of their large apical vesicles, although the same fixation and staining method was used for both fungi. This may reflect differences in vesicle content such as hydrolytic or wall synthesizing enzymes. However many investigators have noticed that the appearance of the cytoplasmic vesicles varies according to the fixation and staining method used; in aldehyde-osmium fixed hyphae, the vesicles contain electron dense material which may be dispersed or condensed (Grove, 1978; Ramadani, 1978).

The preferential accumulation of these vesicles in the apical region and during the initiation and growth of branches, may also suggest their involvement in wall formation. This suggestion is in agreement with the conclusion of Girbardt (1969); Grove *et al* (1970), for morphologically similar vesicles in other fungi.

Presence of large and small vesicles throughout the cytoplasm may indicate that the site of their production may be in the more mature regions of the hyphae, as was suggested by Trinci (1978) in that these vesicles are formed in remote subapical regions of the hypha, although no evidence for the origin of vesicles from any structure could be observed in

this study. A sharp decrease in vesicle concentration from hyphal apex observed in the test fungi was also observed by Girbardt (1969) in *Polystictus versicolor* and by Collinge *et al* (1978) in *Penicillium chrysogenum*. Thus the decline in the rate of wall synthesis from tip to base of the extension zone is reflected in a comparable gradient in vesicle concentration in the tip, this, in turn, may reflect a gradient in the rate at which vesicles fuse with the protoplasmic membrane (Gooday and Trinci, (1980).

In *P. blakesleeanus*, multivesicular bodies were observed in young and old regions of the hypha, whilst in *M. mucedo* these structures were only found in older regions of the hypha. These structures have already been reported in *P. blakesleeanus* sparangiosphore by Peat and Banbury (1967), Marchant *et al* (1967) who observed these vesicular bodies in the apical region of stage 1 sparangiosphore. They were, however, more commonly found towards the base of the growing zone of stage IVb, in association with the endoplasmic reticulum. However, these multivesicular bodies were only reported in the older regions of *Fusarium culmorum* and *Coprinus lagopus* hyphae, and were absent from *Pythium ultimum* (Marchant *et al*, 1967). These authors therefore associated these structures with the deposition of chitin microfibrils since they were absent from *Pythium* hyphae.

It is generally considered that rapid expansion of the primary wall and plasma membrane which occurs during tip growth is always associated with vesicles (McClure *et al*, 1968; Girbardt, 1969; Grove *et al*, 1970), which carry to the tip

hydrolytic enzymes together with wall synthesizing enzymes. These fuse with the plasma membrane, liberating their contents into the wall. Hydrolytic enzymes continuously loosen the wall at the tip and permit the insertion of new wall material (Bartnicki-Garcia, 1973). A large potential of wall lytic activity in hyphal apices has been observed by many investigators (Robertson, 1958, 1959, 1965; Park and Robinson, 1966b, Green, 1969). The release of lytic activity during growth must be a gradual process delicately co-ordinated with wall synthesis. The balance between synthesis and lysis of wall polymers can easily be disturbed and shifted in favour of lysis by a variety of external stimuli, resulting in violent disintegration of the hyphal apex (caused by either an increase in wall lysis or a decrease in wall synthesis), or under certain conditions by the formation of large apical swelling (Bartnicki-Garcia, and Lippman, 1972a, b; Rizvi and Robertson, 1965).

Bursting of hyphal tips by flooding with water or hypotonic solution (Robertson, 1958, 1959) might appear as an osmotic phenomenon. However Bartnicki-Garcia and Lippman (1972a) indicated that the above phenomenon (flooding with water), in addition to lowered pH or heating involve an active chemical process of apical wall weakening. This is indicated by the fact that temperature coefficient of osmotically-induced lysis is in the region 1.3-2.1 rather than 1.0 which would be expected if the process was purely osmotic. They, therefore, suggested that enzymic processes which weaken the wall are involved in tip bursting. In contrast flooding with divalent metal ions, Mg^{2+}, Mn^{2+} or Ca^{2+}, may cause only a partial in-

crease in wall lytic activity insufficient to weaken the wall to its bursting point but enough to temporarily increase the plastic area of the growing apex thereby giving rise to a swollen tip (Bartnicki-Garcia and Lippman, 1972a).

In higher plant cells wall extension is stimulated by acid condition and inhibited by Ca^{2+} ions (Ray and Baker, 1965), whilst acid solution caused the tips of fungal hyphae to burst (Bartnicki-Garcia and Lippman, 1972a). Likewise, Park and Robinson (1966a) had earlier suggested a rationalization of the action of acidic pH, pointing out that it could prevent the formation of new cross-linked material at the apex, whilst not affecting lysis and so leading to bursting. However Dow and Rubery (1975) found a reversible and antagonistic effect of H^+ and Ca^{2+} on the integrity of the tip wall of *Mucor rouxii*, which is possibly due to the modification of the physical state of the acidic polymer (mucoran), found in the hyphal tips, and not to a cleavage of covalent bonds. They suggested that H^+ ions cause the wall matrix to become less viscous so that the wall becomes more extensible, whilst Ca^{2+} ions cause the wall matrix to become more viscous so that the wall becomes more rigid. They pointed out also that such effects could be of general importance in those fungi with acidic polysaccharides in their hyphal walls.

The observation that bursting is not always strictly apical but may occur subapically or laterally, suggests that the lytic activity is not necessarily restricted or fixed to the apical dome wall. Since the highest concentration of vesicles occurs in the apical dome (Girbardt, 1957, 1969; Bracker, 1971), this would be the zone most severely and

rapidly degraded and hence the most frequent site of bursting. However, vesicles may also migrate to a subapical region causing a cytoplasmic discharge immediately below the apical dome or even further behind on the lateral walls. Some of the observed discharges of cytoplasm through lateral hyphal walls were probably at sites where branches were about to emerge and where the enzymatic machinery for apical growth was already congregated. The occurrence of spontaneous bursts of hyphal tips of *P. blakesleeanus* during normal growth of hyphae (undisturbed colony), suggest that the postulated balance between wall synthesis and wall lysis must be rather precarious and can readily drift in the direction of lysis.

Temporarily arrestment of hyphal tips of *M. mucedo* upon flooding with liquid medium, followed by apical swelling, indicate that metabolic activities of the apex did not stop, *i.e.* metabolism continued without extension, and hence developed the apical swelling. This was confirmed by the ultrastructural studies which showed a large accumulation of vesicles with nearly all other cytoplasmic components at the swollen tip. Later on the swollen tip produced two or more apical branches at the sites of vesicle accumulation. Trinci (1979) has reported that apical branches may be formed when the rate of supply of components required for primary wall growth exceeds the rate at which they can be incorporated into the tip wall.

The greater resistance of aquatic fungi tested, to bursting and/or hyphal tip swelling may reflect a greater metabolic stability of the apical walls of these fungi, or may reflect

the adaptation to their natural environment, particularly the case of *Allomyces arbuscula* which is known to contain chitin in its wall. On the other hand hyphal tips of *P. blakesleeanus* and *M. mucedo* may represent a weak microfibrillar aggregation or orientation since it has been reported that aggregation together with the characteristic interlacing of the microfibrils are both responsible for establishing and providing the strength of the chitin which occupies the innermost region of the wall and the extreme tip (Burnett, 1979). However, no studies on microfibrillar orientation are available relating to vegetative hyphae of *P. blakesleeanus* and *M. mucedo*, except the microfibrillar orientation of rapidly expanding Phase 1 aporangiophore of *P. blakesleeanus* (Roelfsen, 1950a,b, 1958). Therefore, more studies may be needed to investigate the possible alterations imposed on the ultrastructural architecture of wall under flooding. However, no accurate comparison relating responses to flooding, could be presented here due to the different culture media used to grow the two groups of fungi. This fact may also be the reason behind these phenomena, taking into account the possibility that the culture conditions might affect wall thickness, physical and chemical properties of the wall polymers (Miles, 1981) and thus the structure and strength of the wall. In fact, 1% malt extract liquid medium did not support any growth of *P. blakesleeanus* and *M. mucedo* at all temperatures tested, between 18-30^{o}C, whereas on solid malt extract very sparse colonies were developed at room temperature (20±2).

Additionally, nothing could be detected from the ultrastructural studies that may allow an interpretation of tip bursting or swelling and apical branching of the tested hyphae. However, the possibility of the involvement of imbalance between wall synthesis and wall lysis is rather acceptable, since bursts of hyphal tips were also recorded during normal growth of hyphae of *P. blakesleeanus*.

CHAPTER THREE

PROTOPLASTS REGENERATION USING IMMUNOLOGICAL TECHNIQUES

3.1 Introduction

The production of protoplasts in fungi has simplified the study of their biochemistry, morphology and genetics; it is based on the assumption that they are physiologically normal, retaining all the properties of the intact cells from which they derived, including the ability to make new cell wall. Therefore, since the regeneration process in most fungal protoplasts takes place very slowly (Villanueva and Garcia Acha, 1971), it is possible to follow the individual stages of the biosynthesis of the new wall and may demonstrate potential differences in wall formation process between cellulose and chitin-walled fungi. Svoboda *et al* (1969) stated "It is possible in these protoplasts to trace a gradual construction of single wall components, their arrangement in a complex structure, the regulatory mechanism of these processes and their relation to the other cell structure".

Studies on development of new wall materials during regeneration in fungi have been accomplished by many investigators using fluorescent brighteners, such as Calcofluor (Peberdy and Gibson, 1971; Gull *et al*, 1972; Peberdy and Buckley, 1973; Saadi, 1980).

The successful application of the immunofluorescent technique in the observation of the sites of cell wall extension in the yeast *Schizosaccharomyces pombe* by May (1962), suggested that this method may be useful in studying sites of cell wall extension. Subsequently investigation of the growth of filamentous fungi using IMF technique have been made by Goos and Summers (1964) on *Candida albicans* and two

species of the fungus *Fusarium*. With *C. albicans*, it was noted that wall material of the parent cell is incorporated into the wall of the daughter cells or into hyphal walls when they are produced. With *Fusarium* species, however, they found antigenic dissimilarity in the walls of hyphae and conidia during conidial germination and conidiation. Fultz and Sussman (1966) also reported antigenic differences in the walls of hyphae and rhizoids of *Allomyces macrogynus* using the IMF technique, and observed changes in the structure of mycelial walls with age. Marchant and Smith (1968) demonstrated apical growth of hyphae of *Fusarium culmorum* using this method and observed serological differences between various parts of the growing hypha (hyphal apex and older regions of the hypha). Hunsley and Kay (1976) also used this method in their study on surface distribution of wall components using antibodies to specific wall fractions.

The use of IMF technique in the study of wall regeneration by protoplasts may be useful if wall specific antisera could be prepared. However, little is known about the localization of the active antigenic substances in the organisms nor has their part in eliciting an immunological response been defined. In order to find to what extent antigens exist in the cytoplasm and whether they differ from surface antigens, Hearn and Mackenzie (1979) prepared antisera against wall fractions and protoplasts from *Aspergillus fumigatus*. Their results suggest that there was a common antigenic component both in the wall and the sub-cellular material. But, however, extracts from the wall and protoplasts contain antigens which differ both in immunological reactivity and in chemical structure. Studies

by Wilson and Hearn (1982) on wall-free cytoplasmic extracts obtained from protoplasts, and on wall fractions of *Aspergillus fumigatus*, by the Enzyme-Linked-Immunosorbent Assay (ELISA), have shown that the protoplasts contained substances which differed antigenically from surface-located structure.

Although protoplasts from fungi have been widely produced using wall lytic enzymes (references below) or mechanically (Banbury personal communcation and Saadi, 1980), mechanical and other non-enzymatic methods have not been used extensively. This may be because they are specific for a particular oganism or parts of organism such as sporangiophores. Additionally the technique is time consuming to obtain large amounts of protoplasts and there are some doubts regarding their physiological state. Most workers have favoured the use of lytic enzymes for protoplast isolation, these generally being the most rapid for bulk extraction (Peberdy, 1979b).

The first observations of the protoplast formation in filamentous fungus *Neurospora crassa* were reported by Emerson and Emerson (1958) and Bachmand and Bonner (1959), who used a snail juice enzyme preparation. Subsequently protoplasts have been prepared successfully from a number of chitin-walled fungi by the action of:

(1) Snail juice preparation on: *Fusarium culmorum* (Aguirre and Villanueva, 1962), *Trichothecium roseum* (Garcia Acha and Villanueva, 1963), *Geotrichum candidum* (Gull *et al*, 1972), *Dactylium dendroides* (Amaral *et al*, 1983).

(2) By lytic complexes preparation from *Streptomyces* species: on *Fusarium culmorum* (Aguirre and Villanueva, 1962, Lopez-Belmonte *et al*, 1966, and Garcia Acha *et al*, 1966), *Aspergillus nidulans* (Peberdy and Gibson, 1971), *Geotrichum candidum* (Dooijewaard-Kloosterziel *et al*, 1973).

(3) By the extracellular enzyme preparation from the culture filtrate of *Trichoderma harzianum* (ex *T. viride*) on *Schizophyllum commune* (De Vries and Wessels, 1972, 1973) *Aspergillus fumigatus* (Hearn and Mackenzie, 1979, Wilson and Hearn, 1982), *Phycomyces blakesleeanus* (Saadi, 1980).

(4) By a combination of lytic enzymes (cellulase) plus snail juice preparation or streptomyces sp. complexes on: *Penicillium chrysogenum* (Anné *et al*, 1974).

(5) By commercial lytic enzymes: *e.g.* cellulase, Novozyme 234 and some others less effective enzymes from fungi: *Acremonium chrysogenum*, *Aspergillus nidulans*, *A. niger*, *Penicillium chrysogenum*, *Volvoriella volvacea* and *Saccharomyces cerevisiae* (Hamlyn *et al*, 1981).

However, few reports on the production of protoplasts from cellulosic Oomycetes have been made. Bartnicki-Garcia and Lippman (1966) produced protoplasts from *Phytophthora* spp. Sietsma *et al* (1967) from *Pythium* PRL 2142, Sietsma *et al* (1969) from *Apodachlya brachynema*, *Dictyuchus sterile* and *Pythium* PRL 2142, by inducible lytic enzyme complexes from *Streptomyces* spp. and Sietsma and De Boer (1973) from *Pythium* PRL 2142 by the combination of helicase and cellulase.

Purification of the lytic complexes from *Streptomyces spp.* have yielded three β-D-glycanases: exolaminaranase, endo-

laminaranase and cellulase; the combined action of these three enzymes with lipase produced protoplasts from the Oomycetes as reported above (Sietsma *et al*, 1969). Both lipase and endo-laminaranase were not essential but aided protoplast formation. Hasegawa and Nordin (1969) reported the presence of an inducible α-1,3-glucanase in the culture filtrate of *Trichoderma viride*.

Pretreatment of cells with certain thiol compounds or addition of these compounds to the lytic media has been reported to improve the efficiency of protoplast formation (Villaneuva and Garcia Acha, 1971; Gull *et al*, 1972; Sietsma and Boer, 1973; Torres-Bauza and Riggsby, (1980). This effect may be related to the reduction of disulfide bonds in wall protein so that opening of the molecules occurred which allowed penetration of the lytic enzymes (Peberdy, 1979b).

Many other factors were also found to affect protoplast release, *e.g.* physiological age of culture, osmotic stabilizer, nature of the culture medium and pH value. Several reviews have been published on the preparation of fungal protoplasts (Villanueva and Garcia Acha, 1971; Peberdy, 1972, 1976, 1979b).

Since the first reports of the isolation and characterization of fungal protoplasts, an extensive literature has accumulated about their regenerative properties. Emerson and Emerson (1958), Bachman and Bonner (1959), described reversion of *Neurospora crassa* protoplasts to typical mycelial growth when transferred to a suitable liquid or solid nutrient medium. Garcia Acha *et al* (1966) stated that mycelium, originating from an isolated protoplast after transfer to fresh

nutrient medium was able to sporulate normally; these reversion cultures were indistinguishable from the original culture in their growth habit, morphology, chemical composition, sensitivity to strepzyme RA and productivity of protoplasts.

Two patterns of morphological development are found in protoplast reversion in the mycelial fungi. In one form, protoplasts give rise to abnormally shaped germ tubes, which resemble chains of budding cells and ultimately change to normal hyphae at their tips. This pattern of development was found in *Aspergillus nidulans* (Peberdy and Gibson, 1971), *Geotrichum candidum* (Dooijewaard-Kloosterziel *et al*, 1973), *Penicillium chrysogenum* (Anné *et al*, 1974). An apparent variation on this pattern was described in *Trichoderma viride*, where the protoplast, having produced the abnormal bud-like structure, lyse at the tip and a normal hypha grows from the original protoplast (Benitez *et al*, 1975). The second type, and totally different form of development, was described in *Rhizopus nigricans* (Gabriel, 1968) and later in *Schizophyllum commune* (De Vries and Wessels, 1975). In the latter the protoplasts first develop a wall which maintains a spherical shape, thus forming a primary cell which later produces a normal germ tube. In some fungi, *e.g. Pythium* both types of development leading to the formation of normal hyphae have been described (Sietsma and De Boer, 1973), and *Fusarium culmorum* (Garcia Acha *et al*, 1966). Reviews on protoplast regeneration and wall biogenesis have been presented by Peberdy (1979a,b).

Besides other classical diagnostic procedures, serological, techniques have been used with advantage as an additional and perhaps

more rapid tool in the study of taxonomic relationships among fungi, and in providing a better understanding of the phylogenetic relationship of morphologically different fungi (Madhosingh, 1964; Hornok, 1980; Chard *et al*, 1983), presumably these techniques could also be useful in determining antigenic relationship between chitin and cellulose-walled fungi.

Immunofluorescent staining techniques have been used in the diagnosis and identification of human fungal pathogens (Kaplan and Kaufman, 1961; Gordon, 1962; Aldoory and Gordon, 1963). Application of this method to the detection of particular species of fungi and in particular plant pathogens are reviewed by Preece and Cooper (1969).

Several reports have been published linking the chemical structure of the antigens of fungi with immunological activity using the immunodiffusion technique. Mannans and mannose-containing polysaccharides such as galactomannans and rhamnomannan were found to be the major cell wall polysaccharide antigens of fungi (Hayashi *et al*, 1978a). Galactomannans have been obtained from many species of fungi such as *Trichophyton*, *Cladosporium*, *Penicillium* and *Aspergillus* (Gander,1974). Hayashi *et al* (1978a) isolated the serologically active substance from *Absidia cylindrospora* and found it to be fucomannan-peptide, composed of fucose and mannose and a very small amount of protein. Miyazaki *et al* (1979) indicated that α-1-6 linked mannopyranosyl residues might play an important role as the common determinant of the serologically active substance of *Absidia cylindrospora*, *Mucor hiemalis* and *Rhizopus nigricans*. Mitchell and Hansenclever (1970) also suggested

that α-1-2 and α-1-3 linkages of the mannan of *Candida stellatoidea* were important factors in the antigen-antibody interaction, but that α-1-6 linkages were not significant.

Many investigators have indicated a cross-reactivity among diagnostic antigens from different fungal classes, as a result of the presence of shared antigenic determinants. Antigenically active galactomannan from *Aspergillus fumigatus*, cross-reacted with similar structures present in *Candida albicans*, *Trichophyton rubrum*, *Saccharomyces cerevisiae* and *Penicillium* sp. (Suzuki, *et al*, 1967; Azuma *et al*, 1971). Preece and Cooper (1969) indicated a cross-reaction of *Mycosphaerella melonis* (Ascomycete) with *Botrytis cinerea* (fungi imperfecti). However, Halsall (1976) prepared cytoplasmic and cell wall antigens and antisera from four *Phytophthora* species and cell wall antigens from two *Pythium* species. Immunodiffusion of the *Pythium* and *Phytophthora* cell wall antigens showed that the two *Pythium* spp. did not cross-react with *Phytophthora* cell wall antisera. Immunodiffusion analysis of both cell walls and cytoplasmic analysis of *Phytophthora* revealed some degree of specificity between species but not between A1 and A2 mating types in *Phytophthora cinnamomi*. Species specificity was improved by using indirect fluorescent antibody technique after cross absorption of the antisera. Hayashi *et al* (1978, a,b) reported a cross-reaction of antisera prepared against the extracellular nondialyzable fraction of *Absidia cylindrospora* (Fucomannan-peptide), with antigenic fraction from four *Mucor* spp. and *Rhizopus nigricans*, but no cross-reaction with the purified mannan of *Candida albicans* and *Saccharomyces cerevisiae*.

In the first part of this investigation, antisera were raised against some members of chitin and cellulose-walled fungi. In addition, protoplasts from a member of each group of fungi were also prepared in an attempt to compare the regeneration process and trace the construction of wall components based on the property of adsorption of a particular antiserum, specific to the cell wall by measuring the specificity or intensity of fluorescence using the IMF technique. Additionally, antisera were also used to investigate the antigenic relationships between the two groups of fungi (Homologous and heterologous antigen-antibody reaction).

3.2 Materials and Methods

3.2.1 Organisms

Phycomyces blakesleeanus and *Saprolegnia parasitica* were used as members of chitin and cellulose-walled fungi respectively, for protoplasts studies.

Antisera were raised against three chitin-walled fungi, *Mucor mucedo, Penicillium waksmanii, Phycomyces blakesleeanus,* and two cellulose-walled fungi, *Pythium debaryanum,* and *Seprolegnia parasitica.* All fungi were the same strains as used previously except *P. waksmanii* which was originally isolated from sea weeds *Eucheuma striatum* Schmidzt (Dewey *et al,* 1983).

3.2.2 Buffers and solutions

A - Phosphate Buffer Saline PBS pH 7.2: 500cm^3 x 10 stock solution was prepared as follows:

F - Destain solution:

Methanol	50%
Acetic acid (glacial)	7%

made up to final volume with distilled water.

G - Gull *et al* (1972) osmotic stabilizer solution:

Tris	1.2g
EDTA $Na_2 . H_2O$	0.03g
Mannitol (final conc. 0.8M)	148.6g
β-Mercapto ethanol (final conc. 2.5mM)	

The solution was made up to one litre with distilled water and pH was adjusted to 7.0 with N-HCl.

3.2.3 Protoplasts preparation

Eighteen to twenty-four hours old cultures of *P. blakesleeanus* and *S. parasitica*, grown on PYG liquid medium on shaker at room temperature were used. The mycelium was harvested by filtration on Whatman No.1 filter paper, washed three times with distilled water and once with 0.01M phosphate buffer pH 6.0. Mycelium was resuspended in $2cm^3$ of a solution containing osmotic stabilizer in 0.01M phosphate buffer at pH 6.0, plus 10mg lytic enzyme. Cellulase (Onozuk R-10, Yakult Japan) for *S. parasitica* and Novozyme 234 (Novoenzyme Products Ltd., Windsor, England) for *P. blakesleeanus*. Ampicillin was added at a concentration of $100\mu g/cm^3$ to prevent bacterial growth. This solution was placed in $25cm^3$ vials and incubated on a shaker at room temperature. Samples of the digested suspension were examined at 1-6 hr. intervals. The protoplasts were separated from the mycelial debris by

repeated centrifugation at low speed, 500-1000xg for 10 min. Protoplasts were washed three times in 0.01M phosphate buffer pH 6.0 containing osmotic stabilizer.

For regeneration of protoplasts, the normal growth medium either liquid or semisolid (solidified with 0.7% agar) was used at room temperature. A small volume of this medium was pipetted onto a sterilized slide in a Petri dish containing wet filter paper. A few drops of protoplast suspension was added to the medium and regeneration was followed by examining the slides at intervals. Samples from the liquid regenerating medium were also examined. 0.1% Calcofluor white ST (CYANAMID) in phosphate buffer 0.01M pH 6.0 was used as a brightener to check the absence of wall materials from protoplasts. Samples of protoplasts were also stained with the homologous antisera by the immunofluorescent technique. Slides were examined by UV fluorescence microscopy using a combination of filters BG_3, K 460. Different osmotic stabilizers were used for production of protoplasts: NaCl 0.4M, NH_4Cl 0.4M, $MgSO_4$ 0.6M and Gull *et al* (1972) solution (described under materials).

3.2.4 Antigen preparation

Antigens were prepared from fungi: *M. mucedo*, *P. waksmanii*, *P. blakesleeanus*, *P. debaryanum* and *S. parasitica*, grown in liquid cultures under the same conditions as stated previously, $5cm^3$ of spore suspension was used as inoculum (except for *P. debaryanum* where 10-12 of 6mm diameter discs from a slightly submarginal area of a mature colony were used) in 100ml PYG liquid medium in $250cm^3$ Erlenmeyer flasks, incubated

in static conditions at 24°C for 2 days (3 days for *P. debaryanum*). The following procedure was used for the preparation of antigens from all five fungi tested: Mycelia were harvested from liquid cultures on Whatman No.1 filter paper, by suction filtration, washed three times with distilled water and once with PBS pH 7.2. Mycelia from 4-5 flasks of each fungus were combined. The extraction method of Johnson *et al* (1982) was used. Mycelia were frozen with liquid air and ground with a mortar and pestle with 15cm^3 PBS pH 7.2. The slurry was centrifuged at 10,000xg for 10 min. This process was repeated twice and the final pellet was resuspended in 10cm^3 PBS and lyophilized. Protein content of the lyophilized material was extracted with 1N NaOH and estimated by a modification of Lowry *et al* (1951) (Methods 1).

3.2.5 Immunization

An emulsion for the immunization was prepared as follows (Herbert, 1973): one cm^3 of Freunds complete adjuvant (modified *M. butyricum*, Calbiochem), was mixed with 5mg antigen (ground lyophilized mycelia), dissolved in 1cm^3 PBS pH 7.2. Antigens were mixed with the solution by repeatedly sucking into and expelling from a disposable 2cm^3 syringe. A complete emulsion was obtained by using short bursts (30 sec.) of ultrasonication in an MSE ultrasonic power unit. Temperature was maintained low by surrounding the vials with crushed ice. The emulsion was tested to determine its integrity, by allowing a few drops to fall into cold water in a Petri dish. The first drop usually spread over the surface, but subsequent drops remained as discrete white drops below or on the surface:

if a true emulsion had been formed. A second emulsifier, $2cm^3$ of saline containing 2% Tween 80 were added to the emulsion to prevent re-aggregation or release of antigen. The mixture was then shaken vigorously by hand or mixed by repeatedly sucking and expelling from a syringe. The best emulsions were very viscous.

Pre-immune control serum was collected from New Zealand white rabbits, by bleeding from the ear. Rabbits were immunized, following the immunization Protocol below, $4cm^3$ antigen preparation was used each time:

1 - one cm^3 antigen emulsion was injected intramuscularly into each hind limb and $2cm^3$ subcutaneously into several sites along the back;

2 - second injection, same as above (1), 3 weeks later;

3 - first bleeding from the ear, two weeks after the second injection;

4 - second bleeding as above, 10 days after the first bleeding;

5 - a booster injection same as above (1), was given a week after the second bleeding;

6 - two weeks after boosting, rabbits were exsanguinated, blood was collected, refrigerated overnight at 4^oC, the serum was decanted from the clots, clarified by centrifugation at 2000xg on an MSE bench centrifuge and stored at -20^oC.

3.2.6 Indirect immunofluorescent antibody technique

Slide cultures of fungi, prepared by inoculating a small block of solid PYG medium, on a sterile glass microscope

slide, with mycelium around the sides. The inoculated slides were incubated inside a sterile Petri dishes containing wet filter papers. The preparations were left at room temperature (22±2°C) for 2-3 days. The agar blocks were discarded and the mycelia, which were easily located at the site of the two inoculated edges of the agar, remained adherent to the glass slide. Slides were dried at room temperature, dipped in fixative (Ethanol: chloroform: formalin, 6:3:1) for 3 min. dipped in 95% methanol for 4 min., washed with distilled water and dried completely at room temperature. Homologous antisera at appropriate dilutions with PBS (pH 7.2), were added, left to react for 30 min. in moistened Petri dishes, washed by three 5 min. changes with PBS, pH 7.2 followed by removal of surplus moisture. Commercial goat anti-rabbit gamma globulin conjugated with fluorescein isothiocyanate (FITC) (MILES-YEDA LTD), diluted 1:20 with PBS was added to the slides, left to react for 30 min. in moistened Petri dishes, washed by three 5 min. changes of PBS pH 7.9, mounted in PBS: glycerol solution (PBSx2 at pH 7.9 with equal volume of glycerol), slides were then examined in a Leitz ortholux microscope (Leitx Wetzlar) equipped with vertical illuminator (50w mercury lamp), a combination of filters BG12, K510 were used and evaluated for yellow green fluorescence. Photographs were taken with XP1 400-ASA film. (Numbers on scale bars represent micrometers). Antigenicity of fungal hyphae was tested with homologous antisera diluted serially in Eppendorf centrifuge tubes as follows: 1:8, 1:16, 1:32, 1:64 and 1:128. Cross-reactions were also employed using each fungus against heterologous antisera prepared, in addition to several other Oomycetous fungi listed in the results were tested.

Test controls were set up as follows:

(1) Fixed mycelium + specific (homologous) undiluted antiserum - FITC.

(2) Fixed mycelium - specific (homologous)AS + FITC.

(3) Fixed mycelium + pre-immune undiluted serum + FITC.

(4) Fixed mycelium only without staining.

3.2.7 Immunodiffusion test

Fungal antigens used in the immunodiffusion treatments were prepared in four ways as follows:

(1) extraction of mycelia (0.1 gm wet wt. mycelium cultured under the same conditions as used in preparation of initial antigens for injecting the rabbits), with 2ml PBS pH 7.2, after freezing with liquid air and grinding;

(2) extraction of frozen mycelia (prepared as above) with $2cm^3$ 0.05M ammonium carbonate containing 0.5% Triton-X100 at pH 8.0 (Hearn and Mackenzie, 1979);

(3) extraction of frozen mycelia with 2% SDS in PBS pH 7.2;

(4) culture filtrate from cultures used as above.

Gel diffusion tests were determined by the method of Ouchterlony (1949), using a medium made as follows: 1g agarose (agarose 15) dissolved in $50cm^3$ distilled water by boiling, to this warm agarose solution, $50cm^3$ of Tris-EDTA-Borate buffer pH 8.6 were added followed by thorough mixing. Fifteen cm^3 aliquots of agar mixture were pipetted into immunodiffusion plates on a levelling table, allowed to solidify and left overnight at 4^oC. A pattern of six peripheral wells (diameter 0.4mm, 50µl capacity), 0.3mm from a central well were cut using a template. Agar from each well was removed

with a pipette attached to a water vacuum system. The outer wells were filled each with 50µl of the appropriate antigenic extract, and the central well with the homologous or heterologous antiserum (performed in a cold room on a levelling table). Undiluted and diluted antisera and antigens (1:2, 1:4, 1:8) were used. Plates were left for 24 to 48 hr. at 4°C.

3.2.8 Staining of immunodiffusion gels

After observing the precipitin lines, gels were carefully removed from the plates and treated as follows:

1 - washed with distilled water in a plastic box for 5-15 min.;
2 - arranged on a clean glass plate and blotted between layers of tissue or filter papers under a heavy weight for 30 min. to remove the excess of antigens and antisera;
3 - blotted gels were washed in 20 mM borate buffer pH 8.0 for 5 min.;
4 - washed with 0.9% sodium chloride 2x15 min.;
5 - transferred to 1% agarose coated glass plates (plates 15x15 cm coated with 15cm^3 gel buffer medium used for immunodiffusion test);
6 - dried at room temperature for 24 hr. or longer, and blotted again for 15 min.
7 - the glass plates dried by a hair drier for 2-3 min.;
8 - stained in coomasie blue for 15-30 min.;
9 - destained with destaining solution for 15-30 min. or until the precipitin lines were clear;
10 - the plates dried again under hair drier and examined.

3.2.9 Agglutination test

Immunodiffusion reactions were substantiated by agglutination tests. Equal volumes of SDS extract (50µl) of each fungus were mixed with their corresponding antisera in Durham agglutination tubes, covered and incubated in a water bath at 37°C for 2 hr. and refrigerated overnight. In positive tubes where agglutination had taken place, cloudy precipitin bands could be seen when the tubes were subjected to transmitted light.

3.2.10 Concentration and purification of antisera

To obtain clearer precipitin lines in the immunodiffusion the weak antisera, obtained from *Mucor mucedo*, and *Phycomyces blakesleeanus*, were concentrated by the method of Marchant and Smith (1968) as follows: antisera were placed in sealed dialysis tubing and granular sucrose packed around them. The tubes were then dialyzed for 1.5-2 hr. against PBS pH 7.2 to remove any sucrose.

An attempt to increase the specificity of all the antisera was done by using the following purification procedure (Joustra and Lundgren, 1969): antisera were first dialyzed against distilled water for 12 hr. at 4°C in 3cm diameter dialysis tubing, and then against sodium acetate 50mM pH 5 for 24 hr. They were redialyzed against distilled water for 2x12 hr. and dialyzed further against sodium acetate 50mM pH 5 for 24 hr. Antisera were centrifuged at 16000xg for 30 min., supernatants were adjusted to 40% relative atmosphere and left at 4°C for 2 hr. Following further centrifug-

ation as above the precipitates were dissolved in 20mM borate buffer at pH 7.5-8, containing 0.9% sodium chloride plus 0.02% sodium azide, and were dialyzed against the same buffer for 24 hrs. Antisera were then tested by the immunodiffusion method.

3.2.11 Antigen-Antibody blocking reaction

The specific reactions of fungal antigens with their respective antisera were blocked with reagents below, using fixed mycelial preparations from slide sultures. Slides were overlaid with:

(1) 2% SDS in PBS;

(2) 10mg/cm^3 concanavalin A;

(3) 5% mannose in PBS,

incubated at room temperature for 2 hr. followed by several washings with PBS, pH 7.2., and stained as stated previously (immunofluorescent technique).

3.2.12 Preparation of pure cell walls

Pure cell walls were prepared from both fungi using the same procedure as follows: three-day old cultures were harvested by filtration, washed 3 times with distilled water. Mycelium (4-5g wet wt.) was frozen with liquid air and ground by a mortar and pestle, suspended in 5cm^3 distilled water and centrifuged at 2000xg on an MSE bench centrifuge for 10 min. The pellet was washed 8-10 times with distilled water followed by centrifugation as above. The pellet was then given an ultrasonic treatment at full power for 5 min. in an MSE Ultrasonic Power unit at temperature maintained low by surrounding

the vials with crushed ice. This procedure was repeated 4 times. The pellet was then washed 5 times again with distilled water. Microscopic examination of the pellet showed that it consisted of small pieces of cell wall materials; staining with lactophenol cotton blue failed to determine any residue of cytoplasm.

3.2.13 Adsorption of antisera

An attempt was made to obtain by adsorption and elution, wall specific antisera, that would react only with cell wall as follows: One cm^3 of undiluted antiserum was adsorbed against an excess of the appropriate pure fungal cell walls (0.15-0.2g wet wt.), preparations were incubated for 2 hr. at room temperature. Centrifuged at 2000xg for 10 min. and the supernatant was discarded. The walls were resuspended and incubated in $1cm^3$ PBS at pH 5, left overnight at 4^oC. Preparations were centrifuged as above. The wall free supernatant was neutralized with 0.5M NaOH and tested against both protoplasts and freshly prepared cell walls.

3.3 Results

3.3.1 Observations on Protoplasts Production

Although some protoplasts were produced from sporangiophores of *Phycomyces blakesleeanus* using the mechanical method , this technique did not yield protoplasts from vegetative hyphae of both fungi tested. This is possibly due to the large size of sporangiophores compared to the small vegetative hyphae belonging to these fungi. However, the

isolation of protoplasts from fungi, using lytic enzymes is now a well established technique (Peberdy, 1979b). Therefore this method was tested. Novozyme 234 and cellulase, both inexpensive commercial lytic enzymes were recommended by Dr. J.F. Peberdy of Nottingham University (Personal communication), as used by Hamlyn *et al* (1981). These were used for the production of protoplasts from *P. blakesleeanus* and *S. parasitica* respectively and proved successful.

3.3.2 Morphological features of Lysis and Protoplasts Production

With *P. blakesleeanus* the first observable change in mycelium on incubation with the lytic enzyme was swelling of the hyphal tips (Fig. 19A), followed by the start of protoplast release from the tips about 3-4 hr. after incubation. Protoplast release was not accompanied by a general degradation of hyphal walls; hyphae remain undissolved even after long incubation with lytic enzyme. Protoplasts of *P. blakesleeanus* were found to be of different sizes (12-21μm) (Fig. 19B,D). With *S. parasitica* during the lytic digestion with cellulase enzyme, the mycelium underwent extensive fragmentation 2-3 hr. after incubation and this resulted in protoplasts release from the open ends of fragments (Fig.20A). Nearly all protoplasts were more or less similar in size (14-17μm).

3.3.3 Factors affecting Protoplast Production

3.3.3.1 Osmotic stabilizer: several osmotic stabilizers were tested for their ability to affect protoplast release and to maintain protoplast stability. These were NaCl 0.4M, NH_4Cl 0.4M, $MgSO_4$ 0.8M and Gull *et al* (1972) stabilizer solution. Significant release of protoplasts was only observed with $MgSO_4$. To test the effect of molarity of $MgSO_4$ on the release of protoplast, lytic solutions containing 0.1-1.0M of the stabilizer were tested with the pH maintained at 6.0. Best results were obtained using 0.5-0.8M $MgSO_4$, with maximum yield at 0.6M. At 1M $MgSO_4$ the hyphae became plasmolyzed and the cytoplasm was divided into small spheres which were not released from the mycelium. Therefore 0.6M $MgSO_4$ was used throughout the work.

3.3.3.2 Enzyme concentration: the effect of both novozyme and cellulase concentrations on the release of protoplasts from the two fungi, was also studied. The number of protoplasts released increased with increasing concentration of enzyme up to 10mg/ml. In addition increasing concentration up to this level shortened the time needed to produce large numbers of protoplasts. Further increase up to 20mg/ml did not show any significant difference in number and time to release protoplasts.

3.3.3.3 Age of culture: the influence of culture age at harvesting on the yield of protoplast, was marked. Maximal yield was observed after 15 hr. of incubation falling sharply when the mycelium was grown for more than 24 hr.

3.3.4 Protoplast isolation and examination

Separation of protoplasts from the rest of the mycelial debris was difficult especially with *S. parasitica* in which small hyphal fragments were produced. In addition, the variable sizes of *P. blakesleeanus* protoplasts complicated the isolation of pure protoplasts. In order to overcome these problems the following methods were employed:

1 - centrifugation at 2000xg for 5 min.;
2 - filtration through sintered funnel without pressure followed by centrifugation as above;
3 - filtration through two layers of muslin followed by centrifugation;
4 - repeated centrifugation at low speed (under methods).

The last method was the most efficient resulting in minimal of mycelial fragments.

Protoplasts were washed with 0.6M $MgSO_4$ in 0.01M phosphate buffer pH 6; stained with calcofluor and examined by fluorescent microscopy. Preparations which failed to fluoresce, clearly indicated the absence of cell wall materials. In addition, calcofluor stained-preparations were submitted to osmotic shock by dilution with water, the protoplasts were lysed releasing intracellular vacuoles. Suspension of protoplasts could be maintained at $4^{o}C$ without serious loss in viability up to 24 hr.

3.3.5 Regeneration of Protoplasts

In previous IMF studies, when homologous antisera to *S. parasitica* and *P. blakesleeanus* were reacted with their

respective hyphae, both cell walls and cytoplasmic materials fluoresced, indicating that the antisera were not wall specific. This was confirmed when these antisera were reacted with their respective protoplasts and pure wall fragments. In addition intracellular material obtained from burst protoplasts showed very bright fluorescence. Therefore an attempt was made to make the antisera specific for cell walls only (containing cell wall specific antibodies only), by adsorbing the whole antiserum with pure cell wall preparations, or with protoplasts after centrifugation. Unfortunately the protoplast preparations were not one hundred per cent free from cell wall fragments as mentioned before. Therefore adsorption and elution from pure cell wall fraction was tried. However, the antibodies were readily adsorbed by cell walls; placement of these walls in acidic solution did not result in their release. Thus it was impossible to carry on studying cell wall synthesis and protoplast regeneration by the serological method. Protoplast regeneration was therefore followed by light microscopy. Nearly all the various stages of regeneration process of *P. blakesleeanus* and *S. parasitica* protoplasts were observed. About 2-3m of incubation in the regeneration medium the majority of protoplasts of both fungi began to develop a protrusion which increased in volume (Fig.19D, E, Fig. 20C). Later the complete regeneration occurred. In *P. blakesleeanus* (advanced stage of regeneration), the protoplast gave a series of yeast-like forms (Fig.19F,G) grouped in a chain, finally producing a germ tube more or less similar to the normal hypha, leading to a well developed normal colony (Fig. 19G).

Fig. 19: Protoplast of *Phycomyces blakesleeanus* (light microscopy):

A - General picture of early stage of protoplast release from hyphae incubated in a lytic medium, showing terminal swelling of hypha (arrow) and empty cell walls or hyphae remaining in the preparation.

B - Aggregation of free protoplasts, note the different sizes.

C - F Protoplast regeneration:

C - Unregenerated protoplast, 1 hr. after incubation in regenerating medium.

D - Protoplast showing early stage of regeneration, 2 hr. after incubation in regenerating medium.

E - Regenerated protoplast, 4 hr. after regeneration.

F - Regenerated protoplast, 10 hr. after regeneration showing the establishment of a distinct chain of yeast-like forms, and early development of a terminal normal hypha.

G - 30 hr., microcolony developed from regenerated protoplast.

A, E, F, G X 463

B, C, D X 690

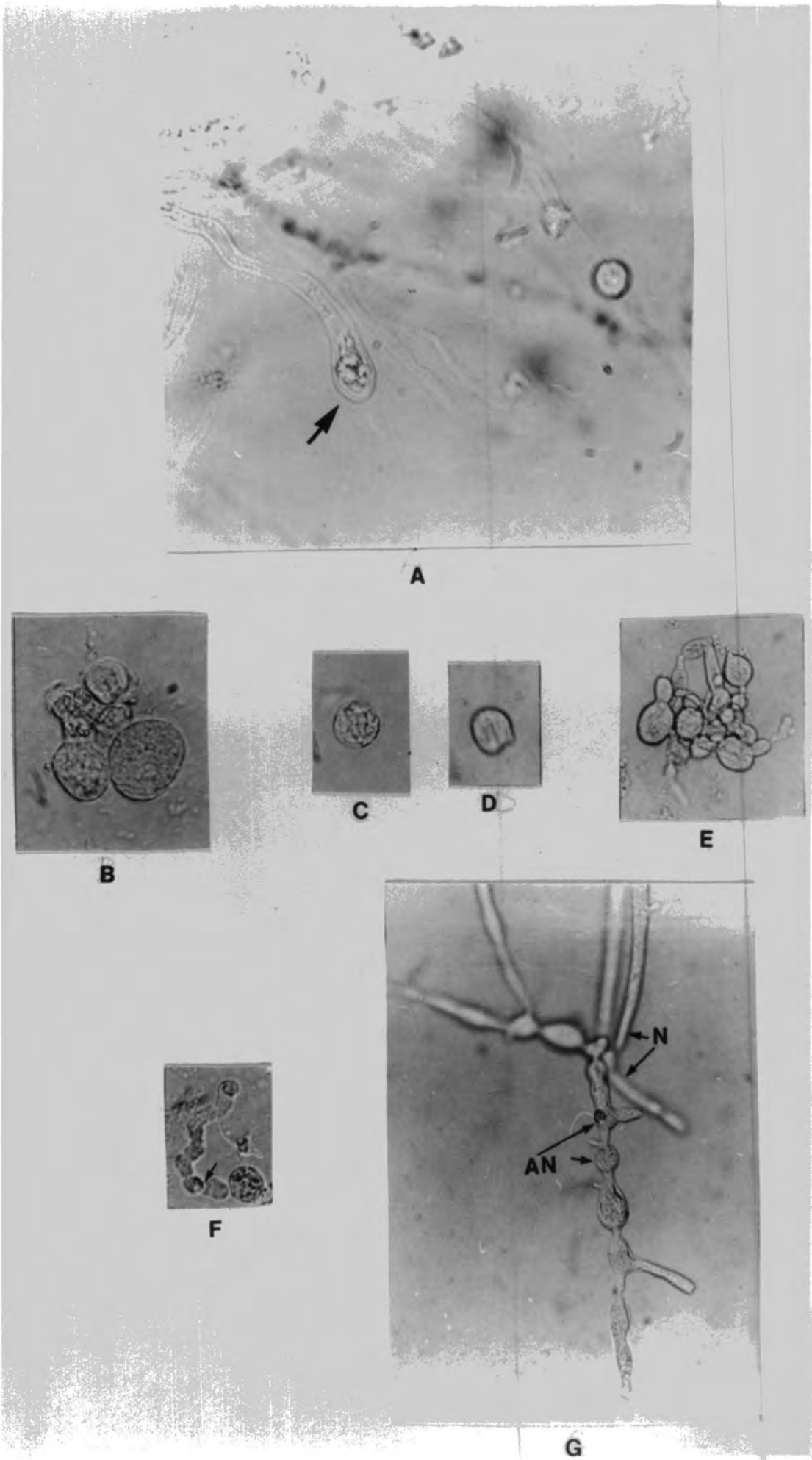

A
B
C
D
E
F
N
AN
G

Fig. 20: Protoplasts of *Saprolegnia parasitica* (light microscopy X 463.

A - General picture of protoplasts release from fragmented hyphae (arrow) in the lytic medium.

B - E Protoplast regeneration process.

B - Unregenerated protoplast, 1 hr. after incubation in regenerating medium.

C - Protoplast, 4 hr. after incubation in regenerating medium, showing development of abnormal hypha.

D - Protoplast, 10 hr. after incubation in regenerating medium, note the establishment of normal hyphae (arrows) from the abnormal hyphae.

E - Protoplast, 12 hr. regeneration, showing a well developed normal hypha and branch from the abnormal one.


A
B
C
AN
N
D
AN
N
E

In *S. parasitica*, protoplasts developed an abnormal, very thin hyphae, sometimes branched with a swollen tip. Later on a normal hypha developed from the abnormal one (Fig. 20D) and continued to grow as a normal hypha (Fig. 20E).

3.3.6 Indirect immunofluorescent technique (IMF)

Results of immunofluorescent reactions were recorded in a range from ± (slightly visible) to 4+ (extreme brilliant yellow green). Test bleeds were done five weeks after the first injection, satisfactory antisera for fluorescent antibody studies were obtained from the injection of *Penicillium waksmanii*, *Saprolegnia parasitica* and *Pythium debaryanum*, *i.e.* contained enough antibodies to stain homologous antigen +2 to +3 when used undiluted. However only a very weak (slightly visible) reaction with *Mucor mucedo* and *Phycomyces blakesleeanus* antisera was detected. In addition very weak precipitin lines were obtained by the immunodiffusion test (IMD) even with the antisera of fungi that gave a good reaction with the IMF test above; therefore further injections (under methods) were carried out until a satisfactory IMF and IMD reactions were obtained.

Antisera from final bleed (approximately 8 weeks after first injection) were diluted up to $^{1}/_{128}$, to eliminate most of the heterologous staining. Table (15) shows the intensity of fluorescence of homologous antigen-antibody reactions with the range of diluted antisera. *Penicillium waksmanii*, *Saprolegnia parasitica* and *Pythium debaryanum* showed a very bright fluorescence with the diluted antisera

TABLE 15 Intensity of the immunofluorescence of homologous antigen-antibody reaction by the IMF technique

Antigens	Dilutions of Antisera					
	undiluted	1/8	1/16	1/32	1/64	1/128
Mucor mucedo	+	+	+	±	-	-
Pencillium waksmanii	++++	++++	+++	+++	++	++
Phycomyces blakesleeanus	+	+	+	±	-	-
Pythium debaryanum	++++	++++	+++	++	++	++
Seprolegnia parasitica	++++	++++	+++	+++	++	++

up to 1/32, (+4 to +3) (Fig. 21, 22, 23). Higher dilution showed a slight reduction in the fluorescence intensity up to 1/128. Whereas the reaction of *M. mucedo* and *P. blakesleeanus* (Fig. 24, 25) was weak even with the undiluted antisera (+1), no fluoresence could be detected on dilution higher than 1/32. Therefore 1/32 diluted antisera of *P. waksmanii*, *S. parasitica* and *P. debaryanum*, and 1/16 dilution of *M. mucedo* and *P. blakesleeanus* antisera were used as standards for the comparison of homologous and heterologous staining reactions (Table 16).

Antisera from each of the five fungi were cross-reacted by the IMF technique with the hyphae of all the five test fungi and in addition with several other Oomycetes listed in Table (16). The results showed a slight cross-reaction between the chitinous fungi *P. waksmanii*, *M. mucedo* and *P. blakesleeanus* (between ± and +), whereas a very detectable, sometimes an extreme cross reaction was noticed between the cellulose wall fungi (between ± and 4+) (Fig. 26-29). Other

TABLE 16 Intensity of the immunofluorescence of homologous and/or heterologous antigen-antibody reaction by IMF technique

Fungal antigen	Diluted antisera					
	Control	M.m.^{o}	P.w.$^{\Delta}$	P.b.^{o}	P.d.$^{\Delta}$	S.p.$^{\Delta}$
Chitin-walled fungi						
Mucor mucedo	-	+	+	+	±,+	±,-
Penicillium waksmanii	-	±	+++	±,+	-,±	-
Phycomyces blakesleeanus	-	+	+	+	+	-
Cellulose-walled fungi						
Achlya beneckii	-	-	-,±	±,+	+	±,+
Aphanomyces cladogamus	-	+,±	+,++	±	++	++++
Aplanopsis spinosa	-	++	+++	++	++	+++
Apodachlya brachynema	-	±,+	-,±	±,-	+	++++
Phytophthora parasitica	-	-	-,±	-	+	+,++
Pythium debaryanum	-	+	±	-	+++	++
P. middletonii	-	+	+	-,±	++	++
P. ultimum	-	-,±	-,±	-	+++	+
Saprolegnia ferax	-	±,+	±	-	+	++
S. parasitica	-	+,±	+	-	++	+++
S. terrestris	-	-	-	-	+	++
Sapromyces elongatus	-	±	±	±	+++	++++

M.m. : *Mucor mucedo*

P.w. : *Penicillium waksmanii*

P.b. : *Phycomyces blakesleeanus*

P.d. : *Pythium debaryanum*

S.p. : *Saprolegnia parasitica*

$^{\Delta}$ $^{1}/_{32}$ diluted antisera

^{o} $^{1}/_{16}$ diluted antisera

Oomycetes tested, especially *Apodachlya brachynema*, reacted more strongly with antiserum to *S. parasitica* than with antiserum to *P. debaryanum* (Fig. 30-33, 35). In fact three Oomycetous fungi, *Aphanomyces cladogamus*, *Apodachlya brachynema* and *Sapromyces elongatus* showed a greatest cross reaction (4+), with antiserum to *S. parasitica*, than was found in the homologous reaction (+3). Very weak cross reactions were observed between chitin and cellulose-walled fungi (- to 1+) (Fig. 36, 37), except for *Aplanopsis spinosa* which reacted fairly strongly (non-specifically) (2+ to 3+) with all the antisera (Fig. 34).

Hyphae stained by the IMF technique were equally fluorescent over the entire surface, *i.e.* a uniform fluorescence at the hyphal wall periphery was detected, except the cross-reaction of *P. debaryanum* with *M. mucedo* antiserum in which two types of reactions were observed (Fig. 38,39), a uniform fluorescent reaction and a spotted reaction in which the reactive antigens appeared to be concentrated in an alternating pattern within each of the hyphae.

Spores of *P. waksmanii*, *M. mucedo* and *P. blakesleeanus* showed a brighter fluorescence than vegetative hyphae. In addition, extracellular secretions of some hyphae of *S. terrestris*, *Apodachlya brachynema* and *Aphanomyces cladogamus* showed antigenic reactions being detectable as fluorescent halo surrounding the hyphae (Fig. 28, 32).

3.3.7 Blocking reactions

In an attempt to identify the reactive antigens in fungi showing a strong positive reaction, SDS, CoA and

Mannitol blocking reagents were used to block the antigen-antibody reaction. SDS is an anionic detergent and powerful dissociating agent, splits all except covalent bonds (Maizel, 1971), ConA is a lectin binds specifically with α-D-mannose, α-D-glucose and closely related sugars. Results were shown in Table (17), no effect of SDS treatment was observed on *P. waksmanii* fluorescence intensity, whereas a considerable effect, nearly complete blocking was observed with hyphae of *S. parasitica*. With *P. debaryanum* the remaining reactive antigen appeared as distinct spots along the hyphae (Fig.42).

TABLE 17. Effect of blocking reagents on the homologous antigen-antibody reaction, stained by the IMF technique

Blocking reagent	Antigens		
	P.d.	P.w.	S.p.
CoA	++	++	+,++
Mannose	+++	++	±,+
SDS	± patches	+++	±,-

\- invisible
± slightly visible
\+ visible
++ bright
+++ very bright

A slight effect or reduction of fluorescence intensity was observed with CoA on the three fungi tested. Using mannose, a range of reactions from non(*P. debaryanum*), slightly (*P. waksmanii*) to a considerable reduction of fluorescence

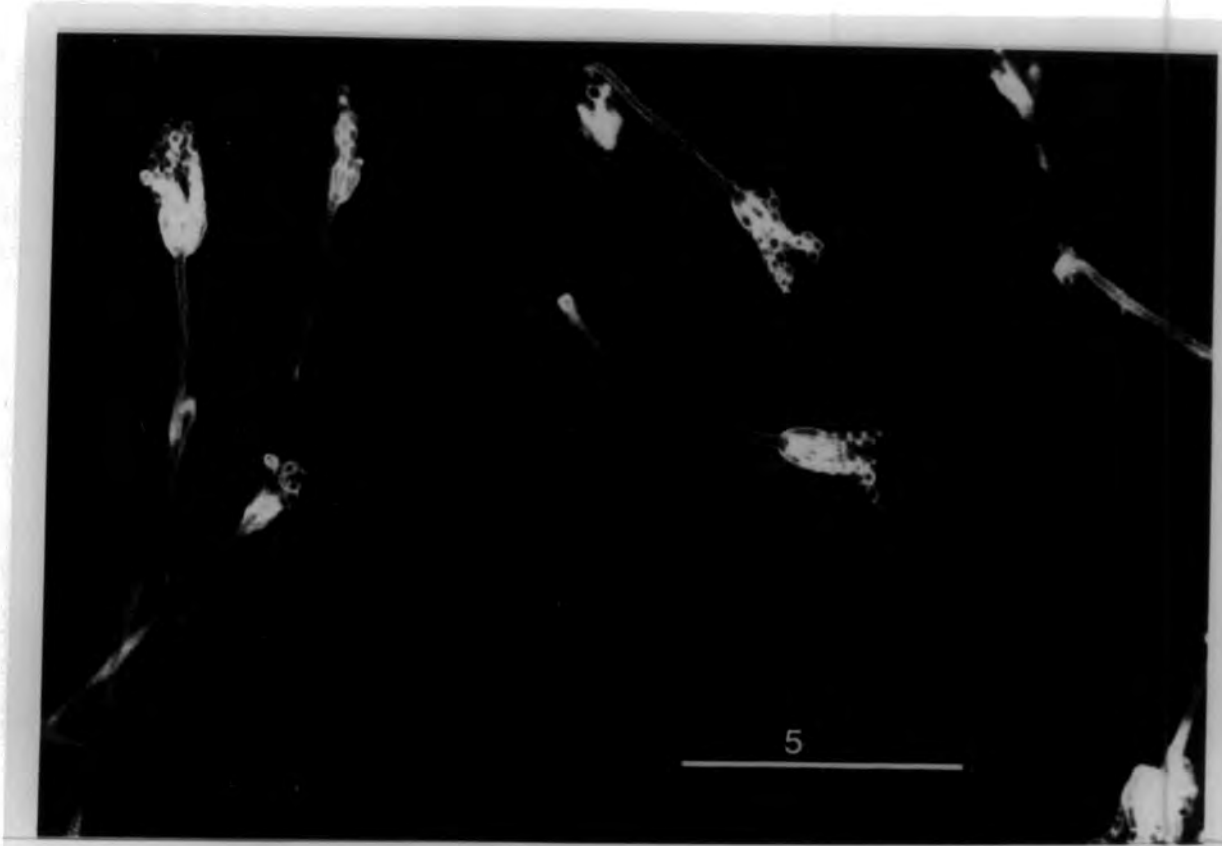
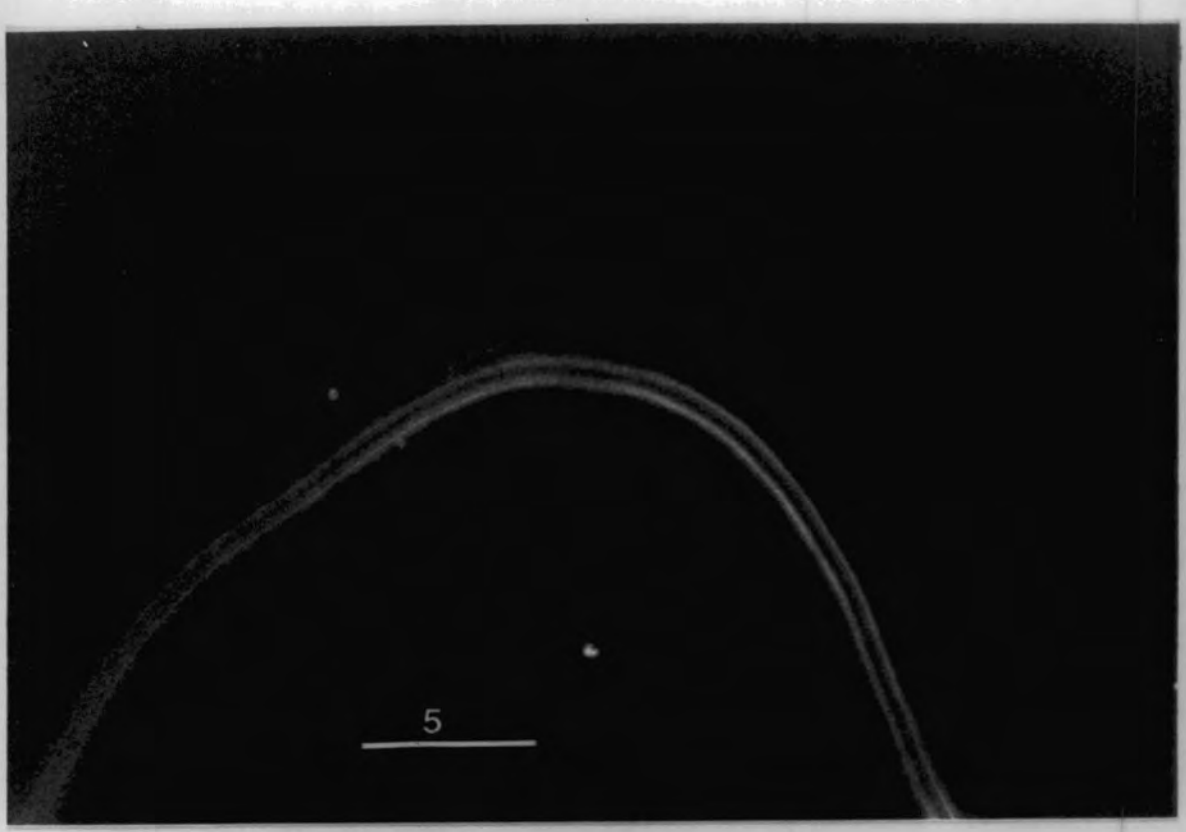


Figure 21 Indirect immunofluorescent micrograph of a homologous antigen-antibody reaction of hypha and conidial heads of Penicillium waksmanii. 1/32 AS dilution, X690

Fig. 22: Indirect immunofluorest micrograph of homologous antigen-antibody reaction of a hypha of *Saprolegnia parasitica*, $^{1}/_{32}$ AS dilution. X463.

Fig. 23: Indirect immunofluorescent micrograph of homologous antigen-antibody reaction of a hypha of *Pythium debaryanum*. $^{1}/_{32}$ AS, X 463.


5

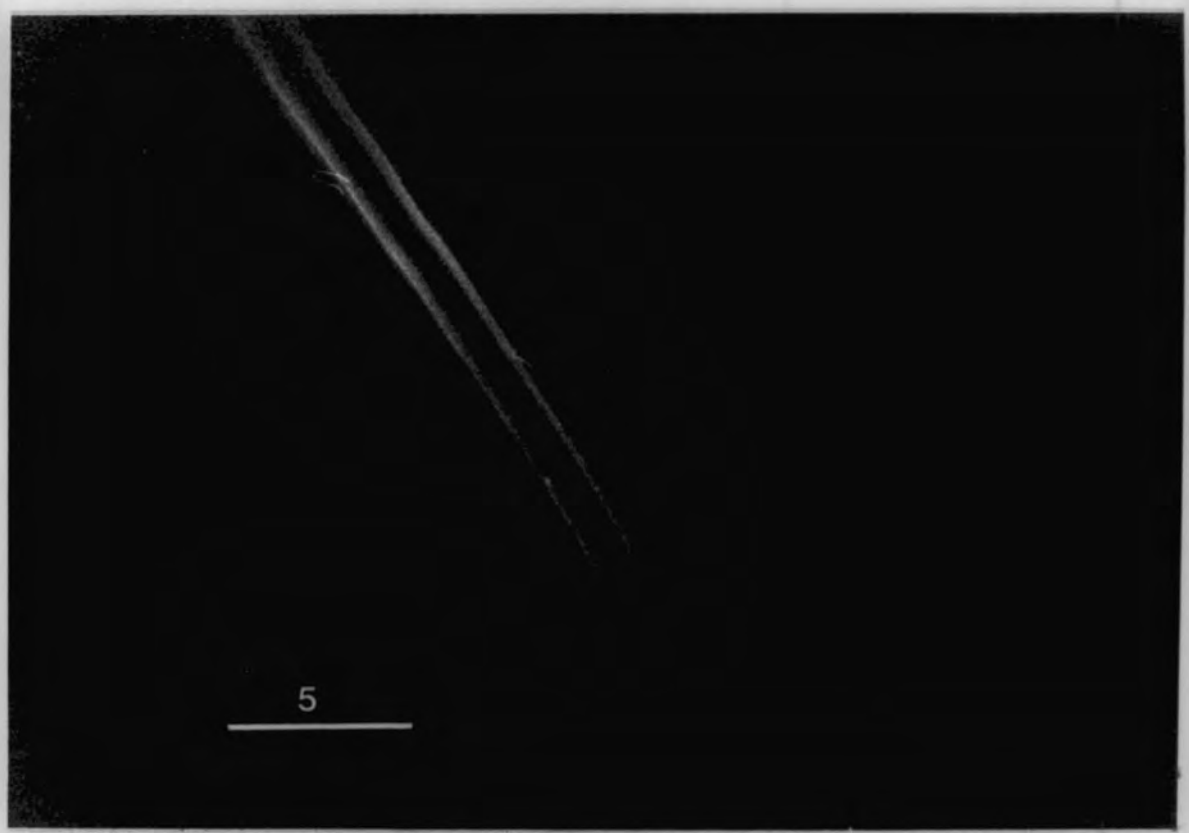

5

Fig. 24: Indirect immunofluorescent micrograph of homologous antigen-antibody reaction of sporangiophores and sporangia of *Mucor mucedo*. $^{1}/_{16}$ AS, X463.

Fig. 25: Indirect immunofluorescent micrograph of homologous antigen-antibody reaction of sporangiophores and sporangia of *Phycomyces blakesleeanus*. $^{1}/_{16}$ AS, X463.


5

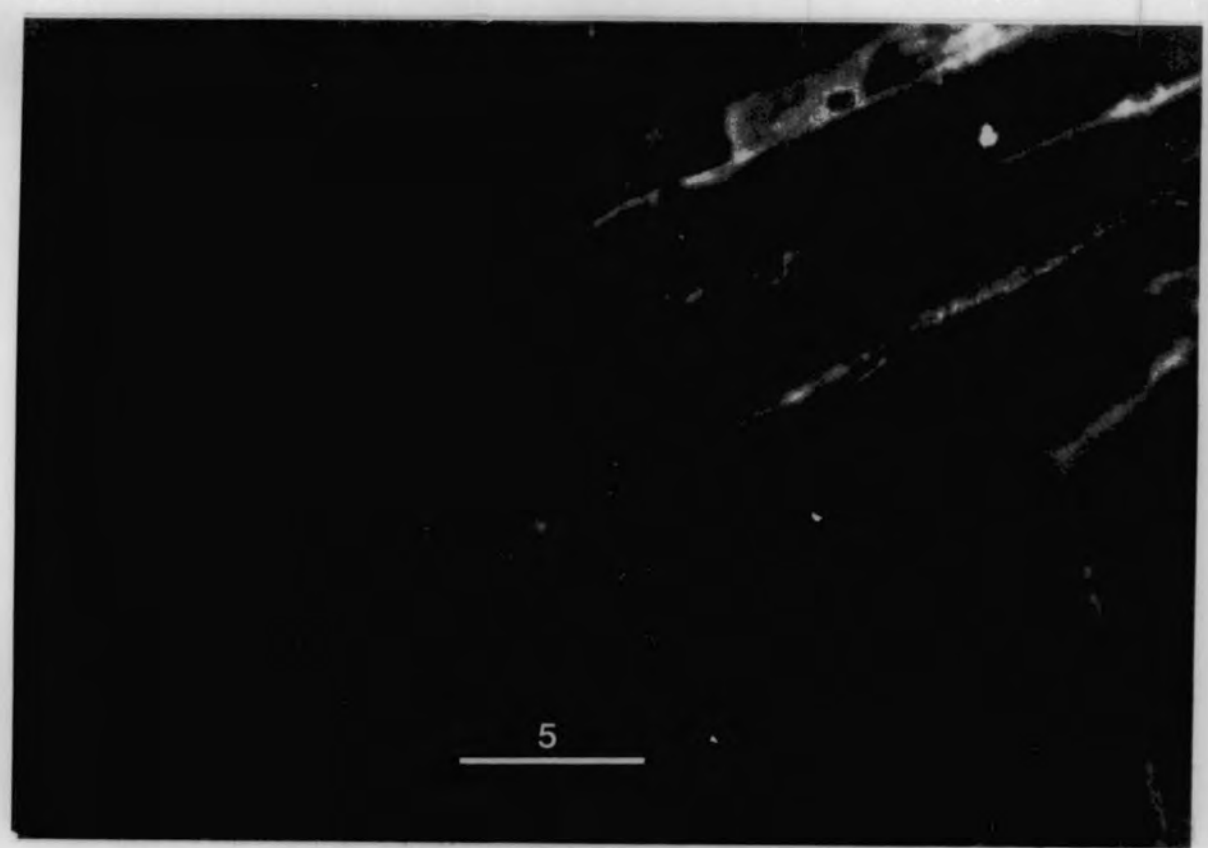
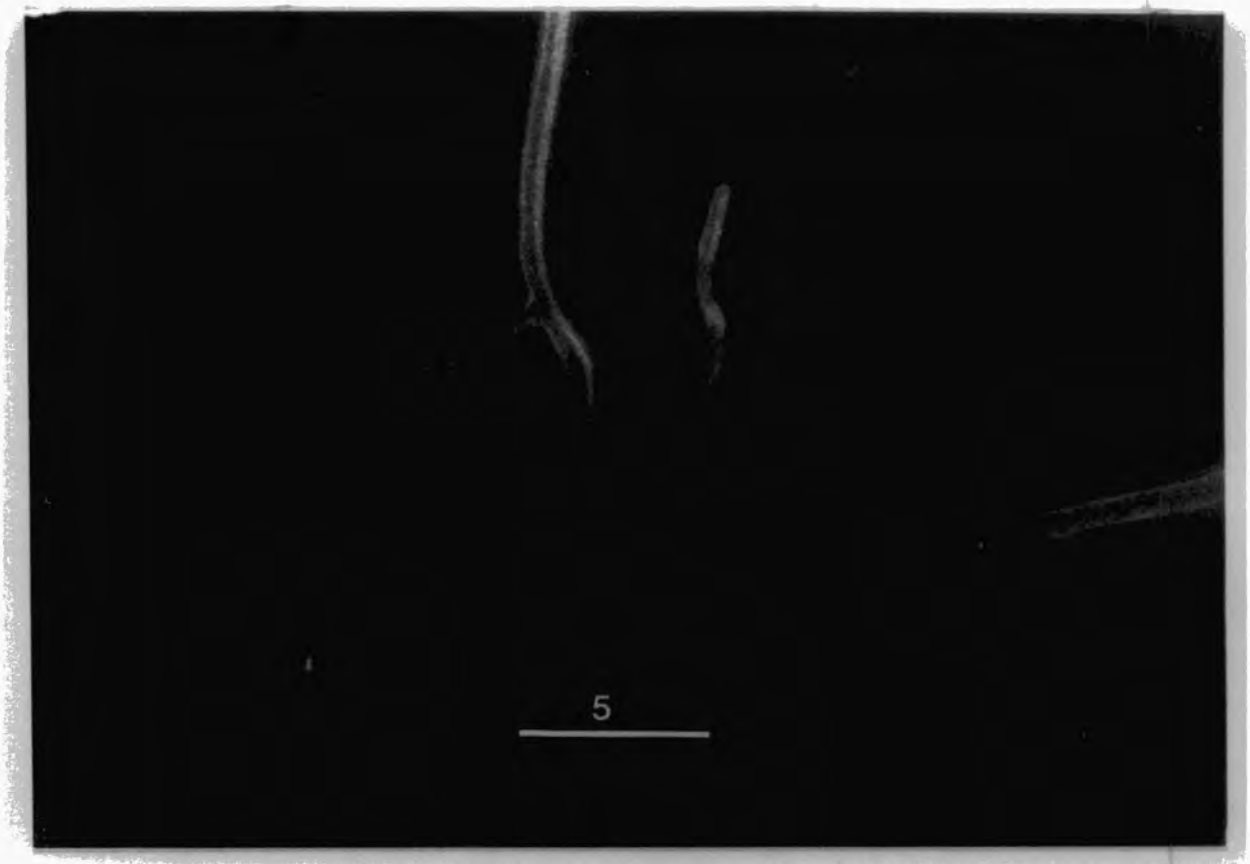

5

Fig. 26: Indirect immunofluorescent micrograph of *Pythium debaryanum* hyphae cross-reacted with *Saprolegnia parasitic* antiserum. $^{1}/_{32}$ AS dilution, 463.

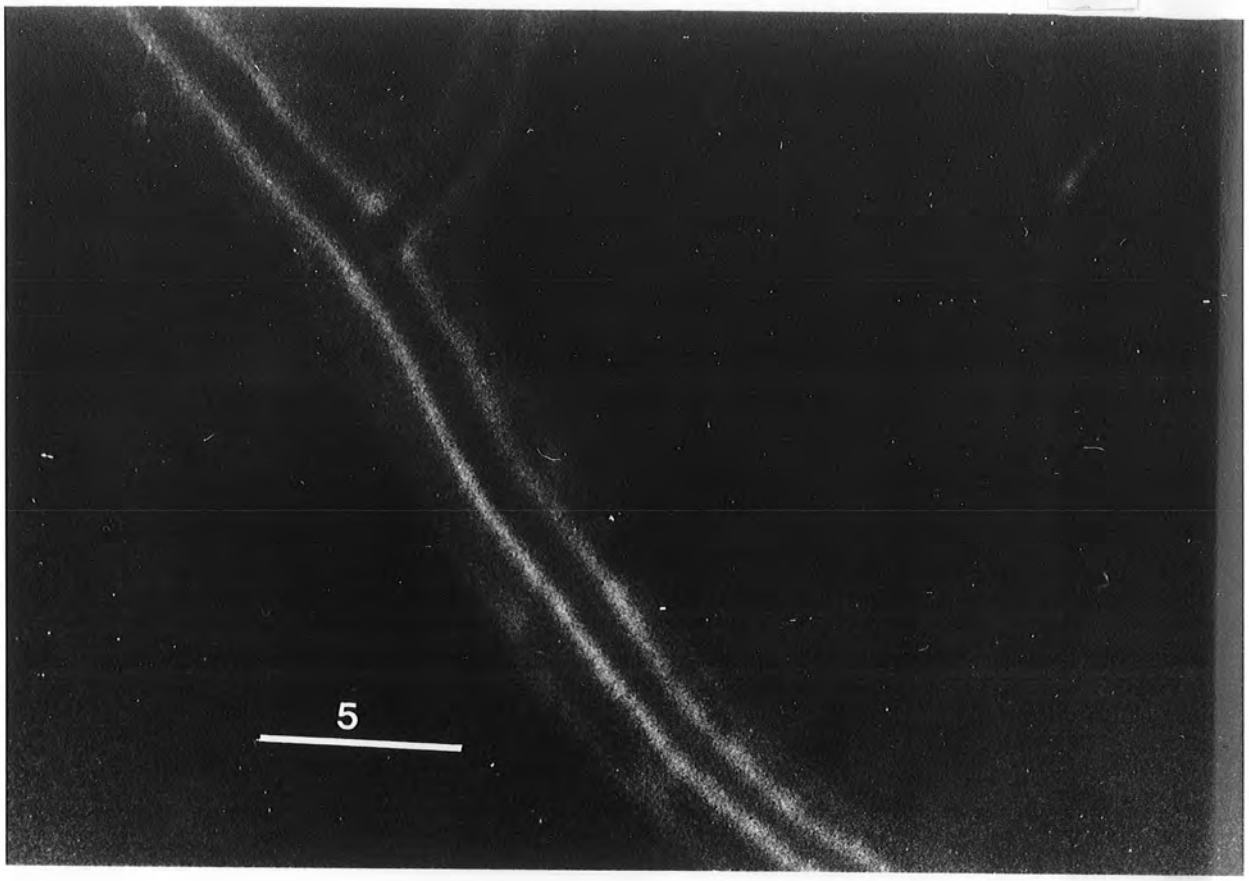
Fig. 27: Indirect immunofluorescent micrograph of *Phytophthora parasitica* hyphae cross-reacted with *Saprolegnia parasitica* antiserum. $^{1}/_{32}$ AS dilution, X463.


5


10

Fig. 28: Indirect immunofluorescent micrograph of *Saprolegnia terrestris* cross-reacted with *Saprolegnia parasitica* antiserum. $^{1}/_{32}$ AS dilution, X463.

Fig. 29: Indirect immunofluorescent micrograph of *Achlya beneckii* hyphae cross-reacted with *Pythium debaryanum* antiserum. $^{1}/_{32}$ AS dilution, X463.


5

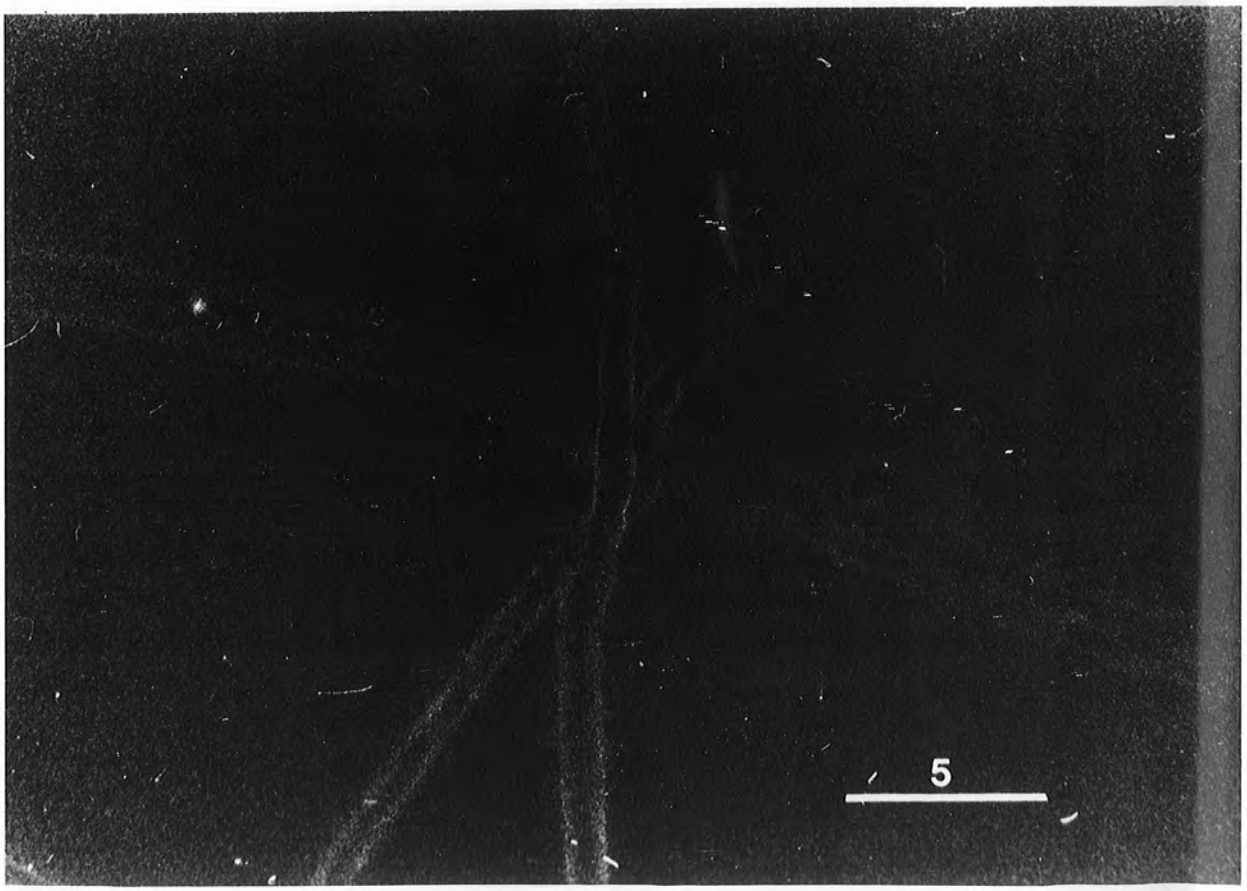
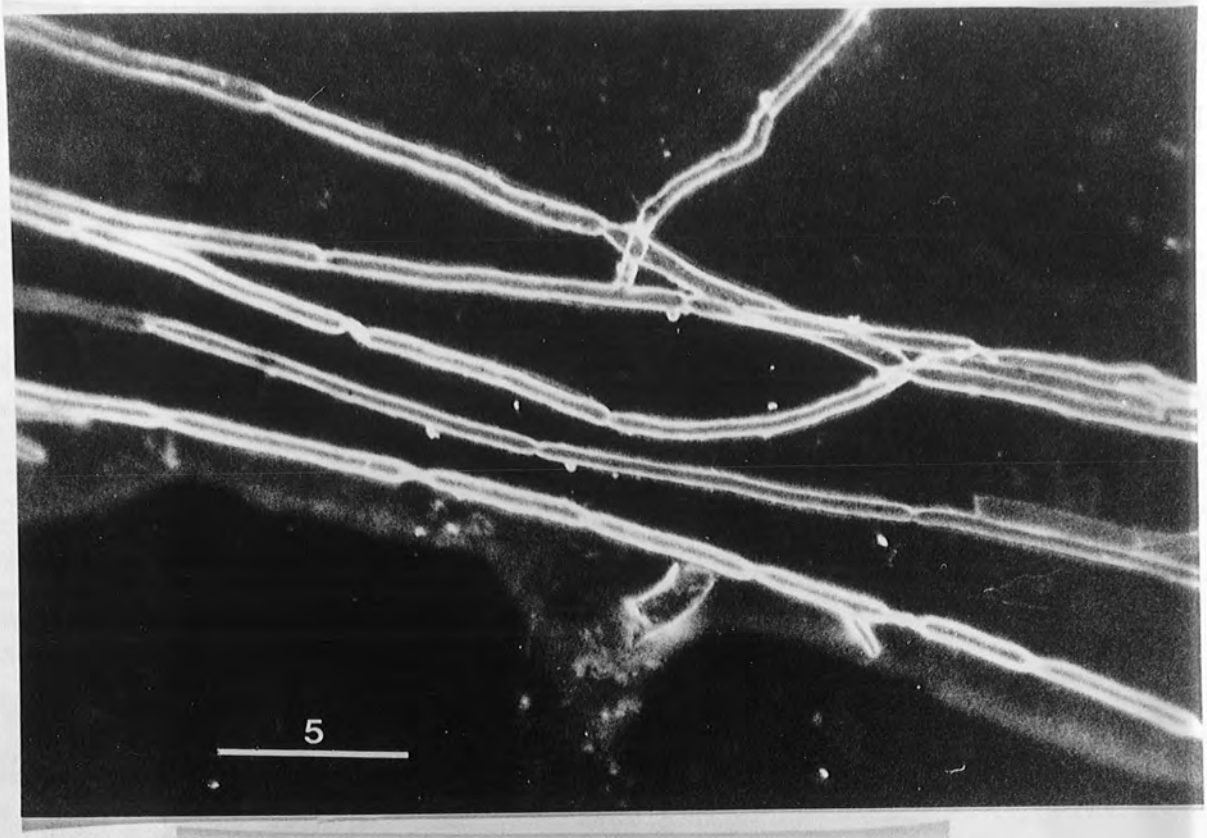

5

Fig. 30: Indirect immunofluorescent micrograph of *Apodachlya brachynema* hyphae cross-reacted with *Saprolegnia parasitica* antiserum. $^{1}/_{32}$ AS dilution, X463.

Fig. 31: Indirect immunofluorescent micrograph of *Sapromyces elongatus* cross-reacted with *Saprolegnia parasitica* antiserum. $^{1}/_{32}$ AS dilution, X463.


5

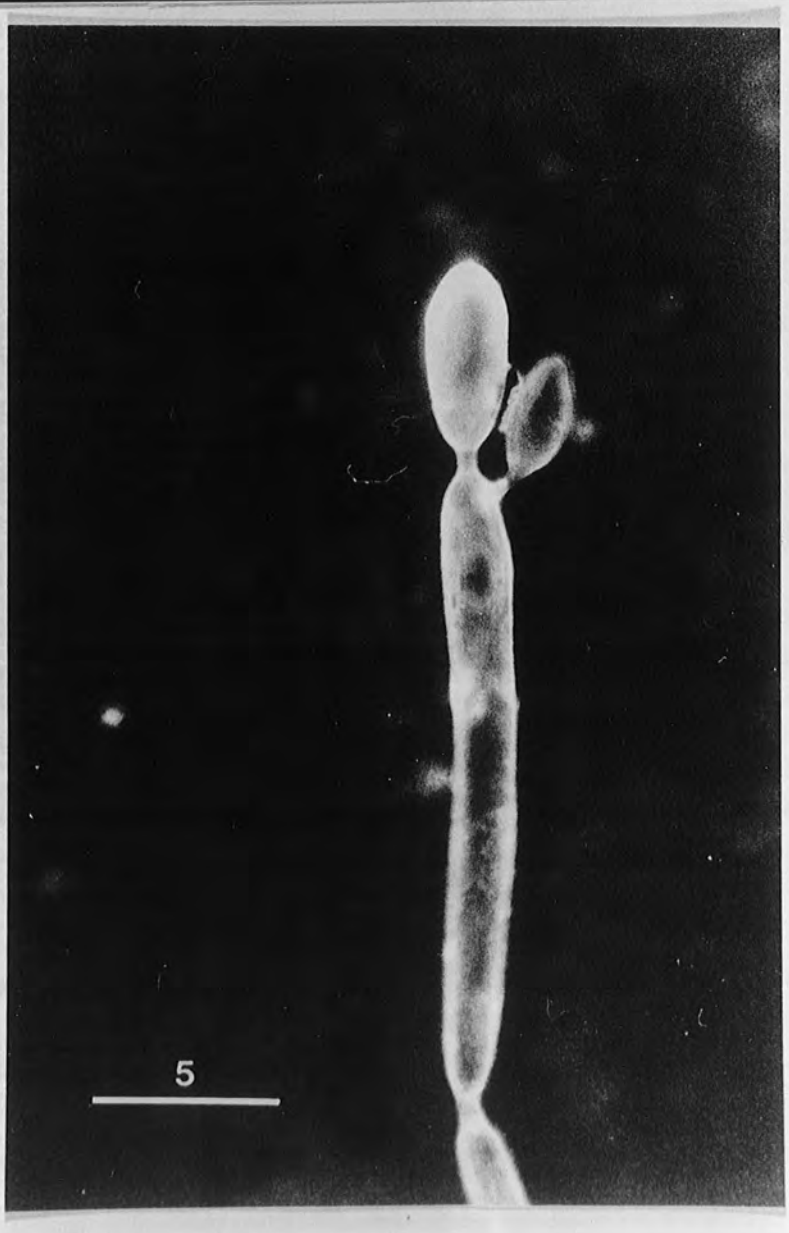

5

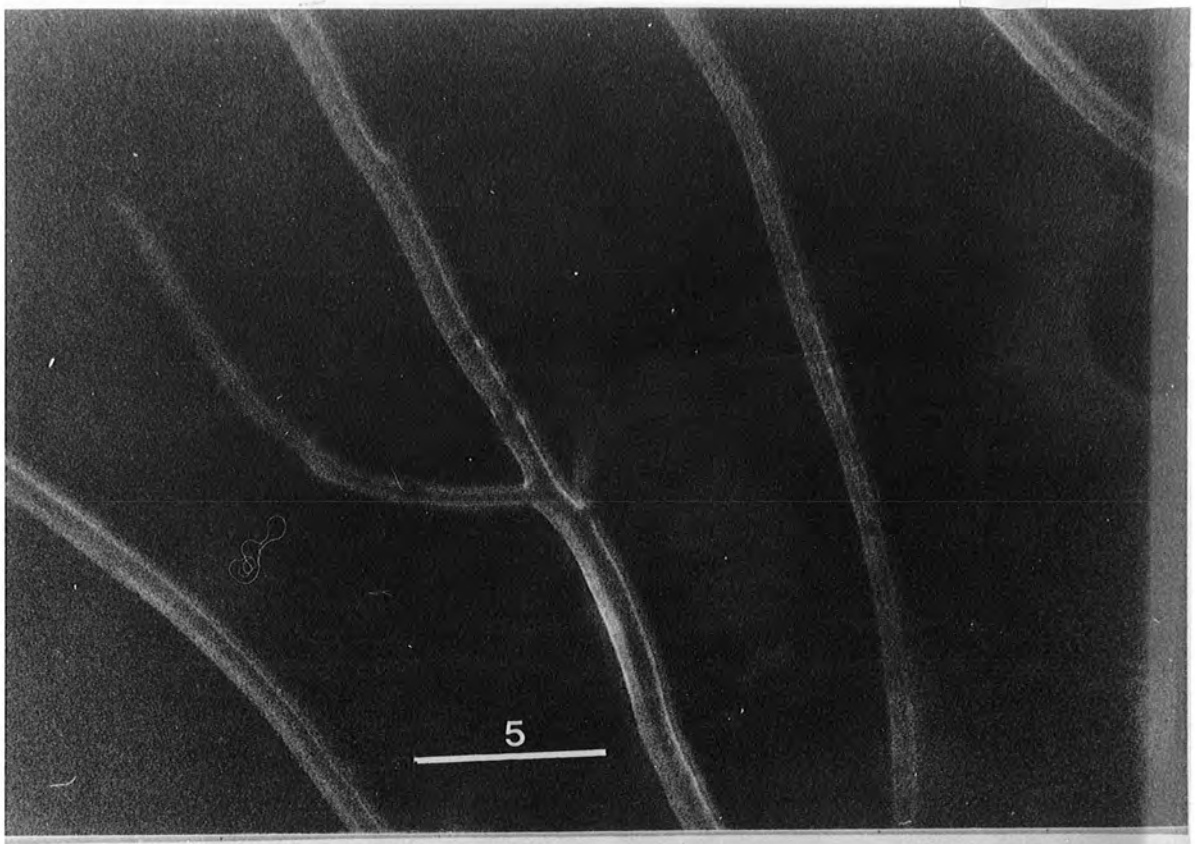
Fig. 32: Indirect immunofluorescent micrograph of *Aphanomyces cladogamus* hyphae cross-reacted with *Saprolegnia parasitica* antiserum. $^{1}/_{32}$ AS dilution, X432.

Fig. 33: Indirect immunofluorescent micrograph of *Aphanomyces cladogamus* hyphae cross-reacted with *Pythium debaryanum* antiserum. $^{1}/_{32}$ AS dilution, X463.


5
5

Fig. 34: Indirect immunofluorescent micrograph of *Aplanopsis spinosa* hyphae cross-reacted with *Mucor mucedo* antiserum. $^{1}/_{16}$ AS dilution, X463.

Fig. 35: Indirect immunofluorescent micrograph of *Aplanopsis spinosa* hypha cross-reacted with *Pythium debaryanum* antiserum. $^{1}/_{32}$ AS dilution, X690.


5

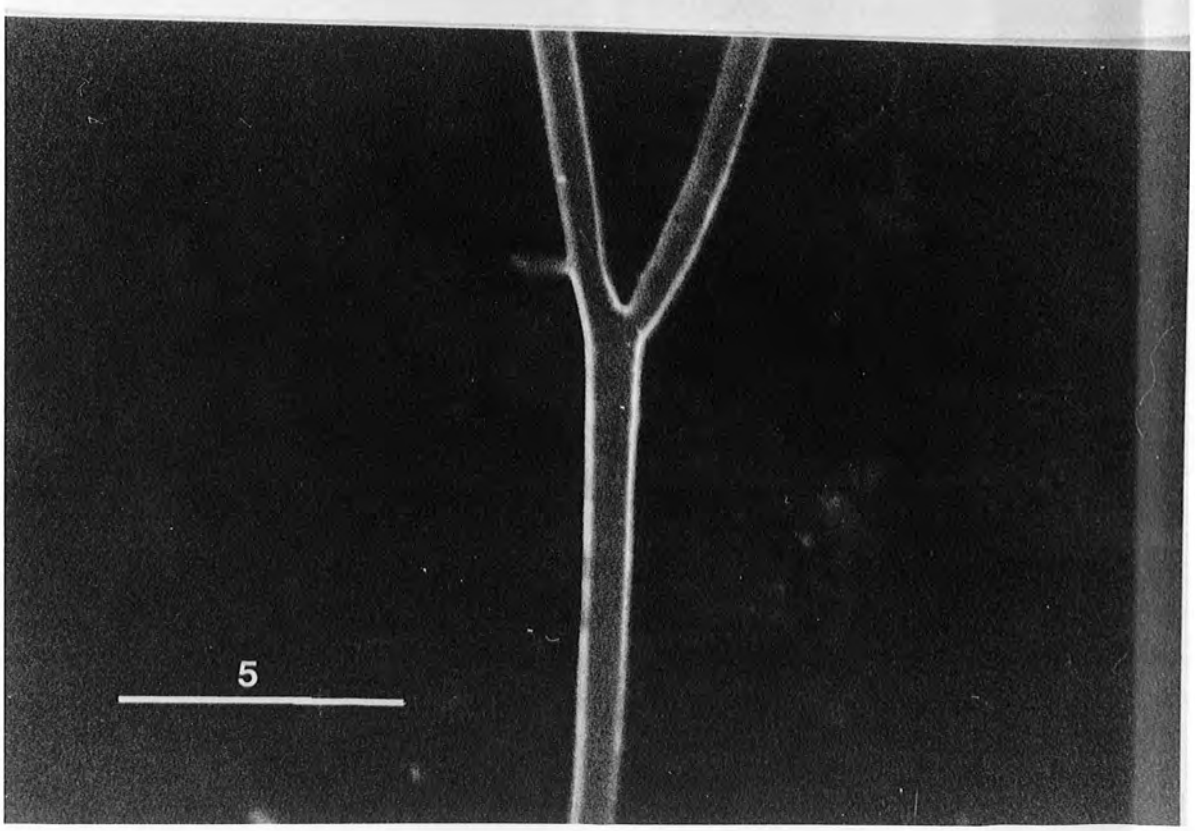

5

Fig. 36: Indirect immunofluorescent micrograph of *Aphanomyces cladogamus* hyphae cross-reacted with *Penicillium waksmanii* antiserum. $^{1}/_{32}$ AS dilution, X463.

Fig. 37: Indirect immunofluorescent micrograph of *Aphanomyces cladogamus* hyphae cross-reacted with *Mucor mucedo* antiserum. $^{1}/_{16}$ AS dilution, X463.

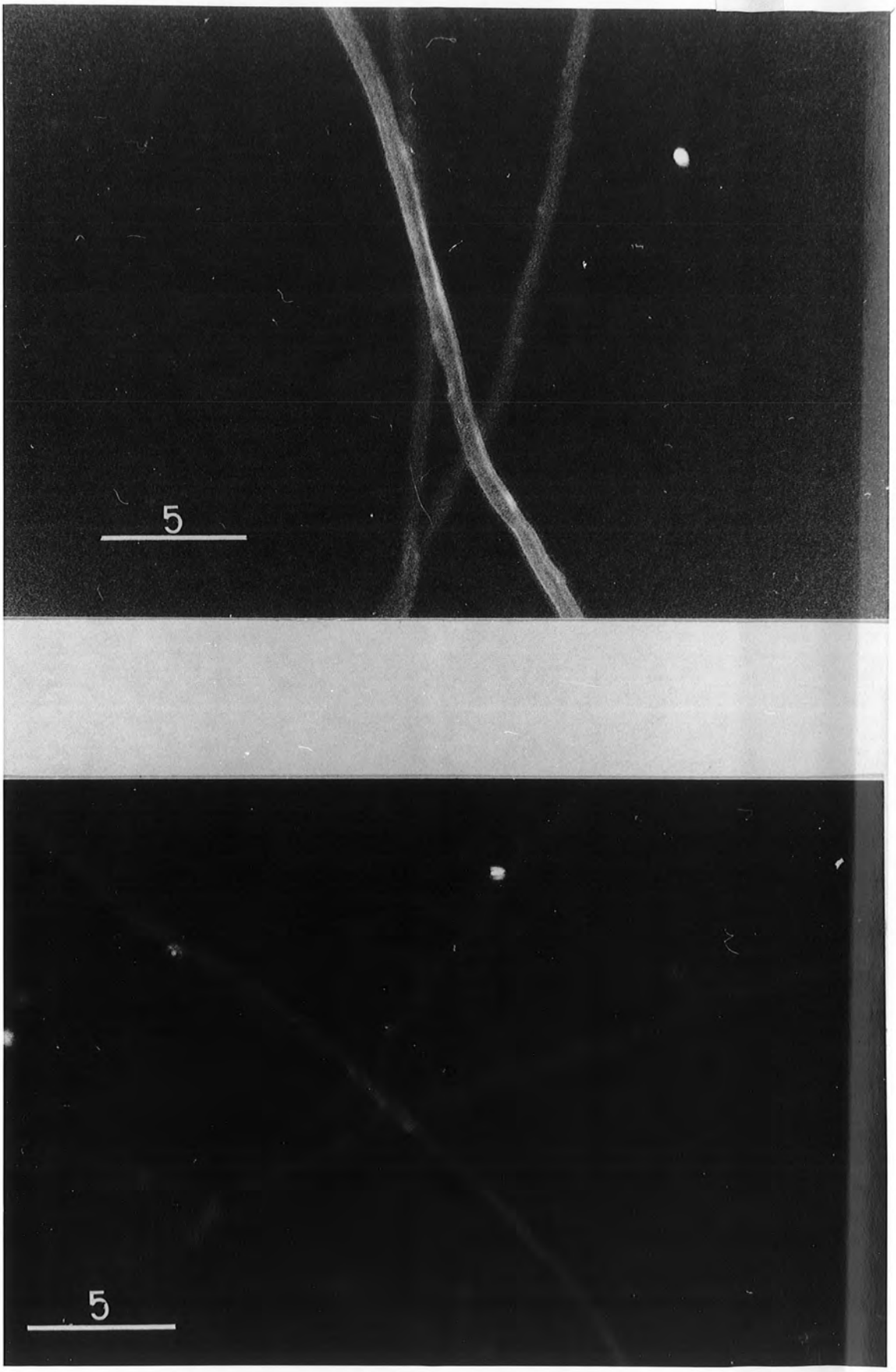
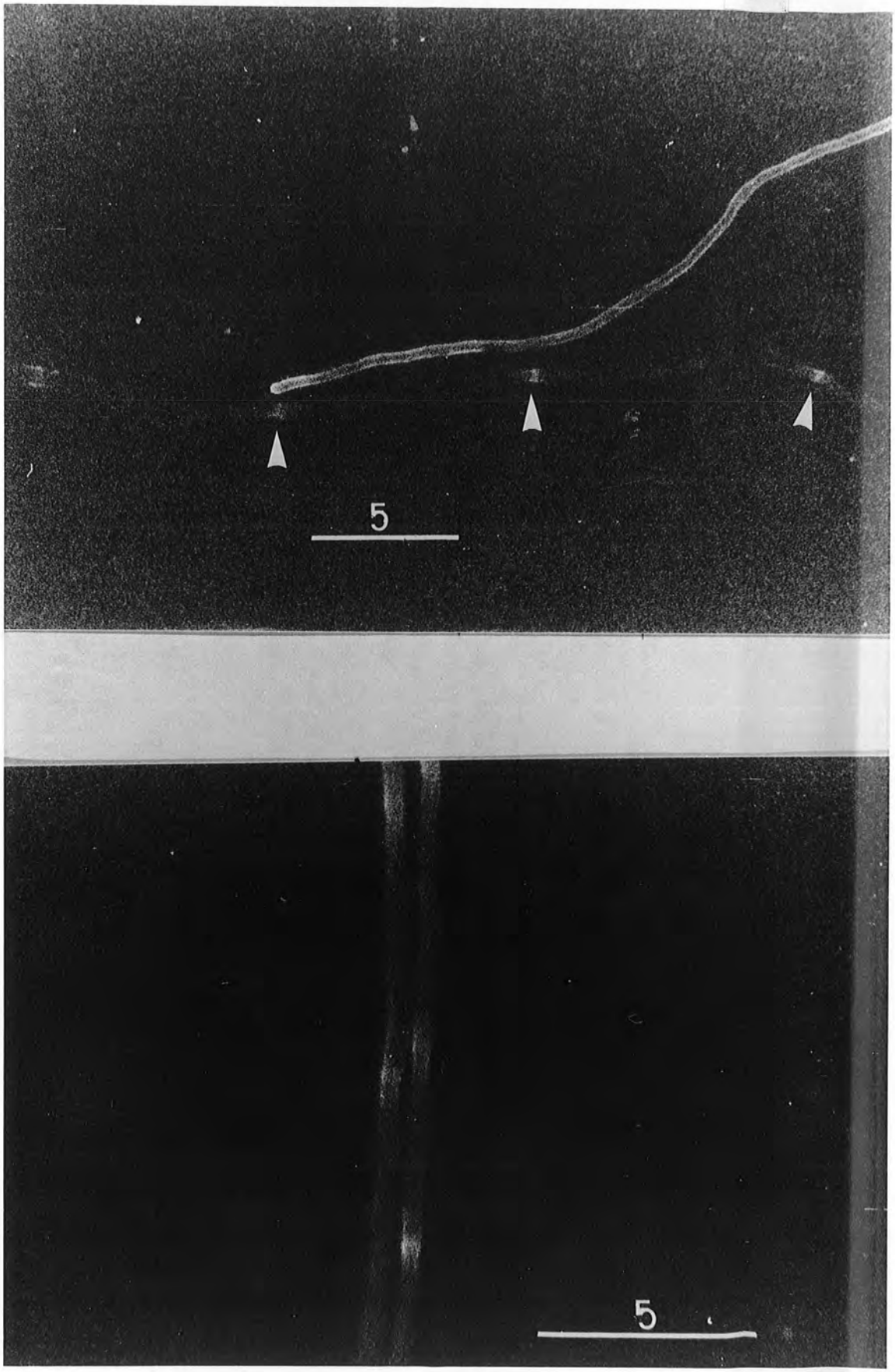

5
5

Fig. 38: Indirect immunofluorescent micrograph of two hyphae of *Pythium debaryanum* cross-reacted with *M. mucedo* antiserum, showing two types of reactions, a uniform and a spotted one (arrows). $^{1}/_{16}$ AS dilution, X463.

Fig. 39: Indirect immunofluorescent micrograph of *Pythium debaryanum* hypha cross-reacted with *Mucor mucedo* antiserum, showing the spotted reaction. $^{1}/_{16}$ dilution, X690.


5
5

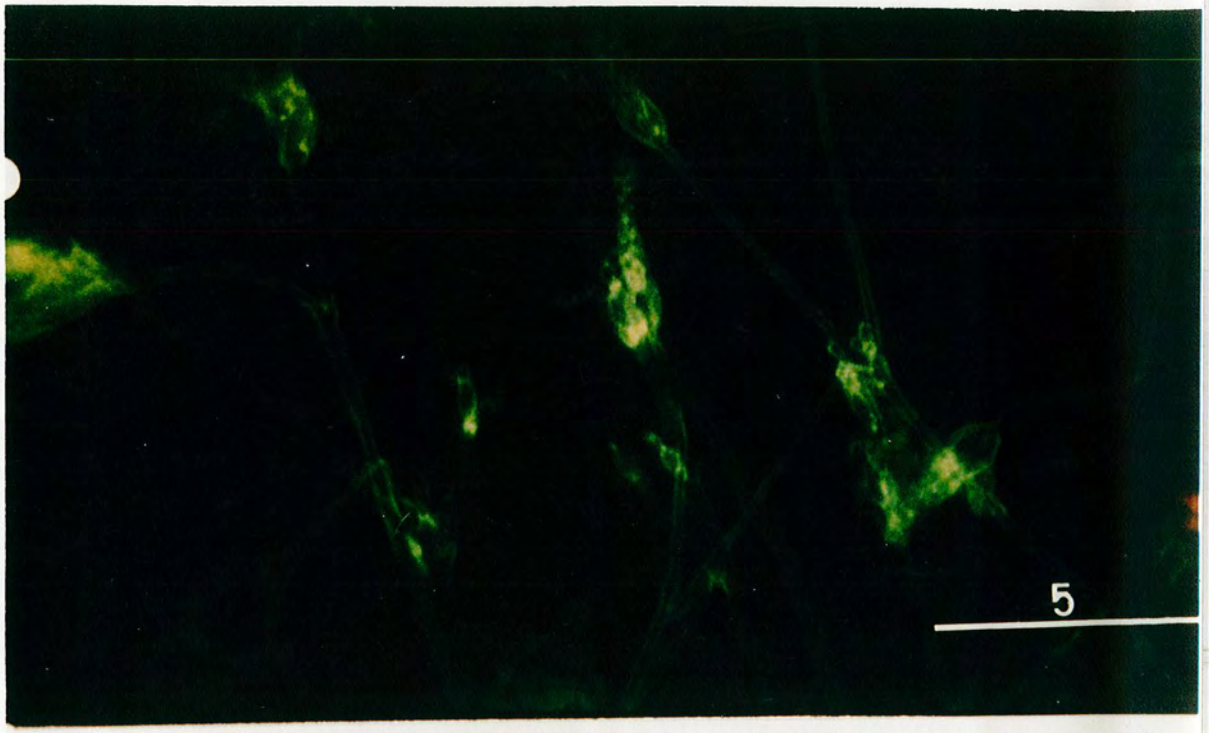
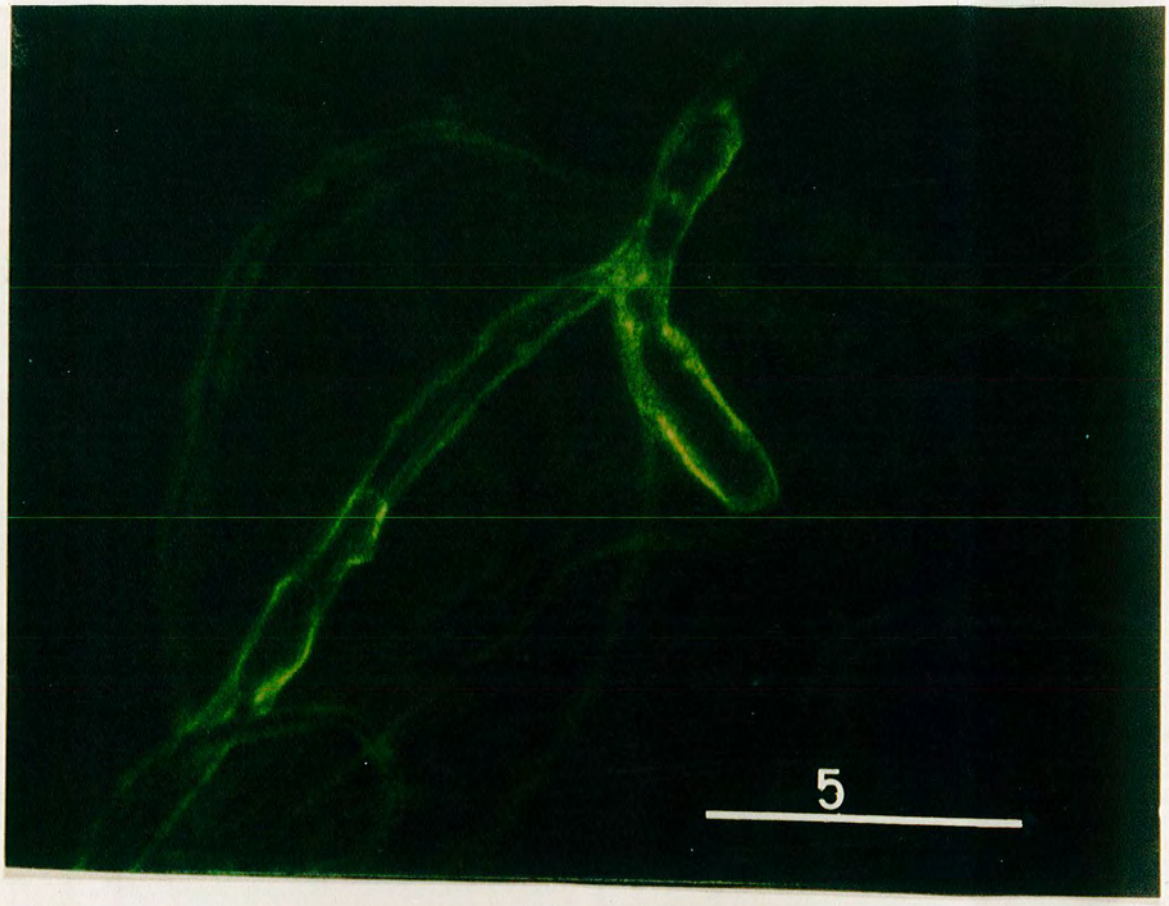
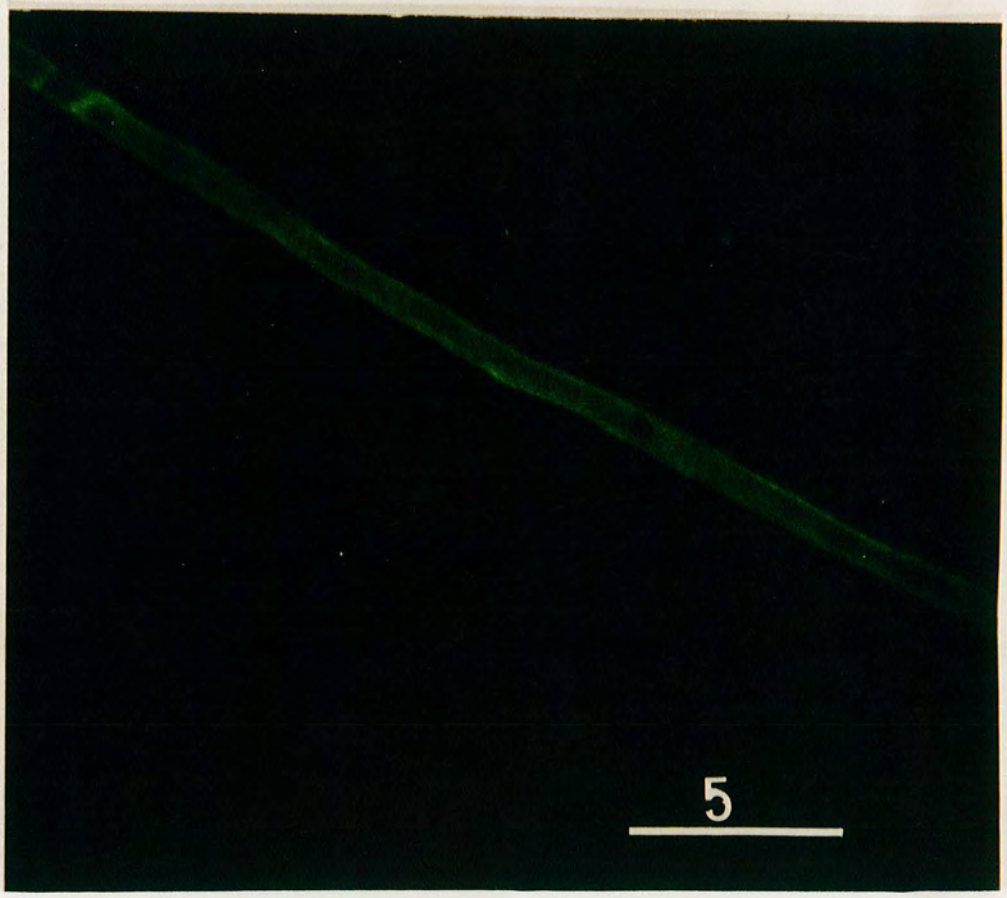


Figure 40 Yellow-green immunofluorescence of Pénicillium waksmanii hypha and conidial heads. 1/32 AS dilution, X690


5


5

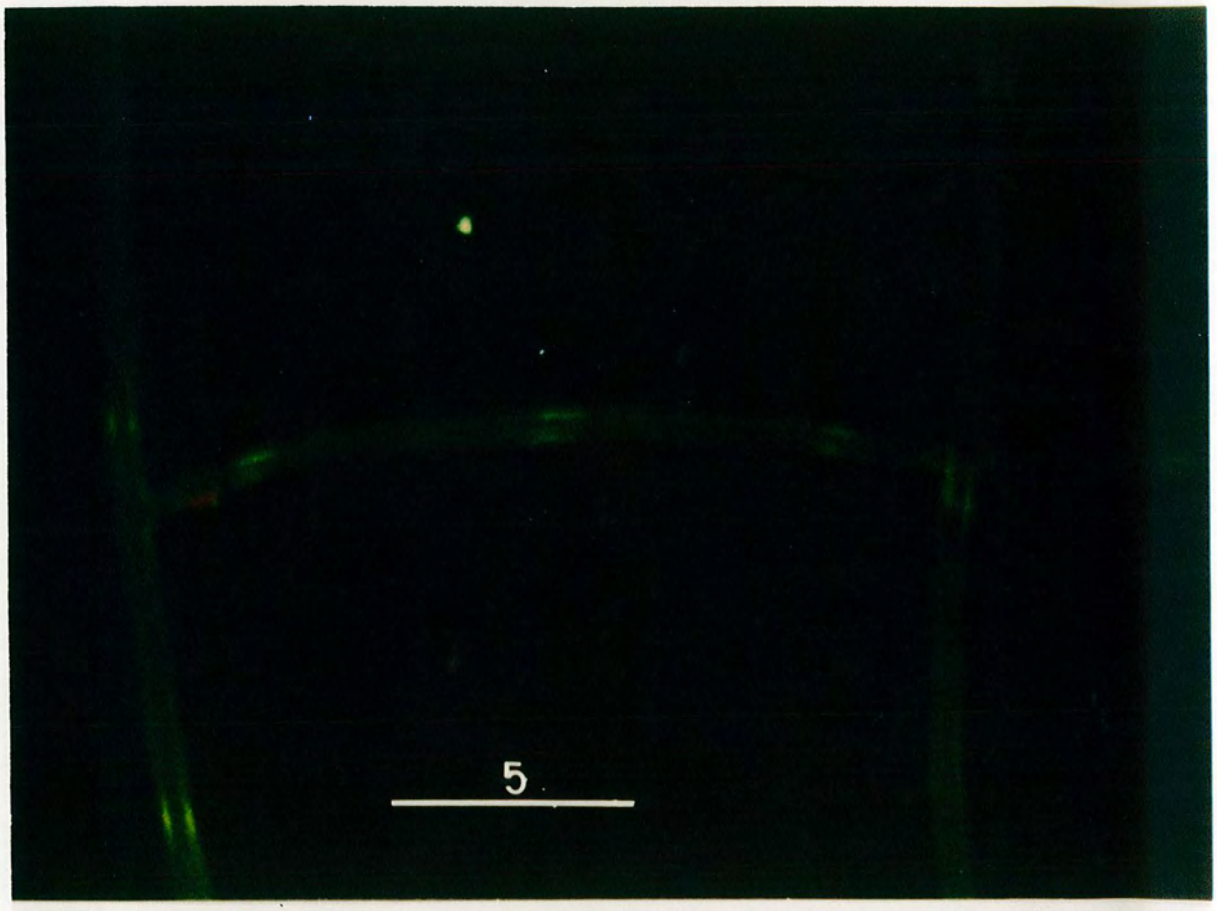


Figure 42 Blocking of a homologous sereological reaction of Pythium debaryanum hypha by prior treatment with SDS, X690

(S. parasitica). Therefore, the general conclusion from these results is that the antigen-antibody reaction of the cellulose-walled fungi was remarkably blocked by SDS and mannose (*S. parasitica* only) treatment.

3.3.8 Immunodiffusion test (IMD)

Immunodiffusion results were paralleled with the IMF staining results in that the suitable antigen-antibody reaction bands were only obtained with *P. waksmanii*, *S. parasitica* and *P. debaryanum*, whereas a very faint reaction was shown by *M. mucedo* and *P. blakesleeanus*. Undiluted antisera were tested against extracts of hyphae using PBS, SDS and Triton, in addition to culture filtrate.

P. waksmanii antiserum reacted with homologous SDS extract, two bands were developed, one band revealed a very strong reaction, whereas in reaction with the PBS and Triton extracts, only one band was detected (Fig. 43: 1A). No reaction was observed between culture filtrate and antiserum, *i.e.* no reactive extracellular antigens was found in culture filtrate.

Antisera to *M. mucedo* and *P. blakesleeanus* failed to show any detectable bands when allowed to react with their homologous extracts (Fig. 43: 2A, 5A), except for a semi-circular band surrounding the antiserum well of *M. mucedo*. Dilution of antisera and antigen extracts to the extent mentioned under methods, did not improve or reveal any precipitating antibodies.

Reaction of *S. parasitica* extracts with homologous antiserum revealed very strong detectable bands, two bands for

each extract (Fig. 43: 3A), except the culture filtrate, where no reaction could be detected.

P. debaryanum (Fig. 43: 4A) showed one band for each extract tested. An SDS extract of this fungus also revealed a very strong precipitin line. SDS proved to have the maximum ability to extract antigens; PBS and Triton generally gave weaker reactions. Therefore, SDS extracts of fungi were used for cross-reaction studies.

No cross-reaction was observed within the chitin-walled fungi (Fig.43: 1B, 2B, 5B), although a weak reaction was detected by the IMF technique. In contrast very strong reactive bands were observed within cellulose-walled fungi, especially when *S. parasitica* SDS extract was cross-reacted with *P. debaryanum* antiserum, where two bands could be detected (Fig.43, 4B). This reaction was also confirmed when the reverse antigen-antibody reaction was tested. A very faint precipitin band was also noticed between *M. mucedo* extract and *P. debaryanum* antiserum (Fig. 43: 4B).

Undiluted antisera only gave positive precipitin bands in the reactions with their heterologous antigens. Dilution of antisera to the extent mentioned previously (methods) had weakened the precipitin bands of the homologous reaction, high dilution did not reveal any reactive bands.

Many difficulties were encountered when staining the antigen-antibody reaction bands revealed by the IMD test. These were thought to be due to the impurities in the antisera, it was difficult to differentiate the bands, noticed before staining, from the dark stained background. Therefore,

an attempt was made to purify the antisera by dialysis against distilled water and several different buffers (under methods). The results of this purification resulted in the loss of some activity detected as a reduction in the intensity of precipitin bands or loss of some bands seen before purification. An attempt was also made to concentrate the weak antisera against *M. mucedo* and *P. blakesleeanus*. No specific bands could be seen before or after concentration but the very dark ring around the antiserum well remained.

Fig. 43: Immunodiffusion pattern of the reactions of undiluted antisera against antigens extracted from mycelia with PBS, SDS, Triton-X100, in addition to culture filtrate.

1 - *Penicillium waksmanii* antiserum (Pw)

A - Homologous reaction

(a) PBS

(b)(e) SDS

(c)(f) Triton-X100

(d) Culture filtrate

B - Homologous and heterologous reactions using SDS extracts of:

(g) *Penicillium waksmanii*

(h) *Phycomyces blakesleeanus*

(i) *Pythium debaryanum*

(j) *Saprolegnia parasitica*

(k) *Mucor mucedo*

2 - *Mucor mucedo* antiserum (Mm)

A -)
) as above
B -)

3 - *Saprolegnia parasitica* antiserum (Sp)

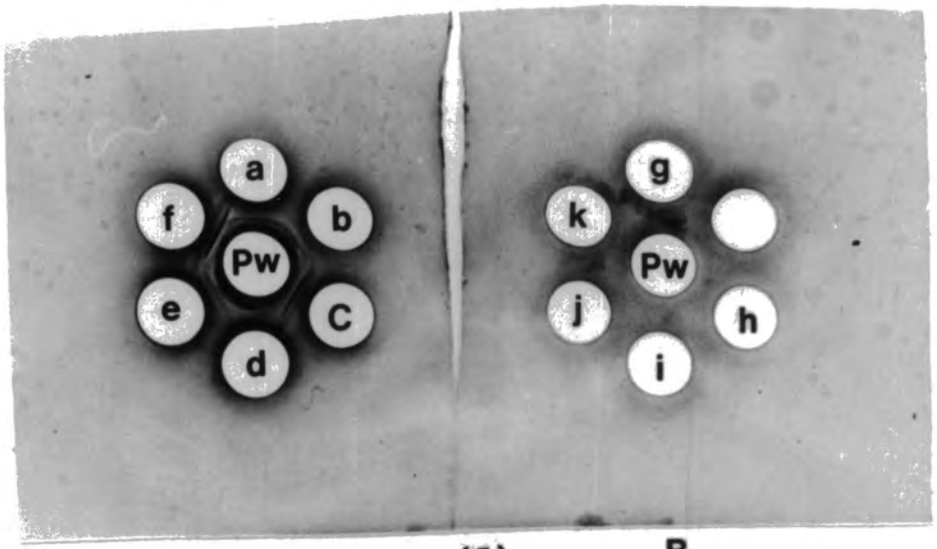
A -)
) as above
B -)

4 - *Pythium debaryanum* antiserum (Pd)

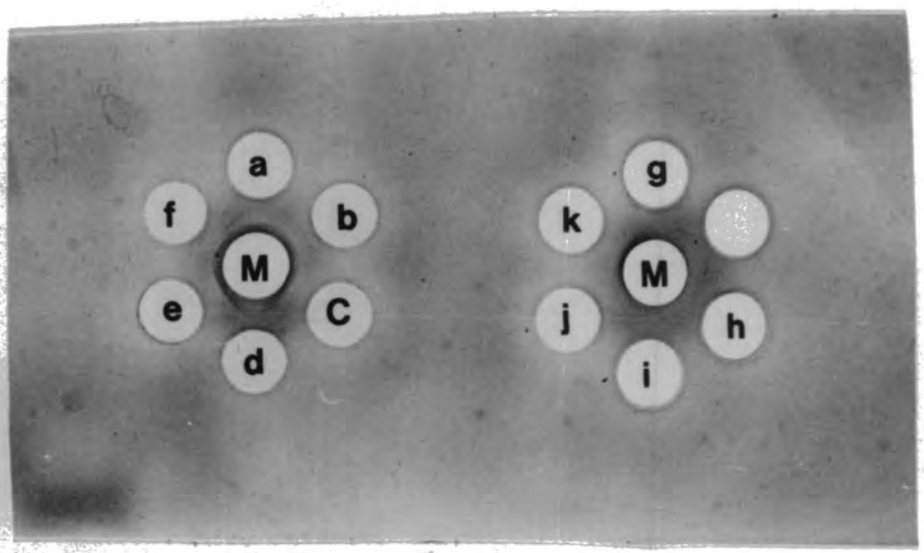
A -)
) as above
B -)

5 - *Phycomyces blakesleeanus* (Pb)

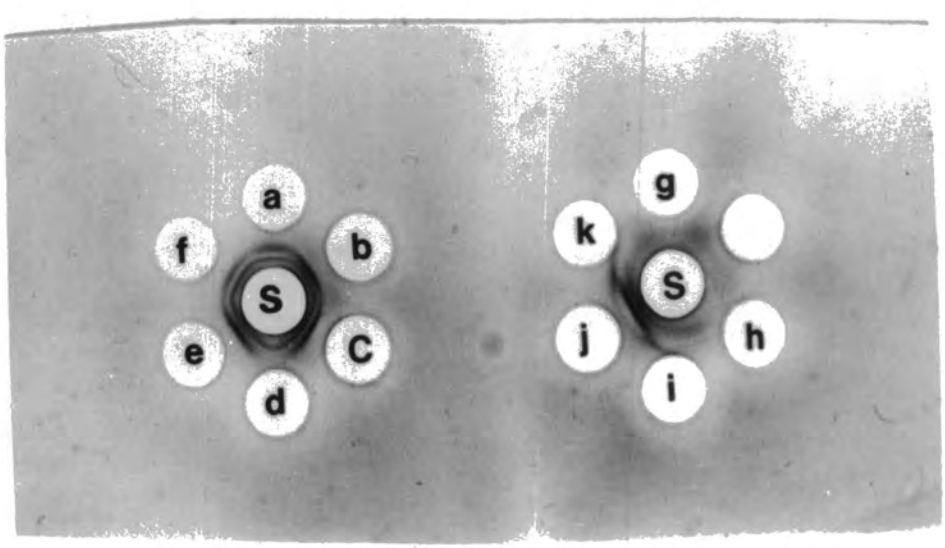
A -)
) as above
B -)



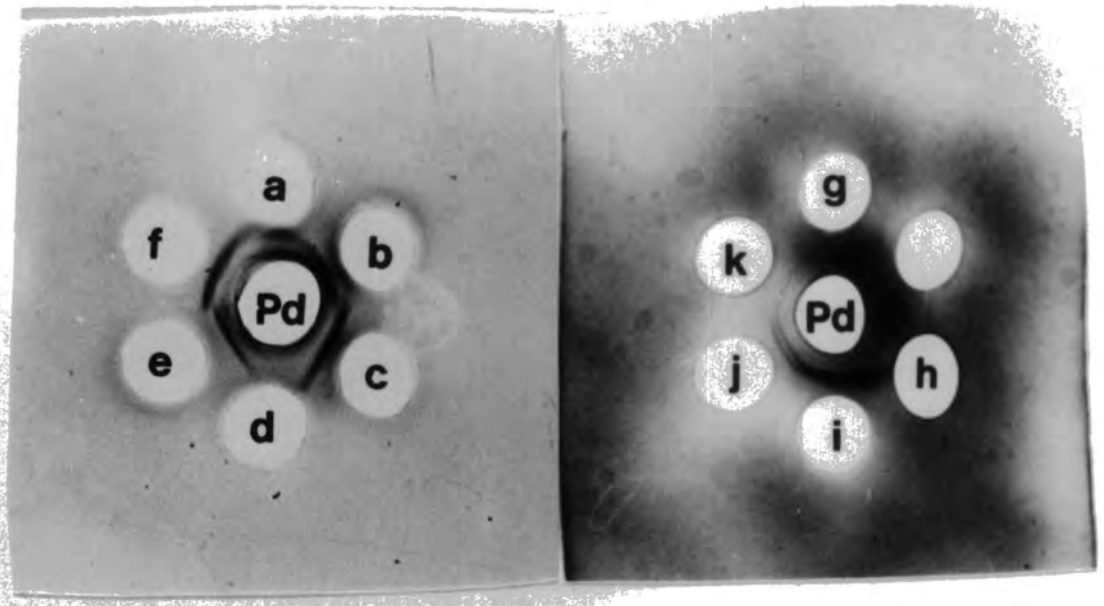
A (1) B



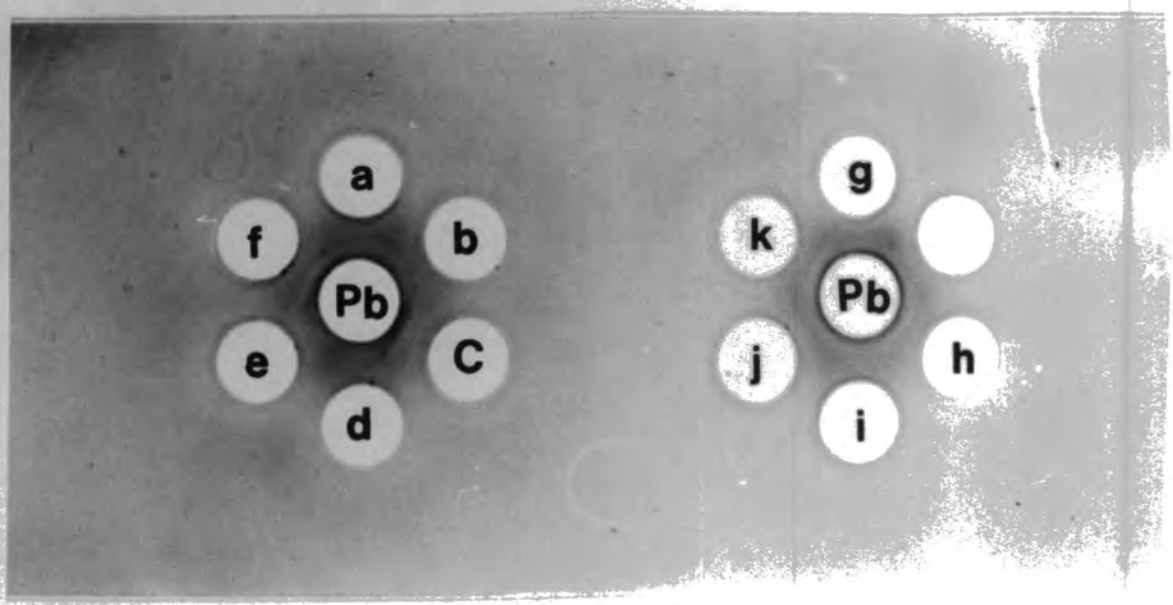
A (2) B



A (3) B



A (4) B



A (5) B

3.4 Discussion

The effectiveness of commercial enzyme novozyme 234 and cellulase in releasing protoplasts from chitin and cellulose-walled fungi respectively, was confirmed in this study. Although it has been reported that specific enzymes alone are generally ineffective in producing protoplasts from fungi, Sietsma *et al* (1967) failed to obtain protoplasts from *Pythium* sp. using cellulase. This may not be surprising in view of the complexity and diversity of hyphal wall composition (Bartnicki-Garcia, 1968). However, Hamlyn *et al* (1981) pointed out that many fungi contain chitin and glucan as the major cell wall components, and enzymes hydrolysing these polymers may be present as side activities in commercial enzyme preparations such as novozyme 234. This enzyme has been shown to contain a very high proteolytic activity, in addition to chitinase, α-D-glucanase, β-D-glucanase, as side enzymatic activities. Additionally commercial cellulase preparation have been shown to contain α-D-glucanase, β-D-glucanase with little chitinase activity (Hamlyn *et al* 1981).

The effective release of protoplasts from only young mycelia (15-24 hr. old), was possibly because the walls of these hyphae are more susceptible to lysis; because melanin is absent or present at low concentration (Carter and Bull, 1969; Bull, 1970 a,b). An alternative explanation, given by Bartnicki-Garcia and Lippman (1972a) is that the activity of wall-bound lytic enzymes in young hyphae, thought to be used by the fungus in wall assembly, may be high and this enhances lytic effect of the exogenous enzymes.

Swelling of hyphae with associated release of protoplasts from *P. blakesleeanus* was also observed in *Fusarium culmorum* (Aquirre *et al*, 1964), and in *Aspergillus nidulans* (Peberdy and Gibson, 1971). Additionally the release of different sizes of protoplasts from *P. blakesleeanus* has also been detected and explained by Peberdy and Gibson (1971), as a reflection in differences in susceptibility of the different regions of hyphae to the lytic enzymes: the small protoplasts being released from the terminal region and the large ones from the older parts.

The ability of the osmotic stabilizer, $MgSO_4$ to release and stabilize protoplasts was also observed by Sietsma *et al* (1967), Peberdy (1979b), Amaral *et al* (1983). Peberdy indicated that this ability of $MgSO_4$ might be due to the involvement of cation binding to the hyphae. Whereas Amaral *et al* pointed out that the effect of this stabilizer on the release of protoplasts is not entirely osmotic.

Described pattern of protoplasts regeneration of *P. blakesleeanus* was more or less in agreement with type of regeneration of other filamentous fungi, as *Neurospora crassa* (Bachmann and Bonner, 1959), *Fusarium culmorum* (Garcia Acha *et al*, 1966). *Aspergillus nidulans* (Peberdy and Gibson, 1971) and *Pythium* sp. (Sietsma and De Boer, 1973). Whereas pattern of protoplast regeneration observed for *S. parasitica* was very different; a primary very thin hypha with a swollen tip originated from the protoplast and this gave rise to a normal hypha. Apparently, this type of regeneration has never been recorded in the literatures for any fungus.

Unsuccessful attempts to prepare wall specific antisera only, by adsorption onto protoplasts or reverse adsorption onto pure wall fractions followed by elution, may be due to the existence of common antigenic components both in the wall and in the subcellular materials (protoplasts), *i.e.* existence of enzyme activity closely associated with the membrane / wall as was suggested by Wessels and Sietsma (1981). This adsorption has never been done before using IMF technique on mixed antiserum. In their work, Hearn and Mackenzie (1979), Wilson and Hearn (1982), have shown that wall-free cytoplasmic extracts obtained from protoplasts differed antigenically and in chemical structure from surface-located structures. However, they did not prepare a purified cytoplasmic and/or wall antiserum from a mixed one.

Immunofluorescent staining technique was more sensitive than immunodiffusion and the tube precipitin test in revealing homologous antigen-antibody reaction of the fungi *P. waksmanii*, *P. debaryanum* and *S. parasitica* at dilutions of antisera up to $^{1}/_{128}$. The IMF sensitivity compared to the other test was also observed by Dewey *et al* (1983). Similar results were found for heterologous reaction with all fungi tested at dilutions of antisera $^{1}/_{16}$ to $^{1}/_{32}$. However, with IMD test undiluted antisera only gave a clear precipitin bands with both reactions (homologous and heterologous).

Presence of cross-reactivities between different genera and species in the same class or between different classes or groups (chitin or cellulose-walled) of fungi observed, indicate the presence of shared antigenic determinants in their walls, as was also detected by several other investigators

(Suzuki *et al*, 1967; Preece and Cooper, 1969; Azuma *et al*, 1971).

Very weak reactions using both test methods were obtained with *P. blakesleeanus* and *M. mucedo*, despite the fact that the protein levels of these antigens injected into the rabbits were 37% and 16% respectively. These results contrast with those obtained with *P. waksmanii*, *S. parasitica*, and *P. debaryanum* where the reactions were very strong at protein content of original antigen of 8, 20 and 20% respectively. These results indicate the possibility that the antigenic activity, especially of *P. blakesleeanus* and *M. mucedo*, was not primarily determined by protein. Hayashi *et al* (1978a) in their study on *Absidia cylindrospora* (member of mucroales) found that the serologically active substance was fucomannan-peptide, composed of fucose and mannose and a very small amount of protein. It was also suggested that this serologically active substance may be loosely bound on the surface of the mycelium and was easily released during extraction of antigen. Miyazaki and Irino (1970) isolated an acidic polysaccharide from alkaline extracts of the cell wall of *M. mucedo* and some other mucorales. This extract had no serological reactivity with anti-wall serum, despite the presence of the fucose-containing polysaccharide (Miyazaki *et al*, 1977). Therefore it appears that the fuco-mannan-peptide of *Absidia cylindrospora* is different from the polysaccharide in the cell wall of *M. mucedo*, such as mucoric acid and mucoran (the acidic polysaccharide) (Bartnicki-Garcia and Reyes, 1978).

As mentioned earlier in the Introduction (3), mannan and mannan-containing polysaccharides were found to be the major

cell wall polysaccharide antigens of fungi. Hayashi *et al* (1978b) reported that the chemical structures of the common immunological determinants of the serologically active substance of mucorales may be different from those of the mannan, since neither purified mannan from *Saccharomyces cerevisiae* and *Candida albicans* nor galactomannan from *Penicillium chrysogenum*, cross reacted with the antiserum of *Absidia cylindrospora* which has specificity only for mucorales. Lack of reactivity of *P. blakesleeanus* and *M. mucedo* antisera may also be due to high levels of cytoplasmic protein present in these antigenic preparations, which gives minimal reaction compared to a highly reactive antigenicity of mycelial walls, which was shown by Wilson and Hearn (1982).

The identities of fungal antigens which reacted strongly with the homologous and/or heterologous antisera were not established in this study. However, the indirect method of blocking these reactions using COA, mannose and SDS gave some indication of the nature of these components. COA which is known to bind with sugar residues and specifically with α-N-acetylglucosaminyl substituents of the wall teichoic acid (Archibald and Coapes, 1972). This blocking reagent showed slight reduction of fluorescence intensity of the three fungi tested, *i.e.* caused a slight aggregation of immunologically active substances, suggesting that walls of these fungi may, however, contain a small proportion of α-linked N-acetylglucosaminyl substituents or the walls contain mainly β-glucosyl substituents.

The blocking reaction in *S. parasitica* by treatment with mannose indicated the presence of this sugar in its cell wall

in higher amounts than in the other two fungi.

The most effective blocking reaction was observed for the homologous antigen-antibody reaction of *P. debaryanum*, when it was treated with SDS, *i.e.* SDS had blocked any possible protein reactive antigen in the cell wall and hence the remaining reactive antigen could be due to glucans, which appeared as distinct points on the surface. It is therefore concluded from the blocking results, that some of the reactive antigens were possibly glycoprotein in nature, with glucan and protein moieties as their active sites. Wessels and Sietsma (1981) have confirmed the presence of glycoprotein in fungal walls.

Two typs of fluorescence reactions (uniform and spotted) were obtained when *P. debaryanum* cross reacted with *M. mucedo* antiserum. The spotted fluorescence reaction was also obtained in the blocking reaction of *P. debaryanum* with SDS, and has been reported previously by Aldoory and Gordon (1963) in older portions of hyphae of *Cladosporium*. They explained it as a decreased affinity of hyphae and spores for fluorescence reagent during ageing, which might be due to masking by other antigens or newly added materials or to enzymatic destruction. However in our study, no spotted reaction was observed with homologous antigen-antibody reaction of *P. debaryanum*, which therefore could not be related to the effect of ageing. In addition Dooijwaard-Kloosterziel *et al* (1973) also found this type of reaction (spotted) in *Geotrichum candidum* and pointed out that this might indicate that glucans are present only at distinct points on the surface or that this polymer is partly

covered with a calcofluor insensitive outer layer (*i.e.* protein).

Attempts using polyclonal antisera to prepare cell wall specific antisera were unsuccessful (reason was discussed earlier). Therefore no attempt was tried to study the effect of PGRS on regeneration of protoplasts, instead the taxonomic relationship between the two groups of fungi was accomplished.

Further studies are needed to find whether the use of monoclonal antiserum specific to chitin or cellulose wall material or to any other wall component could reveal any earlier differences in regeneration process especially with *S. parasitica*, where unusual regeneration process was obtained. It would be interesting also to follow regeneration of protoplasts after treatment with its respective antiserum and staining by IMF technique to see to what extent the cytoplasmic or membrane material incorporate into new wall. Finally it would be worth investigating the effect of PGRS on the early regeneration process of protoplasts, since it is known that these substances act on plasma membrane or within the cytoplasm of higher plant cell.

GENERAL DISCUSSION AND CONCLUSION

Farkas (1979) in his extensive review on fungal cell wall has stated that "The remarkable properties of fungal walls, such as their mechanical strength, morphological features and biological activity are undoubtedly based on their particular chemical composition". Thus much emphasis has been put on the taxonomic value of cell wall composition of fungi (Bartnicki-Garcia, 1968; Wessels and Sietsma, 1981), and when these results are compared, one is struck by the similarities rather than by differences. With the exception of hyphal walls of Zygomycetes and microfibrillar constituent of cell walls (chitin or cellulose), glucan with β(1-3) and β(1-6) linkages seem to occur in the walls of all fungi (Bartnicki-Garcia, 1968; Rosenberger, 1976). This fact was confirmed in this study since cross-reactivities were observed between species and genera of various Oomycetous fungi. On the other hand the lack of, or a weak cross-reactivity between the chitinous fungi, in addition the weak reaction between the two groups of fungi (chitin and cellulose-walled), may also reflect the fact that Zygomycetes have an exceptional cell wall composition than the fungi in all other groups. This difference may also indicate the reasons behind hyphal tip bursting and other reactions obtained as a result of flooding colonies with their liquid culture medium. Likewise, the differences in wall regeneration by protoplasts obtained from representatives of both groups of fungi could be behind this difference. However, no differences were observed as a result of PGRS treatment on vegetative growth of both fungi, since no stimulating effect was obtained with cellulose-walled fungi,

particularly as was expected. This may indicate that these substances specifically act on higher plant cells only even though they do exist in fungi. It is concluded from this study that the relationship between chitin and cellulose-walled fungi is not clear and results may not reflect the function or the involvement of the microfibrils, in particular in these reactions but rather a reflection of combination and interaction of various wall components.

REFERENCES

Adamson, D. (1962) Expansion and division in auxin-treated plant cells. Can.J.Bot. 40, 719-740.

Adebayo, A.A., R.F. Harris and W.R. Gardner (1971), Turgor pressure of fungal mycelia. Trans.Br.Myc.Soc. 57, 145-151.

Aguirre, M.J.R. and J.R. Villanueva (1962), Production of protoplast "like structures" from various species of fungi. Nature (London), 196, 693-694.

Aguirre, M.J.R., I. Garcia Acha, J.R. Villanueva (1964), Formation of protoplasts of *Fusarium culmorum* by strepzyme. Antonie van Leeuwenhoek, 30, 33-44.

Alberghina, L., E. Strutani, M.G. Costantini, E. Martegani and R. Zippel (1979), Regulation of macromolecular composition during growth of *Neurospora crassa*. In fungal walls and hyphal growth, ed. J.H. Burnett and A.P.J. Trinci, Cambridge University Press.

Al-doory, Y. and M.A. Gordon (1963), Application of fluorescent antibody procedures to the study of pathogenic dematiaceous fungi: I. Differentiation of *Cladosporium carrionii* and *C. bantianum*, J.Bact. 86, 332-338.

Amaral, D., D. Kubicki and H.F. Terenzi (1983), Release of protoplasts from the galactose oxidase producing mold, *Dactylium dendroides*, Can.J. Microbiol. 29, 763-766.

Anné, J., H. Eyssen, P. Desomer (1974), Formation and regeneration of *Penicillium chrysogenum* protoplasts, Arch.Microbiol. 98, 159-166.

Archibald, A.R. and H.E. Coapes (1972), Blocking of bacteriophage receptor sites by concanavalin A.J.Gen.Microbiol. 73. 581-585.

Aronson, J.M. (1965), The cell wall. In The Fungi: An Advanced treatise, ed. G.C. Ainsworth and A.S. Sussman, Vol.1, pp.49-76. Academic Press.

Aronson, J.M. (1981), Cell wall chemistry, ultrastructure and metabolism. In Biology of conidial fungi, Vol.2, ed. G.T. Cole and B. Kendrick, pp.459-507, Academic Press.

Aronson, J.M. and L. Machlis (1959), The chemical composition of the hyphal walls of the fungus, Allomyces. Amer.J.Bot. 46, 292-300.

Azuma, I., H. Kimura, F. Hirao, E. Tsubura, Y. Yamamura and A. Misaki (1971), Biochemical and immunological studies on Aspergilli, III, Chemical and Immunological properties of glycopeptide obtained from *Aspergillus fumigatus*. Jap.J. Microbiol. 15, 237-246.

Bachmann, B.J. and D.M. Bonner (1959), Protoplasts from *Neurospora crassa*, J. Bact. 78, 550-556.

Banbury, G.H. (1952), Physiological studies in the Mucorales.II. Some observations on growth regulation in the sporangiophore of *Phycomyces*. J. Exptl.Bot. 3, 86-94.

Bartnicki-Garcia, S. (1966), Chemistry of hyphal walls of *Phytophthora*, J.Gen.Microbiol. 42, 57-69.

Bartnicki-Garcia, S., (1968), Cell wall chemistry, morphogenesis and taxonomy of fungi. Ann.Rev. Microbiol. 22, 87.

Bartnicki-Garcia, S. (1973), Fundamental aspects of hyphal morphogenesis. Symp.Soc.Gen.Microbiol. 23, 245-267.

Bartnicki-Garcia, S. and E. Lippman (1966), Liberation of protoplasts from mycelium of *Phytophthora*. J.Gen.Microbiol. 42, 411-416.

Bartnicki-Garcia S. and E. Lippman (1972a), The bursting tendency of hyphal tips of fungi: presumptive evidence for a delicate balance between wall synthesis and wall lysis in apical growth. J.Gen.Microbiol. 73, 487-500.

Bartnicki-Garcia, S. and E. Lippman (1972b), Inhibition of *Mucor rouxii* by Polyoxin D: Effects on chitin synthetase and morphological development. J.Gen.Microbiol.71, 301-309.

Bartnicki-Garcia, S. and E. Reyes (1968), Polyuronides in the cell walls of *Mucor rouxii*. Biochim et Biophys. Acta, 170, 54-62.

Benitez, T., S. Ramos and I. Garcia Acha (1975), Protoplasts from *Trichoderma viride*: Formation and regeneration. Arch.Microbiol. 103, 199-203.

Bennett, P.A. and M.J. Chrispeels (1972), *De novo* synthesis of ribonuclease and β-1,3-glucanase by aleurone cells of barley. Plant Physiol. 49, 445-447.

Bent, K.J. and A.G. Morton (1964), Amino acid composition of fungi development in submerged culture. Biochem.J. 92, 260-269.

Bhargava, K.S. (1946), Physiological studies on some members of the family Saprolegniaceae. V.Growth Substances. Lloydia, 9, 13-23.

Bonner, J. (1934), The relation of hydrogen ions to the growth rate of Avena coleoptile. Protoplasma, 21, 406-423.

Borrow, A., P.W. Brian, V.E. Chester, P.J. Curtis, H.G. Hemming, C. Henehan, E.G. Jeffreys, P.B. Lloyd, I.S. Nixon, G.L.F. Norris and M. Radley (1955), Gibberellic acid, a metabolic product of the fungus *Gibberella fujikuroi*: some observations on its production and isolation. J.Sci.Food.Agric. 6,

Bowman, B.J. and C.W. Slayman (1977), Characterization of the plasma membrane adenosine triphosphatase of *Neurospora crassa*. J.Biol.Chem. 252, 3357-3363.

Bowman, B.J., F. Blasco and C.W. Slayman (1981a), Purification and characterization of the plasma membrane ATPase of *N. crassa*, J.Biol.Chem. 256, 12343-12349.

Bowman, E.J., B.J. Bowman and C.W. Slayman (1981b), Isolation and characterization of plasma membrane from wild type *N. crassa*, J.Biol.Chem. 256, 12336-12342.

Bracker, C.E. (1967), Ultrastructure of fungi. Ann.Rev. Phytopathol. 5, 343-374.

Bracker, C.E. (1971), Cytoplasmic vesicles in germinating spores of *Gilbertella persicaria*, Protoplasma 72, 381-397.

Brian, P.W., H.G. Hemming and M. Radley (1955), A Physiological comparison of gibberellic acid with some auxins. Physiol. Plant. 8, 899-912.

Brian, P.W., G.W. Elson, H.G. Hemming and M. Radley (1954), The Plant-growth-promoting properties of gibberellic acid, ametabolic product of the fungus *Gibberella fujikuroi* J.Sci. Food Agric. 5, 602-612.

Broughton, W.J. (1968), Influence of gibberellic acid on nucleic acid synthesis in dwarf Pea internodes. Biochim et Biophys. Acta 155, 308-310.

Brunswick, H. (1924), Untersuchungen über Geschlechts und Kernverhaltnisse bei der Hymenomyzetengattung *Coprinus*. In Botanische Abhandlurgen, ed. K. Goebel, Vol.5, Jena: Gustav Fischer (In Gooday and Trinci, 1980).

Bull, A.T. (1970a), Inhibition of polysaccharases by melanin: Enzyme inhibition in relation to mycolysis. Arch.Biochem. Biophys. 137, 345-356.

Bull, A.T. (1970b), Chemical composition of wild type and mutant of *Aspergillus nidulans* cell walls. The nature of Polysaccharides and melanin constituents. J.Gen.Microbiol. 63, 75-94.

Burnett, J.H. (1979), Aspects of the structure and growth of hyphal walls. In Fungal walls and hyphal growth. ed. J.H. Burnett and A.P.J. Trinci, pp.1-26, Cambridge Univ.Press.

Butler, G.M. (1966), Vegetative structure. In the Fungi. Vol.2, ed. G.C. Ainsworth and A.S. Sussman, pp.83-112.Academic Press.

Byres, B. and L. Goetsch (1976), A highly ordered ring of membrane associated filaments in budding yeast. J.Cell. biol. 69, 717-721.

Caldwell, I.Y. and A.P.J. Trinci (1973), The growth unit of the mould *Geotrichum candidum*, Arch.Microbiol. 88, 1-10.

Cantino, E.C. and J.S. Lovett (1960), Respiration of *Blastocladiella* during bicarbonate-induced morphogenesis in synchronous culture. Physiol.plant. 13, 450-458.

Carlile, M.J. (1966), The orientation of zoospores and germtubes, In the Fungus spore, ed. M.F. Madelin, pp.175-186, Proc.18th Symp.Closton Res.Soc., Bristol, England.

Carter, B.L.A. and A.T.Bull (1969), Studies of fungal growth and intermediary carbon metabolism under steady and non-steady state conditions. Biotech.and Bioeng. 11, 785-804.

Castle, E.S. (1958), The topography of tip growth in a plant cell. J.Gen.Physiol. 41, 913-926.

Chard, J.M., T.R.G. Gray and J.C. Frankland (1983), Antigenicity of *Mycena galopus*. Trans.Br. Mycol.Soc. 81, 503-511.

Cleland, R.E. (1970), Protein synthesis and wall extensibility in the Avena coleoptile. Planta 95, 218-226.

Cleland, R.E. (1982), The mechanism of auxin-induced proton efflux. In plant growth substances, ed. P.F. Wareing, pp.23-31. Academic Press.

Cleland, R.E. and D.L. Rayle (1978), Auxin, H^+- excretion and cell elongation. Bot.Mag.Tokyo, Special Issue 1:125-139.

Cohen, J., D. Katz and R.F. Rosenberger (1969), Temperature-sensitive mutant of *Aspergillus nidulans* lacking amino sugars in its cell wall. Nature 224, 713-715.

Collinge, A.J. and A.P.J. Trinci (1974), Hyphal tips of wild type and spreading colonial mutants of *Neurospora crassa*. Arch.Microbiol. 99: 353-368.

Collinge, A.J., E.A. Miles and A.P.J. Trinci (1978), Ultra-structure of hyphae of *Penicillium chrysogenum* Thom from colonies and chemostat cultures. Trans.Br.Mycol.Soc. 70, 401-408.

Cooper, B.A. and J.M. Aronson (1967), Cell wall structure of *Pythium debaryanum*. Mycologia, 59, 658-670.

Cortat, M. (1971), Localization intracellulaire et fonction des β1-3 glucanases lors du bourgeonnement chez *Saccharomyces cerevisiae*, Thése, Zurich (In Févre, 1979).

Cortat, M., Ph.Matile and Kopp (1973), Intracellular localization of mannan synthetase activity in budding baker's yeast. Biochem.Res.Commun. 53: 482-489.

Dabbagh, R., N.F. Conant, H.S. Nielsen and R.O. Burns (1974), Effect of temperature on saprophytic cryptococci: Temperature-induced lysis and protoplast formation. J.Gen. Microbiol. 85: 177-189.

Dargent, R. and J.M. Touzé-Soulet (1976) Sur Ultrastructure des hyphes d'*Hypomyces chlorinus* Tul.Cultive en presence ou en absence de biotine. Protoplasma. 89: 49-71.

Datema, R., H.Van DenEnde and J.G.H. Wessels (1977a), The hyphal wall of *Mucor mucedo*: 1-Polyanionic polymers. Eur.J.Biochem. 80: 611-619.

Datema, R., J.G.H. Wessels and H. Van DenEnde (1977b), The hyphal wall of *Mucor mucedo*: 2-Hexosamine-containing polymers. Eur.J.Biochem. 80: 621-626.

Datko, A.H. and G.A. Maclachlan (1968), Indole acetic acid and the synthesis of glucanases and pectic enzymes. Plant Physiol. 43: 735-742.

De Vries, O.M.H. and J.G.H. Wessels (1972), Release of protoplasts from *Schizophyllum commune* by a lytic enzyme preparation from *Trichoderma viride*. J.Gen.Microbiol. 73: 13-22.

De Vries, O.M.H. and J.G.H. Wessels (1973), Release of protoplasts from *Schizophyllum commune* by combined action of purified 1,3-glucanase and chitinase derived from *Trichoderma viride*. J.Gen.Microbiol, 76: 319-330.

De Vries, O.M.H. and J.G.H. Wessels (1975), Chemical analysis of cell wall regeneration and reversion of protoplasts from *Schizophyllum commune*. Arch.Microbiol.102: 209-218.

Dewey, F.M., K.A. Donnelly and D. Foster (1983), *Penicillium waksmanii* isolated from a red sea weed *Euchema striatum*. Trans.Br.Mycol.Soc. 81: 433-434.

Dick, M.W. (1960), Morphology and taxonomy of the Oomycetes, with special reference to Saprolegniaceae, Leptomitaceae and Pythiaceae. II. Cytogenetic systems. New Phytol. 71: 1151-1159.

Dietrich, S.M.C. (1975), Comparative study of hyphal wall components of Oomycetes: Saprolegniaceae and Pythiaceae. Anais Acad.Brasil Sci., 47: 155-162.

Dofour, I.P. and A. Goffeau (1980), Molecular and Kinetic properties of the purified plasma membrane ATPase of the yeast *Schizosaccharomyces pombe*. Eur.J.Biochem. 105: 145-154.

Dooijewaard-Kloosterziel, A.M.P., J.H. Sietsma and J.T.M. Wouters (1973), Formation and regeneration of *Geotrichum candidum* protoplasts. J.Gen.Microbiol. 74: 205-209.

Dow, J.M. and P.H. Rubery (1975), Hyphal tip bursting in *Mucor rouxii*: Antagonistic effects of calcium ions and acid. J.Gen.Microbiol. 91: 425-428.

Ekundayo, J.A. and M.J. Carlile (1964), The germination of sporangiospores of *Rhizopus arrhizus*: spore swelling and germ-tube emergence. J.Gen.Microbiol. 35: 261-269.

Emerson, R. (1958), Mycological organization. Mycologia 50: 589-621.

Emerson, S. and M.R. Emerson (1958), Production and reversion of protoplast-like structures in the osmotic strain of *Neurospora crassa* Proc.Nat.Acad.Sci., U.S.A. 44: 668-671.

Farkaš, V. (1979), Biosynthesis of cell walls of fungi. Microbiol.Rev. 43: 117-144.

Févre, M. (1979), Glucanases, glucan synthases and wall growth in *Saprolegnia monoica*. In Fungal walls and hyphal growth, ed. J.H. Burnett and A.P.J. Trinci. Cambridge Univ. Press.

Févre, M. and M. Rougier (1978), Synthése de la paroi apicale des hyphes de *Saprolegnia monoica*: données cytologiques et radio autographiques. Colloque de la Société Francaise de Microscopie Electronique. Nancy 1978, (In Burnett and Trinci, 1979).

Filner, P. and Varner, J.E. (1967), A test for *de novo* synthesis of enzymes: density labelling with H_2O^{18} of barley α-amylase induced by gibberellic acid. Proc.Nat.Acad.Sci., U.S.A. 58: 1520-1526.

Fultz., S.A. and A.S. Sussman (1966), Antigenic differences in the surfaces of hyphae and rhizoids in *Allomyces*. Science, 152: 785-787.

Gabriel, M. (1968), Formation and regeneration of protoplasts in the mold *Rhizopus nigricans*, Folia Microbiol.13:231-234.

Gander, J.E. (1974), Fungal cell wall glycoproteins and peptidopolysaccharides. Ann.Rev.Microbiol.28: 103-119.

Garcia Acha, I. and J.R. Villanueva (1963), Differences in the mode of action of strepzyme and *Helix pomatia* enzyme preparations on *Trichothecium roseum* spores, Nature.200: 1231.

Garcia Acha, I., F. Lopez-Belmonte and J.R. Villanueva (1966), Regeneration of mycelial protoplasts of *Fusarium culmorum*. J.Gen.Microbiol. 45: 515-523.

Giles, K.W. and A. Myers (1966), The effects of giberellic acid and light on RNA, DNA and growth of the three basal internodes of dwarf and tall peas. Phytochem. 5: 193-196.

Girbardt, M. (1957), Der spitzenkörper von *Polystictus versicolor*. Planta 50: 47-59.

Girbardt, M. (1969), Die Ultrastruktur der apikal region von pitzhyphen. Protoplasma, 67: 413-441.

Glaston, A.W. and W.K. Purves (1960), The mechanism of action of auxin. Ann.Rev.Plant Physiol.11: 239-276.

Gooday, G.W. (1968), Hormonal control of sexual reproduction in *Mucor mucedo*. New Phytol. 67: 815-821.

Gooday, G.W. and A.P.J. Trinci (1980), Wall structure and biosynthesis in fungi. In the eukaryotic microbial cell, ed. G.W. Gooday, D.Lloyd and A.P.J. Trinci, pp.207-251. Symposia of the Society for General Microbiology 30.

Goos, R.D. and D.F. Summers (1964), Use of fluorescent antibody techniques on the observation of morphogenesis of fungi. Mycologia, 56: 701-707.

Gordon, M.A. (1962), Differentiation and classification of yeasts by the coons fluorescent antibody technique. In Fungi and fungous diseases, ed. Gilbert Dalldorf, pp.207-219, Charles C. Thomas, Springfield.

Green, P.B. (1969), Cell morphogenesis. Ann.Rev.Plant Physiol. 20: 365-394.

Green, P.B. (1974), Morphogenesis of the cell and organ axis - biophysical methods. Brookhaven Symp. in Biology, 25, 166-190. (In Gooday and Trinci, 1980).

Griffin, D.H. and C. Breuker (1969), Ribonucleic acid synthesis during the differentiation of sporangia in the water mold. *Achlya*. J.Bact. 98: 689-696.

Grove, S.N. (1978), The Cytology of hyphal tip growth. In the filamentous fungi, ed. J.E. Smith and D.R. Berry, Vol.3, pp.28-50, Wiley.

Grove, S.N. and C.E. Bracker (1970), Protoplasmic organization of hyphal tips among fungi: Vesicle and spitzenkorper, J.Bact. 104: 989.

Grove, S.N., C.E. Bracker and D.J. Morre (1970), An ultra-structural basis for hyphal tip growth in *Pythium ultimum*. Amer.J.Bot. 59: 245-266.

Gruen, H.E. (1959), Auxins and fungi. Ann.Rev. Plant Physiol. 10: 405-440.

Gull, K. (1975), Cytoplasmic microfilament organization in two Basidiomycetes fungi. J.Ultrastruct.Res. 51: 226-232.

Gull, K. and A.P.J. Trinci (1974), Detection of areas of wall differentiation in fungi using fluorescent staining. Arch.Microbiol. 96: 53-57.

Gull, K., P.M. Moore and A.P.J. Trinci (1972), Preparation of protoplasts from *Geotrichum lactis* and use of fluorescence to detect cell walls. Trans.Br.Mycol.Soc. 59: 79-85.

Hager, A., H. Menzel, A. Krauss (1971), Versuche und hypothese zur primärwirkung des auxins beim strechung swachstum. Planta 100: 4775.

Hale, M.G., C.W. Roane and M.R.C. Huang (1962), Effect of growth regulators on size and number of leaf spots and on oxygen uptake and extension growth of Colcoptile section of corn inbred lines K_{41} and K_{44}. Phytopathol. Abst. 52:188.

Halsall, D.M. (1976), Specificity of cytoplasmic and cell wall antigens from four species of *Phytophthora*. J.Gen. Microbiol. 94: 149-158.

Hamlyn, P.F., R.S. Bradshaw, F.M. Mellon, C.M. Santiago, J.M. Wilson and J.F. Peberdy (1981), Efficient protoplast isolation from fungi using commercial enzymes. Enzyme Microb.Technol. 3: 321-325.

Hasegawa, S. and J.H. Nordin (1969), Enzymes that hydrolyze fungal cell wall polysaccharides:1. Purification and properties of an endo-α-D-(1-3)-glucanase from *Trichoderma viride*. J.Biol.Chem. 244: 5460-5470.

Hayashi, O., H. Yamada, T. Yadomae and T. Miyazaki (1978a), Purification and partial characterization of the ser ologically active substance from *Absidia cylindrospora*. J.Gen. Microbiol. 106: 289-295.

Hayashi, O., T. Yadomae, H. Yamada and T. Miyazaki (1978b), Cross-reactivity of antiserum to *Absidia cylindrospora* among some Mucorales and other fungi. J.Gen.Microbiol. 108: 345-347.

Hearn, V.M. and D.W.R. Mackenzie (1979), The preparation and chemical composition of fractions from *Aspergillus fumigatus* wall and protoplasts possessing antigenic activity. J.Gen.Microbiol. 112: 35-44.

Heath, I.B., J.L. Gay and A.D. Greenwood (1971), Cell wall formation in the Saprolegniales: Cytoplasmic vesicles underlying developing walls. J.Gen.Microbiol. 65: 225-232.

Herbert, W.J. (1973), Mineral-oil adjuvants and the immunization of laboratory animals. In Handbook of Experimental Immunology, ed. D.M. Weir. Appendix 2, Blackwell Scientific. Publ.

Hornok. L. (1980), Serotaxonomy of *Fusarium* species of the section *Gibbosum* and *Discolor*. Trans.Br.Mycol.Soc. 74:73-78.

Horowitz, N.H., G. Charlange, G. Horn and N.P. Williams (1976), Isolation and identification of the conidial germination factor of *Nrueospora crassa*. J. Bact. 127: 135-140.

Hunsley, D. and J.H. Burnett (1970), The ultrastructural architecture of the walls of some hyphal fungi. J.Gen. Microbiol. 62: 203-218.

Hunsley, D. and D. Kay (1976), Wall structure of the *Neurospora* hyphal apex: Immunofluorescent localization of wall surface antigens. J.Gen.Microbiol. 95: 233-248.

Jacobsen, J.V. and J.E. Varner (1967), Gibberellic acid-induced synthesis of Protease by isolated aleurone layers of barley. Plant Physiol. 42: 1596-1600.

Jennings, D.H. (1973), Cations and filamentous fungi: invasion of the sea and hyphal functioning. In Ion transport in Plants, ed. W.P. Anderson, pp.323-335, Academic Press.

Jennings, D.H. (1979), Membrane transport and hyphal growth. In Fungal walls and hyphal growth, ed. J.H. Burnett and A.P.J. Trinci, pp.279-294. Cambridge Univ. Press.

Johnson, M.C., T.P. Pirone, M.R. Siegel and D.R. Varney (1982), Detection of *Epichloë typhina* in tall Fescue by means of Enzyme-linked-immunosorbent-assay. Phytopathol. 72: 647-650.

Jones, R.L. (1973), Gibberellins: their physiological role. Ann.Rev.Plant Physiol. 24: 571-598.

Jones, R.L. and J.V. Jacobsen (1978), Membrane and RNA metabolism in the response of aleurone cells to gibberellic acid. Bot.Mag.Tokyo, Special issue 1:83-99.

Joustra, M. and H. Lundgren (1969), Preparation of freeze dried monomeric and immunochemically pure human immunoglobulin. XVII, Annual colloquim "Protides of the biological fluids", Brugge, 17: 511-515.

Kaplan, W. and L. Kaufman (1961), Cell wall replication in *Streptococcus pyogenes*. Science, 135: 722-723.

Katz, D. and R.F. Rosenberger (1971a), A mutation in *Aspergillus nidulans* producing hyphal walls which lack chitin. Biochim. Biophys.Acta 208: 452-460.

Katz, D. and R.F. Rosenberger (1971b), Lysis of an *Aspergillus nidulans* mutant blocked in chitin synthesis and its relation to wall assembly and wall metabolism. Arch.Mikrobiol. 80: 284-292.

Key, J.L. and J.B. Hanson (1961), Some effects of 2,4-dichlorophenoxy acetic acid on soluble nucleotides and nucleic acids of soyabean seedlings. Plant Physiol. 35: 145-152.

Klein, D.T. (1962), Effect of growth-regulators on mutants of *Neurospora crassa*. Physiologia plantarum, 15: 239-245.

Koch, A.L. (1982), The shape of the hyphal tips of fungi. J.Gen.Microbiol. 128: 947-951.

Lang, A. (1966), Gibberellin and regulation of meristematic activity. In differentiation of apical meristems and some problems of ecological regulation of development in plants. pp.51-66, Academic Press.

Lang, A. (1967), Relations among cell growth, DNA synthesis and gibberellin action. Ann.N.Y.Acad.Sci. 144: 180-190.

Leelavathy, K.M. (1966), Studies on some aspects of the rhizosphere mycoflora of the important fodder grasses of Uttar Pradesh. Ph.D. Thesis, Hindu Univ. India, (In Leelavathy, 1969).

Leelavathy, K.M. (1969), Effects of growth regulating substances on fungi. Can.J. Microbiol. 15: 713-721.

Leonian, L.H. and V.G. Lilly (1937), Is Heteroauxin a growth promoting substance? Amer.J.Bot. 24: 135-137.

Lien, T., R. Pettersen and G. Knutsen (1971), Effects of indole-3-acetic acid and gibberellin on synchronous cultures of *Chlorella fusca*. Physiol.Plant. 24: 185-190.

Lopez-Belmonte, F., I. Garcia Acha and J.R. Villaneuva (1966), Observations on the protoplasts of *Fusarium culmorum* and their fusion. J.Gen.Microbiol. 45: 127-134.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.O. Rondall (1951), Protein measurement with the folin phenol reagent. J.Biol.Chem. 193: 265-275.

Madhosingh, C. (1964), Aserological comparison of three *Fusarium* species. Can.J. B ot. 42: 1143-1146.

Makarem, E.H. and N. Aldridge (1969), The effect of gibberellic acid on *Hansenula wingei*. Can.J.Microbiol.15: 1225-1230.

Mandels, G.R. (1953), A rapid cell volume assay for fungi toxicity using fungus spore. J.Bact. 65: 16-26.

Marchant, R. and D.G. Smith (1968), A serological investigation of hyphal growth in *Fusarium culmorum*. Arch.Microbiol. 63: 85-94.

Marchant, R., A. Peat and G.H. Banbury (1967), The ultrastructural basis of hyphal growth. New Phytol. 66: 623-629.

Matile, P., M. Cortat, A. Wiemken and A. Frey-Wyssling (1971), Isolation of glucanase-containing particles from budding *Saccharomyces cerevisiae*. Proc.Nat.Acad.Sci. U.S.A., 68: 636-640.

Masuda, Y. (1978), Auxin-induced cell wall loosening. Bot.Mag. Tokyo, Special issue 1: 130-133.

May, J.W. (1962), Sites of cell wall extension demonstrated by the use of fluorescent antibody. Exp.Cell.Res. 27:170-172.

McClure, W.K., D. Park and P.M. Robinson (1968), Apical organization in the somatic hyphae of fungi. J.Gen.Microbiol. 50: 177-182.

Mehrotra, B.S. (1951), Effect of certain hormones on growth and reproduction of some species of *Phytophthora*. Current Sci. 5: 131-132.

Miles, E.A. (1981), Morphology and physiology of *Penicillium chrysogenum* and *Geotrichum candidum* in batch and continuous culture. Ph.D. Thesis, University of London.

Miller, C.O. (1956), Similarity of some kinetin and red light errects. Plant Physiol. 31: 318-319.

Mishra, N.C. and E.L. Tatum (1972), Effect of L. Sorbose on Polysaccharide synthetase of *Neurospora crassa*. Proc.Nat. Acad.Sci. U.S.A., 69: 313-317.

Mitchell, W.O. and H.F. Hansenclever (1970), Precipitin inhibition of *Candida stellatoidea* antiserum with oligosaccharide obtained from the parent mannan. Infection and Immunity, 1: 61-63.

Mitchell, J.W. and P.C. Marth (1946), Germination of seeds in soil containing 2,4-dichlorophenoxy acetic acid. Bot.Gaz. 107: 408-416.

Miyazaki, T. and T. Irino (1970), Acidic polysaccharides from the cell wall of *Absidia cylindrospora, Mucor mucedo* and *Rhizopus nigricans*. Chemical and Pharmaceutical Bulletin. Tokyo, 18: 1930-1931.

Miyazaki, T. and O. Hayashi, Y. Ohshima and T. Yadomae (1979), Studies of fungal polysaccharides: the immunological determinant of the serologically active substances from *Absidia cylindrospora, Mucor hiemalis* and *Rhizopus nigricans*. J.Gen. Microbiol. 111: 417-422.

Miyazaki, T., I. Suzuki, H. Yamada, T. Yadomae, Y. Kumazawa and K. Mizunoe (1977), Studies on fungal polysaccharides, XXI, Serologically active substance(s) from *Absidia cylindrospora*, Kitasato Arch.Exp. Medicin (In Hayashi *et al*, 1978a).

Mullins, J.T. and E.A. Ellis (1974), Sexual morphogenesis in *Achlya*: Ultrastructural basis for the hormonal induction of antheridial hyphae. Proc.Nat.Acad.Sci. U.S.A. 71: 1347-1350.

Murdia, M.S. (1939), Effect of β-indolyl 3-acetic acid and phenyl acetic acid on the growth of some members of the family Saprolegniaceae, Current Science, 8: 362- 363.

Nakamura, T., K. Tomita, Y. Kawanabe and T. Murayama (1982), Effect of auxin and gibberellin on conidial germination in *Neurospora crassa*. II "Conidial density effect", and auxin. Plant and Cell Physiol. 23: 1363-1369.

Nakamura, T., Y. Kawanabe, F. Takiyamana, N. Takahashi and T. Murayama (1978), Effects of auxin and gibberellin on conidial germination in *Neurospora crassa*. Plant and Cell Physiol. 19: 705-709.

Nielsen, N. (1930), Utersuchungen über einen neuen wachstums-regulierenden st aff: Rhizopin,Jahrb.F.Wiss.Bot. 73:125 (In Banbury, 1952).

Novaes-Ledieu, M., A. Jimenez-Martinez and J.R. Villanueva (1967), Chemical composition of hyphal wall of Phycomycetes. J.Gen.Microbiol. 47: 237-245.

Nutman, P.S., H.G. Thornton and J.H. Quastel (1945), Inhibition of plant growth by 2,4-dichlorophenoxy acetic acid and other plant growth substances. Nature 155: 498-500.

Nysterakis, R. (1954), Résistance de *Neurospora tetrasperma* aux fortes doses d'auxine et relation avec l'hypotheses de l'existance des "metabolic antiauxines". C.R.Acad.Sci. 238: 143 (In Klein, 1962).

Oravec, A.A., N.G. Strovilas, J.L. Beal and A. Tye (1959), Effect of the potassium salt of gibberellic acid on the growth and reproduction rate of *Paramecium multimicronucleatum*. Nature, 184: 1405.

Ouchterlony, O. (1949), Antigen-antibody reactions in gels. Acta Pathologica et Microbiologica Scandinavica, 26:507-515.

Paleg, L.G. (1960), Physiological effects of gibberellic acid: I-On carbohydrate metabolism and amylase activity of barley endosperm. Plant Physiol. 35: 293-299.

Park, D. and P.M. Robinson (1966a), Internal pressure of hyphal tips of fungi and its significance in morphogenesis. Annals Bot. 30: 425-439.

Park, D. and P.M. Robinson (1966b), Aspects of hyphal morphogenesis in fungi. In trends in plant morphogenesis, ed. E.G. Cutter, pp.27-44, Longmans, Green and Co., London.

Parker, B.C., R.D. Preston and G.E. Fogg (1963), Studies of the structure and chemical composition of the cell walls of Vaucheriaceae and Saprolegniaceae. Proc.Roy.Soc.158:435-445.

Pearson, A.J. (1969), The effect of absicic acid on nucleic acid metabolism in plant. Ph.D. Thesis, Univ. of Wales.

Peat, A. and G.H. Banbury (1967), Ultrastructure, protoplasmic streaming, growth and tropism of *Phycomyces* sporangiophores. New Phytol. 66: 475-484.

Peberdy, J.F. (1976), Isolation and properties of protoplasts from filamentous fungi. In Microbial and Plant protoplasts, ed. J.F. Peberdy, A.H. Rose, H.J. Rogers, and E.C. Cocking pp. 39-50, Academic Press.

Peberdy, J.F. (1979a), Wall biogenesis by protoplasts. In fungal walls and hyphal growth, ed. J.H. Burnett and A.P.J. Trinci, Cambridge Univ. Press.

Peberdy, J.F. (1979b), Fungal protoplasts: Isolation reversion and fusion. Ann.Rev.Microbiol. 33: 21-39.

Peberdy, J.F. and C.B. Buckley (1973), Adsorption of fluorescent brighteners by regenerating protoplasts of *Aspergillus nidulans*. J. Gen. Microbiol. 74: 281-288.

Peberdy, J.F. and R.K. Gibson (1971), Regeneration of *Aspergillus nidulans* protoplasts. J.Gen. Microbiol. 69: 325-330.

Pegg, G.F. (1976), Endogenous auxins in healthy and diseased plants. In Physiological plant pathology, ed. R. Heitefuss and P.H. Williams, pp.582-591. Encyclopedia of Plant Physiol. Vol.4, Berlin, Springer-Verlag.

Pegg, G.R. (1981), The involvement of growth regulators in the diseased plant. In: Effects of disease on the Physiology of the growing plant, ed. P.G. Ayres, Cambridge Univ. Press.

Peeters, P.H.J. and G.W.F.H. Borst-Pauwels, (1979), Properties of plasma membrane ATPase and mitochondrial ATPase of *Saccharomyces cerevisiae*. Physiol.Plant. 46: 330-339.

Plomley, N.J.B. (1959), Formation of the colony in the fungus *Chaetomium*. Aust.J.Biol.Sci. 12: 53-64.

Preece, T.F. and D.J. Cooper (1969), The preparation and use of a fluorescent antibody reagent for *Botrytis cinerea* grown on glass slides. Trans.Br.Mycol.Soc. 52: 99-104.

Ramadani, A.S. (1978), Differentiation and ferritin synthesis in *Phycomyces blakesleeanus*: An ultrastructural and Physiological study. Ph.D. Thesis, Durham University.

Ray, P.M. and D.B. Baker, (1965), The effect of auxin on synthesis of oat coleoptile cell wall constituents. Plant Physiol. 40: 353-368.

Rayle, D.L. (1973), Auxin-induced hydrogen ion secretion in *Avena* coleoptile and its implication. Planta 114: 63-73.

Rayle, D.L. and R.E. Cleland (1972), The *in vitro* growth response: relation to *in vitro* growth response and auxin action. Planta 104: 282-296.

Reihardt, M.O. (1892), Das wachsthum der Pilzhyphen. Jahrbucher für Wissenschaftliche Botanik. 23: 479-566 (In Bartnicki-Garcia, 1973).

Richards, R.R. (1949), Responses of representative fungi to certain growth regulating substances. Bot.Gaz.110:523-550.

Rizvi, S.R.H. and N.F. Robertson (1965), Apical disintegration of hyphae of *Neurospora crassa* as a response to L-sorbose. Trans.Br. Mycol.Soc. 48: 469-477.

Robertson, N.F. (1958), Observations on the effect of water on the hyphal apices of *Fusarium oxysporum*. Annals of Botany. 22: 159-174.

Robertson, N.F. (1959), Experimental control of hyphal branching and branch form in Hyphomycetous fungi. J.Linnean Soc. (Botany), 56: 207-211.

Robertson, N.F. (1965), The mechanism of cellular extension and branching. In The Fungi, Vol.1, ed. G.C. Ainsworth and A.S. Sussman, pp.613-624. Academic Press.

Robertson, N.F. (1968), The growth process in fungi. Ann.Rev. Phytopathol. 6:115-136.

Roelofsen, P.A. (1950a), The origin of spiral growth in *Phycomyces* sporangiophores. Recueil des Travaux Botaniques Neérlandais. 42: 72-110.

Roelofsen, P.A. (1950b),Cell wall structure in the growth zone of *Phycomyces* sporangiophores. I. Model experiments and microscopical observations. Biochim et Biophys Acta 6: 340-356.

Roelofsen, P.A. (1958), Cell wall structure as related to surface growth. Acta Botanica Neerlandica, 7:77-89.

Roos, W. (1981), Kinetik und regulation des amino-Saüre-transport in *Penicillium cyclopium*. Thesis Dr. Sci., Martin-Luther Univ. Halle, G.D.R.

Roos, W. and M. Luckner (1984), Relationships between proton extrusion and fluxes of ammonium ions and organic acids in *Penicillium cyclopium*, J.Gen.Microbiol.130: 1007-1014.

Roos, U.P. and G. Turian (1977), Hyphal tip organization in *Allomyces arbuscula*. Protoplasma, 93: 231-247.

Saadi, H.A. (1980), Aspects of the growth and development of the sporangiophore of *Phycomyces blakesleeanus*. Ph.D. Thesis, Durham University.

Sequeira, L. (1973), Hormone metabolism in diseased plants. Ann.Rev. Plant Physiol. 24: 353-380.

Serrano, R. (1978), Characterization of the plasma membrane ATPase of *Saccharomyces cerevisiae*. Molecular and cellular Buochemistry, 22: 1-62.

Shaw, M. and A.R. Hawkins (1958), The physiology of host-parasite relations, V. A preliminary examination of the level of free endogenous IAA in rusted and mildewed cereal leaves and their ability to decarboxylate exogenously supplied radioactive IAA. Can.J.Bot. 36: 1-16.

Shimoda, C. and N. Yanagishima (1971), Role of cell wall - degrading enzymes in auxin-induced cell expansion in yeast. Physiol.Plant, 24: 46-50.

Sietsma, J.H. and W.R. De Boer (1973), Formation and regeneration of protoplasts from *Pythium* PRL, 2142, J.Gen.Microbiol. 74: 211-217.

Sietsma, J.H., D.E. Eveleigh, R.H. Haskins and J.F.T. Spencer (1967), "Protoplasts" from *Pythium* sp. PRL 2142. Can.J. Microbiol. 13: 1701-1704.

Sietsma, J.H., D.E. Eveleigh and R.H. Haskins (1969), Cell wall composition and protoplast formation of some Oomycetes species. Biochim et Biophys.Acta. 184: 306-317.

Slayman, C.W. (1982), Biochemical characterization of the fungal plasma membrane ATPase, an electrogenic proton pump. In Membranes and transport, Vol.1, ed. A.N. Martonosi, pp.479-483, Plenum Press.

Slayman, C.L. and C.W. Slayman (1962), Measurement of membrane potentials in *Neurospora*.Science 136: 876-877.

Smith, J.H. (1923), On the apical growth of fungal hyphae. Ann.Bot. 37: 341-343.

Smith, J.H. (1924), On the early growth rate of the individual fungus hyphae. New Phytol. 23: 65-78.

Smith J.E. and D.R. Berry (1974), Introduction: The conceptual basis of morphogenesis. In an introduction to biochemistry of fungal development. Academic Press.

Smith, J.E., J.G. Anderson, S.G. Deans and D.R. Berry (1981), Biochemistry of microcycle conidiation. In Biology of conidial fungi. Vol.2, ed. G.T. Cole and B. Kendrick, Academic Press.

Spurr, A.R. (1969), A low-viscosity epoxy resin embedding medium for electron microscopy. J.Ultrast.Res. 26: 31-43.

Stork, R. and R.C. Morrill (1977), Nuclei, nucleic acids and protein in sporangiospores of *Mucor bacilliformis* and other *Mucor* species. Mycologia 69: 1031-1037.

Sullia, S.B. (1966), Age as a factor in the rhizosphere mycoflora of sild leguminous plants. Ph.D. Thesis, Banaras Hindu Univ. India (In Leelavathy, 1969).

Suzuki, S., M. Suzuki, K. Yokata, H. Synayama and O. Sakaguchi (1967), On the immunological and biochemical studies of fungi XI. Cross-reaction of the polysaccharides of *Aspergillus fumigatus, Candida albicans, Saccharomyces cerevisiae* and *Trichophyton rubrum* against *Candida albicans* and *Saccharomyces cerevisiae* antiserum. Jap.J. Microbiol. 11: 269-273.

Svoboda, A. (1966), Regeneration of yeast protoplasts in agar gels. Exp. cell Res. 44: 640-642.

Tanimoto, E. and Y. Masuda (1968), Effect of auxin on cell wall degrading enzymes. Physiol. Plant, 21: 820-826.

Thimann, K.V. (1935), On the plant growth hormone produced by *Rhizopus suinus*. J.Biol.Chem. 109: 279-291.

Torres-Bauza, L.J. and W.S. Riggsby (1980), Protoplasts from yeast and mycelial forms of *Candida albicans*. J.Gen.Microbiol. 119: 341-349.

Trinci, A.P.J. (1970), Kinetics of apical and lateral branching in *Aspergillus nidulans* and *Geotrichum lactis*. Trans. Br. Mycol.Soc. 55: 17-28.

Trinci, A.P.J. (1973), The hyphal growth unit of wild type and spreading colonial mutants of *Neurospora crassa*. Arch. Microbiol. 91: 127-136.

Trinci, A.P.J. (1978), Wall and hyphal growth. Sci. Prog. (Oxford), 65: 75-99.

Trinci, A.P.J. (1979), The duplication cycle and branching in fungi. In fungal walls and hyphal growth, ed. J.H. Burnett and A.P.J. Trinci. pp.319-358. Cambridge Univ. Press.

Trinci, A.P.J.and G.H. Banbury (1967), A study of the growth of tall conidiophores of *Aspergillus giganteus*. Trans.Br. Mycol.Soc. 50: 525-538.

Trinci, A.P.J. and K. Gull (1970), Effect of actidione, griseofulvin and triphenyltin acetate on the kinetics of fungal growth. J.Gen.Microbiol. 60: 287-292.

Trinci, A.P.J. and P.T. Saunders (1977), Tip growth of fungal hyphae, J.Gen.Microbiol. 103: 243-248.

Van Laere, A.J., A.R. Carlier and J.A. Van Assche (1977), Cell wall carbohydrates in *Phycomyces blakesleeanus* Burgeff. Arch.Microbiol. 112: 303-306.

Varner, J.E. (1964), Gibberellic acid controlled synthesis of α-amylase in barley endosperm. Plant Physiol. 39 :413-415.

Varner, J.E., G.R. Chandra and M.J. Chrispeels (1965), Gibberellic acid-controlled synthesis of α-amylase in barley endosperm. J.Cell Comp.Physiol. 66: 55-68.

Vaziri-Tehrani, B. (1978), A comparative study of selected cell wall constituents of Oomycetes, Ph.D. Thesis, Reading Univ.

Villanueva, J.R. and I. Garcia Acha (1971), Production and use of fungal protoplasts. In methods in microbiology, Vol.IV, ed. C. Booth, Academic Press.

Vorobiev, L.N. and L. Manusadzianas (1983), Bioelectrical reactions of *Nitellopsis obtusa* induced by indole-3-acetic acid. Physiol.Plant. 59: 651-658.

Wegener, W.S. and H.H. Romano (1963), Zinc stimulation of RNA and protein synthesis in *Rhizopus nigricans*. Science 142: 1669-1670.

Wessels, J.G.H. and J.H. Sietsma (1981), Fungal cell walls: A survey. In Encyclopedia of Plant Physiology, vol.13B, ed. W. Tanner and F.A. Loewus, pp.352-394. Heidelberg: Springer-Verlag.

Wessels, J.G.H., J.H. Sietsma and A.S.M. Sonnenberg (1983), Wall synthesis and assembly during hyphal morphogenesis in *Schizophyllum commune*. J.Gen.Microbiol. 129: 1607-1616.

Wilson, E.V. and V.M. Hearn (1982), A comparison of surface and cytoplasmic antigens of *Aspergillus fumigatus* in an Enzyme-Linked-Immunosurbent-Assay (ELISA). Mykosen 25: 653-661.

Yanagishima, N. (1963a), Effect of auxin and anti-auxin on cell elongation in yeast. Plant and Cell Physiol. 4: 257-264.

Yanagishima, N. (1963b), Strain dependence of the auxin effect in yeast. Plant and Cell Physiol. 4: 349-352.

Yanagishima, N. (1963c) On the respiration-deficient mutation in yeast. Proc.28th Ann.Meet.Bot.Soc.Japan (In Yanagishima 1965)

Yomo, H. (1960), Studies on the amylase activity substance.4. On the amylase activity action of gibberellins. Hakko Kyokaishi 18: 600-602.

Zalokar, M. (1959), Growth and differentiation of *Neurospora* hyphae. Amer.J.Bot. 46: 602-610.