THE EFFECTS OF SUBSTRATE WATER AVAILABILITY ON THE ISOLATION AND GROWTH OF FUNGI

by

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SUMMARY

The work reported in this thesis was carried out to contribute to the knowledge of the effects of substrate water availability or water activity (a_w) on fungal growth parameters and its implications in the preparation of materials susceptible to biodeterioration.

Fungi were isolated from soils of different ecological sites at a range of substrate a_w levels controlled by sodium chloride (NaCl). Three groups of fungi were isolated : firstly, those isolated only at high a_w (a_w about 0.997), secondly, those isolated at high and decreasing a_w (a_w 0.997 to 0.85) and finally, those isolated at only decreased a_w (a_w 0.95 to 0.80). From these isolations, test fungi were selected to study the effects of pH, temperature, exo-enzyme production and biocide efficacy at decreased a_w levels, with glycerol and NaCl as a_w controlling solutes. The linear extension rates of the fungi increased at all test pH values near optimum a_w of growth. Test fungi of the Aspergillus glaucus group were found to be most resistant to low a_w.

Growth and survival of vegetative and fruiting bodies at elevated temperatures were enhanced with the addition of a_w controlling solutes. A. flavus, A. fumigatus displayed high heat resistance and A. amstelodami, A. versicolor and Penicillium citrinum displayed low heat resistance at high a_w levels and vice versa at low a_w levels.

Amylase, lipase and protease activities were studied at lowered a_w, using modifications of the test tube method of Rautela and Cowling. Amylase and protease production in most xerophilic fungi ceased around 0.80 a_w, but lipase production in some xerophilic fungi, including A. glaucus fungi, was up to and including 0.70 a_w with glycerol. Glycerol and NaCl played a subordinate but important role to a_w in the growth rate, enzyme production and heat resistance studies.

Reduced growth and enzyme production of A. amstelodami, A. chevalieri and A. niger at increasing concentrations of captan and verdasan indicated that they were able to detoxify the fungicides to some degree. A significant decrease in potassium ion leakage from fungal membranes was observed at a_w 0.95, decreased with glycerol than at a_w about 1.0. Glycerol probably effected the membrane pore size, thus effecting the permeability of fungicides into the mycelium.

KEY WORDS

Substrate a_w : Fungi : Physiology : Enzymes : Fungicides

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CHAPTER ONE

GENERAL INTRODUCTION

1.1 Introduction

Materials used by man have been subjected to biodeterioration by a wide variety of organisms, including birds, insects, microorganisms, plants and rodents since time immemorial. Recently, the economic impact of microbial damage to numerous complex and highly processed goods introduced into man's economy has increased.

Microorganisms require certain physical and chemical conditions for growth and proliferation. These include adequate moisture, satisfactory temperature, appropriate oxygen levels, suitable pH of the medium and essential nutrients. Microbial growth may be prevented by restricting any of the above requirements or by physical agents, e.g. ultraviolet radiation, heat sterilization and by toxic compounds.

The control of moisture through the regulation of humidity in many applications has proved satisfactory to prevent microbial growth.

1.2 Concept of Water activity (a_w) and its relationship with Relative humidity (RH) and Osmotic Pressure (OP)

There are various water related criteria used in the study of the stability of materials, each having its own limitations. Water content or moisture content measured in various materials will vary due to the hysteresis effect. The reasons of the limited application of moisture content have been listed by Onions et al. (1981). Firstly, the moisture content at a given a_w will be higher when the material desorbs

to equilibrium, and will be lower when the material absorbs to equilibrium. Secondly, with a wide change in temperature, the change in a_w is appreciable while the moisture remains unchanged. Equilibrium relative humidity (ERH) refers to the atmosphere in equilibrium with the material and not the material itself. OP presupposes the presence of a suitable semi-permeable membrane and solute concentration will tell little of the properties of water in the material (Troller and Christian, 1978).

The use of a_w as the most important expression of the water requirements for microbial growth (Scott, 1957) is generally accepted and can be applied to all materials regardless of the manner in which the water is restricted.

Water activity is defined as the ratio of the vapour pressure of water over the substance (p) to the vapour pressure of pure water (p_o) -

$$a_w = \frac{p}{p_o}$$

This ratio is measured at a constant temperature, but in an ideal solution, a_w is independant of the temperature. The effect of temperature on dilute, non-ideal solutions is small, while for concentrated, non-ideal solutions it is significant to the extent that it affects the activity of the solute or solvent. Franks (1975), draws attention to the fact that a_w is essentially a thermodynamic property of a solution and therefore relates to equilibrium situations. Thus, the measurement of a_w is always related to a system in equilibrium.

The ratio of p to p_o is precisely the same as that which is expressed in vapour form as RH (Troller and Christian, 1978). Water activity is therefore numerically equal to RH except that the latter is expressed as a percentage -

$$\% \text{ RH} = a_w \times 100$$

In spite of the lack of a semi-permeable membrane in most circumstances, a_w or water availability is often considered in terms of the OP (π) in atmospheres -

$$\pi = - \frac{RT \log a_w}{\bar{v}}$$

$$\pi = - 4.55 \, T \log a_w$$

where, R = gas constant, 0.8211 atmospheres/^oC

T = absolute temperature in degrees Kelvin

$\bar{v}$ = partial molal volume of water, which for most biological systems is equal to 55.51.

The negative sign indicates that the OP is inversely proportional to a_w, i.e. increase in OP leads to a decrease in a_w and vice-versa.

The use of a_w has practical advantages, including its relative ease of experimental determination, its simple mathematical manipulation and its direct relationship to some easily recognised and measured properties of solutions.

1.3.1 Microbial growth at low a_w levels

Regardless of their ecological and industrial significance, micro-organisms that can thrive at low a_w warrant an investigation because they represent cells that can thrive at a_w levels far lower than cells of any other type of organism.

Only specialized bacteria and perhaps some fungi can truly be termed halophilic. The terms xerophilic, i.e. lovers of dryness and osmophilic, i.e. lovers of high OP are overlapping and imprecise terms. Some microorganisms can be described as 'lovers of low a_w', and in this thesis the term xerophilic is used to indicate fungi capable of growth at low a_w levels.

1.3.2 Bacteria

There are two major types of extremely halophilic bacteria, Halobacterium, which are rod-shaped and lack a conventional cell wall, and Holococcus, which have thick cell walls.

1.3.3 Yeasts

These include the most xerotolerant microorganisms. Xerotolerant yeasts are found in many genera including Brettanomyces, Candida, Debaryomyces, Hansenula, Pichia, Saccharomyces and Torulopsis (Brown, 1976; Pitt, 1975; Scott, 1957; Tilbury, 1976).

Temperature in certain cases plays an important role in the growth of yeasts at varying a_w levels, i.e. some yeast strains exhibit obligate osmophilic characteristics at high temperatures above 30°C. Tilbury (1976), has suggested that this phenomenon may explain why osmophilic yeast spoilage of dried fruits is associated with hot climates.

The existance of obligate osmophilic yeasts is in some dispute. Anand and Brown (1968), found that a selection of osmophilic yeasts did not require a a_w less than that of the basal medium of 0.997. Koh (1975a, b), reported a strain of S. rouxii with a basic requirement for sucrose in excess of 20% w/v in the growth medium.

Compared with halophilic bacteria, osmophilic yeasts do not have an obligate requirement for a specific solute. This could be due to the thick cell walls of yeasts which give them structural integrity whereas, in halophilic bacteria, the cell walls either burst or undergo considerable internal disorganisation.

1.3.4 Fungi - Biodeteriogenic activity of xerophilic fungi

Walter (1931), termed xerophilic fungi as those that were able to grow at a_w levels of 0.90 or lower. Heintzeler (1939), only considered a fungus xerophilic if it was capable of growth below 0.80 a_w. Christian (1963) accepted Heintzeler's definition and considered yeasts to be osmophilic if their lower growth limit was below 0.85 a_w.

Schmiedeknecht (1960), defined a xerophile by the shape of the curve relating to mycelial growth rate to a_w over the entire growth permitting range. Pelhate (1968), went further to define xerophilic fungi as those having maximum growth rates below 0.95 a_w.

Pitt (1975), defined a xerophile as, 'a fungus which is capable of growth, under at least one set of environmental conditions, at a a_w below 0.85'. This definition was adopted in this thesis.

Estimates involving many millions of pounds have been proposed to account for losses due to biodeterioration, but it is difficult to determine the sum for a number of reasons put forward by Eggins (1967). Firstly, the lack of statistics on known biodeterioration because of disinterest in this aspect by biologists; secondly, the reluctance by manufacturers, particularly those in cosmetic, food and pharmaceutical industries to admit to experiencing biodeterioration problems and failure to recognise biodeterioration either due to ignorance or complacency.

Xerophilic fungi are a group of highly specialized microorganisms capable of growth at a_w levels less than 0.85 and are therefore uniquely adapted to low moisture ecological niches. Many are ubiquitous like the Aspergillus, Eurotium and Penicillium species, others such as Eremascus and Monoascus (Xeromyces) are considered to be rare. This may be due to the inadequacy of the isolation media or problems in identification.

Xerophilic fungi present a problem in so far as they are found on a wide variety of substrates and cause a great deal of damage economically and many species produce toxins (Pitt, 1975). The biodeteriorative roles of some of these fungi on various substrates are described below.

Cosmetics and pharmaceutical products by the very nature of their use requires them to be either free of microbial contamination or have a low microbial count. Aspergillus flavus, A. niger and A. tamarii are reported to cause the breakdown of emulsions, leading to the production of acids, odours, unpleasant flavours or discolourations (Manowitz, 1961; Beveridge, 1975).

Dade (1958), found the A. glaucus group, A. fumigatus and A. versicolor to be spoilage agents of optical instruments.

Biodeterioration problems arise at all stages during the processing of leather, but problems involving fungi are considered. Several workers including Orlita (1968), isolated from tanning liquors and finished leather products several fungi, e.g. A. flavus, A. fumigatus, A. niger, A. glaucus, A. ochraceus, A. repens, A. terreus, A. versicolor, Paecilomyces varioti, Penicillium chrysogenum and P. cyclopium. Vegetable tanned leathers can be infected by species of Aspergillus and Penicillium, giving rise to a variety of colour stains.

A. fumigatus and A. niger are implicated in 'slime' formation from certain moist wood pulps. These, along with A. flavus, A. glaucus group and A. versicolor are reported to cause spoilage of paper products and paper boards (Hughes, 1968).

Xerophilic fungi are involved in the spoilage of a wide variety of stored products, such as cereal grains, dairy products, dried fruits, raw sugar, oil seeds and tobacco. The species found in any particular stored product will depend of the nature of the substrate and also the physical factors such as moisture and temperature of the substrate.

A. candidus, A. flavus, A. glaucus group, A. ochraceus, A. restrictus and A. versicolor have been reported on stored cereal grains (Christensen and Kaufmann, 1974). Such infestations cause caking, decay, discolouration, heating, mustiness and loss of germinability.

A number of fungi including A. niger, A. tamarii and Paecilomyces varioti were isolated from heaped Nigerian oil palm fruits by Coursey and Eggins (1961a). The fungi were found to be strongly lipolytic, some producing a lipolytic effect over 2% free fatty acid increase in eight weeks at $\frac{1}{2}$% water content (Cornelius et al., 1965). In the biodeterioration of groundnut oil by aspergilli, Tomlins and Townsend (1968), demonstrated the lipolytic activities of A. flavus and A. niger in the breakdown of oil.

Extremely xerophilic fungi will cause the spoilage of dried fruits due to the very low a_w. On dried prunes, Pitt and Christian (1968), found Xeromyces bisporus and two new species of Chrysosporium to be frequently occuring xerophilic fungi. The ability of these organisms to grow on materials of low a_w could be related to their various enzyme activities.

1.4.1 Possible mechanisms of adaptation to low substrate a_w

It is of paramount importance to understand the mechanisms whereby fungi can withstand low substrate a_w. The mechanisms may involve structural and/or physiological changes in the fungi.

1.4.2 Structural changes

Pitt and Christian (1968), found the aleuriospores of X. bisporus germinated at a_w 0.644. They found that in Eurotium species, the ascospores were more xerophilic than the conidiospores but, the difference between the sizes was negligible.

1.4.3 Physiological changes

When a microorganism is introduced in a new physiochemical environment, it responds essentially in two stages (Brown, 1976). In the first stage, there is comparatively a rapid adjustment in the cell's thermodynamic condition. When the environmental change involves a decrease in a_w, the thermodynamic adjustment involves a transient osmotic stress, the adjustment being modified if the microorganism has an energy source. In the second stage, there are changes in enzyme activities and details of enzyme production. This is followed by modification of biosyntheses and changes in the detail of control of enzyme formation.

Once the second stage is completed and a physiologically steady state is reached, there are two theoretically possible explanations for the

tolerance of or requirement of low a_w (Brown, 1976). Firstly, proteins of tolerant microorganisms are intrinsically better able to function under extreme environmental conditions, e.g. low a_w. Secondly, proteins of tolerant and non-tolerant microorganisms are essentially the same but, in tolerant species, enzymes can function because intracellular conditions are modified so that the inhibitory effect of the environment is diminished.

Several workers including Christian and Waltho (1964), Christian and Hall (1972), have shown that bacteria accumulate potassium ions and amino acids as internal solutes to balance the external high OP environment.

Yeasts produce and accumulate polyols to balance the external environment. Arabitol, erythritol and glycerol are produced by a wide variety of osmophilic yeasts (Nickerson and Carroll, 1945; Spencer and Sallans, 1956; Onishi, 1960). Onishi (1963) found that salt tolerant yeasts do not possess salt tolerant enzymes and concluded that the mechanism of tolerance of the soy yeast, S. rouxii to salt was different from that operating in halophilic bacteria.

Currently, little is known of the mechanism whereby fungi survive low substrate a_w. There is some evidence that xerophilic fungi produce polyols. Lee (1967), suggested that polyols acted as carbohydrate reserves; Corina and Munday (1971), suggested that polyols acted as hydrogen ion acceptors and Brown and Simpson (1973), suggested that polyols acted as 'compatible solutes', which, in high concentrations

allow intracellular enzymes to continue functioning in the same way as enzymes in halophilic bacteria function in the presence of high potassium ion concentrations. The mechanism of fungal survival at low substrate a_w will enable further understanding of the effects of various a_w controlling solutes on fungal growth and the role of internal solutes in protecting enzyme systems, in effecting the penetration of biocides into fungal membranes and in the growth and survival at elevated temperatures.

1.5 Biological methods of producing a controlled range of RH values

In order to condition materials to a standard water content to study the growth of microorganisms, it is essential to control the atmospheric humidity, which is achieved by one of the two methods -

1) Equilibrating samples of the test material with a controlled RH atmosphere, using saturated or unsaturated solutions.

2) By the addition of known concentration/s of various solute/s within the substrate.

Expanding on the two methods of control -

1) Saturated or unsaturated solutions : the samples are exposed to a constant RH and allowed to absorb or desorb water from the air, usually until no further change in weight. For this method, it is necessary

for the equilibrating chamber to be made of non-hygroscopic material, the contents of the chamber brought in thermal equilibrium and constant temperature maintained, salt solution having a large surface area and the prevention of concentration gradients within the solution. This method of control is cheap, convenient and most salts are available in a high degree of purity and are non-volatile and safe to handle (Young, 1967).

Saturated solutions can liberate or absorb relatively large quantities of water without the change in RH, but with unsaturated solutions, large changes will alter the concentration and hence, the RH to an appreciable degree (Young, 1967).

There are two main disadvantages in using this method. Firstly, a stable O_2/CO_2 ratio over the duration of an experiment is difficult to maintain. Although the actual amount of O_2 may not be reduced beyond a threshold level, the build up of CO_2 may inhibit microbial growth. Secondly, this method is not suitable for a_w levels above 0.95. This is because of the nature of the water sorption isotherm, where the rate of change of water content approaches infinity as the a_w approaches one. Scott (1953),

for a brain heart infusion showed that changing from a_w of 0.78 to 0.79, the water content had increased by only 3% of the dry weight, whereas, in changing from a_w of 0.98 to 0.99, the water content had increased by 1050% of the dry weight.

2) Solutes : with the addition of electrolytic, e.g. NaCl, and non-electrolytic solutes, e.g. glycerol, sucrose to water, the a_w is decreased as the water molecules become orientated with respect to solute molecules (Brown, 1976). This method has been employed by several workers including Scott (1953) and since has been used by several workers. Solutes added to foods to decrease the a_w are known as humectants and are discussed in section 1.6. The main advantages of this method are -

a) High accuracy in a_w determinations, the error being proportional to $(1- a_w)$.

b) There is a constant concentration of nutrients.

c) Maintenance of a constant concentration of a particular solute/s at specified concentration/s.

d) The effects of the solute and solvent on the test system may be distinguished by careful study.

e) Unlike the previous method of control, this method is accurate to use at a_w levels above 0.95.

The disadvantages of this method include, firstly, the solute/s may be used in microbial metabolism and secondly, the limitations of the solute/s or humectant/s used in foods (Section 1.6).

1.6 Humectants

They are substances that control the a_w levels in foods for the maintenance of its wholesomeness, safety, texture and for the suppression of undesirable enzymatic and chemical changes.

Salt (NaCl) is traditionally used in high concentrations and has a dehydrating effect on food and inhibits microbial growth due to high OP. The inhibitory concentrations of sugars and polyols on the growth of some microorganisms were found to be related to their molecular weights and the inhibitory effect was believed mainly due to osmotic effect (Barr and Tice, 1957). Sinskey (1976), showed that the antimicrobial effects of a number of aliphatic diols were dependant on the chain length and the position of the hydroxyl groups.

However efficient these compounds, they cause flavour problems at high concentrations, e.g. glycerol - 'spicy' or 'hot' falvours, sugars - too sweet in some applications and salt - too salty. Polymeric compounds are found to be very viscous (Karel, 1976). Combinations

of a variety of humectants are suggested by Karel (1976) to overcome the off-flavour problems, providing each humectant is used at a concentration below the off-flavour threshold.

1.7 Aim of the research programme

The study undertaken was an attempt to understand the effects of substrate a_w on fungal growth limits, its implications in biodeterioration situations and the protection of materials susceptible to biodeterioration.

Soil is one of the main sources of microbial contamination and materials can be subjected to infection under a variety of environmental condition/s such as varying moisture, O_2, pH and temperature levels. Initially, the studies carried out for this investigation concerned the effect of decreasing substrate a_w, using NaCl, on the isolation of fungi from soils of different ecological sites and the succession and/or overlap of various fungal species.

Experiments on test xerophilic and non-xerophilic fungi, implicated in biodeterioration were conducted to enable comparisons to be made with physiological parameters, especially with regards to pH and decreasing a_w, using glycerol and NaCl as controlling solutes.

Moist heat resistance of spores at sub-pasteurisation temperatures by either glycerol or NaCl addition to the heating menstruum was studied in test toxigenic, xerophilic fungi and compared with their hyphal

resistances at elevated temperatures.

Having established the a_w limits within which test fungi were capable of growth, their exo-enzyme production on amylolytic, cellulolytic, lipolytic and proteolytic substrates was tested.

Finally, the activity of two fungicides, captan and verdasan were studied in three xerophilic fungi, in control, i.e. substrate a_w approximately 1.0 and in test, i.e. substrate a_w 0.95, controlled with glycerol, systems. This enabled a comparison of fungicide efficiency between the unchanged osmotic state of the fungal cells and where the osmotic state of the fungal cells was changed.

CHAPTER TWO

ISOLATION OF FUNGI AT DECREASING a_w LEVELS

2.1 Introduction - Isolation of fungi at decreasing a_w

Most microorganisms are invisible to the unaided eye, and in the case of soil fungi, are buried in an opaque matrix. In order to visualize these fungi will inevitably involve experimentation. To an exceptional degree, the basic data of fungal ecology on which all must rest is only as good as the technique used to produce a description of the pattern of fungal activity in space and time. Lists of fungi present in the soil under investigation were produced by earlier isolation techniques. Selection techniques were devised to isolate active mycoflora, e.g. hair colonization technique in controlled relative humidity, Griffin (1963); high NaCl soil plate technique, Chen (1964).

Xerophilic fungi show a very slow rate of growth on ordinary high a_w laboratory media. This would lead to many of the slower growing fungal species, e.g. A. glaucus group, being overgrown by rapidly growing species, or perhaps no growth at all, e.g. X. bisporus will not grow on media with a a_w of more than 0.95 (Scott, 1957).

The nutritional requirements of some xerophilic fungi vary. Harrold (1950), devised a medium with high concentrations of malt extract, yeast extract and sucrose to study species of Eremascus. Frazer (1953), used media with high concentration of malt extract or high concentrations of malt extract and sugar in her studies on X. bisporus. In their studies of the microflora of dried prunes, Pitt and Christian (1968) found that the addition of 0.5% peptone to the growth medium enhanced growth and conidial production in some species. Dallyn and Everton (1969), incorporated high concentrations of glucose, glycerol or sucrose in their isolation media during the investigation of spoilage by X. bisporus.

In some instances, workers have used materials found in the natural habitat of the particular fungus. Skou (1972), added honey to the medium in the studies of the fungus Bettsia alvei Betts. associated with bees and honeycomb.

The soil is one of the main sources of microbial infection. By varying the substrate a_w level, the water available for microbial isolation and growth can be controlled.

2.2.1 Isolation Technique

Although the conventional Warcup soil plate (1950) isolation method was used, it was employed for selecting fungi able to grow at various levels of water availability.

There are problems arising from this method of isolation which must be emphasized. In this technique, 0.01g of soil is incorporated into a_w adjusted, cooled molten agar in a petri dish, incubated at 25°C and the number and identity of the resulting colonies noted. The result is a description of the microflora of the soil in terms of the species present. However, this description cannot be accepted at its face value, i.e. the agar on which the microorganisms have grown will have a specific nutrient composition, pH, a single incubation temperature and solute effects on fungal isolation and growth.

2.2.2 Soil Types

Five soil types were used from different ecological sites, thus ensuring a wide spectrum of fungal isolates.

The percentage moisture content (MC), pH and percentage organic content (OC) of the soil samples were determined as soon as possible after the samples were obtained. Ten replicates were used for each test.

The MC was calculated over the wet or initial weight of the sample:

$$MC = \frac{(Wet - Dry)\ weights}{Wet\ Weight} \times 100$$

Five to six grams of the soil was placed in a preweighed aluminium foil dish and placed in a 105°C oven. The soil sample was dried to a constant weight and cooled in a desiccator. The dry weight of the soil was determined.

The pH of the soil samples were measured on a pH M62 standard electronic pH meter. The soil and distilled deionised water were mixed in a 1 : 1 ratio, i.e. 5g of soil to 5ml of water (Tansey and Jack, 1976).

In order to determine the OC of the soil samples, 2g of an oven dried sample (initial weight) was placed in a preweighed crucible and placed in a furnace at 700°C for 4 hours. The samples were cooled in a desiccator and weighed (final weight).

$$OC = \frac{(Initial - Final)\ weights}{Initial\ weight} \times 100$$

Table 2.1 following lists the details of various soil types.

By varying the substrate a_w level, water availability for microbial isolation and growth can be controlled. The basal isolation medium used was malt extract agar (MEA), modified with glucose and NaCl to produce a range of a_w levels. The isolation a_w levels used were MEA - control, with a a_w about 0.997; NO.97 - MEA with NaCl to produce a a_w of 0.97; NO.95; NO.93; NO.90; NO.87; NO.85; NO.83; NO.80; NO.75 - saturated NaCl agar and malt extract yeast extract + 60% glucose (MY60G) with a a_w of 0.85. Molal concentrations of NaCl used to produce the various a_w levels were calculated from the data of Robinson and Stokes (1959).

0.01g of the soil sample was aseptically placed into a petri dish and 20ml of modified cooled molten agar was poured. The soil particles were dispersed by gently moving the petri dish in a circular motion. For each soil sample at a particular a_w, ten replicates were prepared. In order to prevent drying out during the long incubation periods, the plates were sealed in polythene bags and the bags were placed in aquaria containing water - 100% RH, saturated solutions of K_2SO_4 - 97% RH, $BaCl_2 \cdot 2H_2O$ - 90.3% RH; KCl - 84.2% RH, $(NH_4)_2 \cdot SO_4$ - 80.2% RH and NaCl - 75.1% RH (Young, 1967). The aquaria were incubated at 25°C, $\pm$ 1.5°C. This temperature was satisfactory for the growth of fungal species with growth optima at 18°C to 22°C and 30°C.

The plates were examined at one, two, three, four, eight and twelve weekly intervals. The fungal isolates were aseptically removed and

	S O I L T Y P E S				
	CACTUS GARDENS	ST. PETER'S SOIL - B'HAM	SUTTON PARK - BIRMINGHAM	SWANSEA BEACH - I	SWANSEA BEACH - II
Soil Details	From Birmingham Botanical Gardens. This represents a desert type soil. Temperature about 10°C.	Soil was sieved around rose beds. Heavy clay soil.	Soil from a woodland area with Quercus rober (oak) and Betula pendula (silver birch) litter.	Soil obtained nearer to the shore. Light sandy soil.	Soil obtained further inland. Sandy soil with some black soil.
Moisture Content (%)	7.24	12.24	63.00	0.20	14.97
pH	4.23	6.76	4.25	8.39	7.55
Organic Content (%)	8.06	7.82	36.81	6.95	10.07

F I G U R E 2

TABLE 2.1 : DETAILS OF THE SOIL TYPES

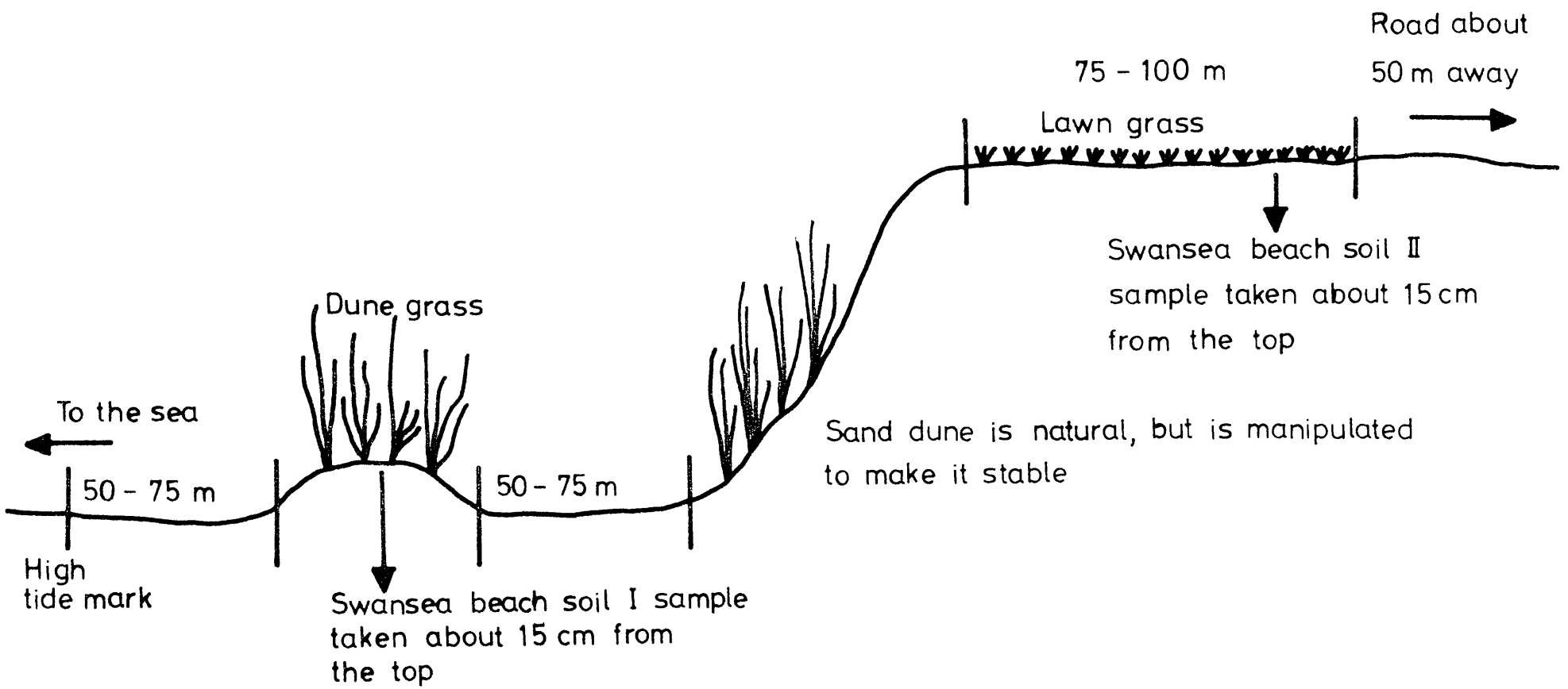


FIGURE 2 : SKETCH OF THE SWANSEA BEACH

plated on Czapek Dox agar + 20% sucrose (Cz20S), MEA and MY40G plates. The recipes of the various media are given in Appendix 1.

2.2.3 Maintenance of Cultures

The isolates were maintained on MEA and MY40G slopes at 12°C to 18°C. The isolates were transferred to fresh slopes after three to four months.

2.2.4 Identification of Isolates

The identifications were made by transferance of fungal growth on to a microscope slide using a needle and by the sellotape technique of Butler and Mann (1959). Literature from Ames (1963), Barnett and Hunter (1972), Gilman (1957), Raper and Fennell (1965), Raper and Thom (1968) and Onions et al. (1981) was used in the identification of the isolates. Many of the identifications were rechecked at the Commonwealth Mycological Institute at Kew.

2.3 Results

Thirty-eight fungal species were isolated from the various isolations, and details of individual isolations from various soil types are listed in Appendix 2. Table 2.2 shows the total fungal species isolated at decreasing a_w and Table 2.3 sets out to compare the fungi obtained at a_w of 0.85 using glucose and NaCl as controlling solutes. The frequency of fungal isolations from all five soil types are seen in Table 2.4

There is a grada tion of fungi isolated at decreasing a_w (Table 2.2). Fusarium spp.,G. roseum, M. plumbeus, M. nigra, Trichoderma spp., Z. moelleri are only isolated on MEA and NO.97,while F. moniliforme and a Phycomycete were isolated on MEA, NO.97 and NO.95.

From the genus Cladosporium, two species were isolated - C. herbarum on MEA and C. sphaerospermum on N .93, NO.90 and NO.87.

All the aspergilli and penicillia isolated, excepting P. canescens, P. chrysogenum, P. frequentans and P. nigricans were not isolated on MEA or NO.97 media, but from NO.95 media and lower. The Penicillium spp were isolated from media with a_w levels not lower than NO.85. A. amsteldomai, A. chevalieri, A. repens and A. ruber were the only fungi isolated at a_w levels of NO.80. No fungi were isolated from NO.75 media after six months.

E. pinetorum was only isolated on MY60G and not from NaCl modified media at any a_w.

From Table 2.4 it is seen that the a_w level for the maximum frequency of isolation varies from isolate to isolate. Generally, the maximum frequency of isolation of most of the Penicillium spp. lies between a_w 0.97 to 0.93 and in most Aspergillus spp. between a_w 0.93 to 0.85.

FUNGI	ISOLATING MEDIA							
	MEA	NO. 97	NO. 95	NO. 93	NO. 90	NO. 87	NO. 85	NO. 83	NO. 80
Cladosporium herbarum									
Fusarium oxysporum									
Fusarium sp.									
Gliocladium roseum									
Mucor plumbeus									
Mycelia sterilia									
Mycogone nigra									
Trichoderma glaucum									
T. koningi									
T. viride									
Zygorhynchus moelleri									
Chaetomium globosum									
Trichoderma lignorum									
Fusarium moniliforme									
Phycomycete									
Cephalosporium curtipes									
Penicillium canescens									
P. chrysogenum									
P. frequentans									
P. nigricans									
Aspergillus chevalieri var. intermedius									
A. niger									
A. ruber									
A. versicolor									
P. brevi-compactum									
Cladosporium sphaerospermum									
P. citrinum									
A. amstelodami									
A. repens									
P. aculeatum									
P. corylophilum									
P. restrictum									
Scopulariopsis brevicaulis									
A. penicilloides									
A. restrictus									

TABLE 2.2 : FUNGI ISOLATED AT VARIOUS a_w LEVELS AT 25° C

FUNGI	ISOLATING MEDIA	
	MY60G	NO.85
Aspergillus chevalieri var. intermedius		
A. versicolor		
Cladosporium sphaerospermum		
Eupenicillium pinetorum		
Penicillium frequentans		
P. nigricans		
A. penicilloides		
A. repens		
A. restrictus		
A. amstelodami		
A. ruber		
P. brevi-compactum		
P. canescens		
P. chrysogenum		

TABLE 2.3 : COMPARISON OF FUNGI OBTAINED AT a_w 0.85 USING GLUCOSE AND NaCl

FUNGAL ISOLATE	a_w								
	MEA	NO. 97	NO. 95	NO. 93	NO. 90	NO. 87	NO. 85	NO. 83	NO. 80
C. herbarum	2								
F. oxysporum	5								
Fusarium sp.	3								
G. roseum	1								
M. plumbeus	4								
M. sterila	2								
M. nigra	3								
T. glaucum	5								
T. koningi	4								
T. viride	8								
Z. moelleri	2								
C. globosum	1	3							
T. lignorum	2	1							
F. moniliforme	5	5	2						
Phycomycete	2	1	15						
C. curtipes	2	14	7	1					
P. canescens	1	-	-	-	-	6	2		
P. chrysogenum	1	8	22	7	13	1	1		
P. frequentans	2	6	19	25	2				
P. nigricans	3	9	21	24	3	8			
A. chevalieri var. intermedius	-	-	2	3	4	5	-	2	2
A. niger	-	-	5	9	2	1			
A. ruber	-	-	2	2	-	-	4	3	2
A. versicolor	-	-	8	9	10				
P. brevi-compactum	-	-	19	4	2	7	1		
C. sphaerospermum	-	-	-	3	4	5			
P. citrinum	-	-	-	4					
A. amstelodami	-	-	-	-	6	8	10	2	2
A. repens	-	-	-	-	9	9	2	1	1
P. aculeatum	-	-	-	-	7				
P. corylophilum	-	-	-	-	12				
P. restrictum	-	-	-	-	4				
S. brevicaulis	-	-	-	-	-	3			
A. penicilloides	-	-	-	-	-	-	3	1	
A. restrictus	-	-	-	-	-	-	5	2	

TABLE 2.4 : ISOLATION FREQUENCY OF FUNGI FROM ALL SOIL TYPES

2.4 Discussion

From the results, there emerges a competitive trend in the isolation of fungi by controlling the substrate available water for microbial growth, i.e. the first instance of isolation of a particular fungus and its isolation up to a certain a_w level. In some instances, the fungi were isolated at a range of a_w levels, but within the range, they were absent at certain a_w level/s, e.g. *P. canescens* was isolated on MEA, NO.87 and NO.85 but not on NO.97, NO.95, NO.93 and NO.90. This absence may be due to the appearance of other fungi and represents a limitation of this isolation technique.

By the addition of known molal concentration of solute/s, the a_w is lowered and the osmotic pressure of the isolating medium is increased. In some instances, high concentrations of the controlling solute can be toxic to a particular fungal isolate. A comparison of the fungi obtained on MY60G and NO.85 did show that *E. pinetorum* was not isolated on media containing NaCl and furthermore, *A. versicolor*, *C. sphaerospermum*, *P. frequentans* and *P. nigricans* were isolated at 0.85 a_w with glucose as the controlling solute, but were only isolated with NaCl as the controlling solute at a_w levels higher than 0.85.

It is interesting to note that within a particular genus, e.g. *Aspergillus* or *Penicillium* different species are isolated and reach maximum isolating frequency at various a_w levels. However, the most striking example is that shown by *Cladosporium*, where *C. herbarum* is only isolated at a maximum a_w (MEA), while *C. sphaerospermum* is only isolated at low a_w levels and is competitive with other xerophiles.

Fungi isolated in these experiments show similar trends to those obtained by previous workers. Fusarium and Penicillium spp. formed the predominant mycoflora of salt marshes and sand dunes, when isolation was carried out using media containing up to and including 10% NaCl (approximately 0.94 a_w). With increasing NaCl content, aspergilli and penicillia become dominant (Pugh, 1962a; Pugh et al., 1963 and Williams, 1968), Griffin (1963) and Chen (1964), at a_w levels of about 0.86 and 0.80 isolated species mainly from the aspergilli and penicillia.

Isolations using the technique of decreasing substrate a_w are extremely important because they provide useful information on the fungi that will invade materials at various a_w or relative humidity levels. A. amstelodami, A. chevalieri, A. repens, A. ruber, A. versicolor, and Penicillium spp. are frequently involved in the deterioration of natural and manufactured products at low a_w and moisture content. Some of the aspergilli, expecially the A. glaucus group and some penicillia are common moulds in stored grain (Christensen, 1957), mildewed fabrics (Galloway, 1935) and deteriorated sugar (Eskin et al., 1971).

As the fungi isolated belong to different taxonomical groups and some clearly isolated at high or low a_w, it would be interesting to study and compare some of the physiological characteristics of xerophilic and non-xerophilic fungi. In the following experiments, the combined effects of pH, solutes and a_w will be studied.

CHAPTER THREE

COMBINED EFFECTS OF pH, SOLUTES AND a_w ON THE GROWTH OF TEST FUNGI

3.1 Introduction

From the previous isolation experiments, selected xerophilic and non-xerophilic fungi were used to study some physiological parameters, chiefly the water availability and its interaction with pH and solutes used for controlling the a_w.

In the following experiments, the model system was the fungus growing on the basal malt extract agar (MEA), modified with an electrolytic (NaCl) and non-electrolytic (glycerol) solute. With each successive hydration shell, the force with which the water molecules are bound to the solute ion or molecule will decrease. A fungus growing on such a medium will compete directly with the solute ion or molecule for the growth requiring water.

Scott (1953) and Christian and Scott (1953) first employed this method to study water relations of Salmonellae, although, many workers previously had used this method to study solute effects on microbial growth. This method is accurate, simple and more time saving than using saturated or unsaturated solutions for controlling the RH of the atmosphere. The latter method has some disadvantages - 1) lengthy periods of equilibration depending on the experimental material, 2) RH gradients within the container, 3) hysteresis effect.

Measurement of radial growth on a solid medium and growth in submerged culture are the two standard methods used to assess fungal growth. Trinci (1969), carried out a number of kinetic studies on the way in which colonies of A. nidulans develop using both methods. He

established that the radius of the colony and the unbranched hyphal length had a constant growth rate, i.e. linear increase with time.

Many workers have used the two-dimensional colony diameter as a growth parameter, e.g. Scott (1953) and Ayerst (1969) to study the effect of a_w and Allsopp (1973) to study the effect of pH.

3.2 Solute and a_w Effects

When using various concentrations of different solutes to control the a_w of the basal medium, it is very important to distinguish between solute toxicity and a_w effects.

Generally, spore germination is less effected by solutes than outgrowth to the vegetable cell, and this in turn is less effected than the vegetative cell growth. Barr and Tice (1957), reported that the inhibitory concentrations of sugars and polyols were found to be related to their molecular weights; lower the molecular weight, lower the inhibition and vice versa, the inhibitory effect largely due to osmotic pressure. It has been postulated that the solute effects a microbial cell primarily through osmotic effects, involving both the cell membrane and osmotic condition of the cell (Mossel and Ingram, 1955; Scott, 1953).

By decreasing the a_w to 0.90, Christian and Waltho (1964) found that the cell water content of the salt tolerant bacterium, Staphylococcus aureus had halved and the concentration of several cell solutes

increased. Low concentrations of ionic salts, but not sucrose caused a release of proteins from Bacillus megaterium cell walls and the walls to contract, indicating that the physiological action of ionic and non-ionic solutes are different (Marquis, 1968).

Several workers, including Horner and Anagnostopoulos (1973) and Pitt and Hocking (1977) have reported that germination time and growth rates of test fungi are effected by the solute type.

Thus, it is important to note that no solute can be regarded as inert in that its only function is to bind to water. It is possible that it can attract other groups in or on the cell besides the hydrogen or oxygen atoms of the water. High solute concentration may therefore have a two-fold effect, firstly, to lower the available water for microbial growth and secondly, the specific action of solute ions or molecules on microbial growth patterns.

3.3.1 Effects of pH on test fungi

In the following series of experiments, the effects of pH at near optimum a_w of growth of sixteen xerophilic and seven non-xerphilic fungi on artificial media were studied.

3.3.2 Materials and Method

The test xerophilic fungi used were : A. glaucus group (A. amstelodami, A. chevalieri, A. repens, A. ruber) A. niger, A. restrictus group

(A. penicilloides, A. restrictus), A. versicolor, C. sphaerospermum, E. pinetorum, P. brevi-compactum, P. chrysogenum, P. citrinum, P. frequentans, P. nigricans, S. brevicaulis and test non-xerophilic fungi used were : C. globosum, C. herbarum, F. moniliforme, F. oxysporum, M. plumbeus, T. glaucum and T. lignorum.

The basal medium used in these studies was 3% MEA. Petri dishes of 9cm diameter containing 15ml of pH and a_w modified MEA were inoculated with 0.5cm plugs of mycelia of 10 - 14 days old cultures, cut using a sterile No. 2 cork-borer. Each plate was centrally inoculated, with the mycelium on the plug facing the agar medium. The plates were sealed in polythene bags and incubated at 25°C, $\pm$ 1.0°C, under conditions of controlled RH. Two colony diameter readings were measured at right angles in each of five replicates, the readings taken at intervals from three to seven days. The mean diametric growth for each fungus at set conditions was calculated over a period of linear growth, excluding early exponential growth and late sub-optimal growth (Trinci, 1971).

The effects of pH on the growth of test fungi were measured at control (MEA) and at approximate a_w levels for growth, i.e. 0.95 and 0.93 for xerophilic fungi and 0.97 for non-xerophilic fungi. The a_w levels were controlled using analytical grades of glycerol and NaCl.

Various buffer solutions were used to control the pH of the media : HCl - KCl buffer - pH2; citrate - phosphate buffer - pH3 to pH7; Tris - HCl buffer - pH8 to pH9 and carbonate - bicarbonate buffer - pH10 (Gomori, 1955). In order to avoid hydrolysis of the agar under acidic conditions, buffer solutions and media of pH2 and pH5 were prepared at double strength, autoclaved separately, cooled to about

45°C and then mixed. From pH6 to pH10, the buffer conditions and media were autoclaved together.

3.3.3 Results

The growth measurements of the test xerophilic and non-xerophilic fungi at pH2 to pH10 are shown in figure 3.1

All the fungal isolates were able to grow over the whole test pH range, excepting S. brevicaulis, which did not grow at pH2 and pH3.

For most of the test fungi, each medium, i.e. MEA, GMEA, and NMEA, had growth optima at different pH values. This was observed in both xerophilic and non- xerophilic fungi.

3.4.1 Combined Effects of pH, solutes and a_w on test fungi

The effects of solutes at lowered a_w were observed at two pH values of pH4.0 and pH6.5, adjusted with citrate-phosphate buffer (Gomori, 1955).

3.4.2 Materials and Method

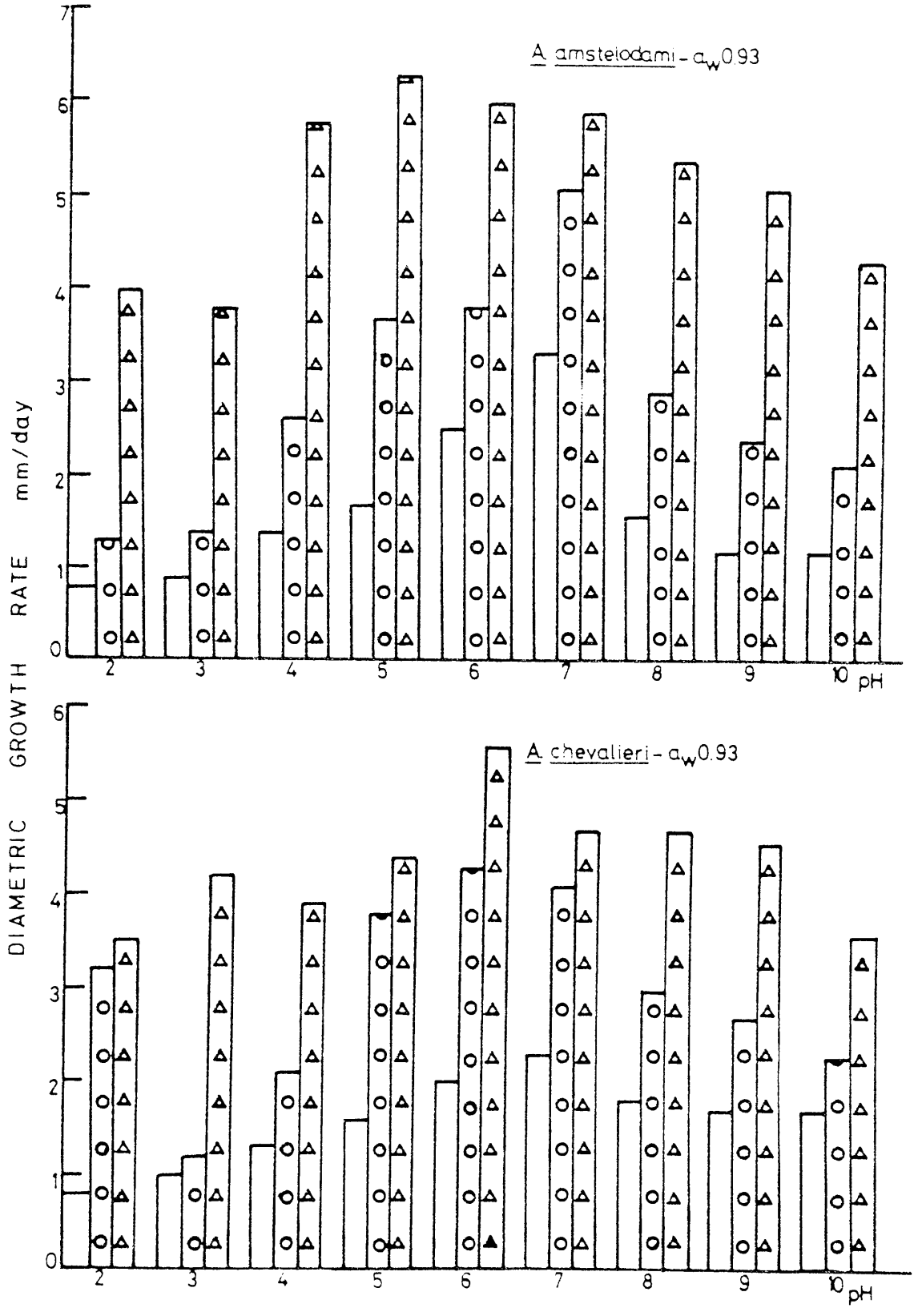
Media of various a_w levels were prepared by the addition of analytical grade of glycerol or NaCl. For xerophiles, media were prepared with glycerol as the controlling solute to give values of a_w over a range of 0.97 to 0.70 and with NaCl as the controlling solute to give values of

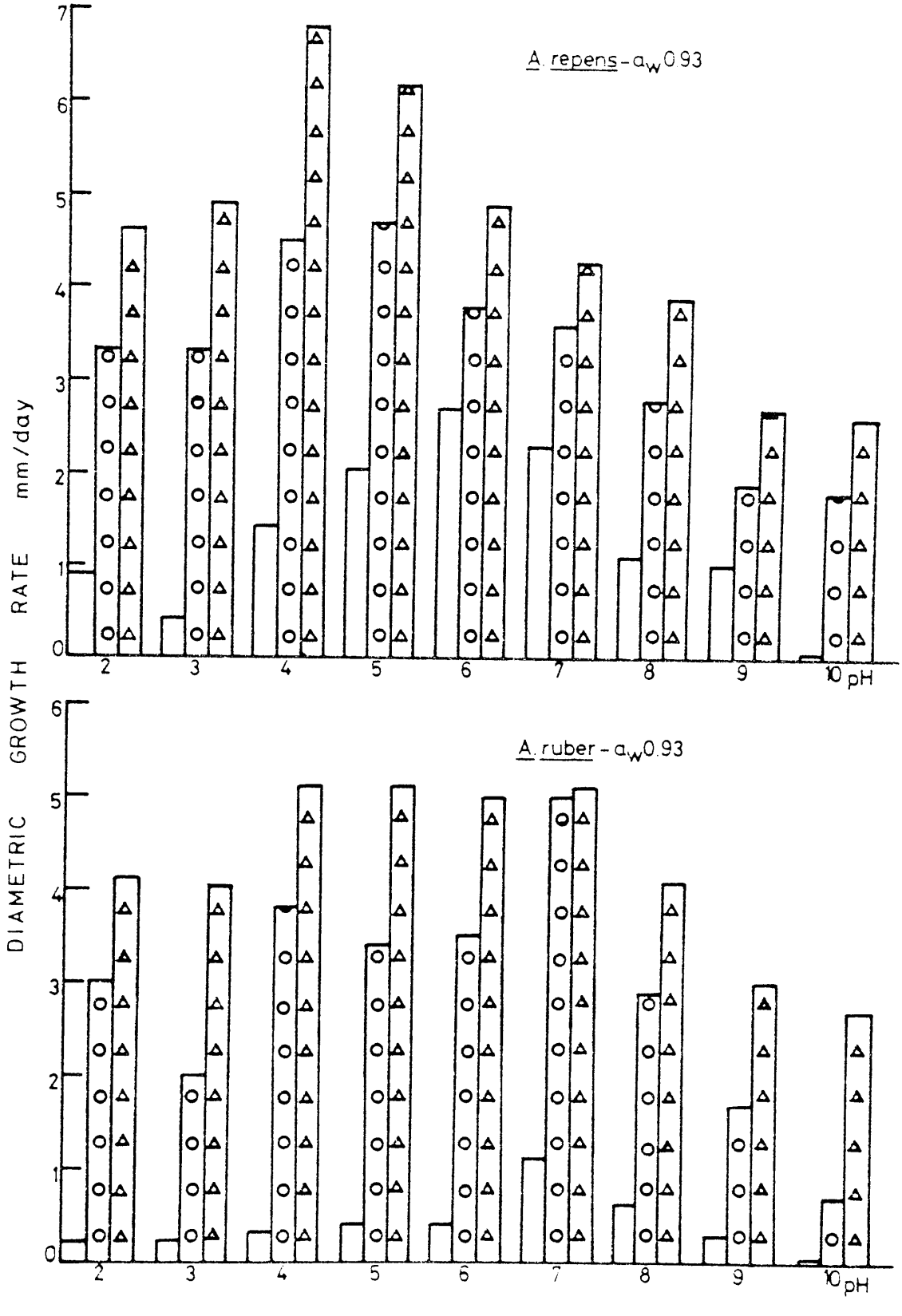
FIGURE 3.1 : THE EFFECTS OF pH OF THE MEDIUM ON GROWTH OF TEST FUNGI USING DIAMETRIC GROWTH RATES

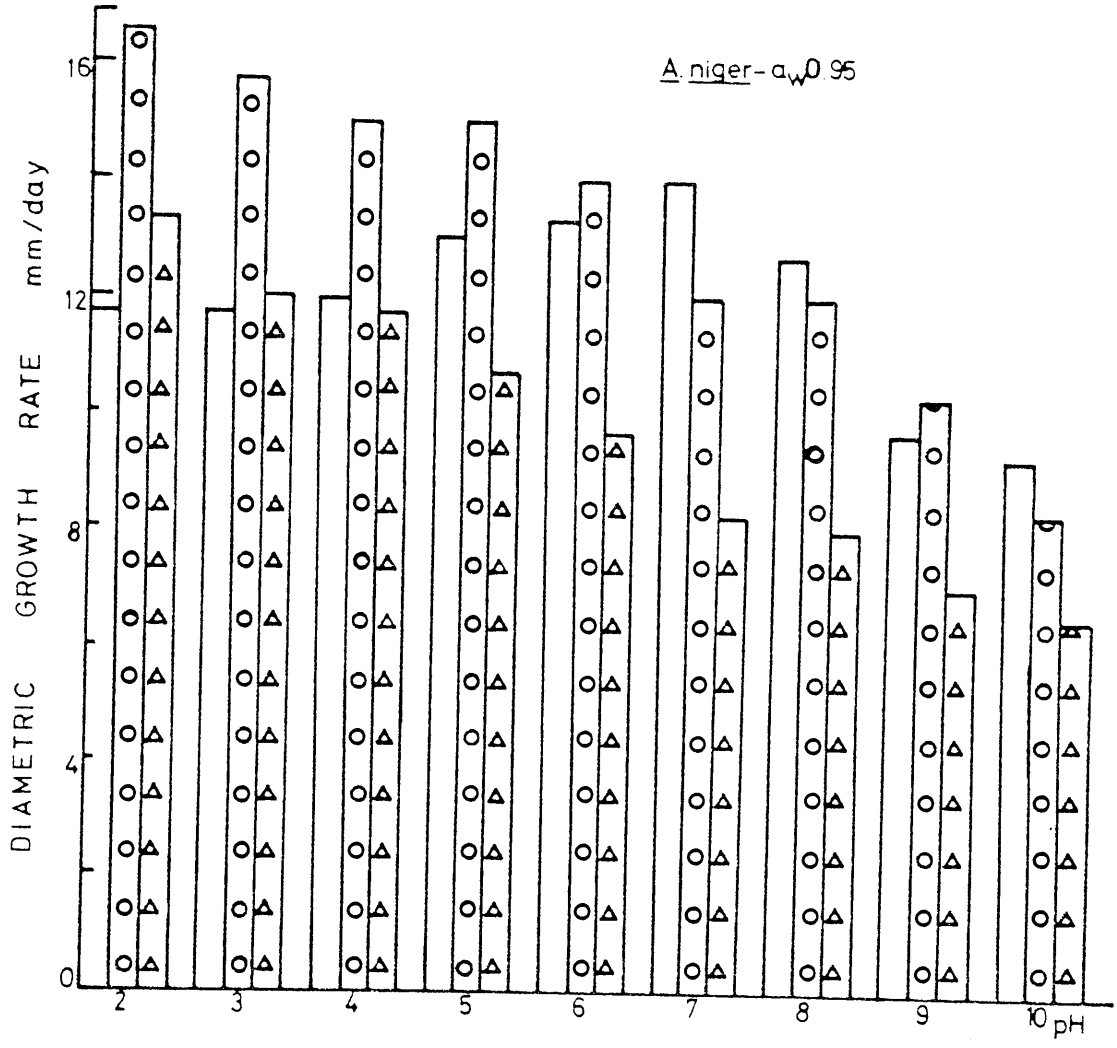
: CONTROL - MEA

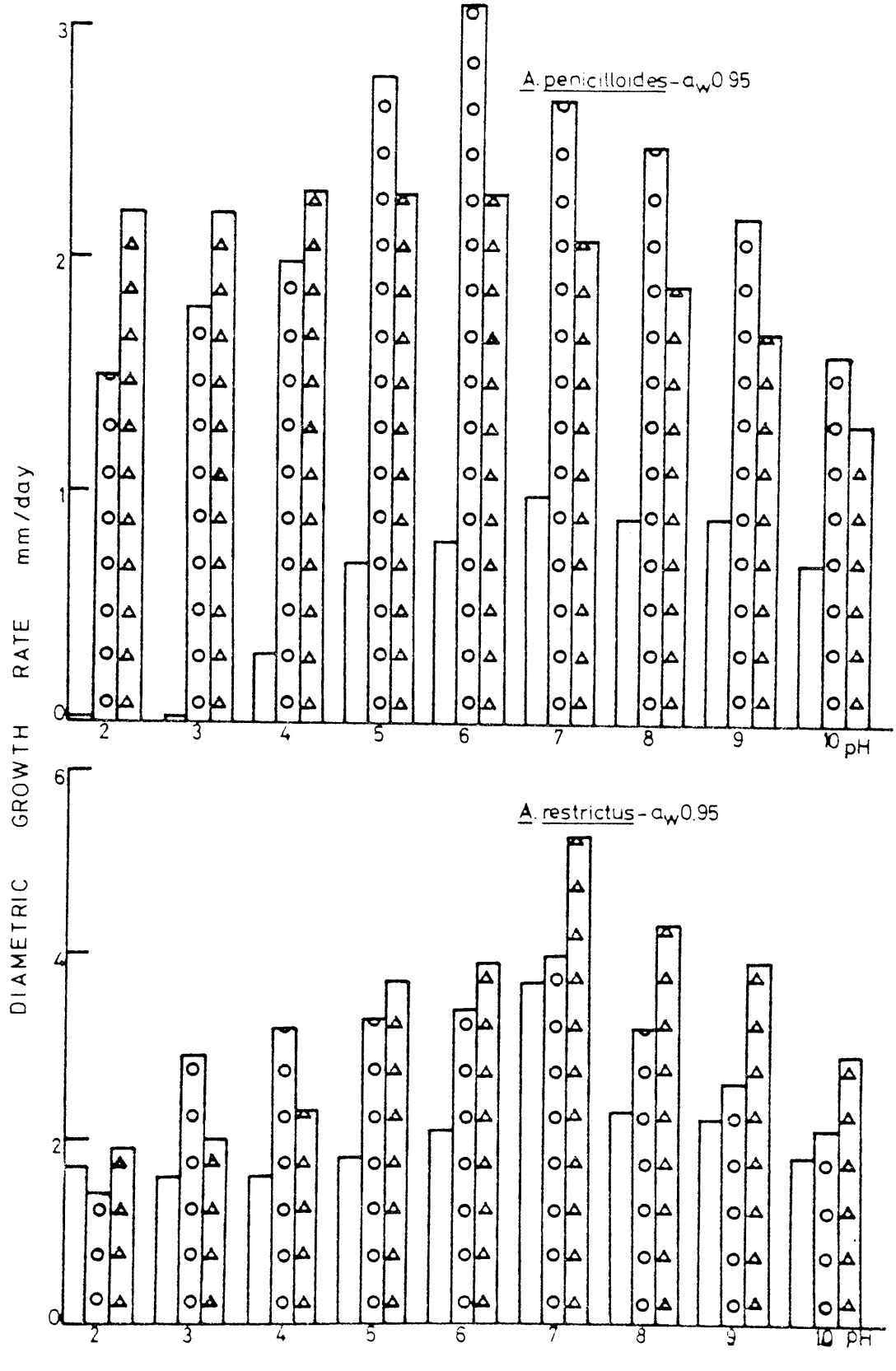
: MEA + GLYCEROL

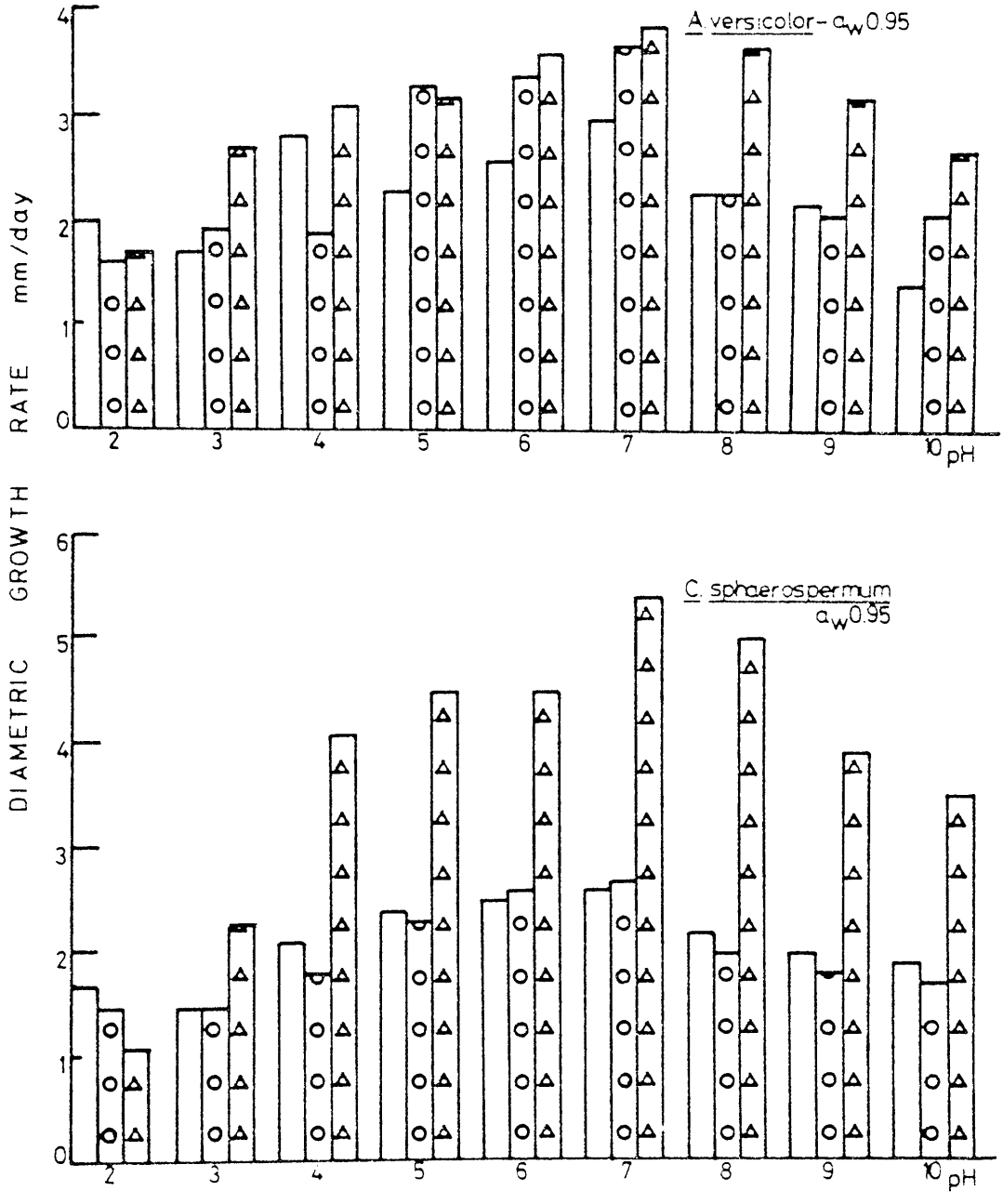
: MEA + NaCl

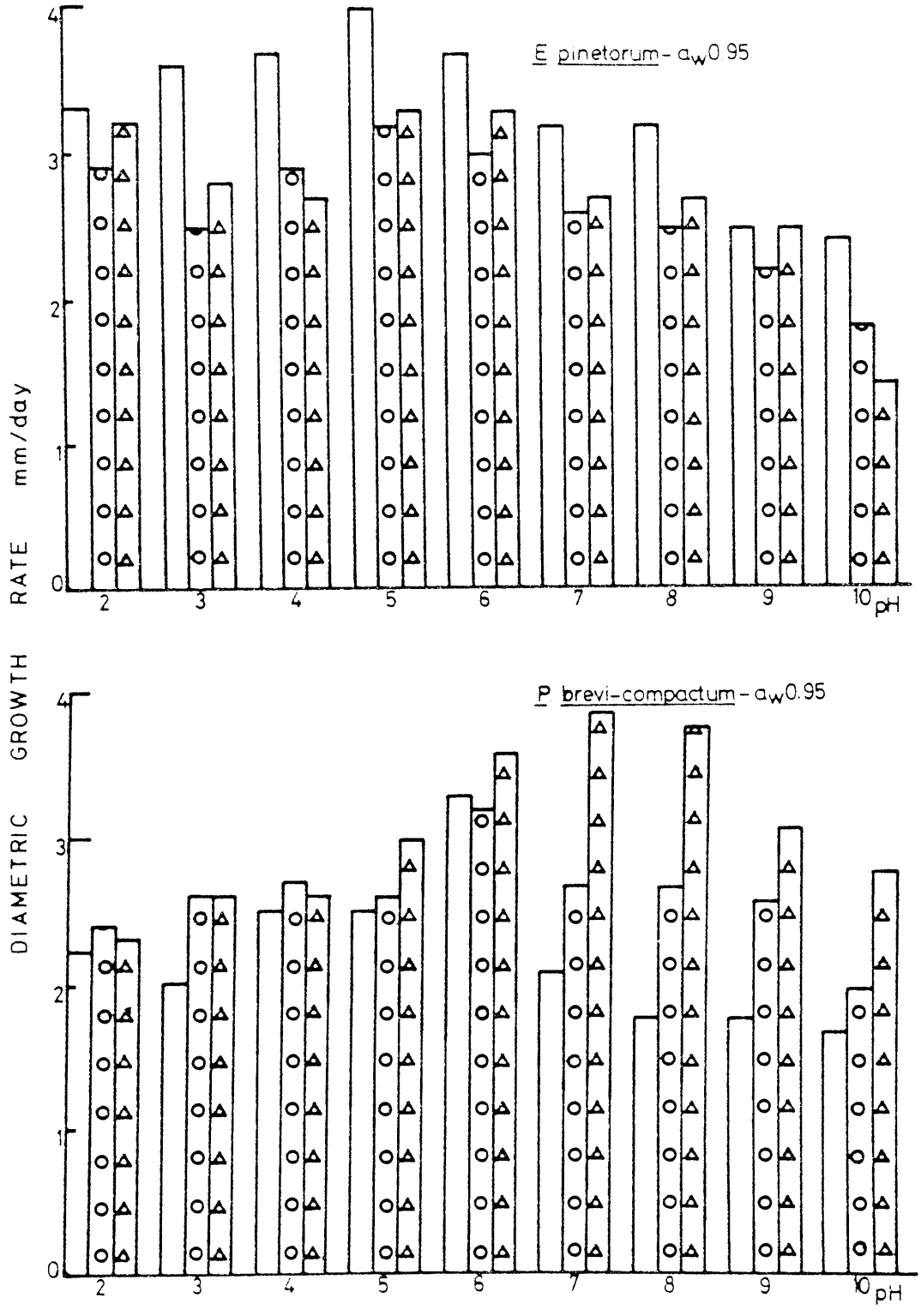

A. amstelodami - a_w 0.93
A. chevalieri - a_w 0.93
DIAMETRIC GROWTH RATE mm/day
pH
0
1
2
3
4
5
6
7
8
9
10

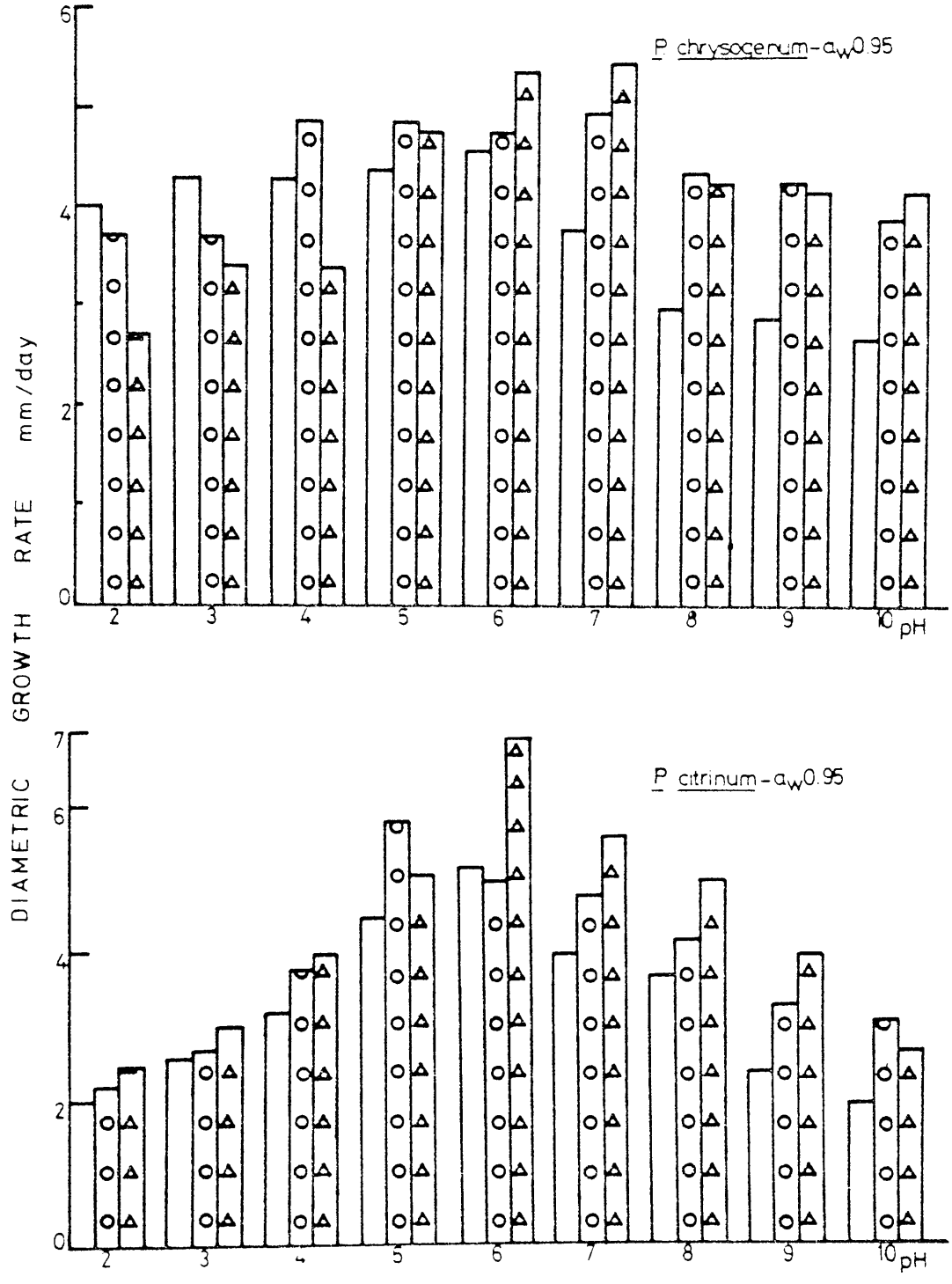

A. repens - a_w 0.93
A. ruber - a_w 0.93
DIAMETRIC GROWTH RATE mm/day
0
1
2
3
4
5
6
7
2
3
4
5
6
7
8
9
10 pH

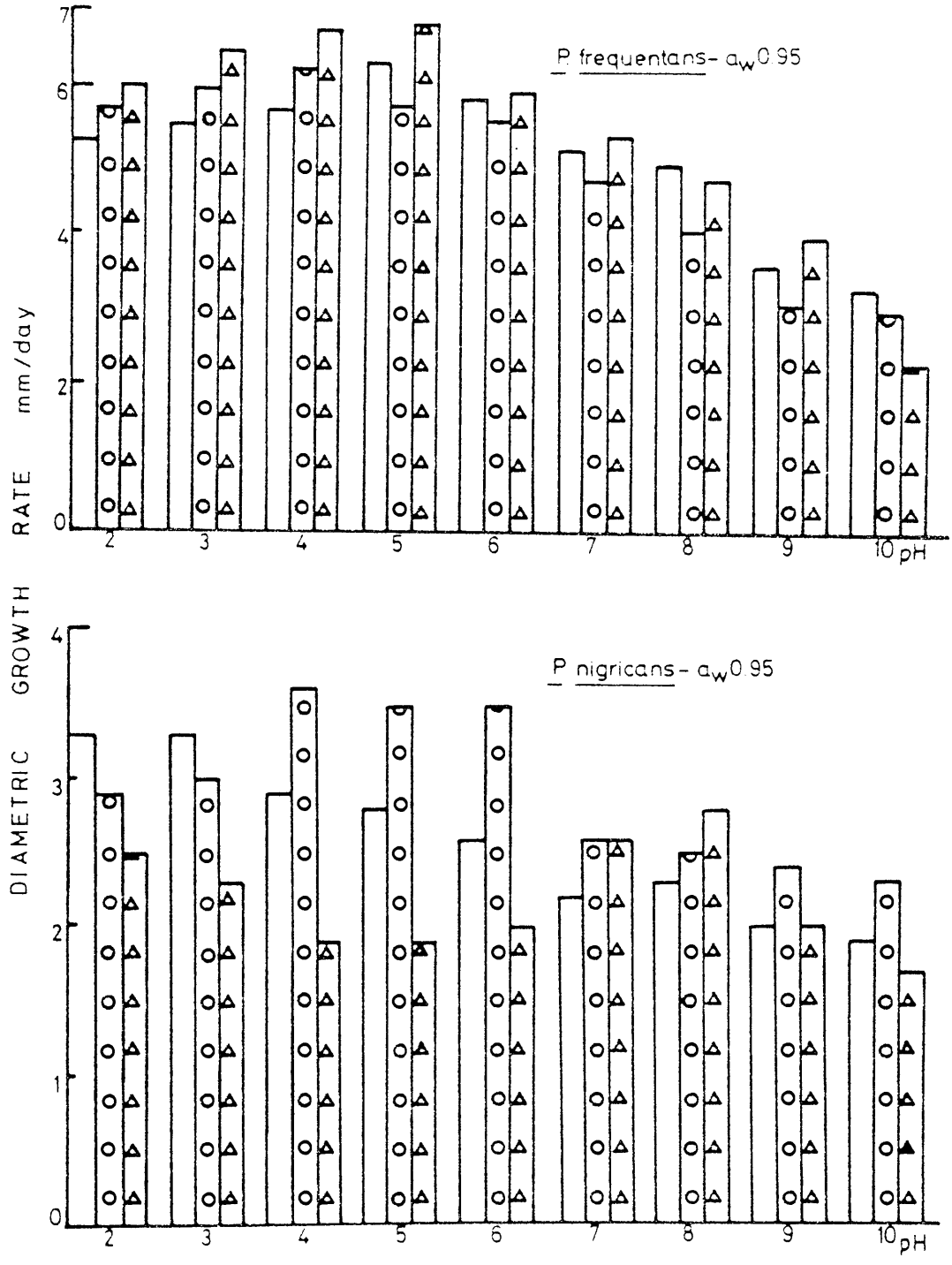

A. niger - a_w 0.95
DIAMETRIC GROWTH RATE mm/day
0
4
8
12
16
2
3
4
5
6
7
8
9
10 pH

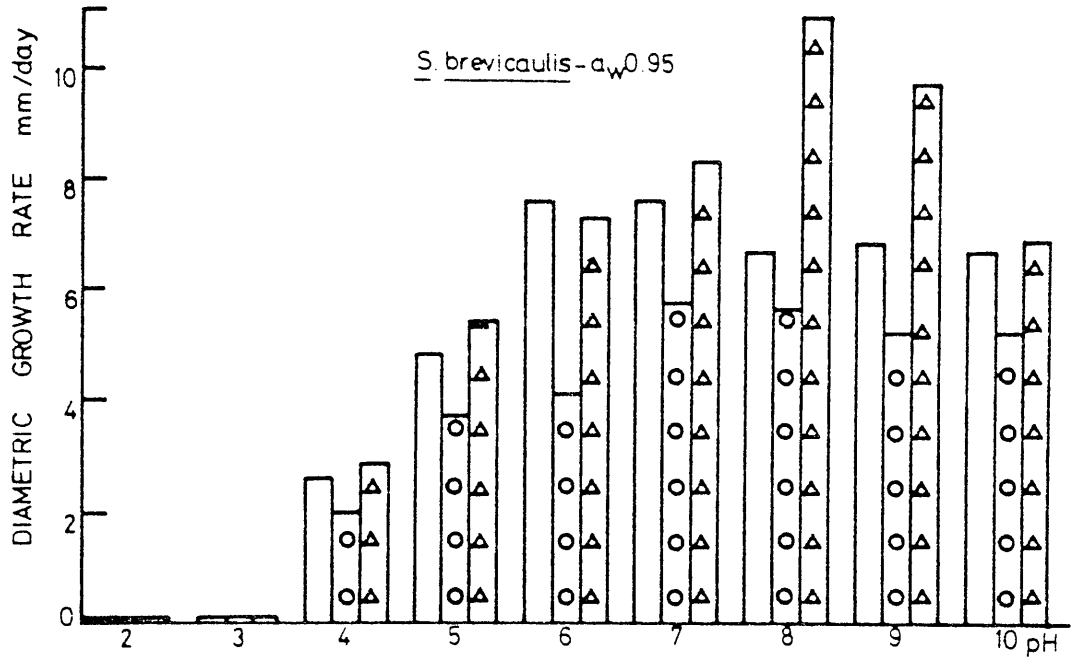

A. penicilloides - a_w 0.95
A. restrictus - a_w 0.95
DIAMETRIC GROWTH RATE mm/day
pH

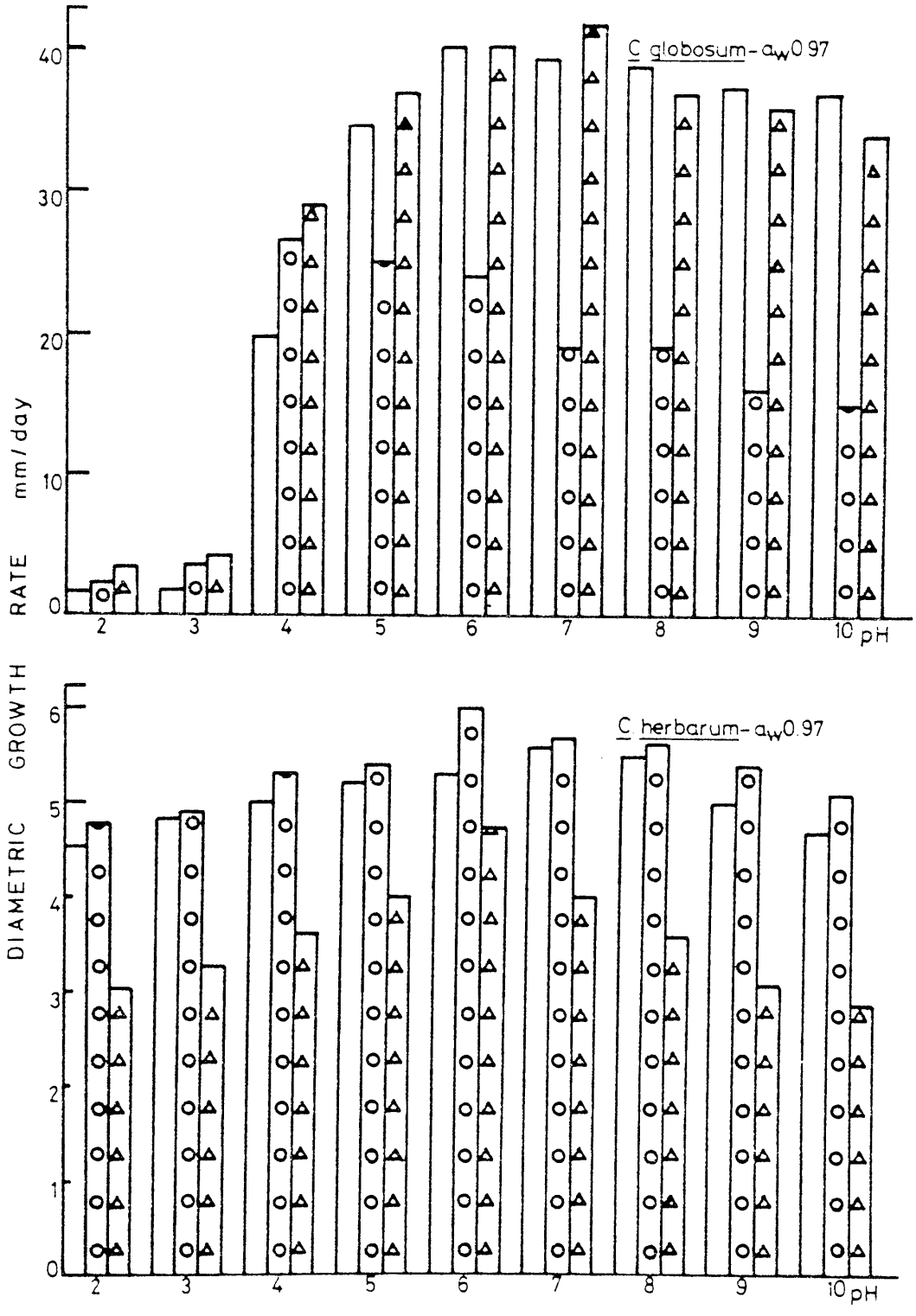

A. versicolor - a_w 0.95
DIAMETRIC GROWTH RATE mm/day
0
1
2
3
4
2
3
4
5
6
7
8
9
10 pH
C. sphaerospermum
a_w 0.95
0
1
2
3
4
5
6
2
3
4
5
6
7
8
9
10 pH

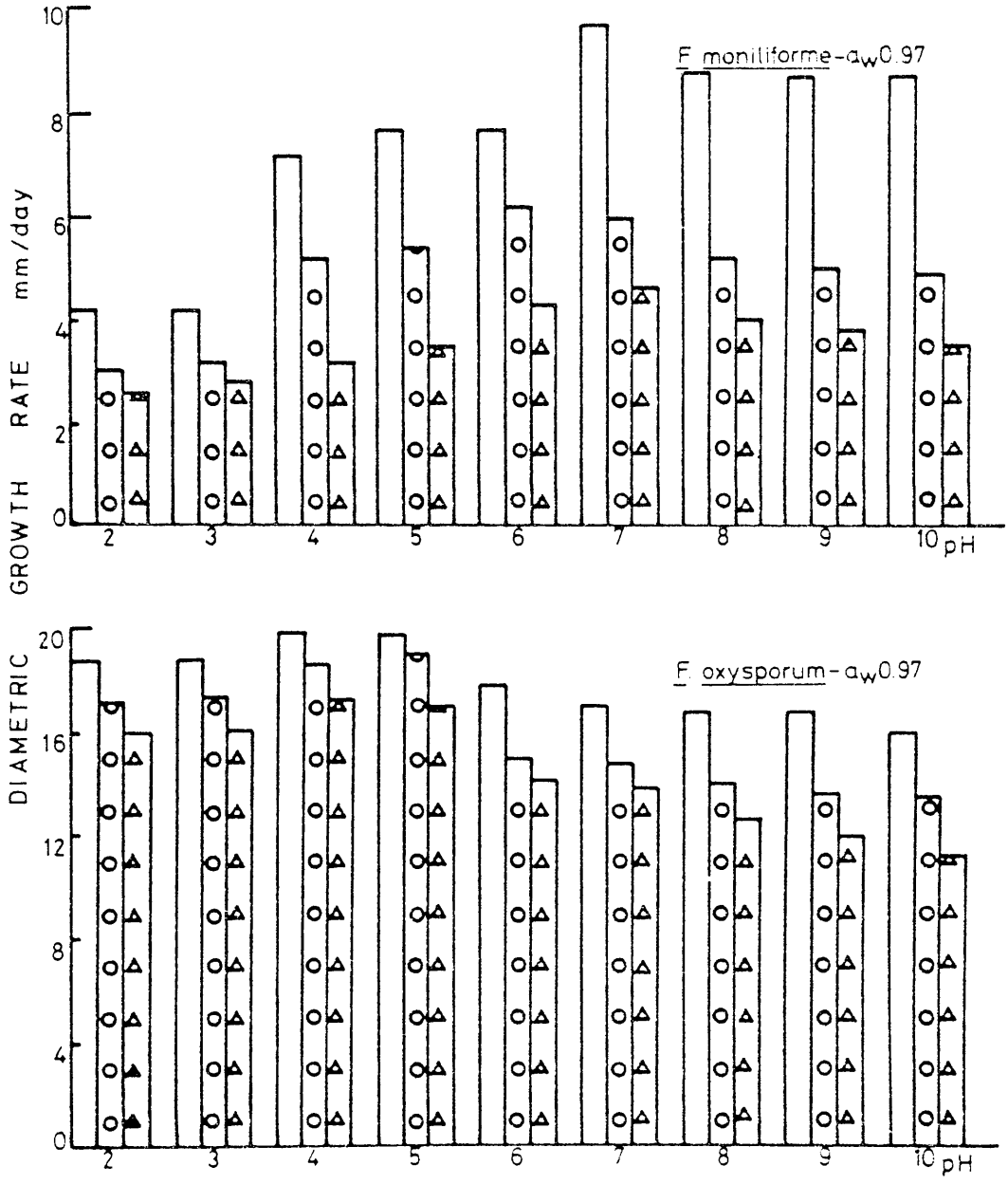

E pinetorum - a_w 0.95
DIAMETRIC GROWTH RATE mm/day
0
1
2
3
4
2
3
4
5
6
7
8
9
10
pH
P brevi-compactum - a_w 0.95
DIAMETRIC GROWTH RATE mm/day
0
1
2
3
4
2
3
4
5
6
7
8
9
10
pH

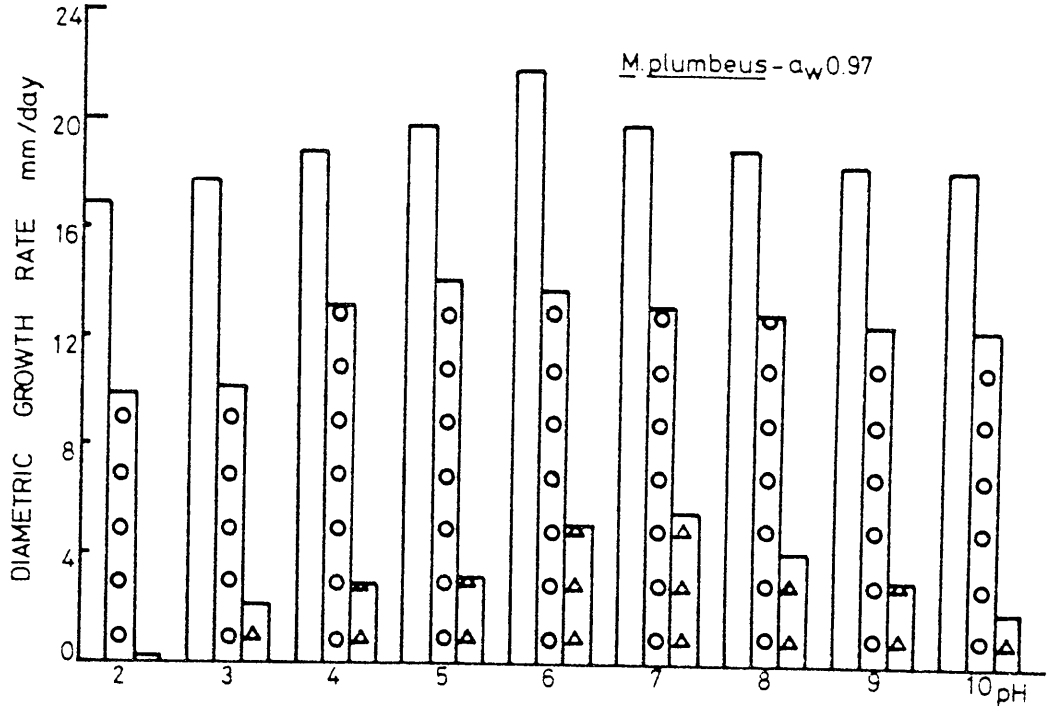

P chrysogenum - a_w 0.95
P citrinum - a_w 0.95
DIAMETRIC GROWTH RATE mm/day
pH
0
2
4
6
7
2
3
4
5
6
7
8
9
10

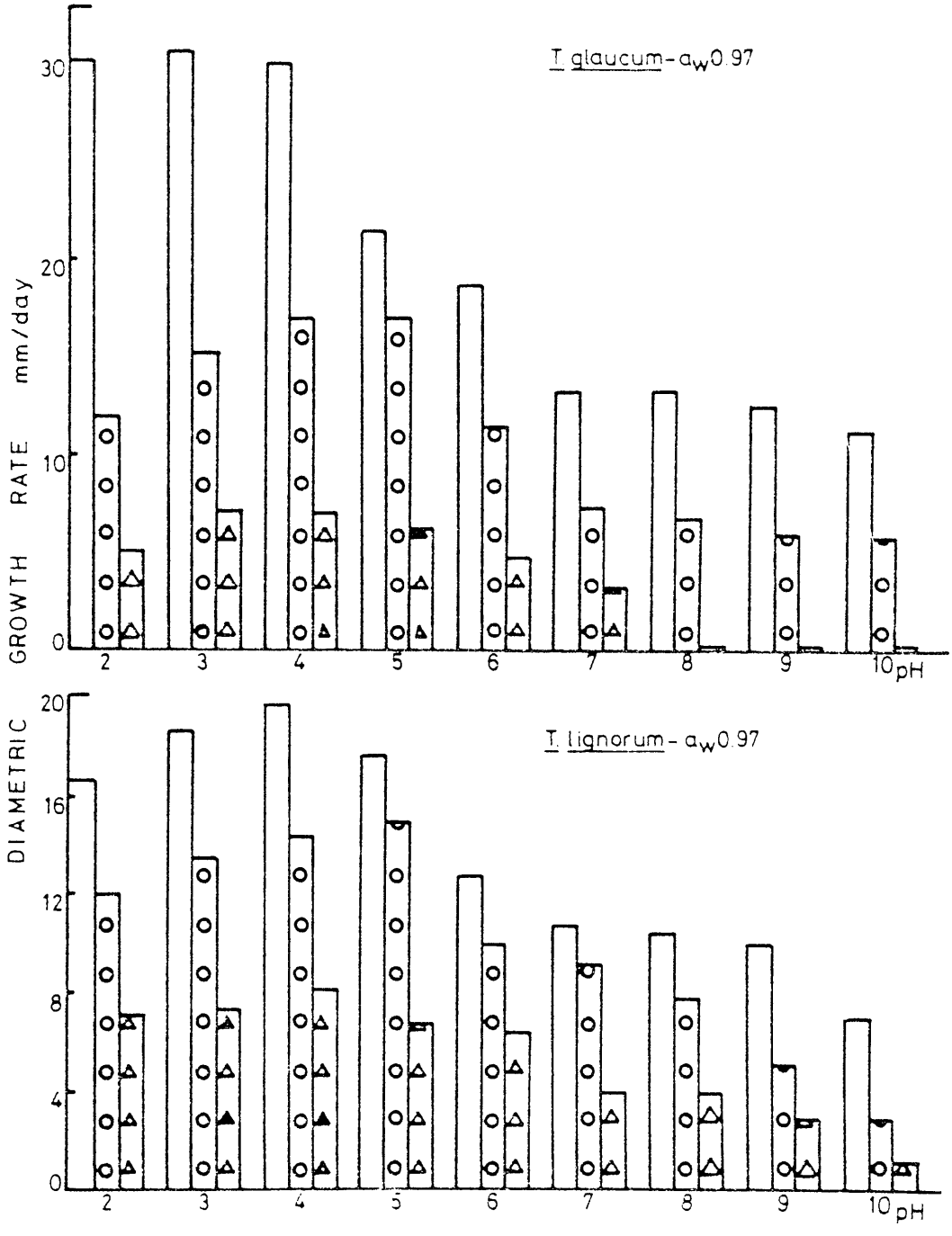

P frequentans- a_w 0.95
RATE mm/day
0
2
4
6
7
2
3
4
5
6
7
8
9
10 pH
P nigricans- a_w 0.95
DIAMETRIC GROWTH
0
1
2
3
4
2
3
4
5
6
7
8
9
10 pH


S. brevicaulis - a_w 0.95
DIAMETRIC GROWTH RATE mm/day
0
2
4
6
8
10
2
3
4
5
6
7
8
9
10 pH


C globosum - a_w 0.97
RATE mm/day
0
10
20
30
40
2
3
4
5
6
7
8
9
10 pH
C herbarum - a_w 0.97
DIAMETRIC GROWTH
0
1
2
3
4
5
6
2
3
4
5
6
7
8
9
10 pH


F moniliforme-a_w0.97
GROWTH RATE mm/day
0
2
4
6
8
10
2
3
4
5
6
7
8
9
10 pH
F. oxysporum-a_w0.97
DIAMETRIC
0
4
8
12
16
20
2
3
4
5
6
7
8
9
10 pH


M. plumbeus - a_w 0.97
DIAMETRIC GROWTH RATE mm/day
0
4
8
12
16
20
24
2
3
4
5
6
7
8
9
10
pH


T. glaucum - a_w 0.97
GROWTH RATE mm/day
30
20
10
0
2
3
4
5
6
7
8
9
10 pH
T. lignorum - a_w 0.97
DIAMETRIC
20
16
12
8
4
0
2
3
4
5
6
7
8
9
10 pH

a_w over a range of 0.97 to 0.80 (Appendix 1). Non-xerophiles were tested over a_w ranges from 0.97 to 0.90 with glycerol and NaCl.

Media with a_w levels greater than 0.95 were sterlized by autoclaving at 115°C for 10 minutes and those below 0.95 were sterilized by steaming at 100°C for 30 minutes. The final pH of the media were checked to give values within $\pm$ 0.2 of the pH values required.

After sterilization, 15ml of the cooled liquid medium was poured into petri dishes in a laminar-flow cabinet to prevent contamination. The plates were maintained inverted in the cabinet without lids until drops of condensate had disappeared from the surface of the medium. The plates were stored under conditions of controlled a_w by using saturated solutions (Young, 1967) or molal glycerol solutions until they were inoculated.

Inoculation, incubation and subsequent colony diameter measurement was similar to that used to study the pH effects. Five replicates were used.

3.4.3 Results

Figure 3.2 shows the growth rate versus the a_w of the xerophilic and non-xerophilic test fungi at pH4 and pH6.5.

Each of the Aspergillus isolates had a characteristic optimum a_w value of growth. The effect of NaCl is very marked, in that no growth was

observed after one month around a_w levels of 0.80. A. niger, A. restrictus and A. versicolor displayed no visible growth at a_w levels around 0.80 with either glycerol or NaCl as the controlling solute. All the aspergilli, excepting A. niger with glycerol and NaCl and A. repens with glycerol as controlling solutes showed a higher growth rate at pH6.5.

Members of the A. glaucus group, i.e. A. amstelodami, A. chevalieri var. intermedius, A. repens and A. ruber, show similar trends in growth patterns, the optimum growth between a_w of 0.97 to 0.90. Excepting A. ruber, they show higher growth rates with NaCl as the controlling solute, although the optimum a_w of growth with NaCl was at a higher value than that when glycerol was used as the controlling solute.

Besides the increase in colony diameter as a parameter of growth, other factors such as the appearance of the colony, production of diffusible pigments into the medium and spore type were observed.

At a_w levels below 0.95, fungi of the A. glaucus group produced loose adventitious hyphal growth and above 0.95 a_w, the colonies have a compact form. This has been found to be a characteristic of this group.

Diffusible pigments produced by some fungi of the A. glaucus group were seen in the media of low a_w. A. chevalieri produced a red diffusible pigment in media with a_w range of 0.85 to 0.77. A. repens and A. ruber produced diffusible brown pigments at low a_w levels.

The amount of pigment produced was dependant on the amount of growth.

There are two spore types, i.e. conidia and cleistothecia, produced by members of A. glaucus group. Generally, more conidial production was observed at a_w levels above and below the optimal growth rates. Near a_w values for optimal growth rates, cleistothecial production was slightly higher than conidial production.

The optimum growth rates of the penicillia were at a_w levels between 0.997 (MEA) to 0.95, higher than those obtained for the aspergilli. Growth was inhibited at a_w levels around 0.80 after one month.

P. chrysogenum, P. citrinum, P. frequentans, P. nigricans and E. pinetorum displayed higher growth rates with glycerol as the controlling solute, while P. brevi-compactum displayed a higher growth rate with NaCl as the controlling solute. In all the penicillia sporulation was slow in media with a_w levels between 0.90 to 0.80.

C. sphaerospermum, at pH4.0 displayed an optimum growth at a_w level of 0.97 and at pH6.5, displayed an optimum growth at a_w level of 0.95 with either glycerol or NaCl. The effect of pH is very marked on the growth of S. brevicaulis at decreasing a_w. Although the optimum growth rate at both test pH values and with either glycerol or NaCl is at a_w 0.97, the growth rate at pH4.0 is reduced at decreasing a_w.

Growth of non-xerophilic fungi is restricted to a_w levels of up to and including 0.90. The effect of solutes on the growth of M. plumbeus and

T. lignorum was very marked. There was a sharp decrease in growth rates with NaCl as the controlling solute. Between species of the same genus, i.e. Fusarium and Trichoderma, there was a variation in the growth patterns. At a_w above 0.95, F. moniliforme with glycerol at both pH4.0 and pH6.5, displayed higher growth rates than with NaCl at pH4.0 and pH6.5, while F. oxysporum, displayed higher growth rates at pH6.5 with either glycerol or NaCl than at pH4.0 with glycerol or NaCl.

T. glaucum displayed a higher growth rate at pH4.0 than pH6.5. With decreasing a_w, there was a decrease in growth rates at both pH values with NaCl as the controlling solute, the fall in growth rate is higher than that with glycerol as the controlling solute. T. lignorum displayed a higher growth rate at pH4.0 with only glycerol as the controlling solute up to 0.93 a_w.

C. herbarum and C. sphaerospermum displayed non-xerophilic and xerophilic tendencies respectively. The growth rates with glycerol as the controlling solute were higher than those with NaCl as the controlling solute in C. herbarum and vice-versa in C. sphaerospermum.

3.5 Discussion

The present method of studying the water relations of the test fungi proved satisfactory. In order to ascertain that there was no loss or gain of water in the growth medium, the a_w of the media below 0.95 were checked by a dew point meter, Protimeter DP680, before and after the experimental period. Ambient temperature, dew point and relative

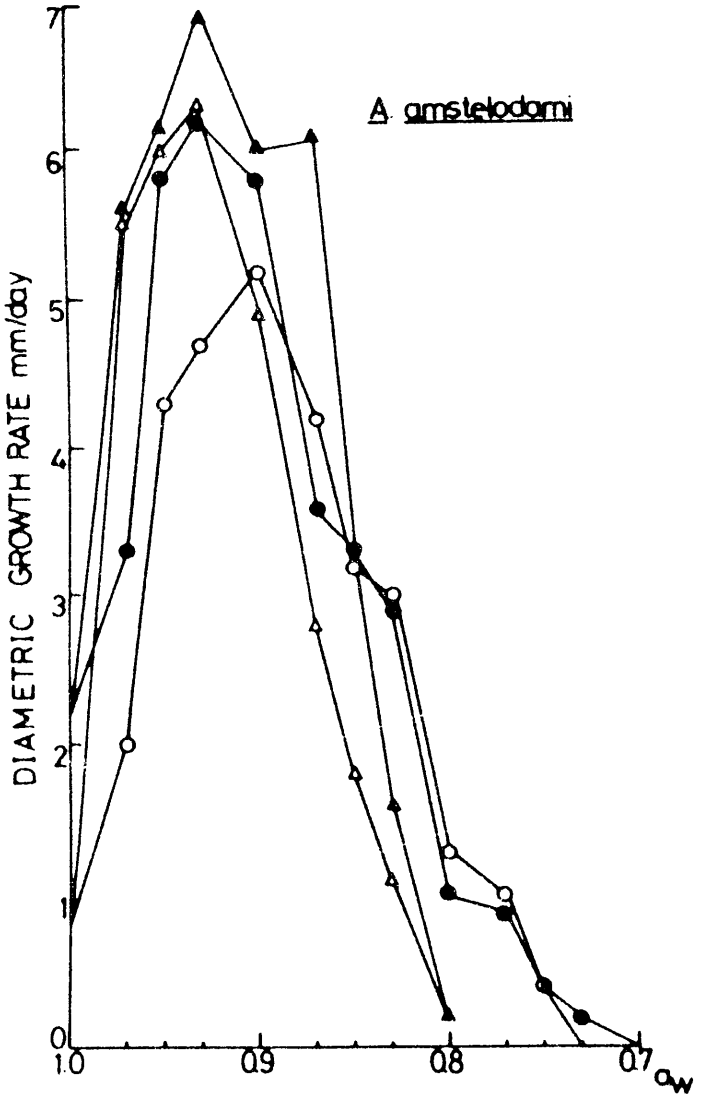
FIGURE 3.2 : COMBINED EFFECTS OF pH, SOLUTES, AND a_w ON GROWTH OF TEST FUNGI USING DIAMETRIC GROWTH RATES

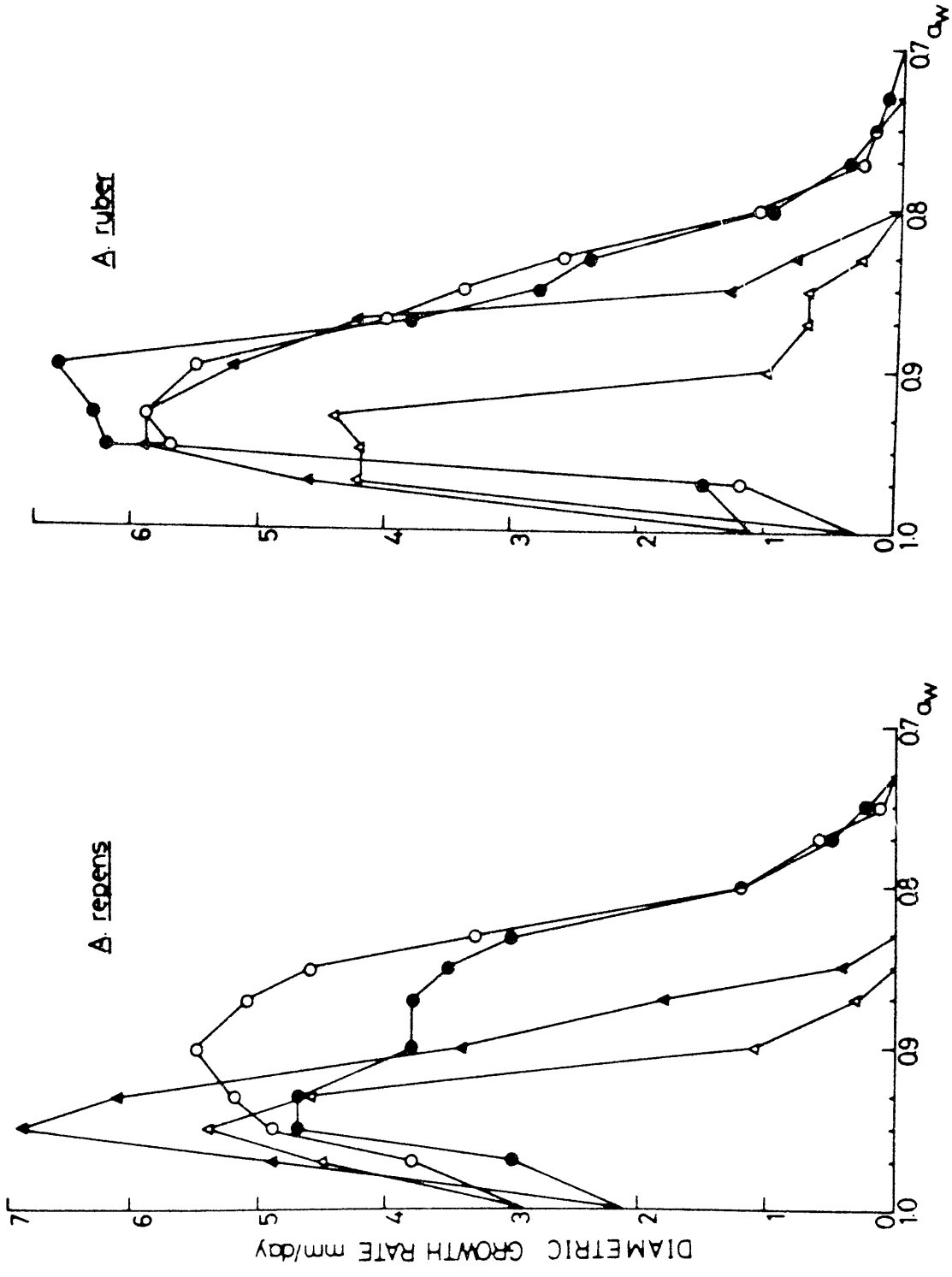
MEA + GLYCEROL pH 4.0

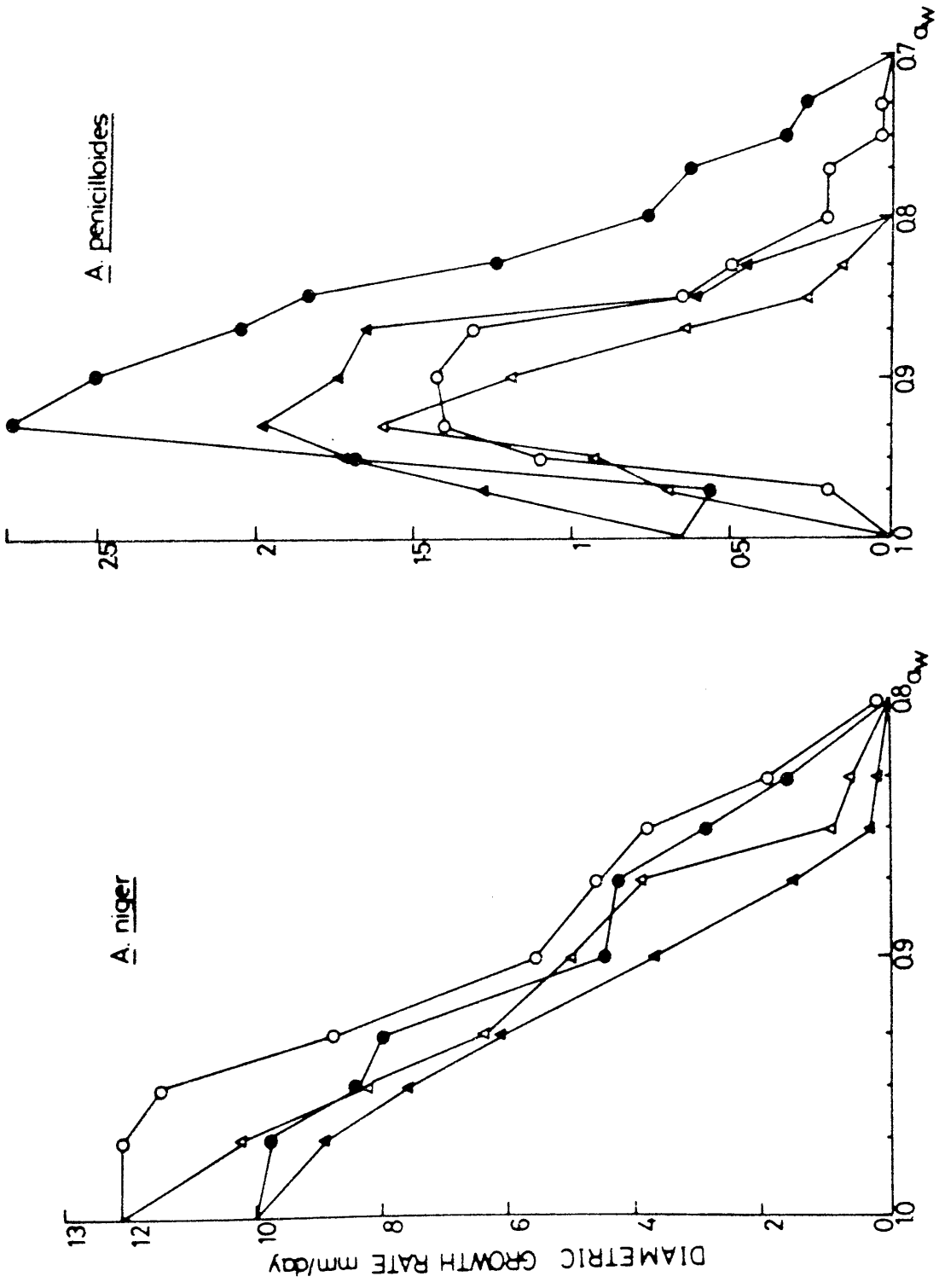
MEA + GLYCEROL pH 6.5

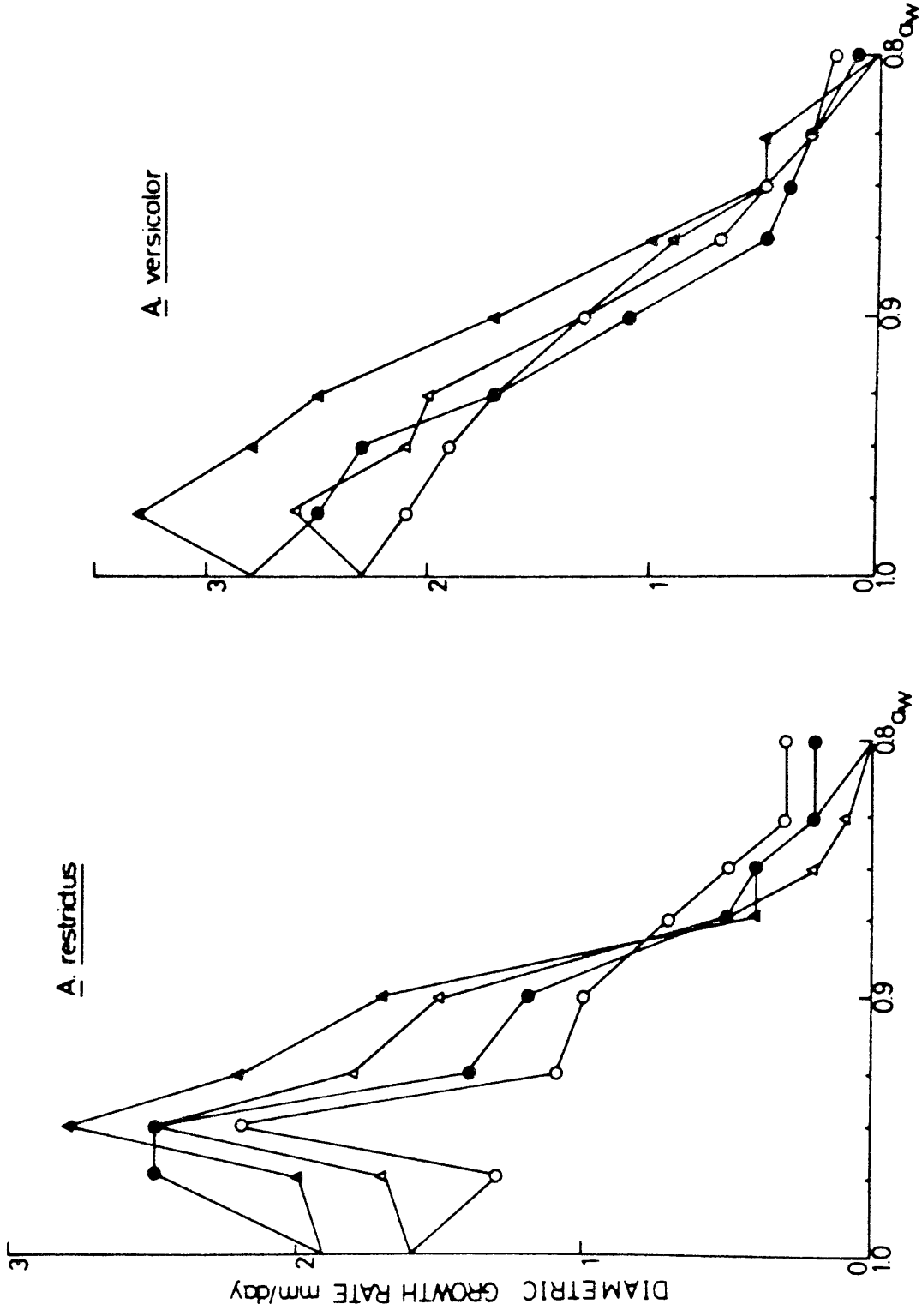
MEA + NaCl pH 4.0

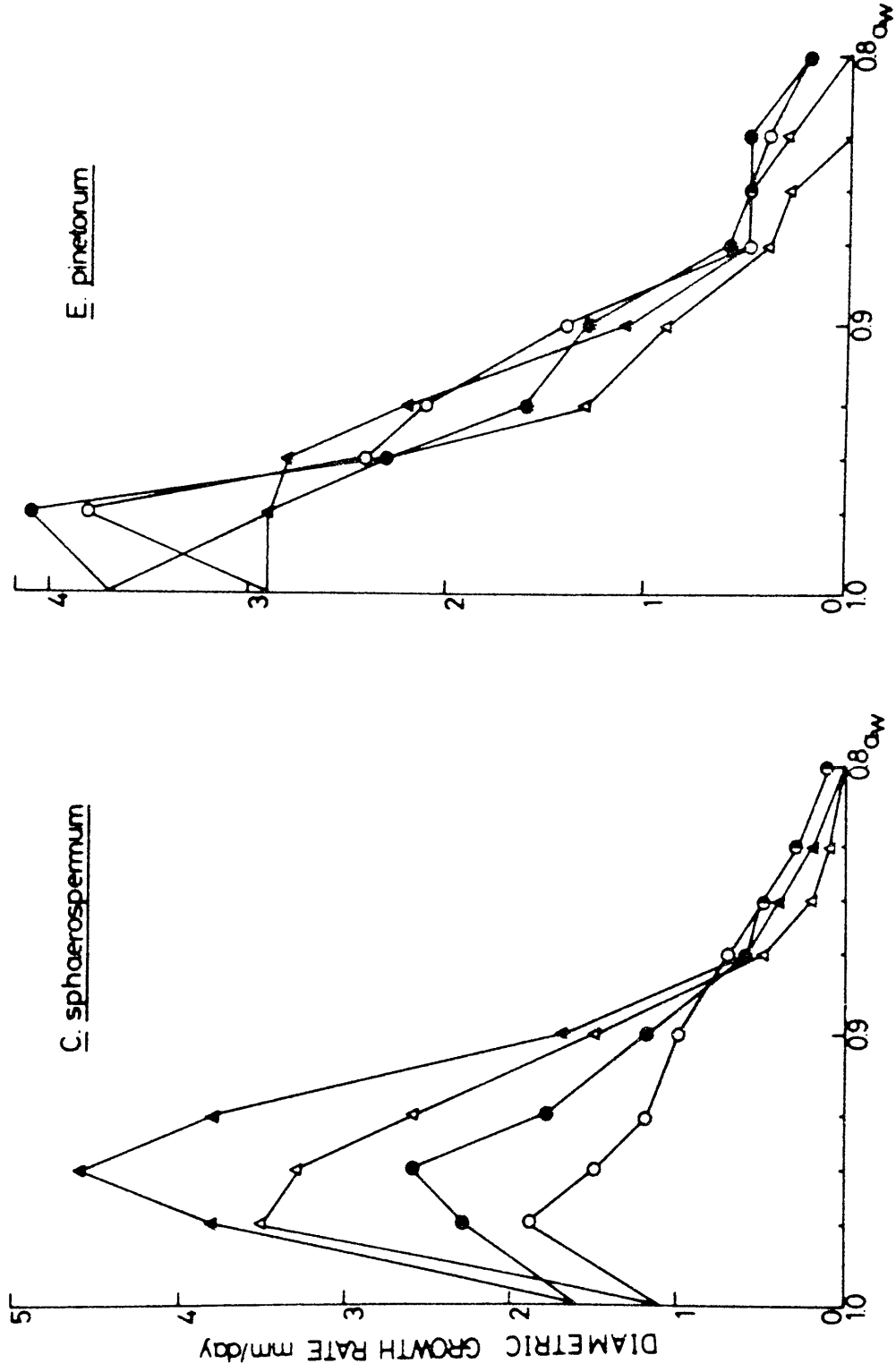
MEA + NaCl pH 6.5

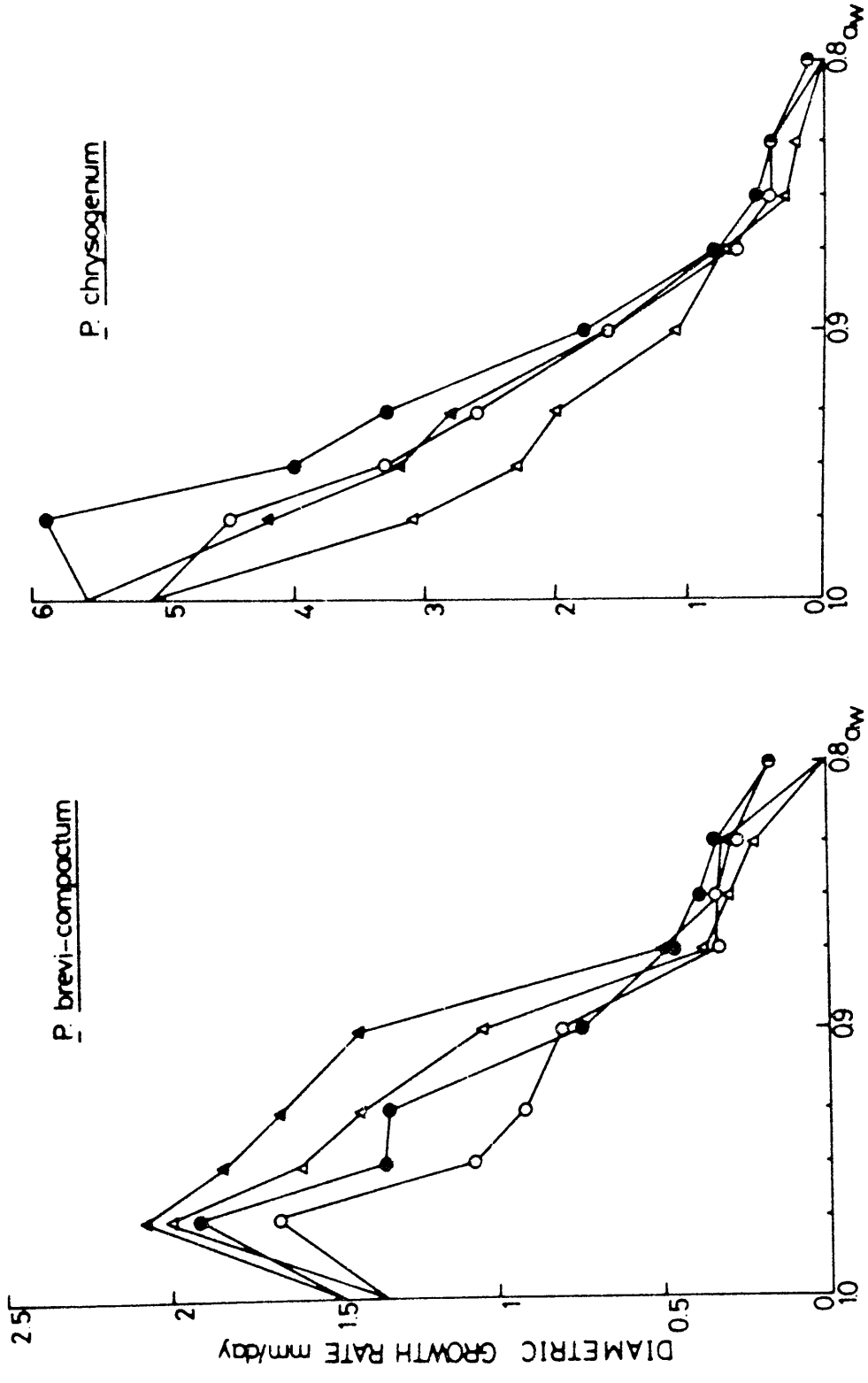


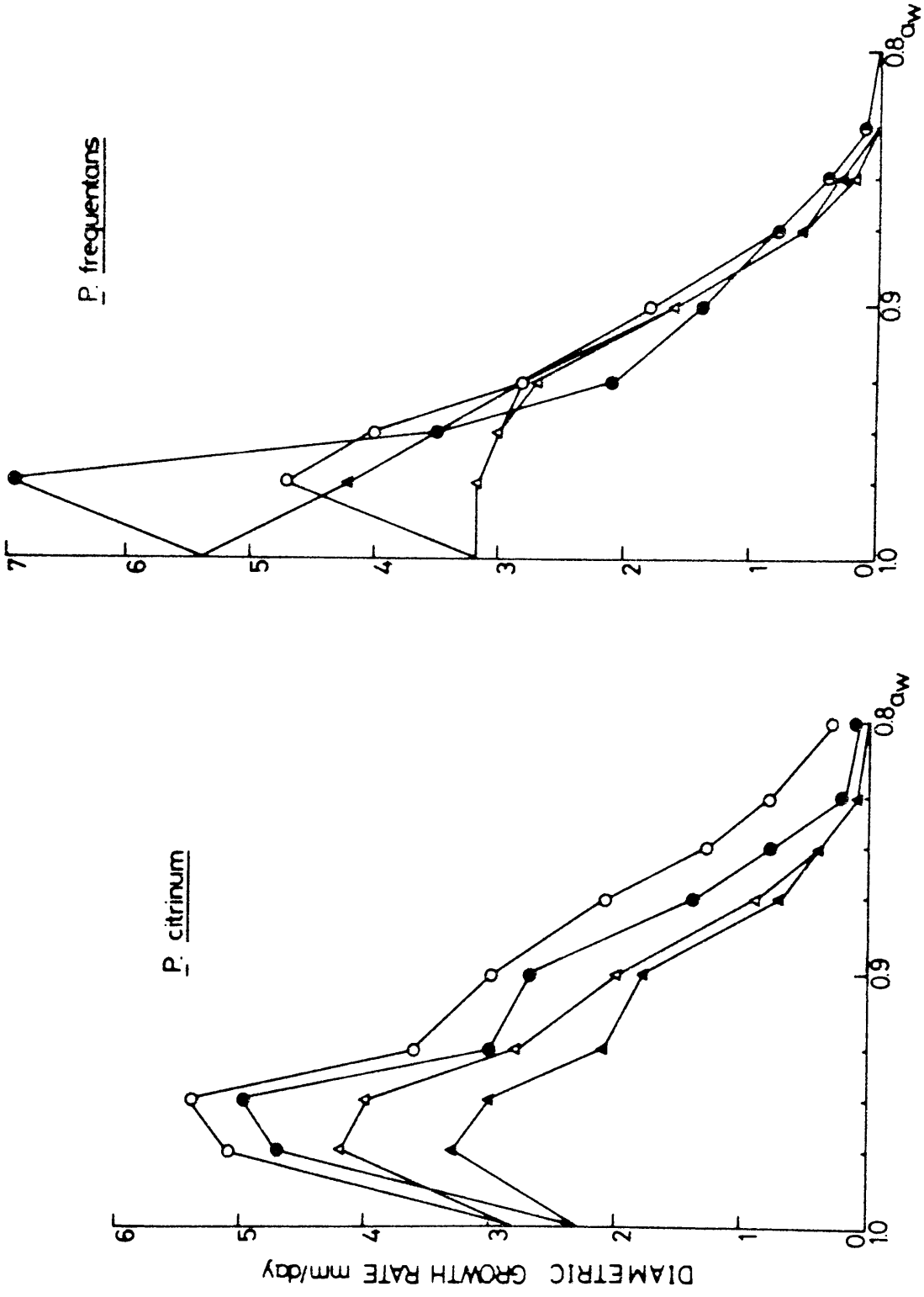

A. repens
A. ruber
DIAMETRIC GROWTH RATE mm/day
0
1
2
3
4
5
6
7
1.0
0.9
0.8
0.7 a_w

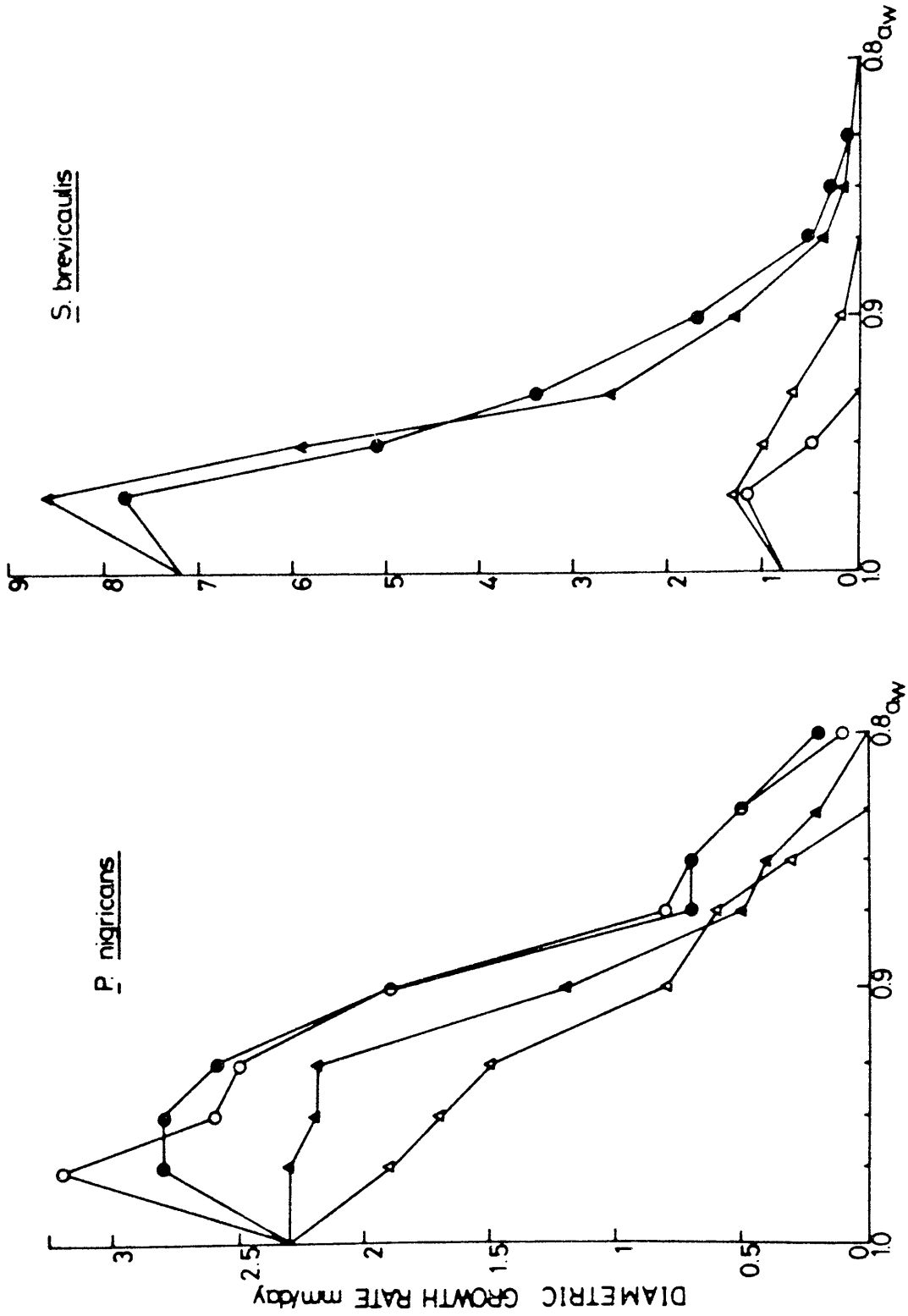

A. penicillioides
A. niger
DIAMETRIC GROWTH RATE mm/day
a_w
a_w

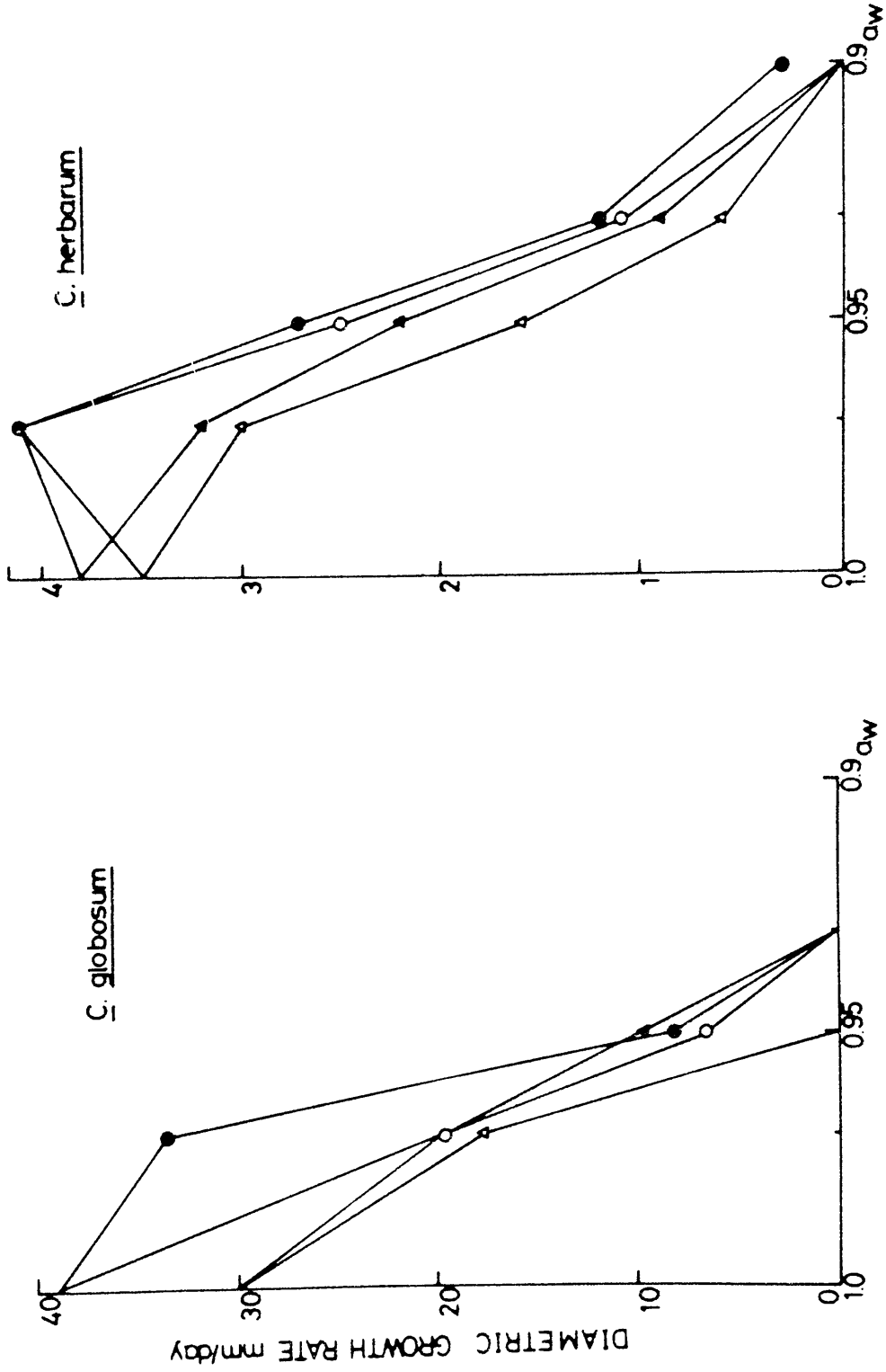

A. restrictus
A. versicolor
DIAMETRIC GROWTH RATE mm/day
0
1
2
3
1.0
0.9
0.8 a_w

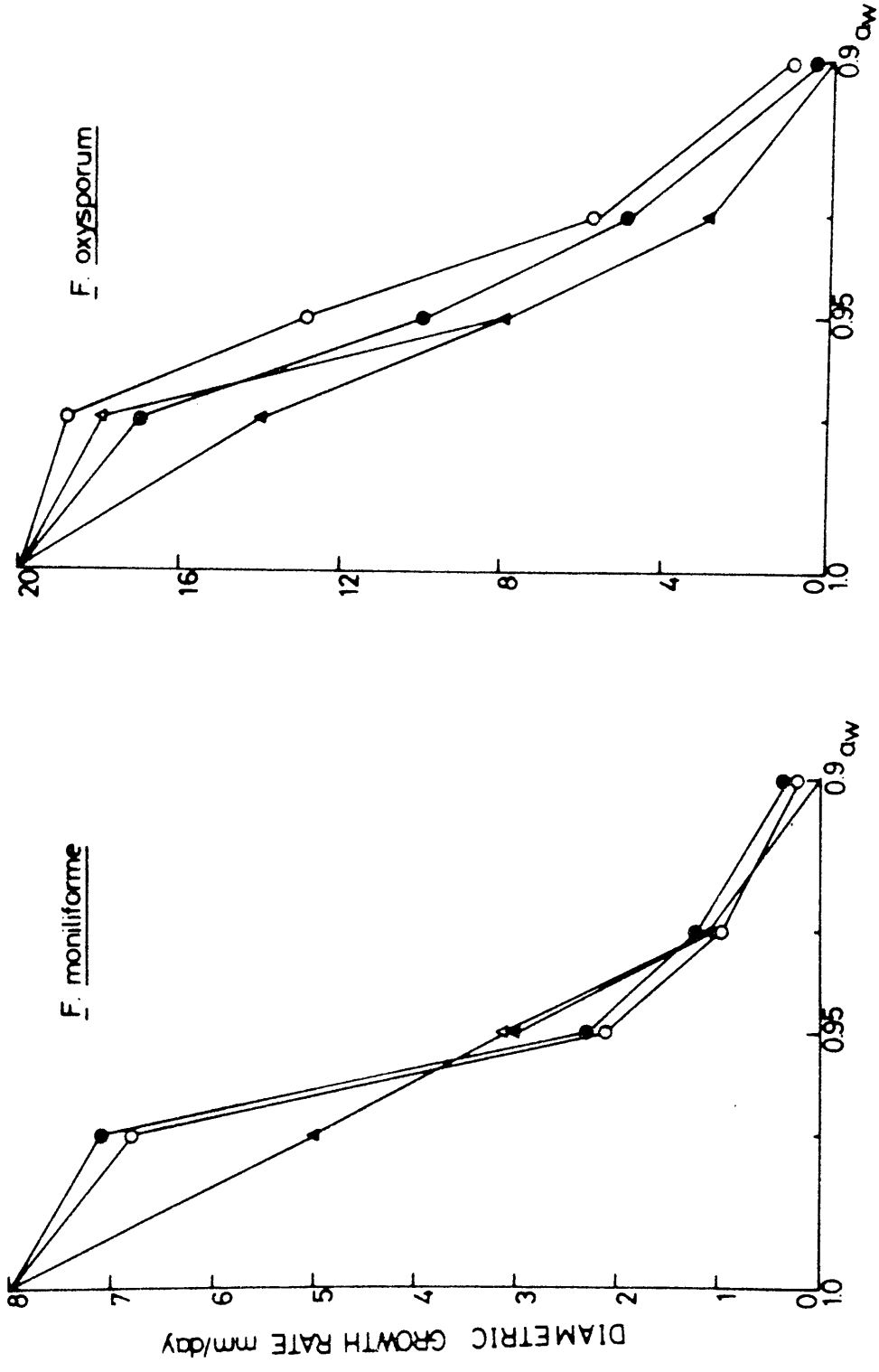

C. sphaerospermum
E. pinetorum
DIAMETRIC GROWTH RATE mm/day
0
1
2
3
4
5
1.0
0.9
0.8 a_w

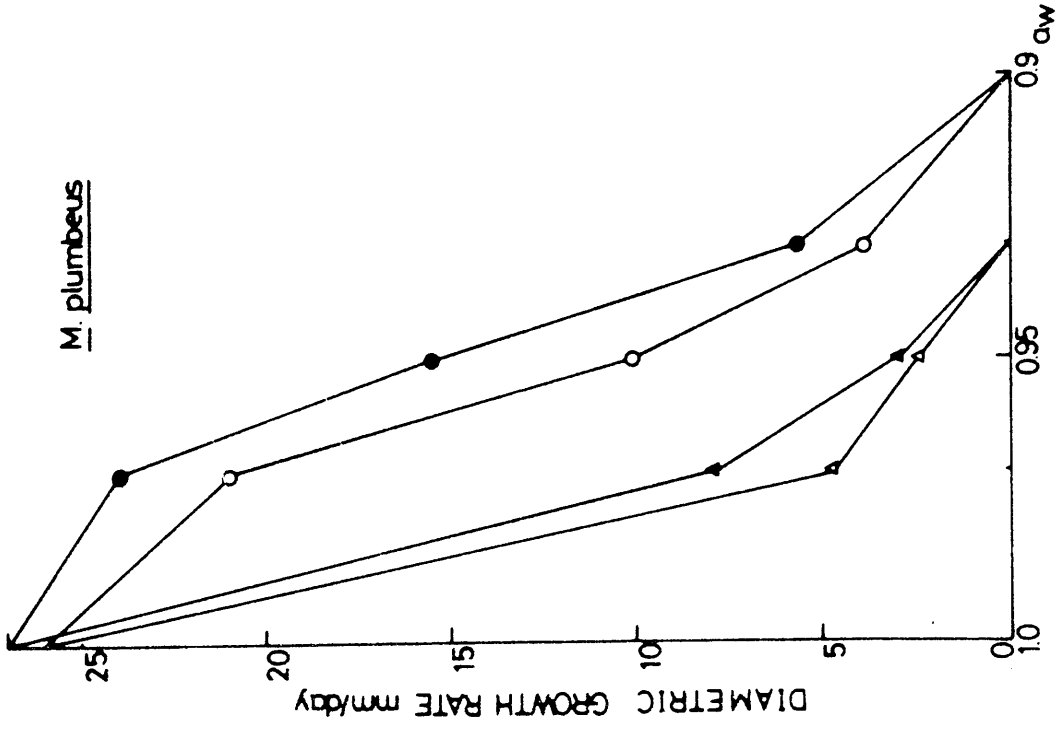

P. brevi-compactum
P. chrysogenum
DIAMETRIC GROWTH RATE mm/day
a_w
0.8
0.9
1.0
0.5
1
1.5
2
2.5
3
4
5
6

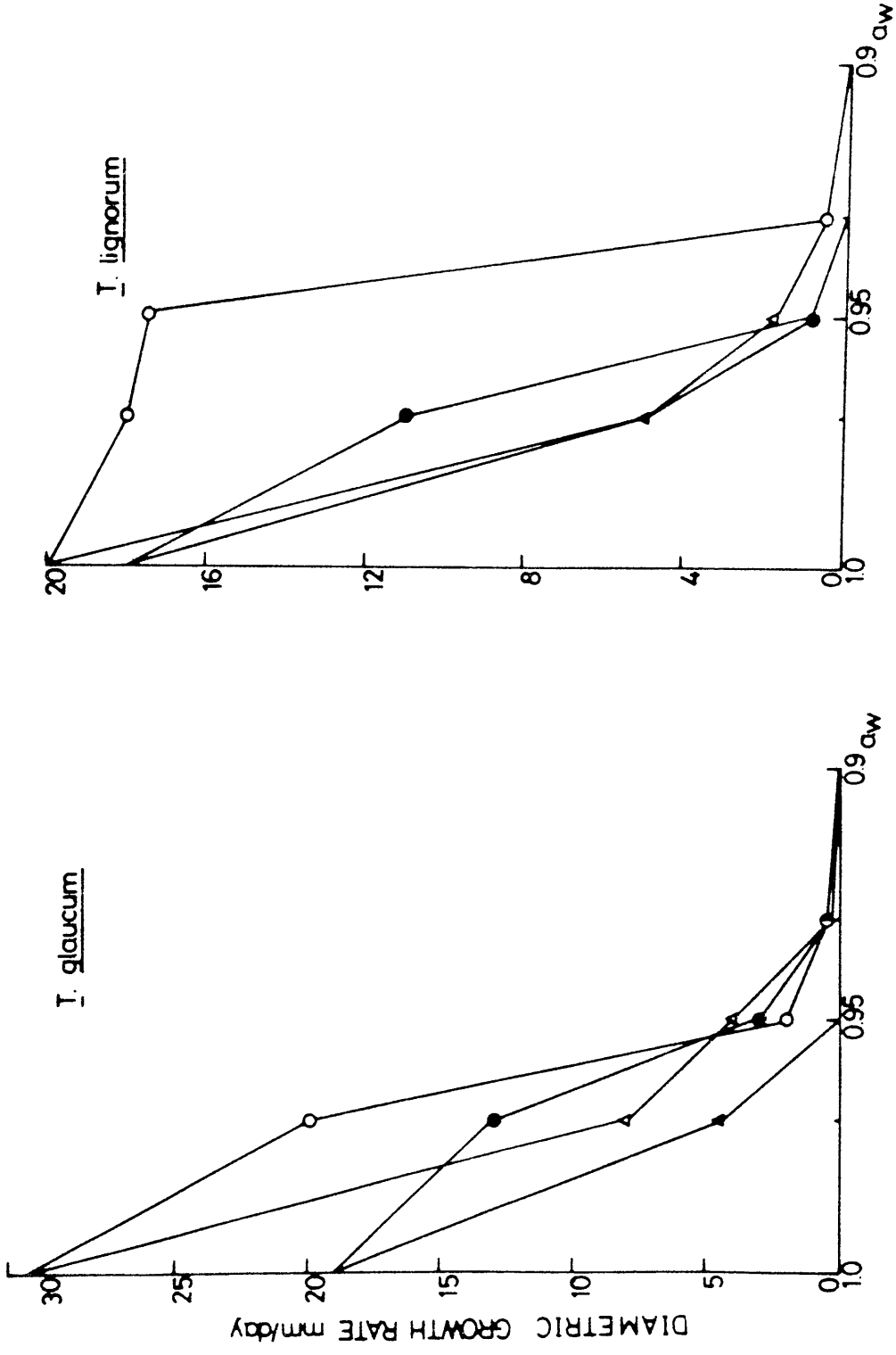

P. frequentans
P. citrinum
DIAMETRIC GROWTH RATE mm/day
0
1
2
3
4
5
6
7
1.0
0.9
0.8 a_w


P. nigricans
S. brevicaulis
DIAMETRIC GROWTH RATE mm/day
0.8 aw
0.9
1.0


C. herbarum
C. globosum
DIAMETRIC GROWTH RATE mm/day
0
1.0
0.95
0.9 aw
10
20
30
40
1
2
3
4


F. oxysporum
F. moniliforme
DIAMETRIC GROWTH RATE mm/day
0
4
8
12
16
20
1
2
3
4
5
6
7
8
1.0
0.95
0.9 aw


M. plumbeus
DIAMETRIC GROWTH RATE mm/day
25
20
15
10
5
0
1.0
0.95
0.9
a_w


T. glaucum
T. lignorum
DIAMETRIC GROWTH RATE mm/day
0
5
10
15
20
25
30
4
8
12
16
20
1.0
0.95
0.9 a_w

humidity were registered and from the values obtained, there was a slight change in the a_w values.

From the results of growth at pH2 to pH10, on MEA and MEA modified media, it is seen that all the isolates were capable of growth, excepting S. brevicaulis, which grew at a pH range from pH4 to pH10. Most isolates displayed growth optima at different pH values in MEA, GMEA and NMEA. Two interesting points emerge : 1) the growth rate is higher over the whole test pH range at near optimum a_w level, 2) over the test pH range, the growth rates of the isolates at optimum a_w values varied with the controlling solute, i.e. glycerol or NaCl.

From the results of the growth at various a_w levels, at pH4 and pH6.5, it is seen that there is decreased growth at decreasing a_w. Non-xerophilic test species had higher growth rates and very narrow optima at high a_w levels, i.e. MEA (0.997) and 0.97. At a_w levels below 0.95, there was a very sharp decrease in growth. These results are supported by those obtained by Luard and Griffin (1981), who also found that large positive turgor potentials were maintained in both drought-sensitive and xerophilic species, even when the external potential severely inhibited growth.

The effect of pH at decreasing a_w is very marked in some isolates, e.g. A. niger, A. penicilloides and S. brevicaulis. In the latter, growth was observed up to a_w 0.83 at pH6.5 and up to 0.90 at pH4.0. It was found that tolerance by fungi to low a_w was increased near the optimum pH for growth (Ingram, 1957).

In some isolates, C. sphaerospermum, M. plumbeus, P. citrinum and P. nigricans, the effects of solutes at decreasing a_w is marked. Pitt and Hocking (1977), obtained very limited growth in xerophytes, C. fastidium and X. bisporus. NaCl intolerance in xerophilic fungi may be due to the failure by the ions to stimulate compatible solute/s production or the ions may alter membrane permeability and thus retain the solute/s. Allaway and Jennings (1970), found that sodium ions caused the loss of polyols from the hyphae of Dendryphiella salina, while calcium ions decreased polyol loss. Other test species were not sensitive to glycerol or NaCl as controlling solutes at decreasing a_w levels. This indicates that the inhibition observed may be due to an osmotic effect rather than the specific solute effect. With the exception of C.fastidium and X. bisporus, Luard and Griffin (1981), observed that most drought sensitive and xerophilic species did not discriminate between glucose, KCl and sucrose in their growth response.

In both xerophilic and non-xerophilic test species, decreasing a_w levels reduced or inhibited sporulation. This can be explained in terms of the lack of nutrients at reduced a_w with solute addition. Hawker (1966), suggested that fungi absorb nutrients in solution and by restricting the a_w or water availability, the nutrient availability will also be restricted. Maximum cleistothecial production in the test A. glaucus group fungi was observed at around optimum a_w for growth while spore production was observed at high and decreasing a_w levels and at low a_w i.e. 0.75 and 0.73, no sporulation was observed. Successive workers, Scott (1957), Kushner (1971) and Griffin (1972), have found that conditions for sporulation, especially sexual sporu-

lation are more exacting than those required by vegetative growth.

Brown (1976), has suggested two possible mechanisms whereby micro-organisms can survive and grow at low a_w. Firstly, the proteins of tolerant microorganisms are different from those of non-tolerant microorganisms. Secondly, proteins of the tolerant species are essentially similar to the non-tolerant species, but the intracellular conditions are modified such that the effect of the environment is diminished. The striking evidence to support his theory of the second mechanism comes from the fact that all the test xerophilic species were capable of growth from a_w values of 0.997 to 0.73 or 0.997 to 0.80, growth being restricted at each a_w extremity.

There is some evidence to show that interdependence of temperature and a_w (Tomkins 1930; Bonner 1948 and Ayerst 1968). Ayerst (1968), recorded a tendency of xerophilic fungi to have low optimum a_w of growth at higher temperature and for the optimum temperature to be at lower a_w. To further study the interrelationship of temperature and a_w, experiments were carried out in the following chapter on hyphal and spore survival of toxinogenic, xerophilic test fungi.

CHAPTER FOUR

HEAT RESISTANCE STUDIES OF XEROPHILIC FUNGI

4.1 Introduction

From previous experiments, it is seen that low a_w levels reduce or inhibit microbial growth. However, by decreasing the a_w the heat resistance of vegetative cells and spores is increased - dry heat being less lethal than moist heat. Successive workers, Corry (1974), Doyle and Marth (1975A, B), Lubienieki-von-Schelhorn and Heiss (1975) have shown that the solute and a_w of the heating menstruum affects the heat resistance of bacterial and fungal spores.

The effects of solutes used in controlling the a_w of the heating menstru are varied. The order of increased resistance at equivalent a_w for several strains of salmonellae was found to be sucrose > glucose > fructose > sorbitol > glycerol and that for strains of Saccharomyces rouxii and Schizosaccharomyces pombe at a_w 0.95 was sucrose > sorbitol > glucose > fructose > glycerol. Doyle and Marth (1975B) found that the heat resistance of conidia of A. flavus and A. parasiticus, between a_w levels of about 0.95 to 0.90 was increased in the order of NaCl > sucrose > glucose. Studies by Beuchat and Toledo (1977) on ascospores of Bassochlamys nivea, showed that the heat resistance was greatly enhanced in grape juice by elevated levels of sucrose.

Solutes within the heating menstruum may influence the microbial cells and spores in several ways and therefore, their effects on heat resistance are not always predictable. Hansen and Rieman (1963), found that for non-sporing organisms, solutes will not only decrease the a_w of the heating menstruum, but will affect the hydration of

microbial proteins and the stability of their enzymes.

The test fungi, A. amstelodami, A. flavus, A. fumigatus, A. versicolor and P. citrinum besides being xerophiles, produce secondary metabolites including mycotoxins.

MYCOTOXINS	FUNGUS	NATURAL OCCURANCE
Sterigmatocystin and derivatives	A. amstelodami +A. versicolor	Green coffee, grains foodstuffs
Aflatoxins	+A. flavus	Corn, cottonseed meal, nuts, rice, sorghum
Tremorgens	A. fumigatus	Mouldy commercial feeds, peanuts, rice
Citrinin	+P. citrinum	Oats, rice, rye, wheat

+ The most important toxin producing species of this table

TABLE 4.1 : FUNGI AND MYCOTOXINS ASSOCIATED WITH VARIOUS AGRICULTURAL COMMODITIES (data from Davis and Diener, 1978).

Thermal inactivation of conidia will not only keep foods from spoiling, but, will also keep them safe, since the growth of these fungi must precede mycotoxin synthesis.

The heat resistance studies carried out here were based upon two criteria, 1) diametric growth rates calculated over a monthly period 2) spore survival.

4.2.1 Effects of Elevated Temperatures on the Mycelial Growth Rates at Decreasing a_w

Materials and Method

All the test fungi, A. amstelodami (ascospores and spores), A. flavus, A. fumigatus, A. versicolor and P. citrinum, were isolated from soil isolations at 25°C, excepting A. flavus and A. fumigatus, which were isolated from all soil types at a_w NO.95 at 45°C to 50°C. Two week old cultures grown on malt extract agar (MEA) and malt extract yeast extract plus 40% glucose (MY40G) were used throughout, excepting for A. amstelodami ascospores where 8 to 9 weeks old cultures were used. This was to ensure ascospore maturation.

The diametric growth rates were measured at 20°C, 25°C, 30°C, 35°C, 40°C, 45°C and 50°C on a basal MEA, at a_w levels of 1.0 (MEA), GO.95, GO.90, GO.85, GO.80, NO.95, NO.90, NO.85 and NO.80. The media were buffered at pH 5.4 using citrate-phosphate buffer (Gomori, 1955). The various a_w plates were prepared, inoculated and measurements were taken as previously shown in Chapter 3. Five replicates were used. There was a small ($\pm$ 0.2) change in pH of various media before and after the experimental period.

The effects of temperature on the a_w of the media controlled by glycerol and NaCl were negligible as shown in the following Table 4.2

SOLUTE	MOLALITY	TEMPERATURE °C					
		25°	30°	35°	40°	45°	50°
Glycerol	3.00	0.94	0.93	0.93	0.93	0.92	0.92
	5.57	0.90	0.89	0.89	0.88	0.87	0.87
	8.50	0.85	0.84	0.84	0.84	0.83	0.83
	11.50	0.80	0.79	0.78	0.76	0.76	0.76
	14.80	0.76	0.75	0.75	0.75	0.75	0.74
	18.30	0.72	0.71	0.71	0.70	0.70	0.70
NaCl	1.40	0.95	0.95	0.95	0.94	0.94	0.94
	2.80	0.91	0.90	0.90	0.89	0.89	0.89
	4.00	0.85	0.84	0.84	0.84	0.83	0.83
	5.00	0.81	0.80	0.79	0.79	0.79	0.78

TABLE 4.2 : WATER ACTIVITY VALUES OBTAINED WITH GLYCEROL AND NaCl AT VARIOUS TEMPERATURES

4.2.2 Results

The diametric growth rates at various a_w and temperatures are shown in figure 4.1. Optimum diametric growth temperature for A. amstelodami was attained between 30°C to 35°C, A. flavus at 35°C; A. fumigatus at 40°C; A. versicolor between 25°C to 30°C and P. citrinum at 30°C. Growth in A. amstelodami, A. flavus and A. fumigatus was observed up to and at 50°C, whereas in A. versicolor and P. citrinum growth was observed up to and at 40°C and 45°C respectively.

At the optimum temperature of growth, there was an increased tolerance to decreasing a_w, controlled with either glycerol or NaCl. In A. flavus

FIGURE 4.1 : THE EFFECTS OF ELEVATED TEMPERATURES AND DECREASING a_w ON GROWTH OF TEST FUNGI USING DIAMETRIC GROWTH RATES

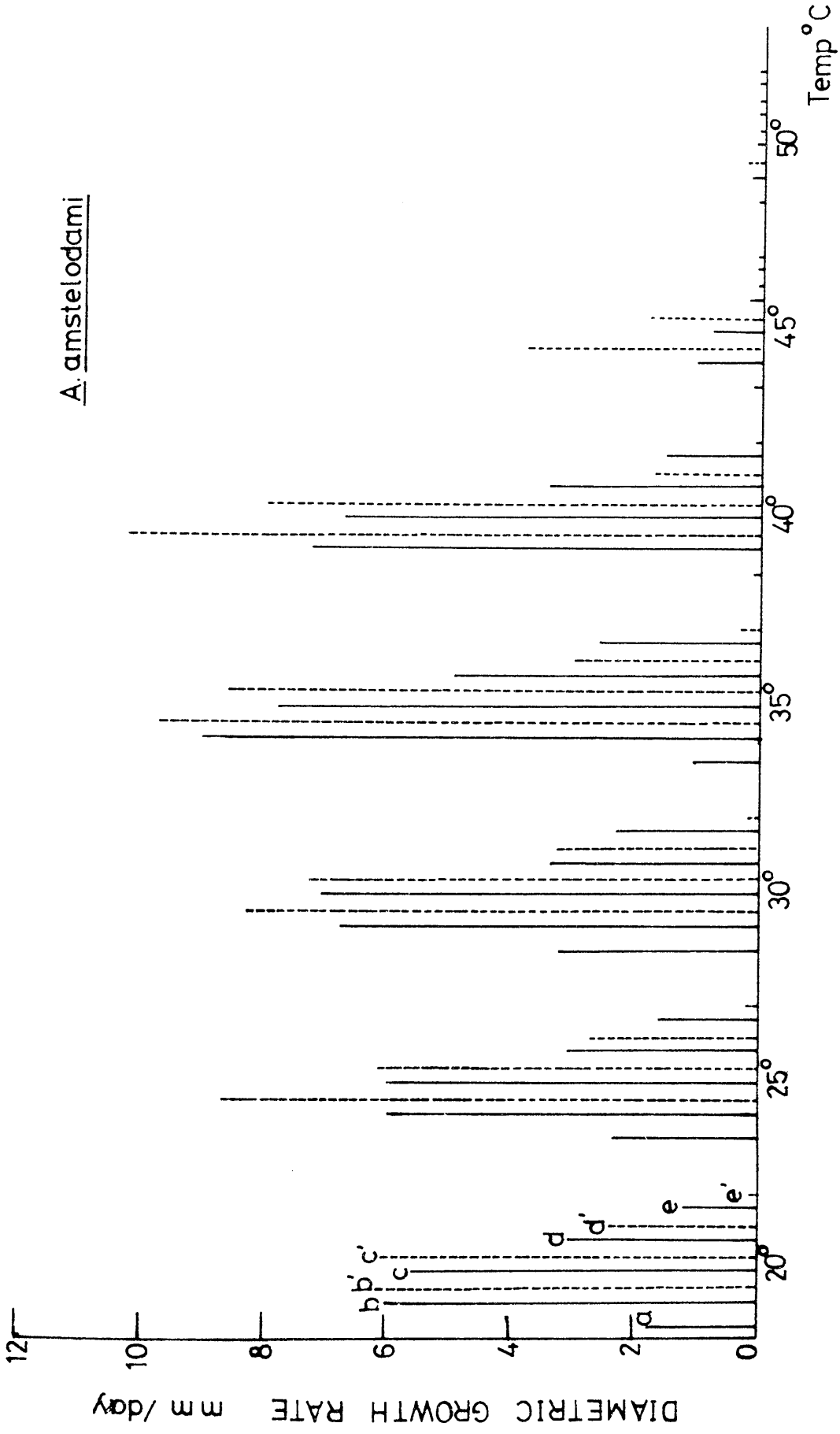
a . MEA - CONTROL

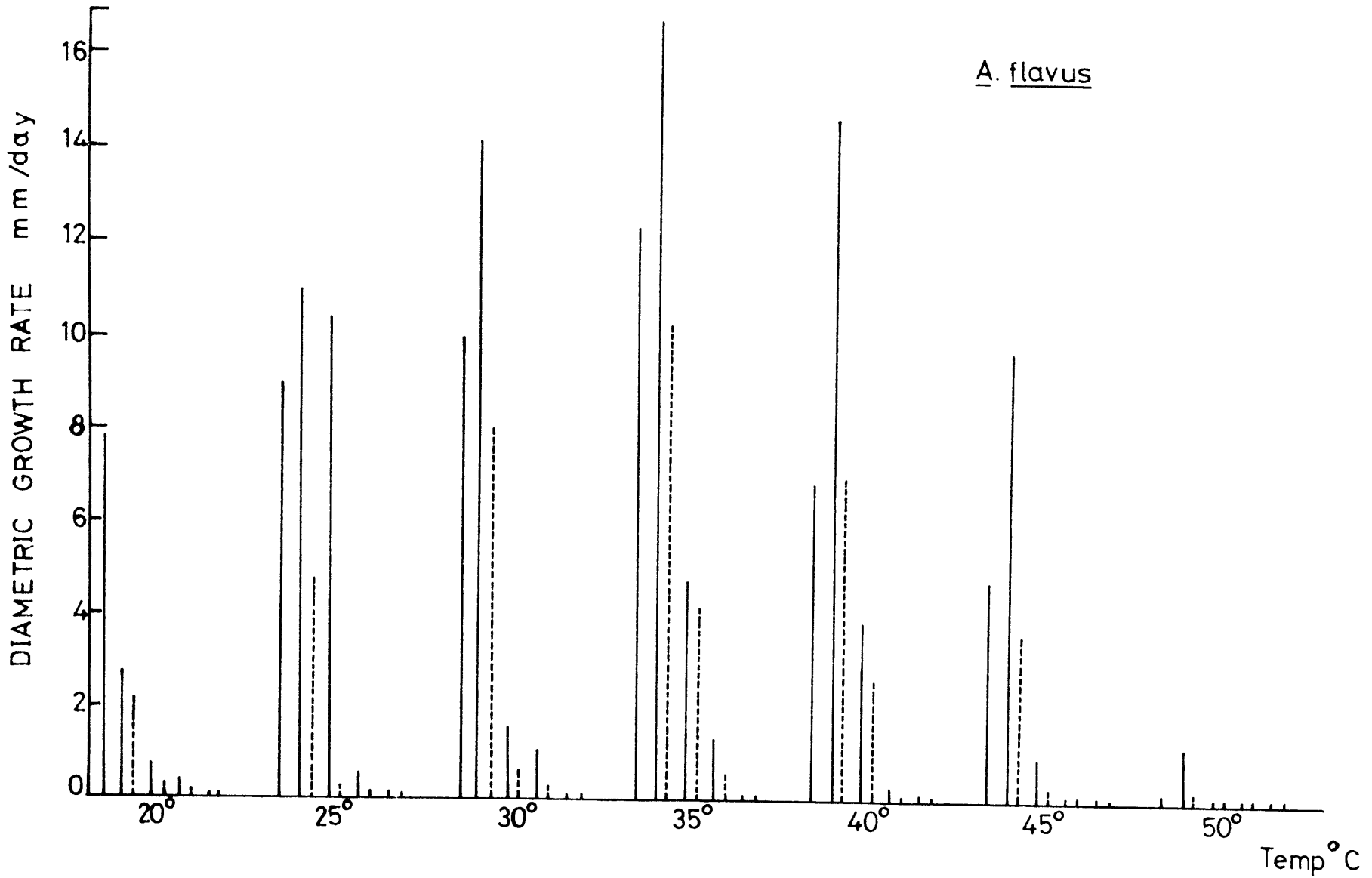
b . MEA + GLYCEROL)
b′ . MEA + NaCl) a_w 0.95

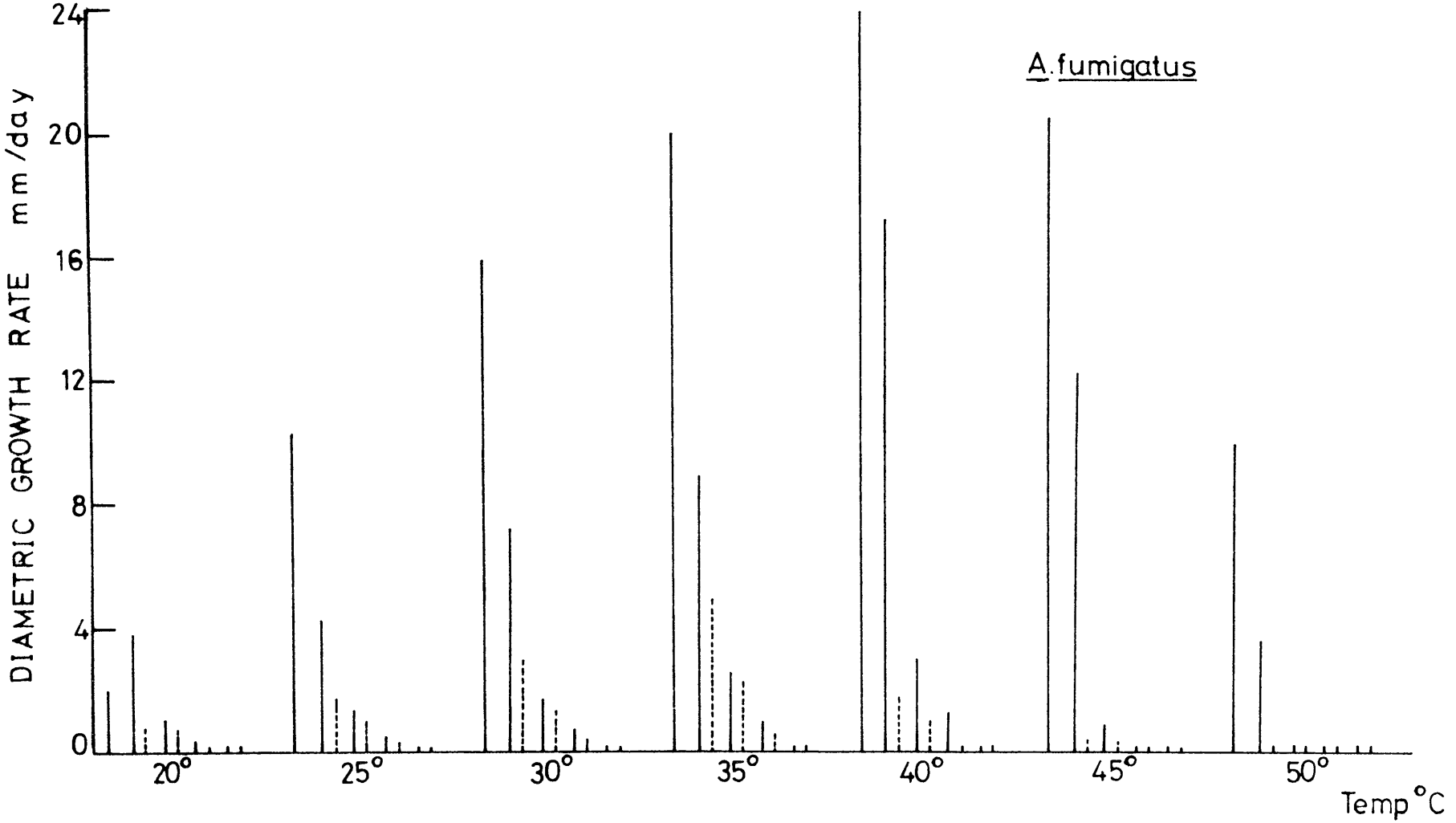
c . MEA + GLYCEROL)
c′ . MEA + NacL) a_w 0.90

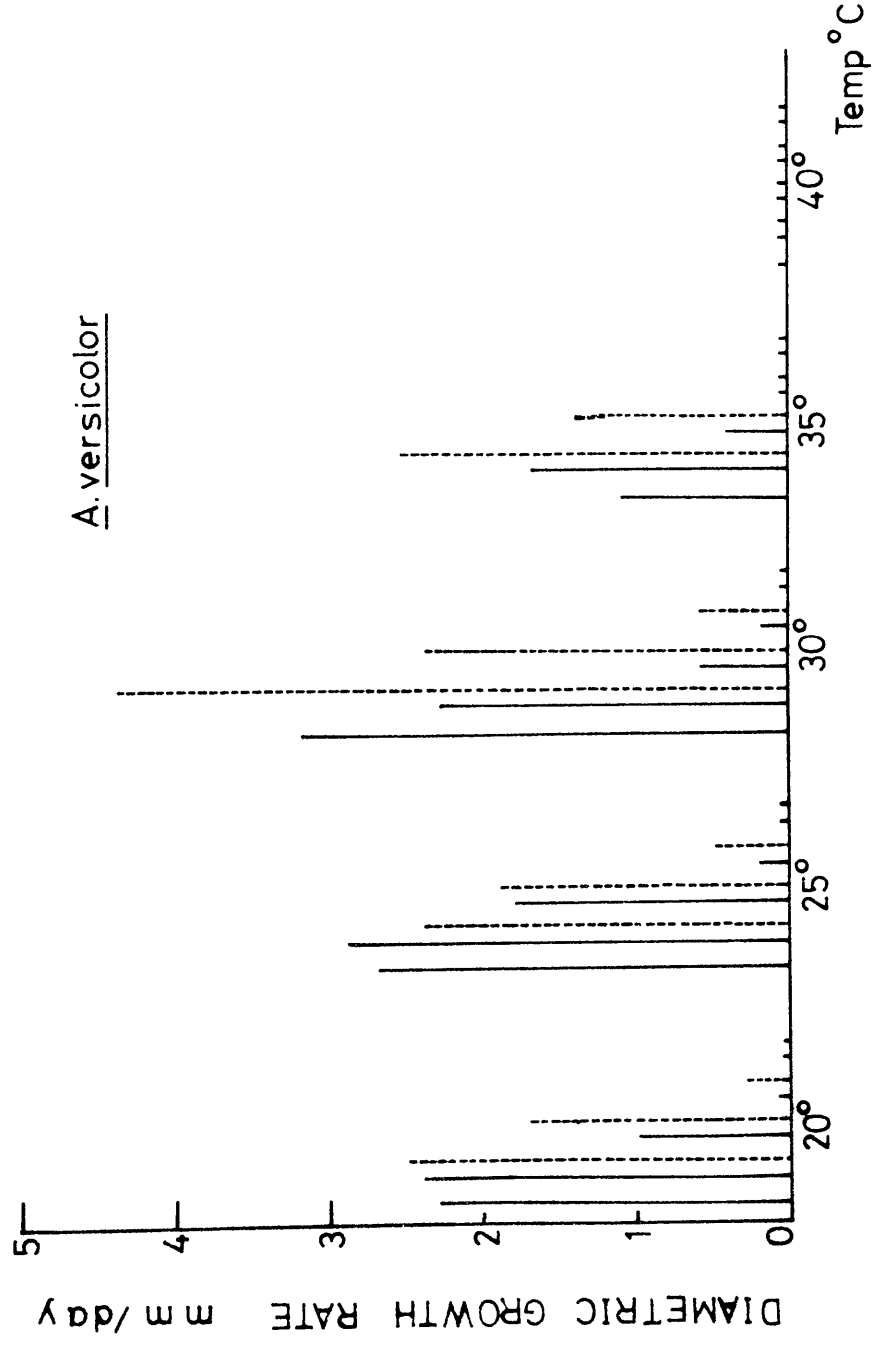
d . MEA + GLYCEROL)
d′ . MEA + NaCl) a_w 0.85

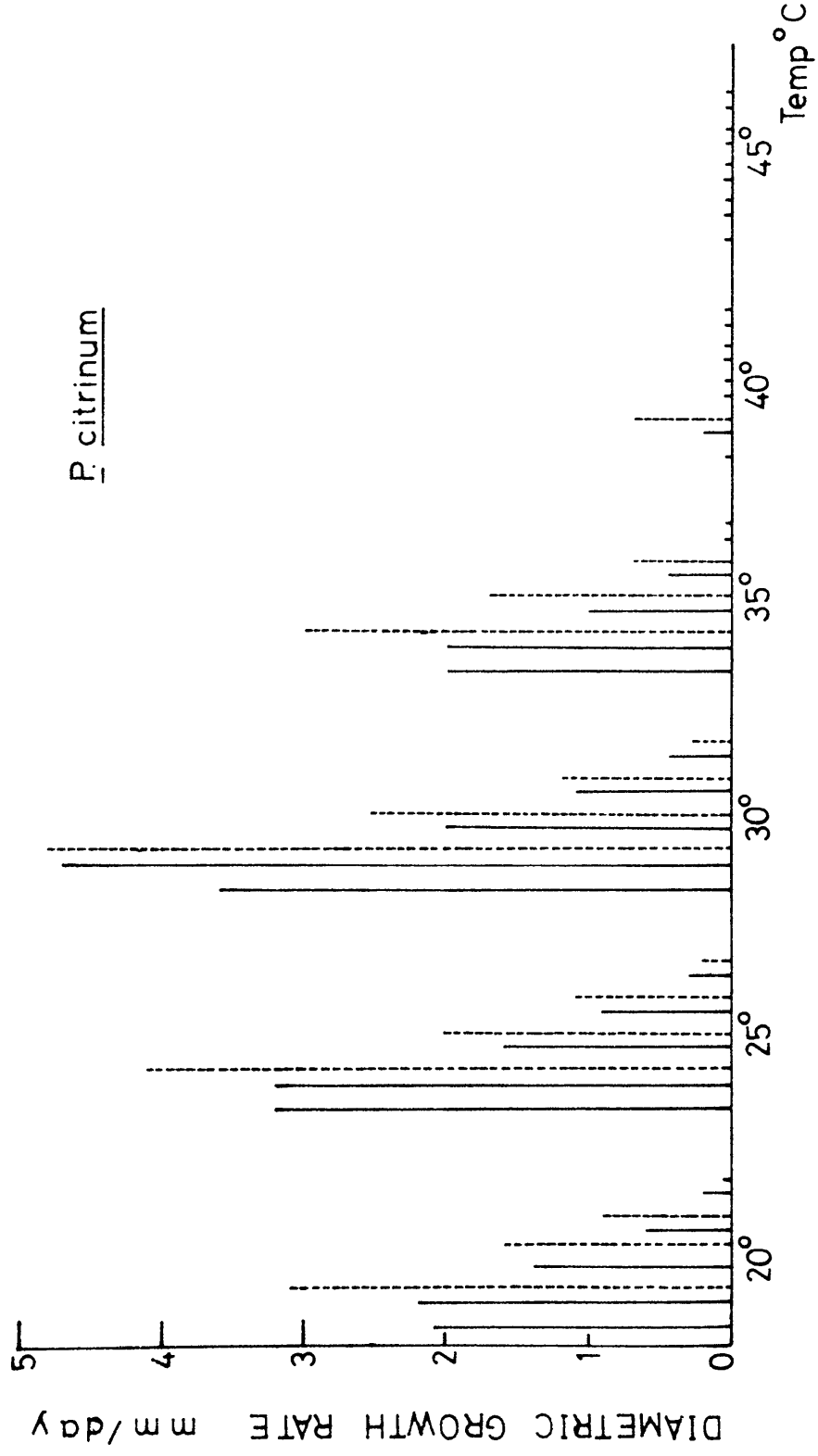
e . MEA + GLYCEROL)
e′ . MEA + NaCl) a_w 0.80


A. amstelodami
DIAMETRIC GROWTH RATE mm /day
Temp °C
0
2
4
6
8
10
12
20°
25°
30°
35°
40°
45°
50°
a
b
b'
c
c'
d
d'
e
e'


A. flavus
DIAMETRIC GROWTH RATE mm /day
0
2
4
6
8
10
12
14
16
20°
25°
30°
35°
40°
45°
50°
Temp°C


A.fumigatus
DIAMETRIC GROWTH RATE mm/day
24
20
16
12
8
4
0
20°
25°
30°
35°
40°
45°
50°
Temp °C


A. versicolor
DIAMETRIC GROWTH RATE mm/day
0
1
2
3
4
5
20°
25°
30°
35°
40°
Temp °C


DIAMETRIC GROWTH RATE mm/day
0
1
2
3
4
5
20°
25°
30°
35°
40°
45°
Temp °C
P. citrinum

and A. fumigatus, the growth at all test temperatures is higher with glycerol as the controlling solute than with NaCl.

The optimum a_w for growth of A. amstelodami, A. versicolor and P. citrinum at all temperatures was at 0.95 with NaCl as the controlling solute; A. flavus was at 0.95 with glycerol, except at 20°C, where optimum growth was observed in control (MEA) plates. Optimum growth in A. fumigatus at all temperatures was seen in control plates.

A. amstelodami, A. versicolor and P. citrinum show higher growth rates at most a_w levels with NaCl in the growth medium. With increasing temperatures of 50°C and 40°C in A. amstelodami and P. citrinum respectively, growth is only recorded at a_w 0.95.

4.3.1 Effects of Solutes, Temperature and a_w on the Viability of Fungal Spores

Materials and Method

The spore inocula were prepared as follows: using a sterile loop, some of the spores or cleistothecia were transferred to a universal bottle containing 10ml of sterile distilled water or 10ml of sterile distilled water with 0.05% of Tween 80 to prevent clumping of the ascospores and spores of A. amstelodami. The inoculum concentration was adjusted using a haemocytometer to give an estimated vaible count

of 10^6 spores per ml, which usually corresponded to a total count of between 1.2 to 9.0 x 10^6 spores per ml. All the spore suspensions were used within 10 to 15 minutes of their preparation. The heating apparatus, shown in Figure 4.2, was adapted from the flask method used for studying the resistance of bacteria to temperatures below the boiling point of water (Stumbo, 1973).

A 500ml three necked flask was used to contain 100ml of the heating menstruum. After sterilization of the flask and 99ml of the menstruum, the assembly was placed in a thermostatically controlled water bath. The whole set-up was allowed to come up to the required temperature. This took about 2 hours. An inoculum of 1.0ml of suspension containing approximately 10^6 spores per ml was introduced to 99ml of the menstruum to give final concentration of approximately 10^4 spores per ml.

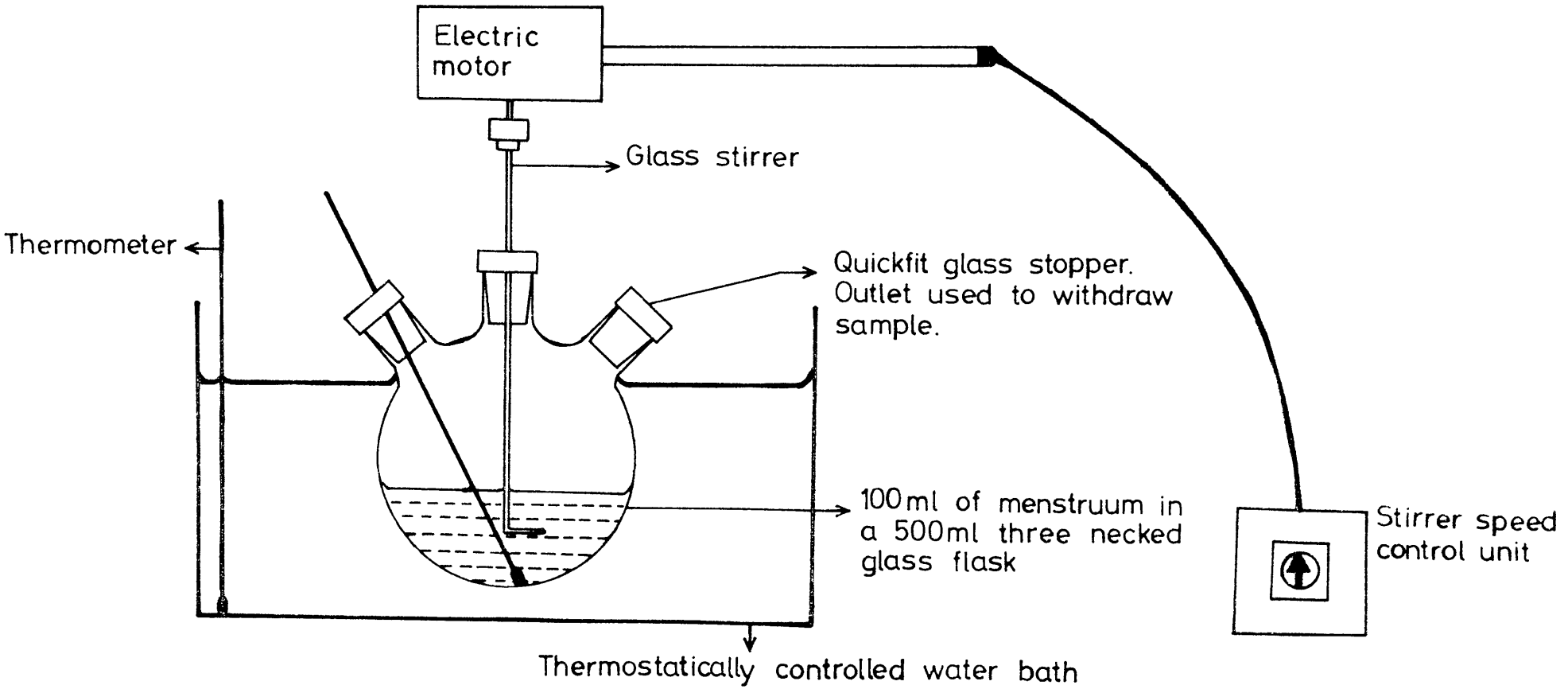


FIGURE 4.2 : HEATING APPARATUS USED FOR SPORE SURVIVAL STUDIES (SCHEMATIC)

The speed of the glass stirer, connected to an electric motor was adjusted so that there was no splashing on the flask wall. The heating menstruum consisted of distilled water, buffered at pH5.4 with citrate-phosphate buffer (a_w approximately 1.0) and modified with either glycerol to give a_w values of 0.95, 0.90, 0.85, 0.80 and with NaCl to give values of 0.95 and 0.90 at temperatures of 45°C, 55°C, and 65°C. Only at 55°C, the a_w of the menstruum was lowered with glycerol to give additional values of 0.75 and 0.70.

At relevant intervals of time, 6ml of the suspension was drawn out using a dispensing pipette. Half of the 6ml suspension, i.e. 1 x 3ml were directly plated out on MEA and MY40G. The petri dishes were gently swirled so that the entire agar surface was covered with the inoculum. The remaining 1 x 3ml suspensions were introduced into 3 universal bottles, each containing 9ml of pH5.4 citrate-phosphate buffer with 0.1% mycological peptone. Serial dilutions of 10^3, 10^2 and 10 of the spore suspensions were prepared and 1ml of these were plated out on the relevant media. Plates of A. amstelodami, A. versicolor and P. citrinum were incubated at 25°C and A. flavus and A. fumigatus plates were incubated at 35°C. Counts of the colonies were taken between 2 - 4 days.

4.3.2 Results

The similogarithmic plots of the number of spores at various time intervals at 45°C, 55°C and 65°C are shown in Figure 4.3.

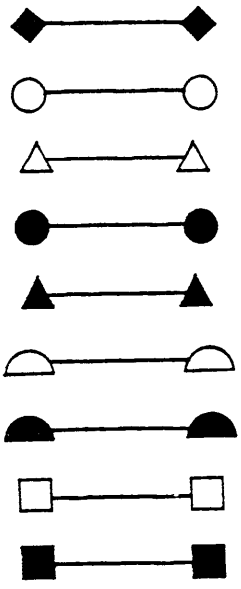
Figure 4.4 shows the D-values against the a_w at 55°C. The D-values were calculated from the previous plots and represent the reduction of the initial spore counts by 90% or by one log cycle.

From the results it is seen that all the spore types were inactivated at all the test subpasteurization temperatures. With the addition of glycerol or NaCl to the heating mentsruum, the heat resistance of the spores is increased. NaCl is found to be more protective during the heat treatments than glycerol at corresponding a_w levels in A. amstelodami, A. versicolor, and P. citrinum, but in A. flavus and A. fumigatus glycerol is more protective than NaCl at corresponding a_w. At 55°C, maximum heat resistance and D-values are observed at a_w 0.85 with glycerol as the controlling solute in all test fungi, excepting A. versicolor and P. citrinum, where maximum heat resistance and D-values are observed at a_w 0.90 with NaCl as the controlling solute. The $D_{55°C}$ values obtained with glycerol of A. amstelodami, A. flavus and A. fumigatus decreased below a_w 0.85, while those of A. versicolor and P. citrinum decreased below a_w 0.90.

With either solute, ascospores of A. amstelodami showed an increased heat resistance at all the a_w levels than the spores, with either solutes. At NO.90, the $D_{55°C}$ values of the ascospores is nearly three times greater than that obtained for the spores.

At 65°C, heat resistance is increased with decreasing a_w. With increasing levels of glycerol or NaCl in the medium, i.e. a_w 0.90, A. flavus and P. citrinum conidia became more heat resistant. Above

FIGURE 4.3 : MOIST HEAT INACTIVATION AT 45°C, 55°C AND 65°C OF ASCOSPORES/ SPORES OF TEST FUNGI AT DECREASING a_w LEVELS



WATER - CONTROL

WATER + GLYCEROL
WATER + NaCl
} a_w 0.95

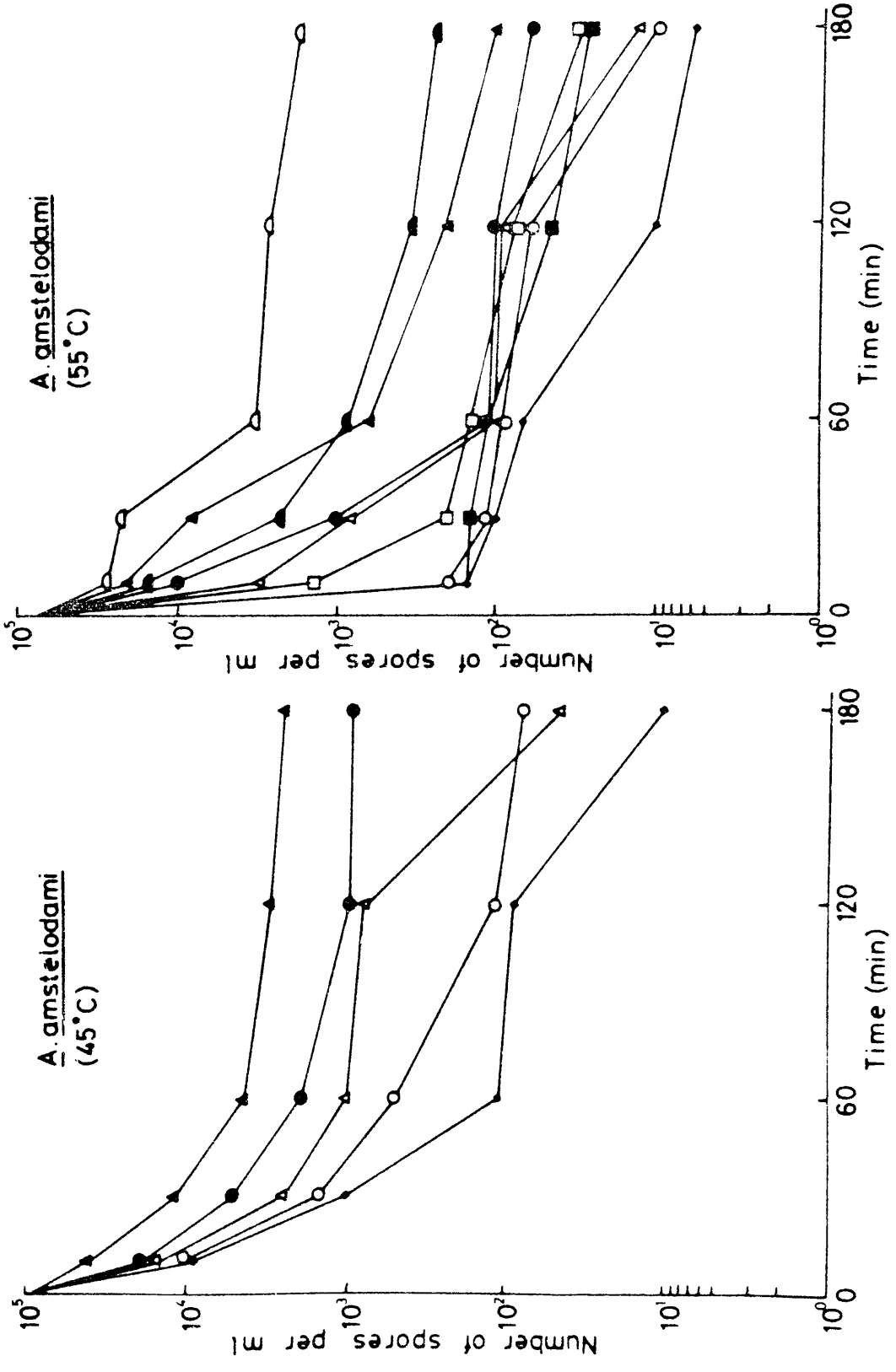
WATER + GLYCEROL
WATER + NaCl
} a_w 0.90

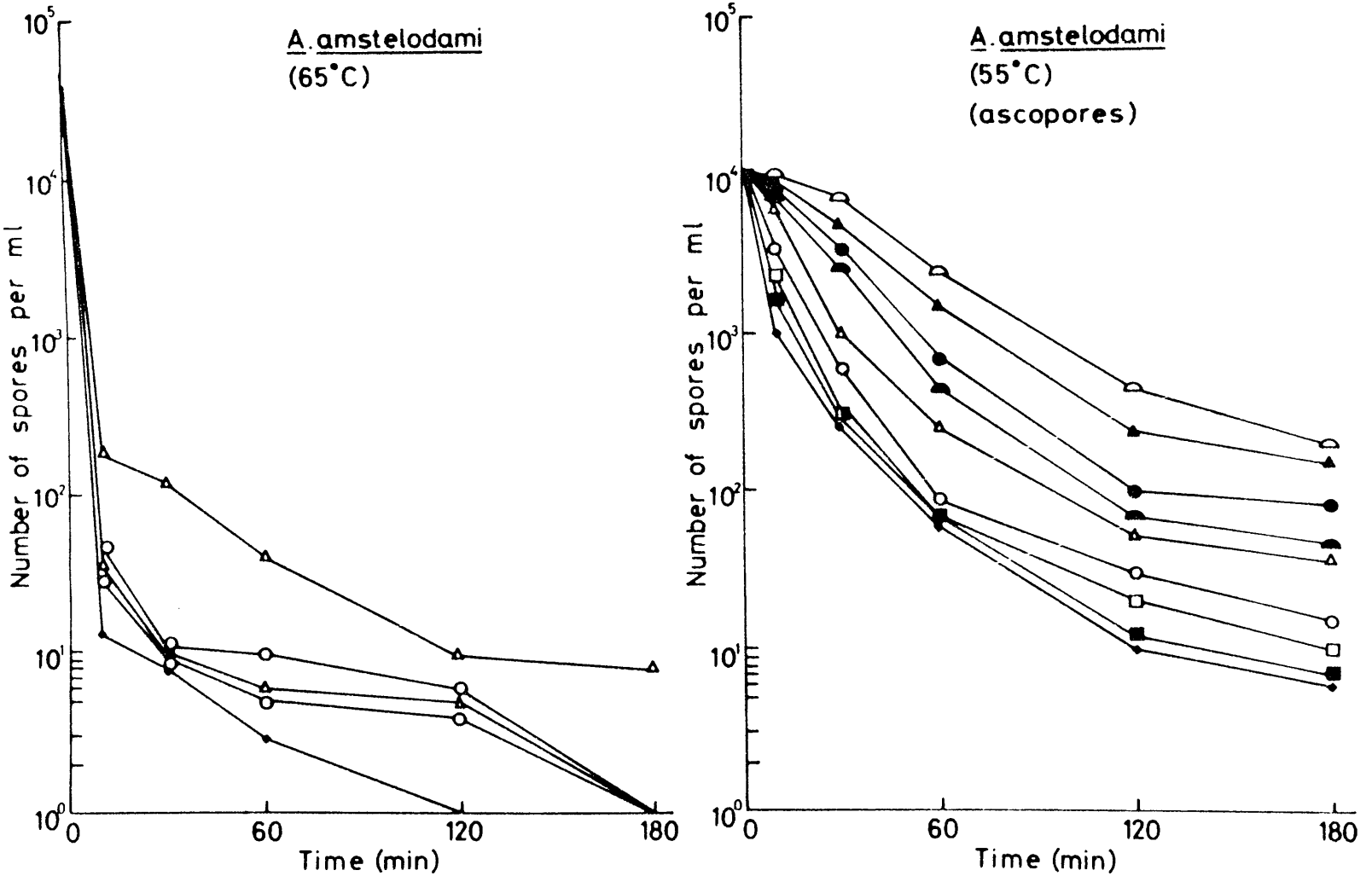
WATER + GLYCEROL - a_w 0.85

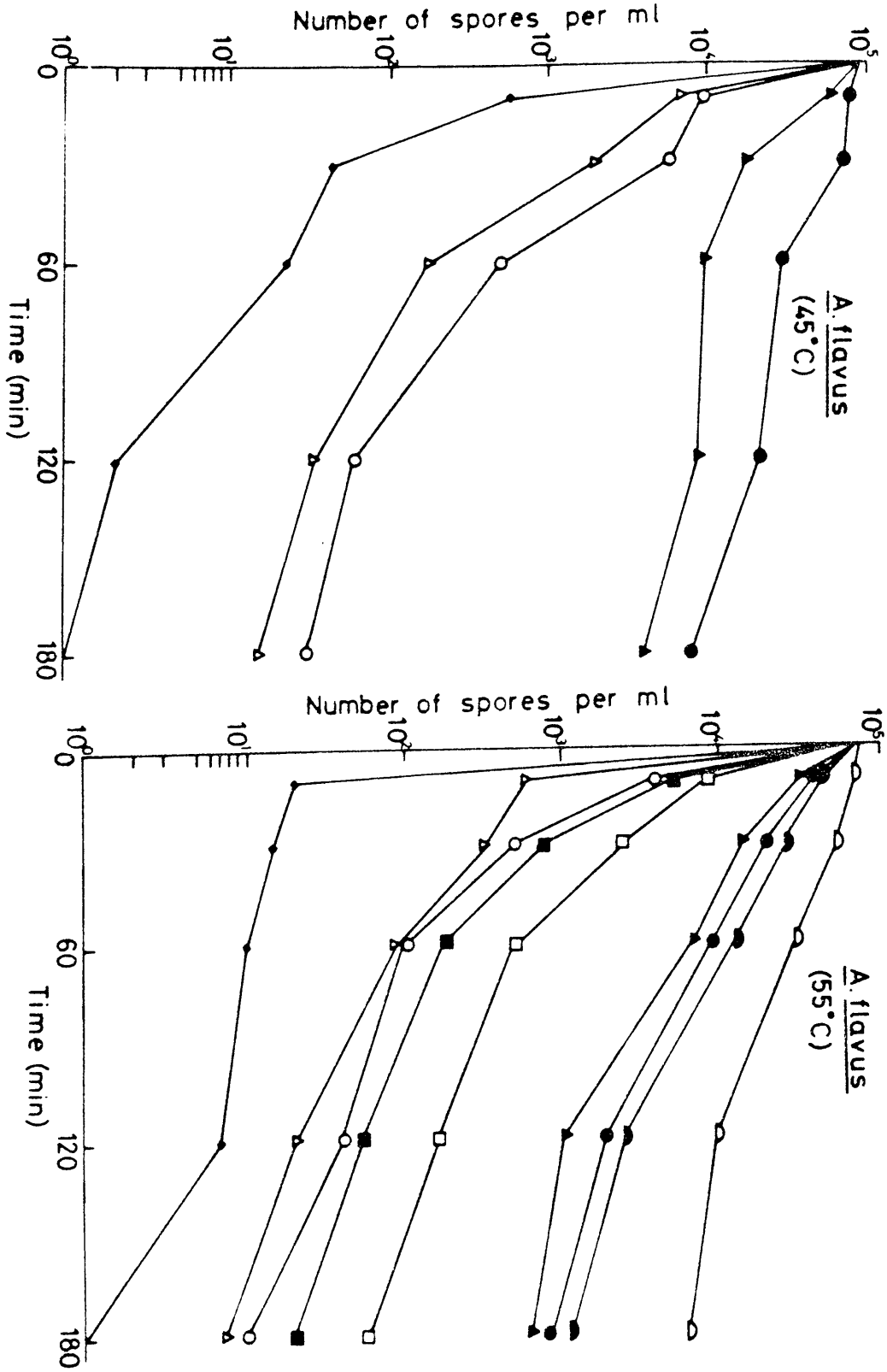
WATER + GLYCEROL - a_w 0.80

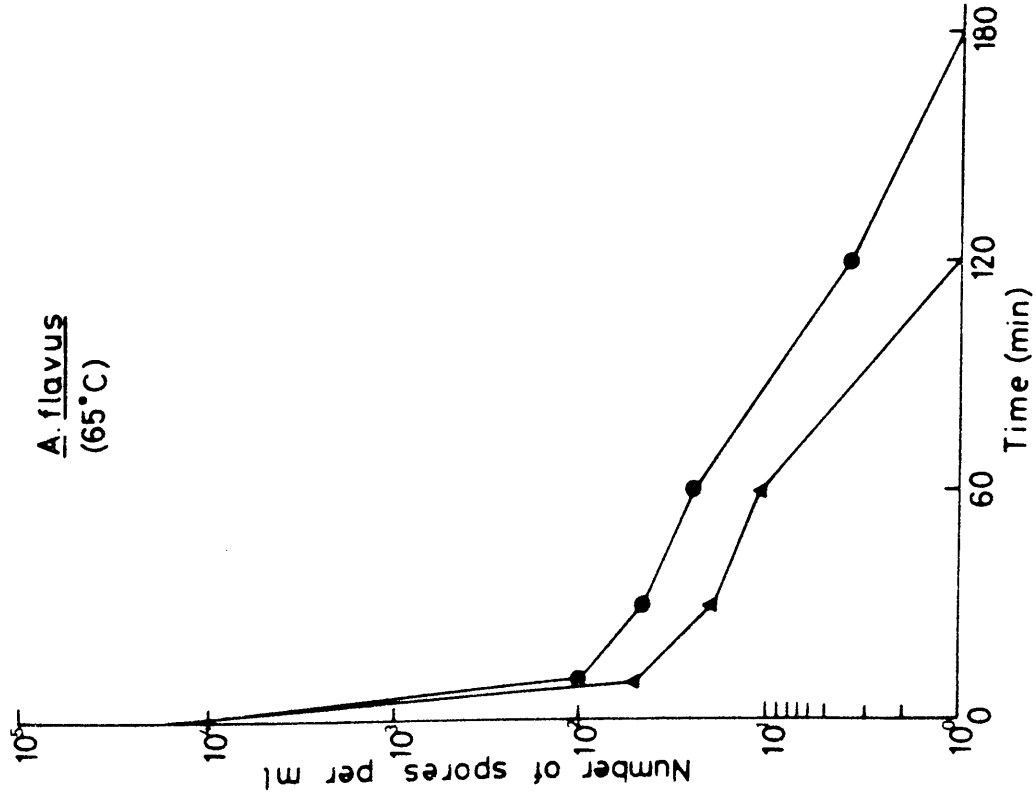
WATER + GLYCEROL - a_w 0.75

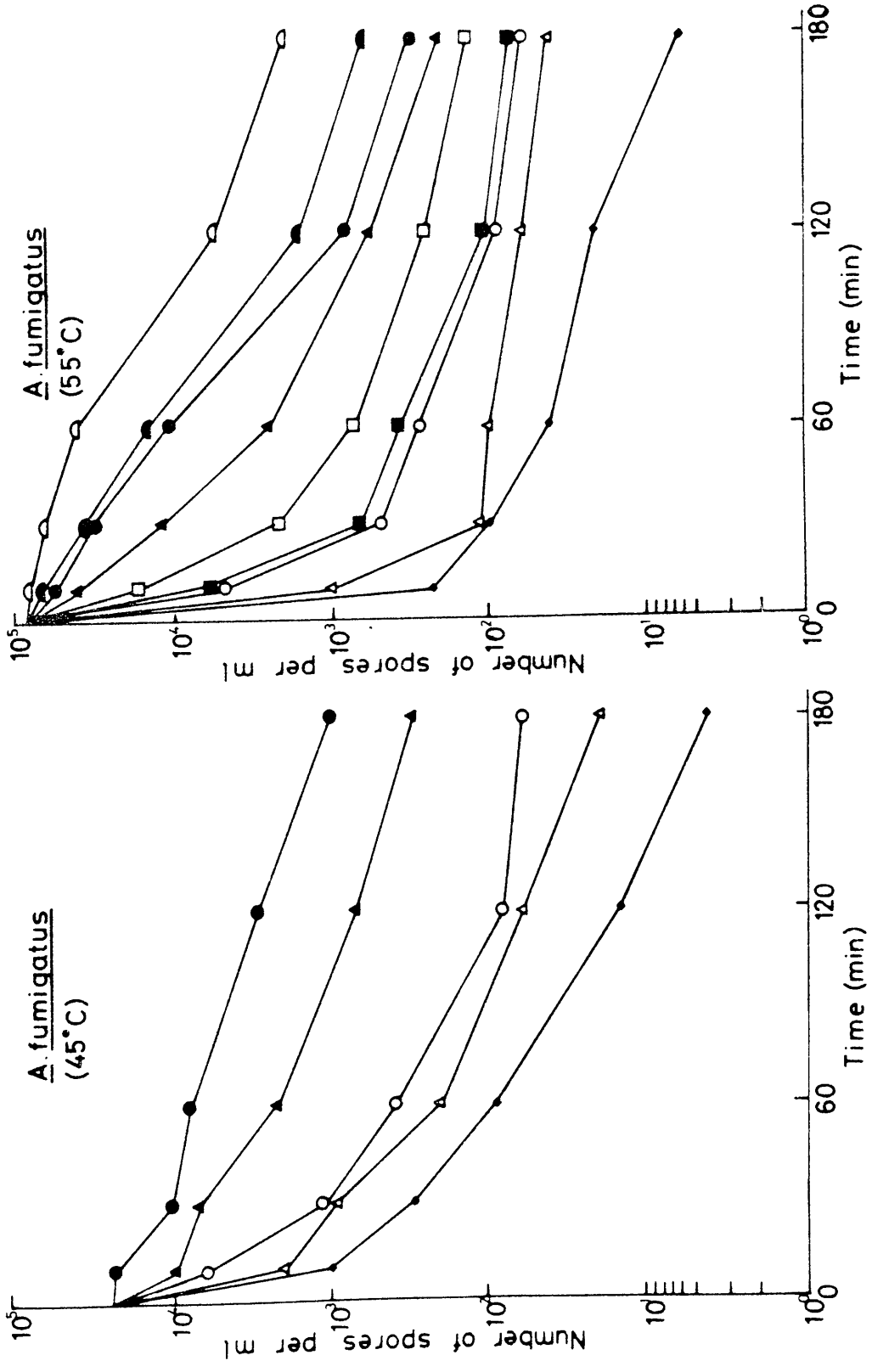
WATER + GLYCEROL - a_w 0.70

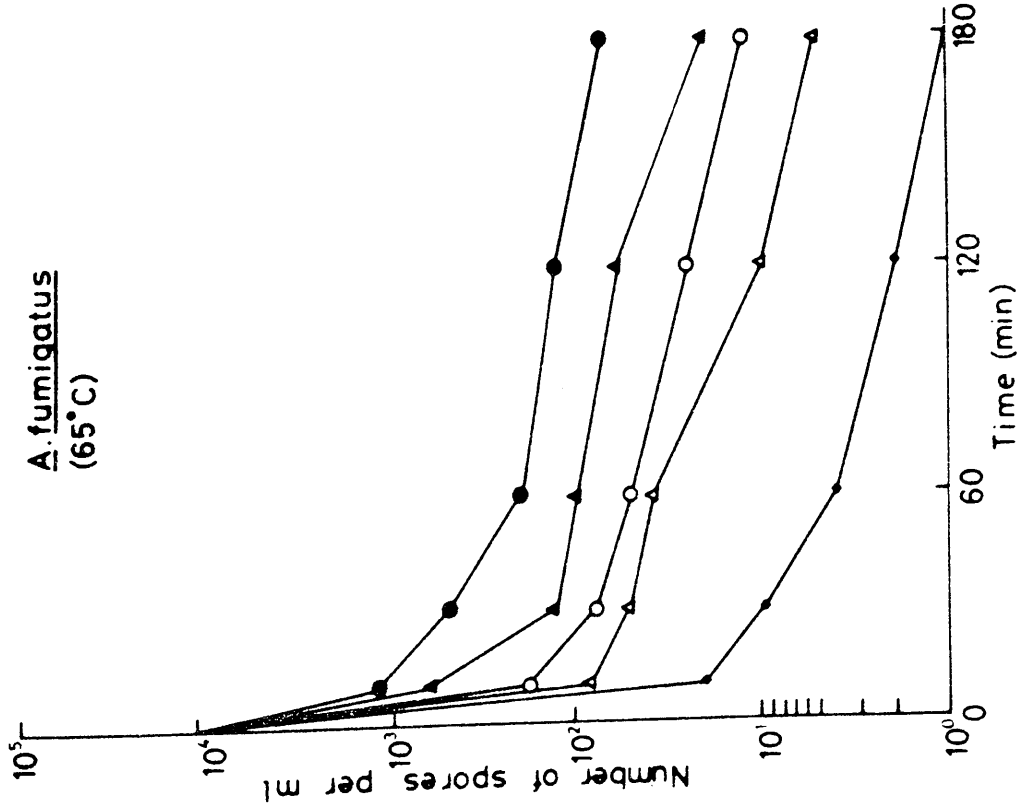

A. amstelodami
(55°C)
A. amstelodami
(45°C)
Number of spores per ml
Time (min)
0
60
120
180
10^0
10^1
10^2
10^3
10^4
10^5


A. amstelodami
(65°C)
Number of spores per ml
Time (min)
A. amstelodami
(55°C)
(ascopores)
Number of spores per ml
Time (min)

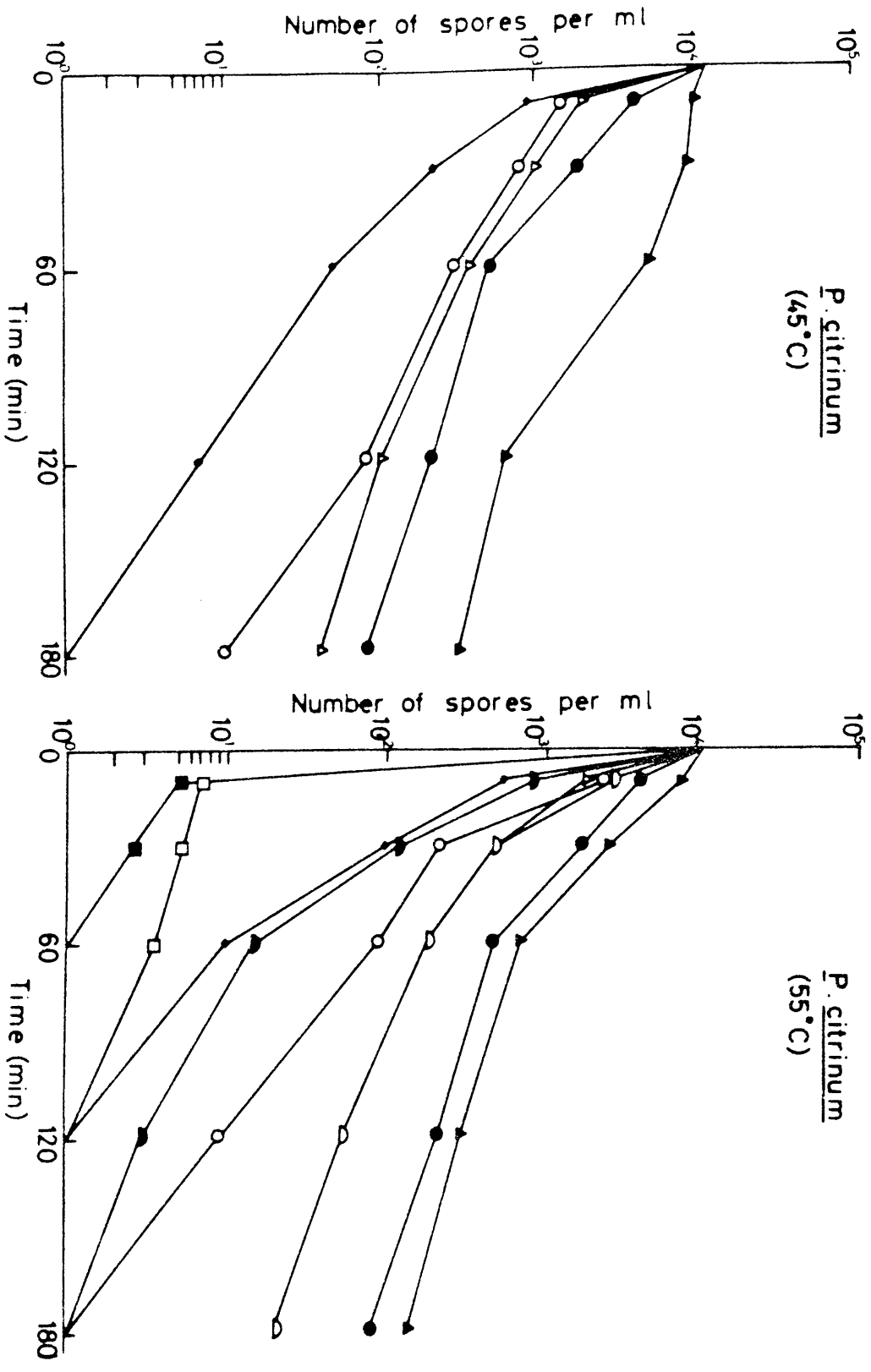

Number of spores per ml
10^0
10^1
10^2
10^3
10^4
10^5
0
60
120
180
Time (min)
A. flavus
(45°C)
Number of spores per ml
10^0
10^1
10^2
10^3
10^4
10^5
0
60
120
180
Time (min)
A. flavus
(55°C)


A. flavus
(65°C)
Number of spores per ml
10^5
10^4
10^3
10^2
10^1
10^0
0
60
120
180
Time (min)


A. fumigatus
(55°C)
A. fumigatus
(45°C)
Number of spores per ml
Time (min)
0
60
120
180
10^0
10^1
10^2
10^3
10^4
10^5


A. fumigatus
(65°C)
Number of spores per ml
Time (min)
10^5
10^4
10^3
10^2
10^1
10^0
0
60
120
180

A. versicolor
(55°C)
A. versicolor
(45°C)
Number of spores per ml
Time (min)
10^0
10^1
10^2
10^3
10^4
10^5
0
60
120
180


Number of spores per ml
10^0
10^1
10^2
10^3
10^4
10^5
0
60
120
180
Time (min)
P. citrinum
(45°C)
Number of spores per ml
10^0
10^1
10^2
10^3
10^4
10^5
0
60
120
180
Time (min)
P. citrinum
(55°C)

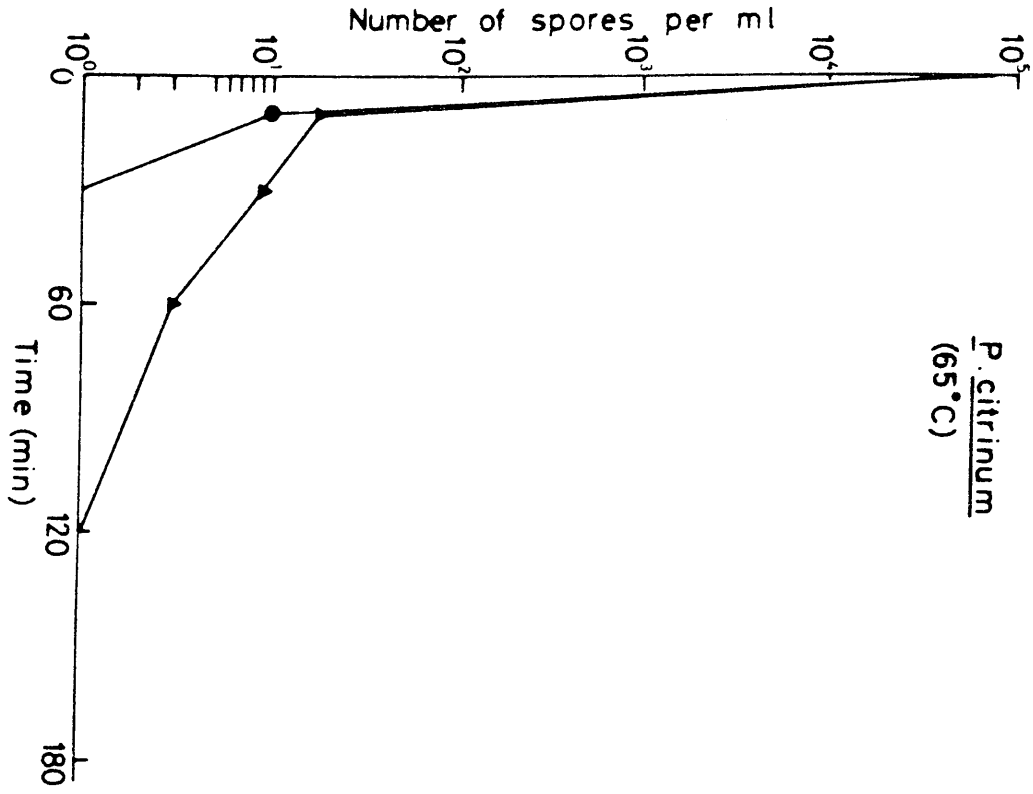
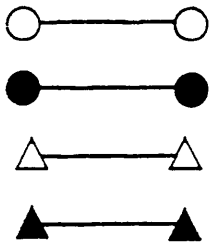

Number of spores per ml
10^0
10^1
10^2
10^3
10^4
10^5
0
60
120
180
Time (min)
P. citrinum
(65°C)

FIGURE 4.4 : $D_{55^{\circ}C}$ VALUES OF TEST FUNGI AT DECREASING a_w



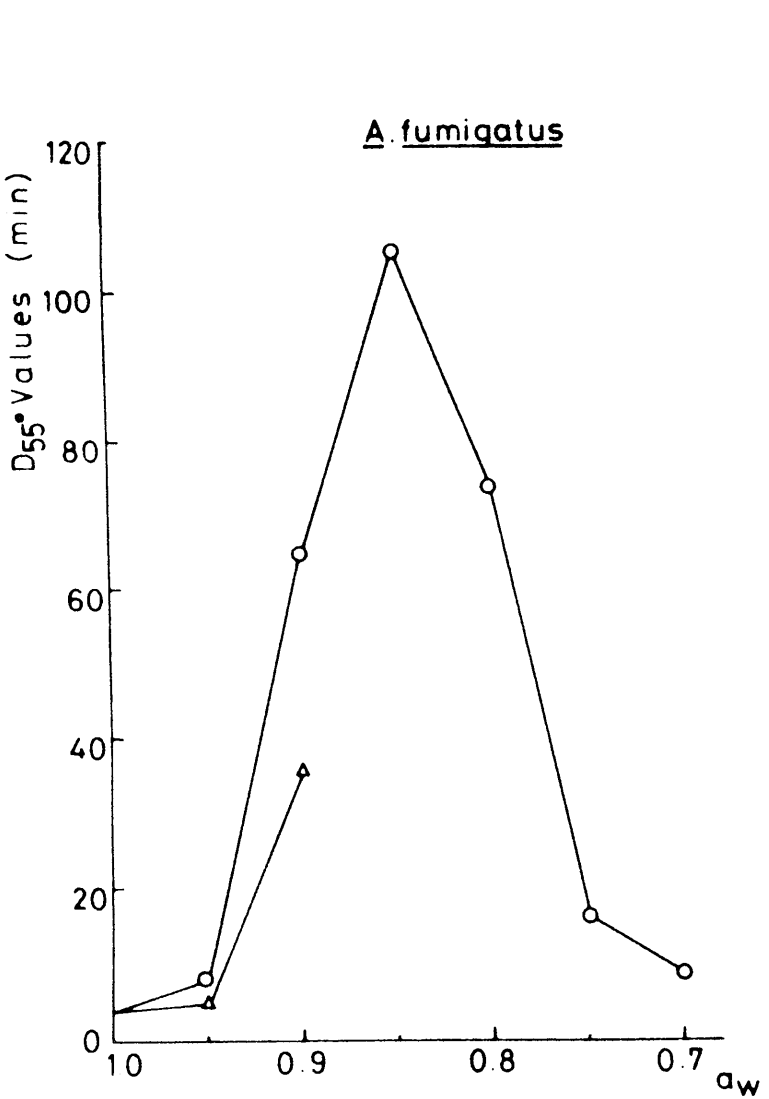
GLYCEROL - SPORES

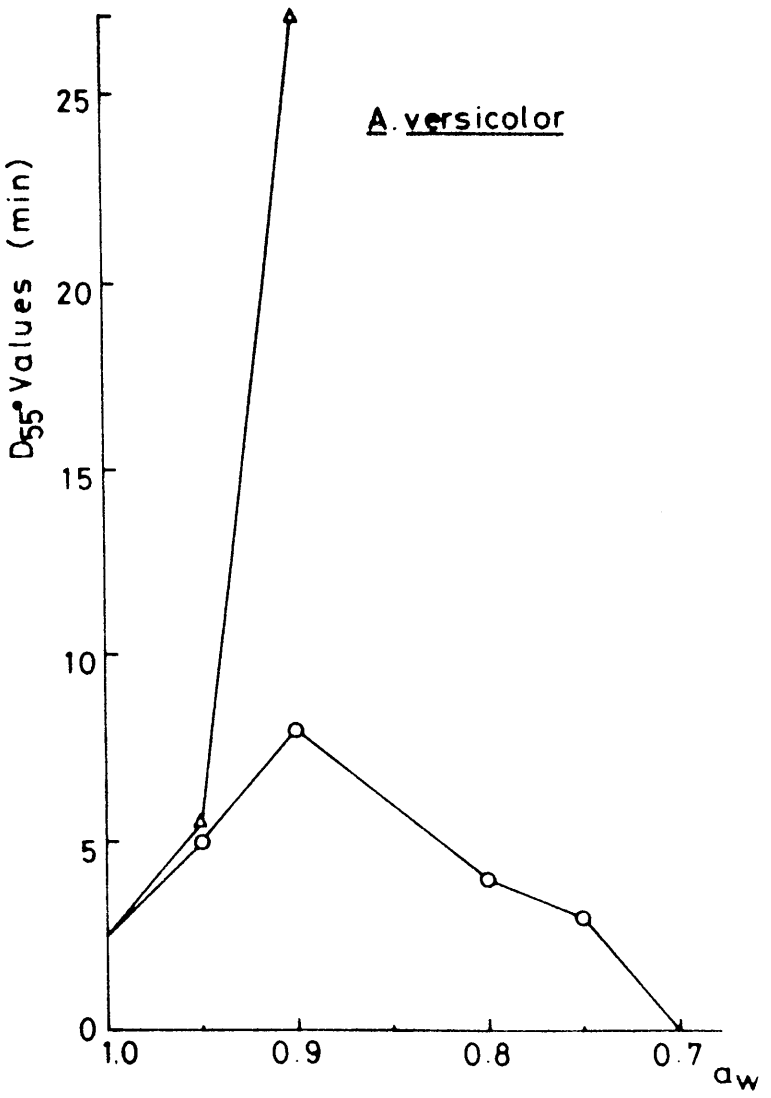
GLYCEROL - ASCOSPORES

NaCl - SPORES

NaCl - ASCOSPORES

A. amstelodami
D55° Values (min)
0
20
40
60
80
1.0
0.9
0.8
0.7
aw
A. flavus
D55° Values (min)
0
40
80
120
160
1.0
0.9
0.8
0.7
aw


A. fumigatus
D_{55}° Values (min)
120
100
80
60
40
20
0
1.0
0.9
0.8
0.7
a_w


A. versicolor
D_{55}° Values (min)
25
20
15
10
5
0
1.0
0.9
0.8
0.7
a_w

P. citrinum
D55°Values (min)
50
40
30
20
10
0
1.0
0.9
0.8
0.7
a_w

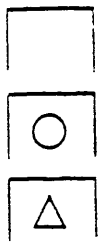
a_w 0.90, the spores lose their viability. Conidia of A. versicolor totally lose their viability at 65°C.

4.4 Discussion

From the results it is seen that hyphal growth is more susceptible to heat than the spores. However, a trend emerges from the results : growth and survival of the vegetative and fruiting bodies at elevated temperatures is enhanced with the addition of solutes in the growth medium. From the results it is seen that the greatest tolerance to low a_w was found at temperatures close to that of the optimum temperature.

A. flavus and A. fumigatus show high heat resistance and A. amstelodami, A. versicolor and P. citrinum show low heat resistance at high a_w levels. At 55°C, and over the a_w range of 0.95 to 0.85, NaCl decreased the heat resistance of the high resistance species, but increased the heat resistance of the low resistance species. Addition of glycerol gave results that were the reverse of those obtained with NaCl. Comparisons of the D-values at 45°C and 65°C of the spores are shown in figure 4.5. Glycerol was found to increase the heat resistance of high a_w resistant A. flavus and A. fumigatus, while NaCl was found to increase the heat resistance of low a_w resistant A. amstelodami, A. versicolor and P. citrinum. Baird - Parker et al. (1967), observed that both glycerol and NaCl increased the heat resistance of the low a_w resistance strains of Salmonellae but decreased the heat resistance of high a_w resistance strains.

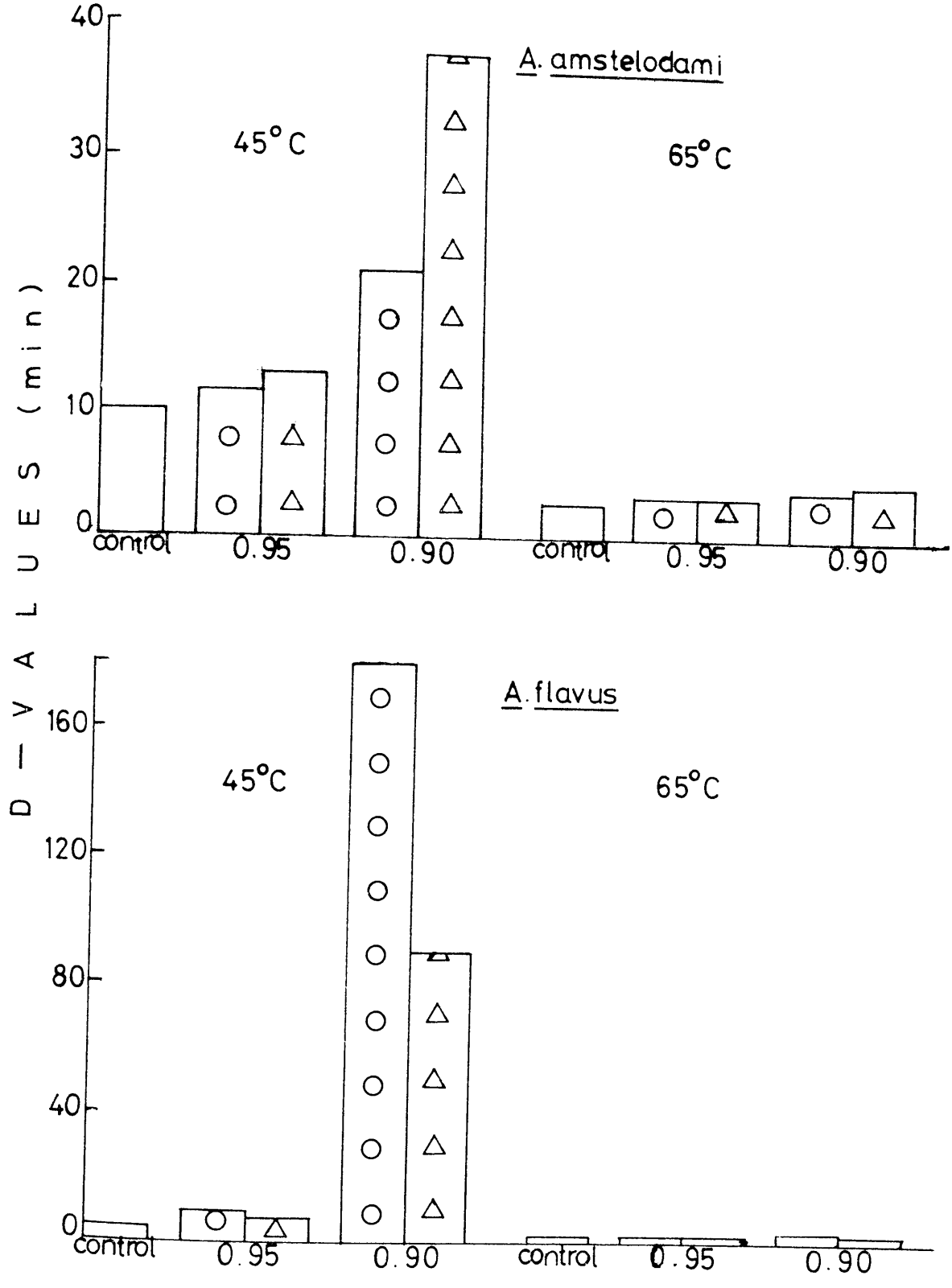
FIGURE 4.5 : COMPARISONS OF $D_{45^{\circ}C}$ AND $D_{55^{\circ}C}$ VALUES OF TEST FUNGI AT DECREASING a_w LEVELS

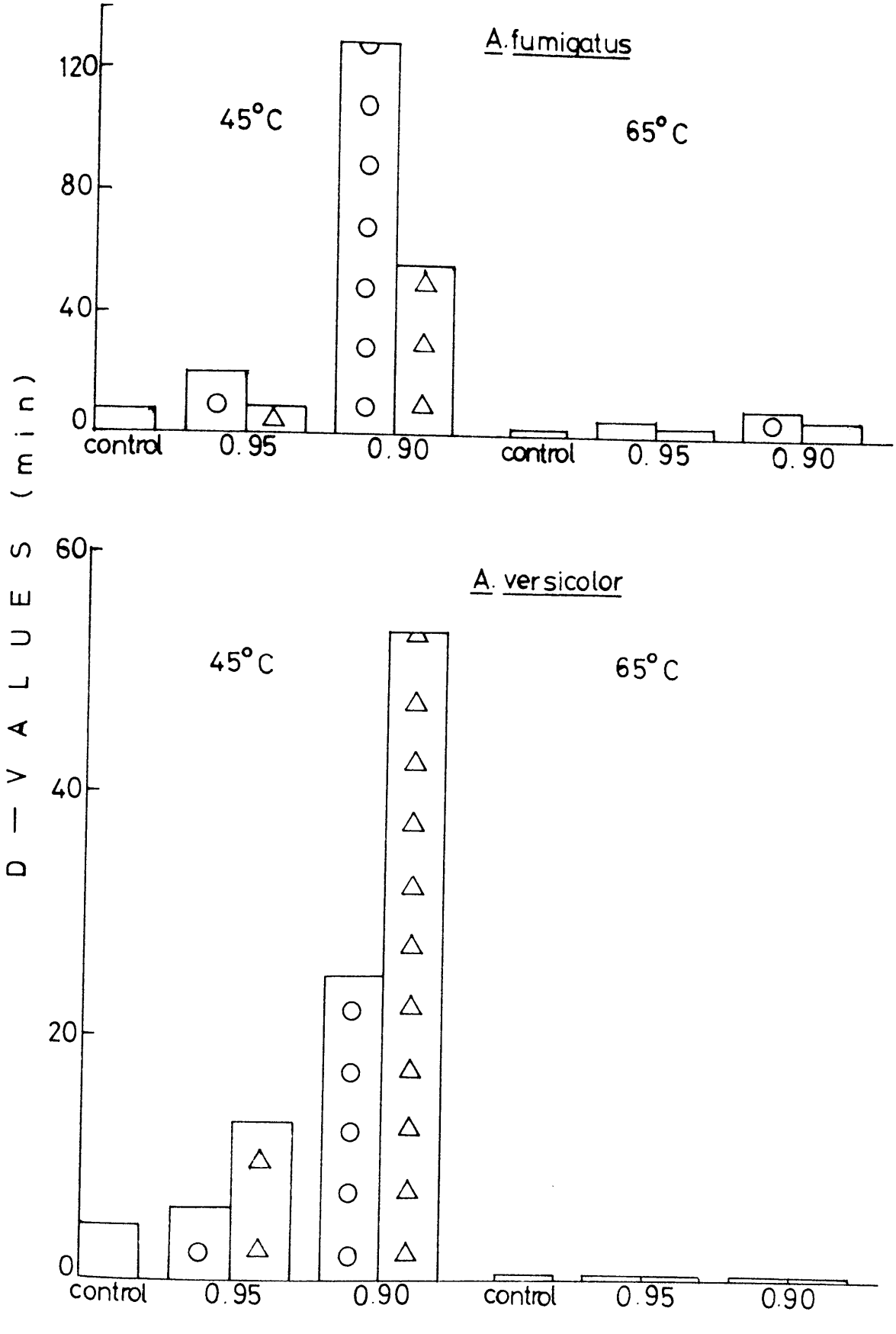


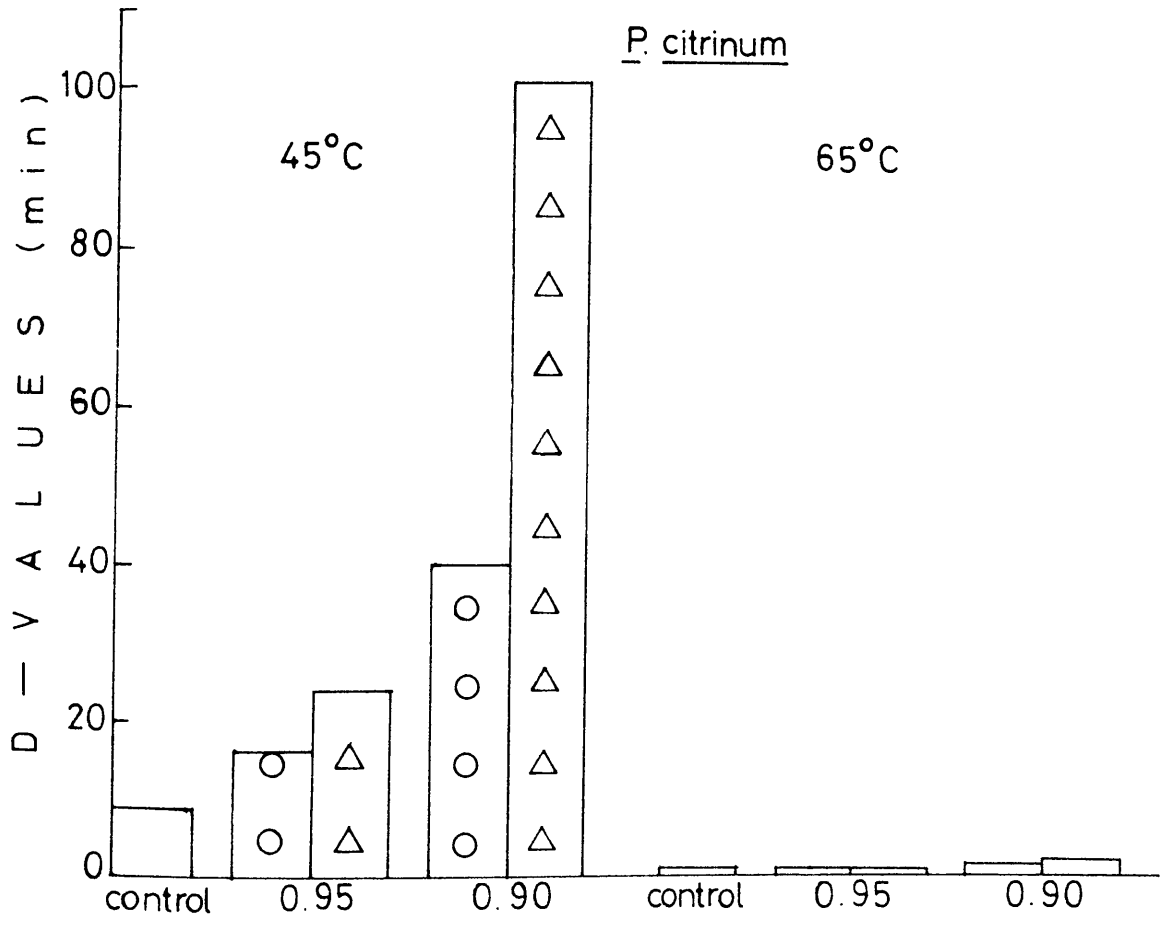
WATER - CONTROL

WATER + GLYCEROL

WATER + NaCl


A. amstelodami
45°C
65°C
D-VALUES (min)
40
30
20
10
0
control
0.95
0.90
control
0.95
0.90
A. flavus
45°C
65°C
160
120
80
40
0
control
0.95
0.90
control
0.95
0.90


A. fumigatus
45°C
65°C
120
80
40
0
control
0.95
0.90
control
0.95
0.90
A. versicolor
45°C
65°C
60
40
20
0
control
0.95
0.90
control
0.95
0.90
D — V A L U E S (m i n)


P. citrinum
45°C
65°C
D — V A L U E S (min)
100
80
60
40
20
0
control
0.95
0.90
control
0.95
0.90

The flask method used was found to be convenient and rapid to determine the heat resistance of the spores of the low resistance fungi. Additional advantages of this method are that the equipment is readily available, easily set-up and the heating and cooling lag periods are negligible.

Glycerol and NaCl failed to protect spores of the test fungi at corresponding a_w to an equivalent degree. This suggests that although a_w may have some effect, other factors such as the chemical nature of the solute play an important role in governing the heat resistance of the spores. Doyle and Marth (1975B) observed that thermal sensitivity was dependant on the age and strain of the fungus, solute controlling the a_w of the heating menstruum and the chemical nature of the buffers used.

Higher viable counts of the A. amstelodami ascospores were obtained than the spores at 55°C. Warcup (1955), found that soil samples heated between 50°C to 75°C gave higher counts of viable ascospores than unheated samples and concluded that ascospore types were activated at sub-lethal temperatures. Pitt and Christian (1970), studying the heat resistance of some xerophilic fungi observed that ascospores were more heat resistant than the spores and A. chevalieri, A. mangini and X. bisporus ascospores remained viable after 10 minutes at 80°C.

The protective mechanism at low a_w to either dry or moist heat is not fully understood and explanations are speculative. As a solute is added to the heating menstruum, the difference of osmotic pressure (OP) between the inside of the spore and menstruum becomes greater. This

discrepancy in the OP may provide a favourable environment for the spore and in the course of which will decrease the leakage of essential spore components during heating. Gerhardt et al. (1964), likened bacterial cell walls to a heterogenous molecular sieve and postulated that the pore sizes of the walls were reduced with cell shrinkage, thus minimizing the loss of the intracellular components on heating.

The difference in OP due to its dehydrating effect may enhance protein stability besides reducing the spore's susceptibility to leakage of essential components. Bateman et al. (1962), comparing the heat resistance studies with those of the biophysical properties of microbial cell water indicated that maximum heat resistance could occur in the region of the water monolayer or BET layer, where the cell water is firmly bound. The water content of the cell at maximum heat resistance may therefore correspond to the optimum water content for heat stability of the protein or other cell component/s whose destruction causes thermal death. This is further supported by the fact that the water sorption isotherms obtained for vegetative bacteria are similar to those obtained for proteins and those for spores were similar to those obtained for wool, where the structure limits water absorption (Bateman et al. 1962; Neihof et al. 1967; Marshall and Murrell 1970).

In the following chapter, the effect of decreasing a_w on the enzyme production is studied.

CHAPTER FIVE

EXO-ENZYME ACTIVITIES OF TEST FUNGI

5.1 Introduction

Until recently (Flannigan and Bana, 1978), little attention has been paid to the production of extracellular enzymes by xerophilic fungi at low a_w.

Enzymes are proteinaceous compounds, catalysing organic reactions in a highly specific and reactive manner. The catalysis is characterized by the binding of the enzyme to the substrate and the breakdown of the enzyme-substrate complex into reaction product/s and enzyme. Water may be involved in both stages, but its main function is to increase the mobility of the substrate and reaction product/s.

At a_w levels below the BET monolayer i.e. water in chemical union to the absorbing surface (Branauer, Emmett and Teller), freely mobile water is not available to carry out reactions, thus, enzyme reactions tend to be suppressed in the lower regions of the sorption isotherm (Figure 5.1).

FIGURE 5.1 : RELATIONSHIP BETWEEN THE HYSTERSIS CURVE AND ENZYME ACTIVITY IN A TYPICAL REACTION SYSTEM (TROLLER AND CHRISTIAN, 1978)

In the present experiments, test Aspergillus species and Penicillium species, showing varying degrees of xerophilism were used along with three non-xerophilic fungi for comparison.

The use of liquid cultures for the detection of enzymes have been used by many workers, including Berger et al. (1936), Mills and Eggins (1974) and Ogundero (1980). The enzymatic assay is carried out either by taking the culture filtrates or grinding the mycelium in a buffer and taking the extract. As stated by Rautella and Cowling (1966), the above methods can produce misleading results of enzymic activities because firstly, the assaying is usually done after a single arbitary time period that may not be optimal for enzyme synthesis of all the organisms. Secondly, positions of the enzyme system may be bound to the fungal mycelium and thus excluded from analysis of the filtrate. Thirdly, denaturization of components, of the enzyme system may occur during processing. Lastly, the hydrolytic action of the enzyme may be prevented from completion by the accumulation of product/s from enzyme action.

Hankin and Anagnostakis (1975) and Flannigan and Bana (1978), grew the fungi in petri dishes on solid agar media and enzyme activity was measured by the diameter of clearance zone around the living colony. The advantages of such a method are that the activities of living cultures and not filtrates are measured at suitable time intervals and involves the use of simple apparatus and assaying techniques. The disadvantages of this system are 1) uneven growth of the fungal colony will result in irregular patterns of clearing, involving an error in

diametric measurements 2) in some instances, the zone of clearance is obscured by the organism 3) semiquantitative assays of enzyme activities from variants of a species can be compared, but not of different species, because the rate of growth of species will vary.

By the use of test tubes to measure the depth of clearance beneath the living cultures, Rautella and Cowling (1966), for determining cellulase activity, overcame the problems encountered in assaying when cultures were grown in a petri dish. The additional advantages of this method include, 1) by using the same set-up, repeated measurements can be taken, thus, minimizing errors due to variations in the set-ups 2) it involves the use of simple equipment, making it ideal for use in large scale screening tests.

This method was modified to determine amylase, cellulase, lipase and protease activities at lowered a_w. The use of test tubes had an additional advantage in measuring enzyme activities at reduced a_w, in that measurements could be taken after a fixed time interval, when fungal growth at reduced a_w was possible.

In the following set of experiments, the effects of pH and a_w on extracellular enzymes of test fungi were studied.

5.2.1 Selection of a Basal Medium

Besides providing a suitable substrate in a medium to detect enzyme activity, sufficient nutrients are necessary for initial growth of a fungus.

Three types of basal media were used with distilled water agar as a control medium. It was important to provide near optimum a_w conditions of growth for the xerophilic fungi. Therefore, each basal medium was also adjusted with glycerol and NaCl to give a_w of 0.95. The basal media used were :

DI	=	Distilled water agar (DWA)	(Appendix 1)
DII	=	DWA + glycerol	" "
DIII	=	DWA + NaCl	" "
MI	=	3% Malt extract agar (MEA)	" "
MII	=	3% MEA + glycerol	" "
MIII	=	3% MEA + NaCl	" "
NI	=	Mineral salts agar (NSA)	" "
NII	=	NSA + glycerol	" "
NIII	=	NSA + NaCl	" "
PI	=	0.5% Mycological peptone + 0.3% Lab-lemco powder agar (PLA)	" "
PII	=	PLA + glycerol	" "
PIII	=	PLA + NaCl	" "

Diametric growth rates were used as the criterion to select a suitable medium. The method used in this experiment was similar to that used in Chapter 3 to determine diametric growth rates. Five replicates were used.

5.2.2 Results

As seen in figure 5.2, the xerophilic fungi A. amstelodami, A. chevalieri, A. niger, A. repens and A. ruber show higher growths at a_w 0.95 than with control media (no additional solute), while A. versicolor and P. citrinum show more growth on control media. On DWA media, growth in A. amstelodami, A. niger, P. citrinum, C. globosum and T. lignorum was extremely sparce. The non-xerophilic fungi (figure 5.2) show maximum growths on control media. With the addition of solutes, the growths were markedly reduced. On MEA and PLA media, there was an overall higher growth, relative to that on DWA and NSA media.

A further experiment using MEA nad PLA was carried out to select a basal medium for enzyme activity.

5.3.1 Comparison of Two Basal Media on Enzyme Production

Amylase, lipase and protease production by the test fungi were compared on a balanced nutrient medium, i.e. MEA and on a limited nutrient medium, i.e. PLA.

Enzyme activity measurements were done using a modified test tube method of Rautella and Cowling (1966). The depth of clearance that developed below the living culture provided a visual measurement of the activity.

FIGURE 5.2 : GROWTH MEASUREMENTS OF TEST FUNGI IN DIFFERENT MEDIA USING DIAMETRIC GROWTH

D. DWA

M. MEA

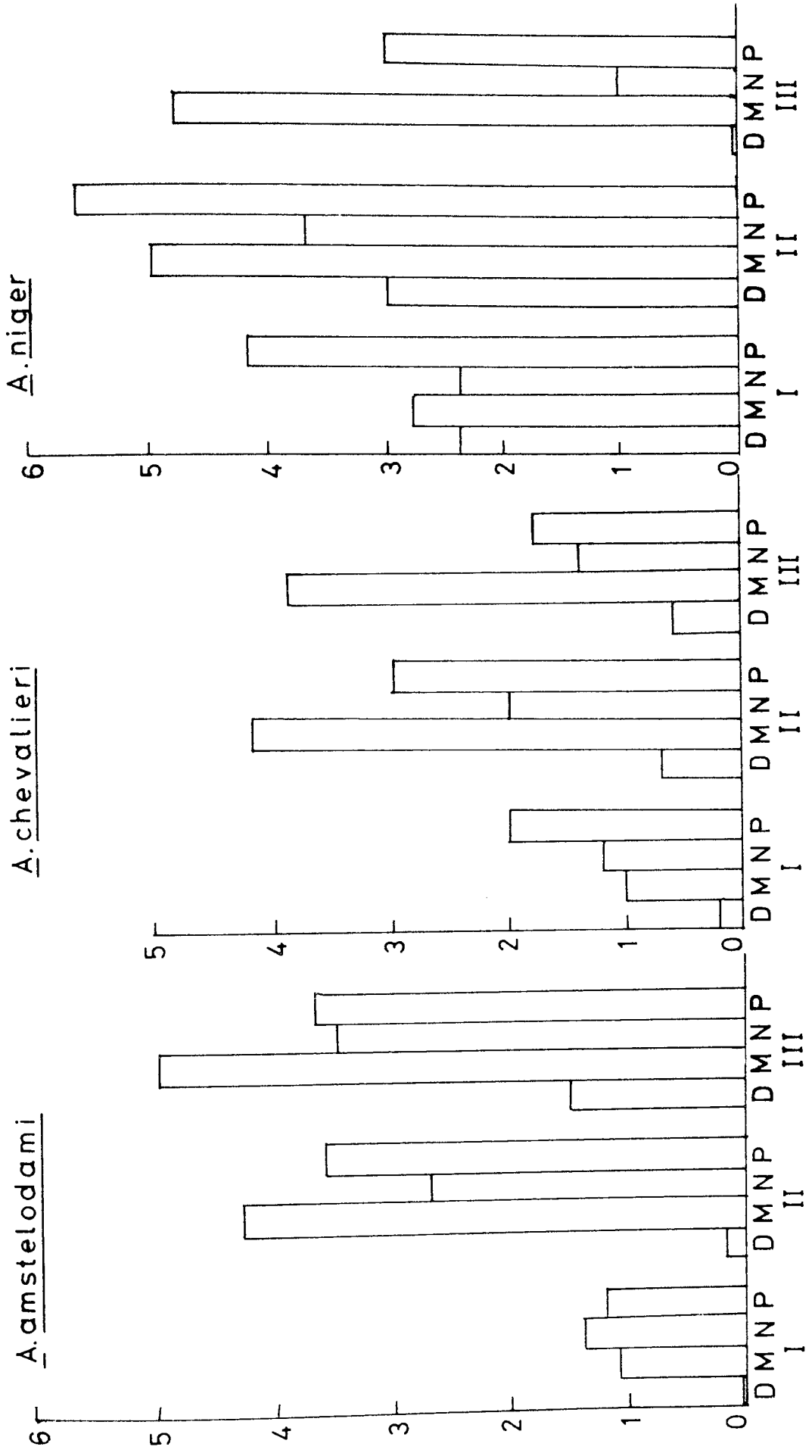
N. NSA

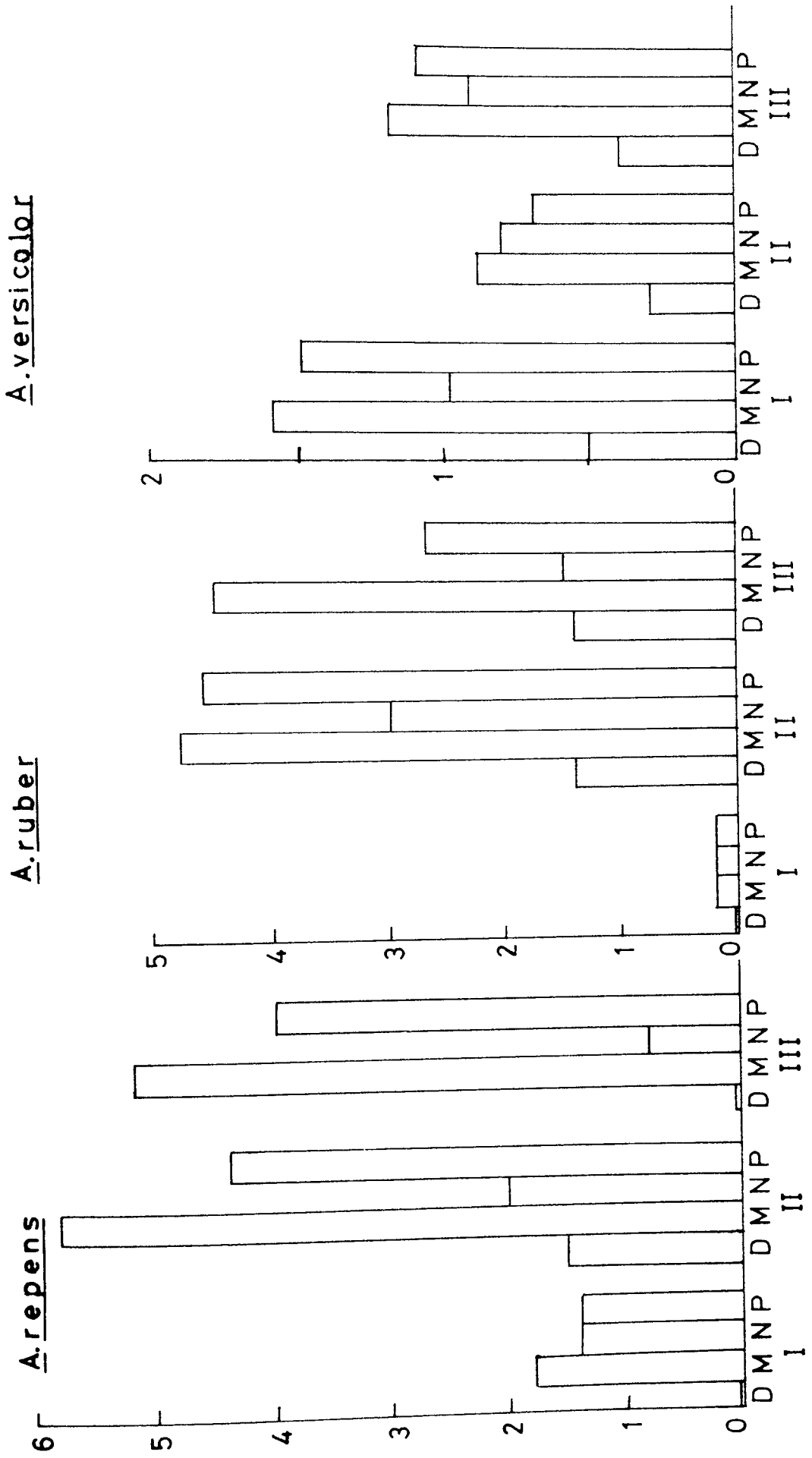
P. PLA

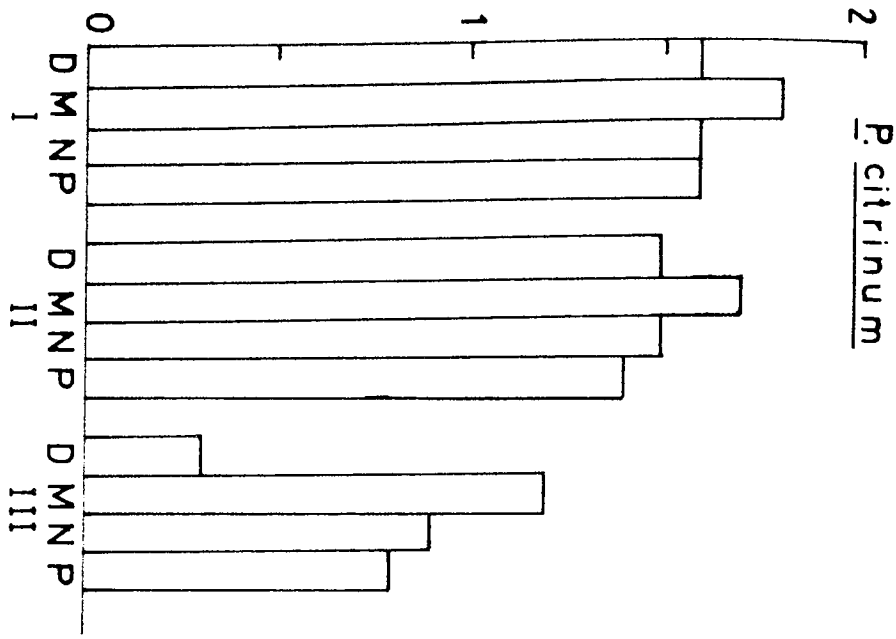
I. a_w APPROXIMATELY 1.0

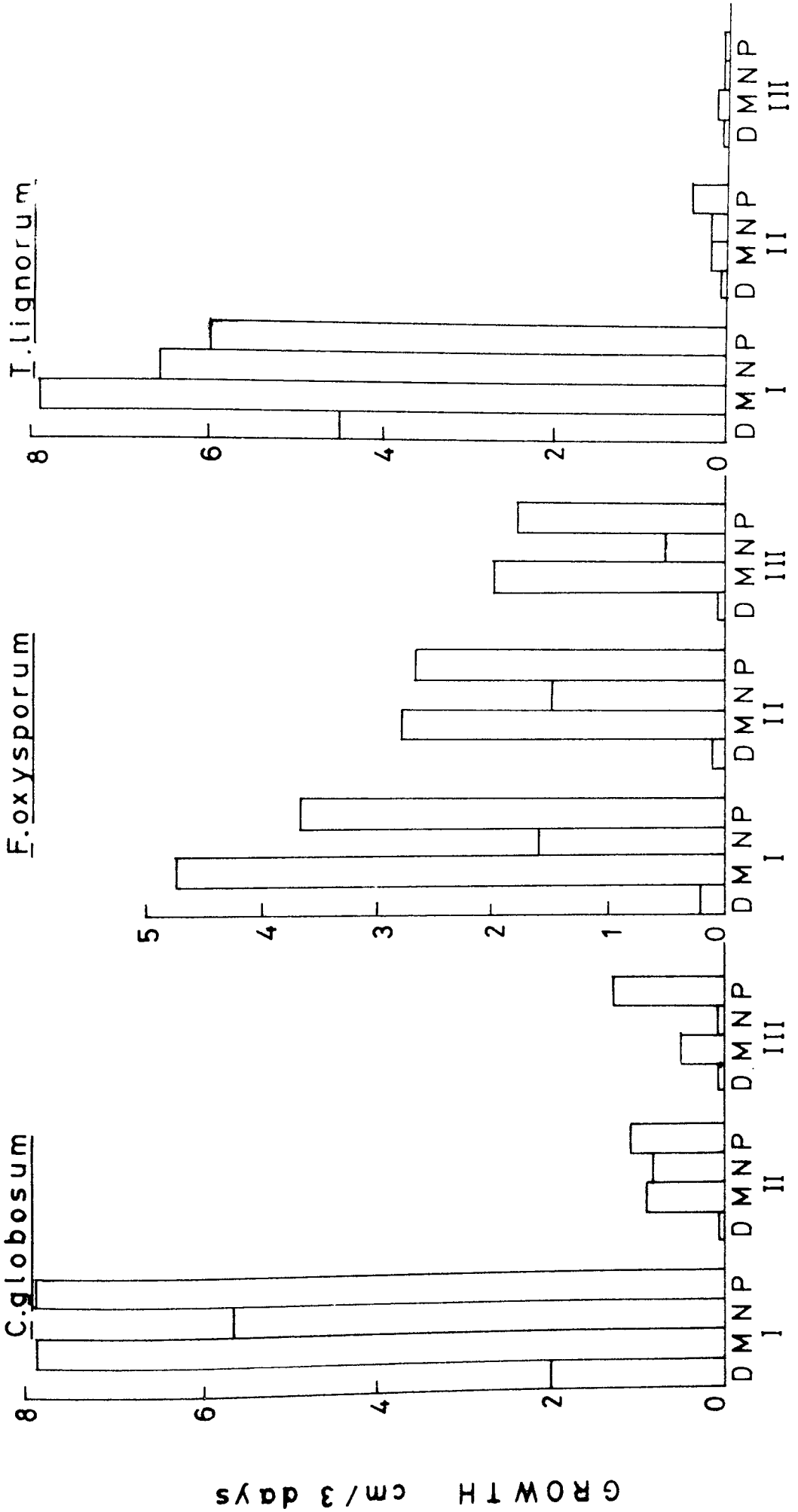
II. a_w 0.95 - GLYCEROL

III. a_w 0.95 - NaCl


A.amstelodami
A.chevalieri
A.niger
GROWTH cm/7 days
0
1
2
3
4
5
6
DMNP
I
II
III


A.repens
A.ruber
A.versicolor
GROWTH cm/7 days
0
1
2
3
4
5
6
DMNP
I
II
III


GROWTH cm/7 days
0
1
2
D
M
N
P
I
II
III
P. citrinum


C.globosum
F.oxysporum
T.lignorum
GROWTH cm/3 days
0
2
4
6
8
0
1
2
3
4
5
DMNP
I
II
III

The ability to degrade soluble starch was the criterion used to determine the ability to produce amylolytic enzymes. The basal medium used contained 0.2% starch (Appendix 5.1). Tributyrin agar (Appendix 5.1) was used to determine lipase production and 0.4% gelatine(Appendix 5.1) was used as the substrate to detect proteolytic enzymes.

Using a sterile automatic pipette syringe, 5ml of sterile cooled liquid test medium was added to heat sterilized (170°C for 3-5 hours), 1.2cm diameter x 10cm length test tubes provided with suitable oxoid caps. The tubes were inoculated in a laminar flow cabinet with very thin plugs of mycelia (0.5cm diameter), from 10 to 14 days old cultures of A. amstelodami, A. chevalieri, A. repens, A. ruber, A. versicolor and week old cultures of A. niger, C. globosum, F. oxysporum, P. citrinum and T. lignorum. The plugs were placed with the mycelium facing the medium. Five replicates were used for each sample. Controls were used without any inoculation. The tubes were incubated at 25°C ($\pm$ 1.0°C) for 2 weeks.

The amount of degraded starch was determined by submerging the agar columns in Lugol's iodine solution (0.5% I_2/KI) for 10 minutes and the depth of clearance in an otherwise blue/black column measured to the nearest 0.5mm. Lipase activities were measured by the clearance in the opaque tributyrin media columns, directly from the test tubes. The protease activities were determined by submerging the agar columns into saturated ammonium sulphate solution ($(NH_4)_2SO_4$) for 2-5 hours.

The depth of clearance in an otherwise opaque column was measured. The agar columns were removed from the tubes by passing water under the agar column using a 15cm long hypodermic needle and a syringe. Safety precautions taken during these procedures and throughout the following experiments were as follows :

1) Plastic gloves were worn when handling agar columns.
2) The contaminated water was autoclaved before it was disposed of.
3) The agar columns were sterilized as soon as the enzyme activities were measured.

5.3.2 Results

Figure 5.3 shows the amylase, lipase and protease activities of the test fungi after 2 weeks on MEA and PLA media.

Generally, enzyme activities on MEA media were significantly lower than on PLA media. The lipase activity differences between the two media was least pronounced for all the test fungi. A. amstelodami, A. chevalieri, A. repens and A. ruber produced no amylases on MEA media. No proteolytic activities were seen in A. amstelodami, A. chevalieri and A. repens, when grown on either MEA or PLA media, while T. lignorum showed no protease activity when grown on MEA medium.

FIGURE 5.3 : ENZYME ACTIVITIES OF TEST FUNGI ON MEA AND PLA MEDIA

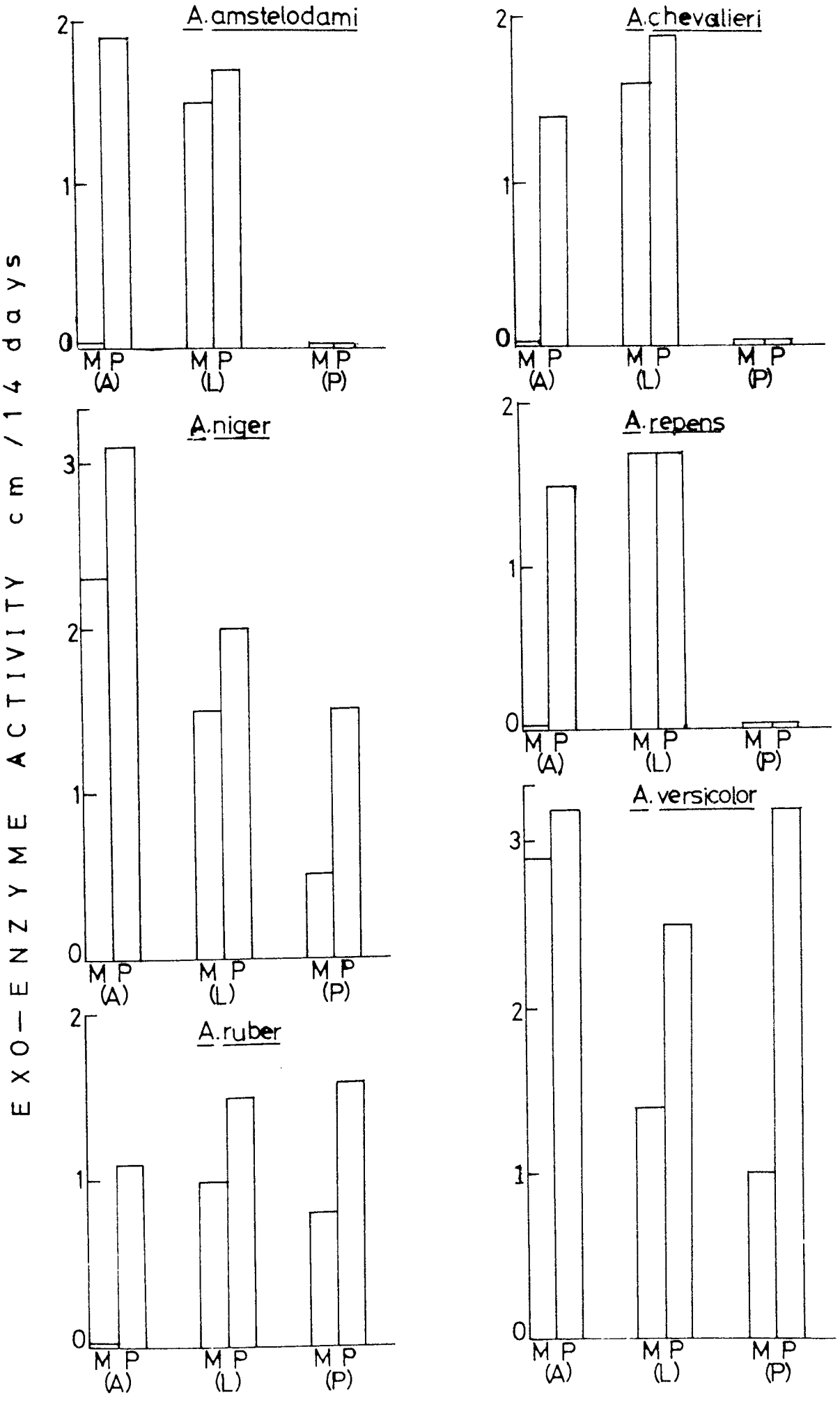
M - MEA

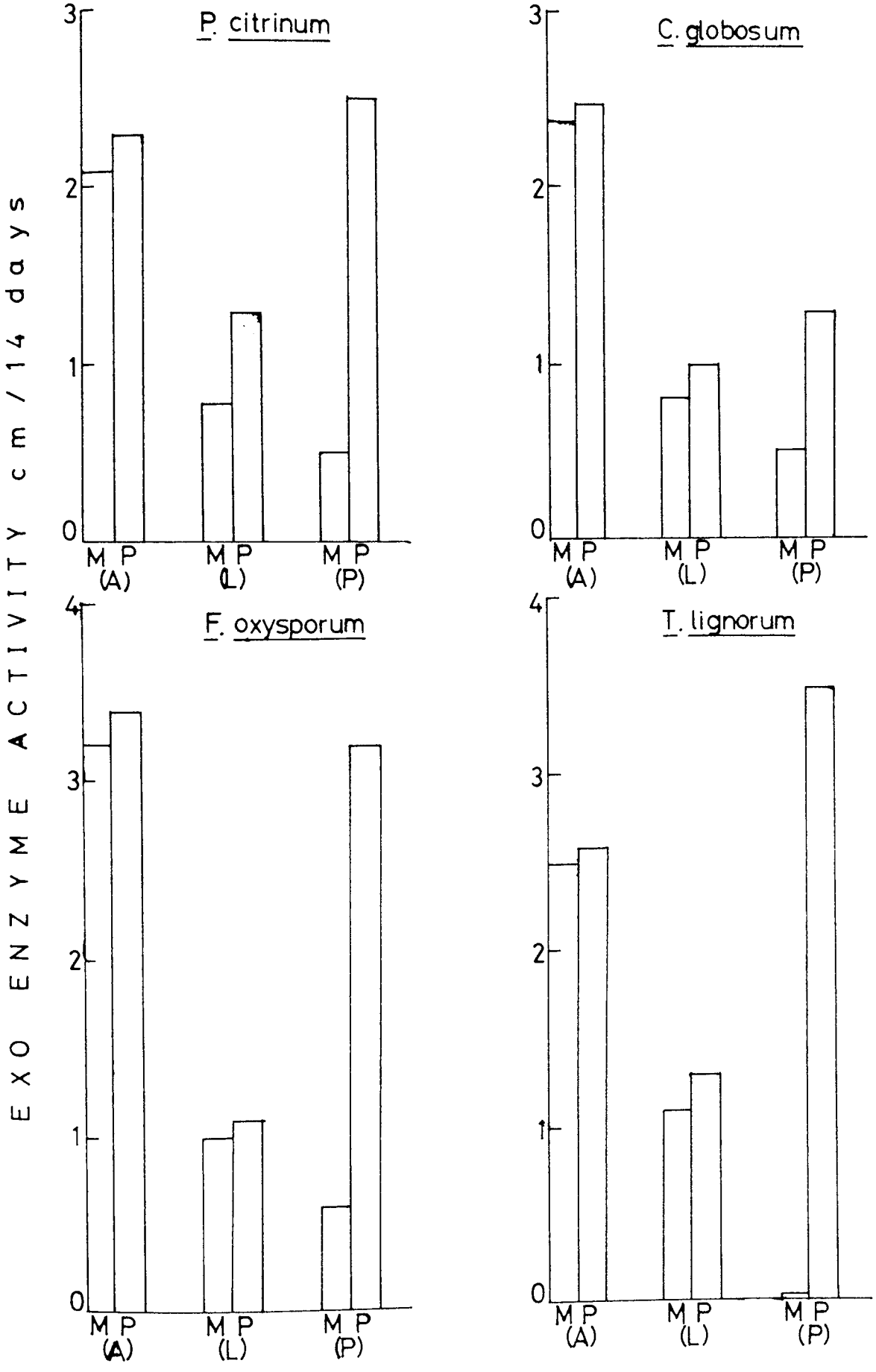
P - PLA

(A) - AMYLASE ACTIVITY

(L) - LIPASE ACTIVITY

(P) - PROTEASE ACTIVITY


EXO—ENZYME ACTIVITY cm / 14 days
A. amstelodami
A. chevalieri
A. niger
A. repens
A. ruber
A. versicolor
M P
(A)
(L)
(P)


EXO ENZYME ACTIVITY cm / 14 days
P. citrinum
C. globosum
F. oxysporum
T. lignorum
0
1
2
3
4
M P
(A)
(L)
(P)

Although, all enzyme activities were reduced on MEA media, growth and sporulation was heavy when compared with that on PLA media.

From these results, PLA was chosen as a suitable basal medium for detection of enzyme activities, and had the advantages of being a simple preparative medium, easily adjustable pH and clearance zones clearly defined.

5.4.1 Effects of pH on Enzyme Activities

The effects of pH2 to pH10 (buffers listed in Chapter 3) on amylase, cellulase, lipase and protease activities of the test fungi were studied to determine the optimum pH values after 2 weeks incubation at 25°C.

Media above pH5 were prepared single strength and autoclaved, while media below and including pH5 were prepared double strength, autoclaved separately and mixed after cooling to about 50°C, thus preventing agar hydrolysis.

The methods of preparation of the test tubes, inoculation and measurement of enzyme activities were similar to those in section 5.3.1. Eggins and Pugh (1962) cellulose agar (Appendix 1) was used to detect cellulase activities. Five replicates were used.

5.4.2 Results

Figure 5.4 shows the effect of pH2 to pH10 on amylase activities of the fungi. The amylase activities were present over the whole pH range, except A. repens and A. ruber, where there was no activity in acidic

(pH2 and pH3) and alkaline (pH8 and pH9) media. A. chevalieri, A. versicolor, P. citrinum, C. globosum and T. lignorum showed no amylase activities at pH10. Amylase activities in A. niger and T. lignorum were high in the acidic and neutral media, i.e. pH2 to pH7, then falling sharply. The maximum amylase activities of the fungi were found to be between pH5 to pH7.

Only A. niger (pH2 to pH5), C. globosum (pH4 to pH7) , F. oxysporum (pH7 to pH8) and T. lignorum (pH2 to pH8) showed cellulase activity.

Lipases were produced by the fungi over the whole test pH range (figure 5.5). The maximum lipase activities were at pH7, except A. amstelodami and A. versicolor, where there was a peak at pH6 and A. chevalieri, where there was a peak at pH5. No visible growth was seen with the naked eye from fungal plugs of A. repens and A. ruber (pH2 to pH6) and C. globosum (pH2 and pH3), although, lipase activities gradually increased with increasing pH.

The effects of pH on protease activities varied considerably in the test fungi (figure 5.6). No protease activities were recorded in A. amstelodami in acidic media, i.e. pH2 to pH5; A. chevalieri in an alkaline medium i.e. pH10 and at any pH in A. repens. The maximum protease activities of the fungi were found to be tetween pH6 to pH8.

Diffusible pigments were produced by some fungi into the enzyme substrates. A. chevalieri produced a brown to brown-yellow pigment into cellulose and tributyrin media at pH3 to pH9 and pH8 and pH9

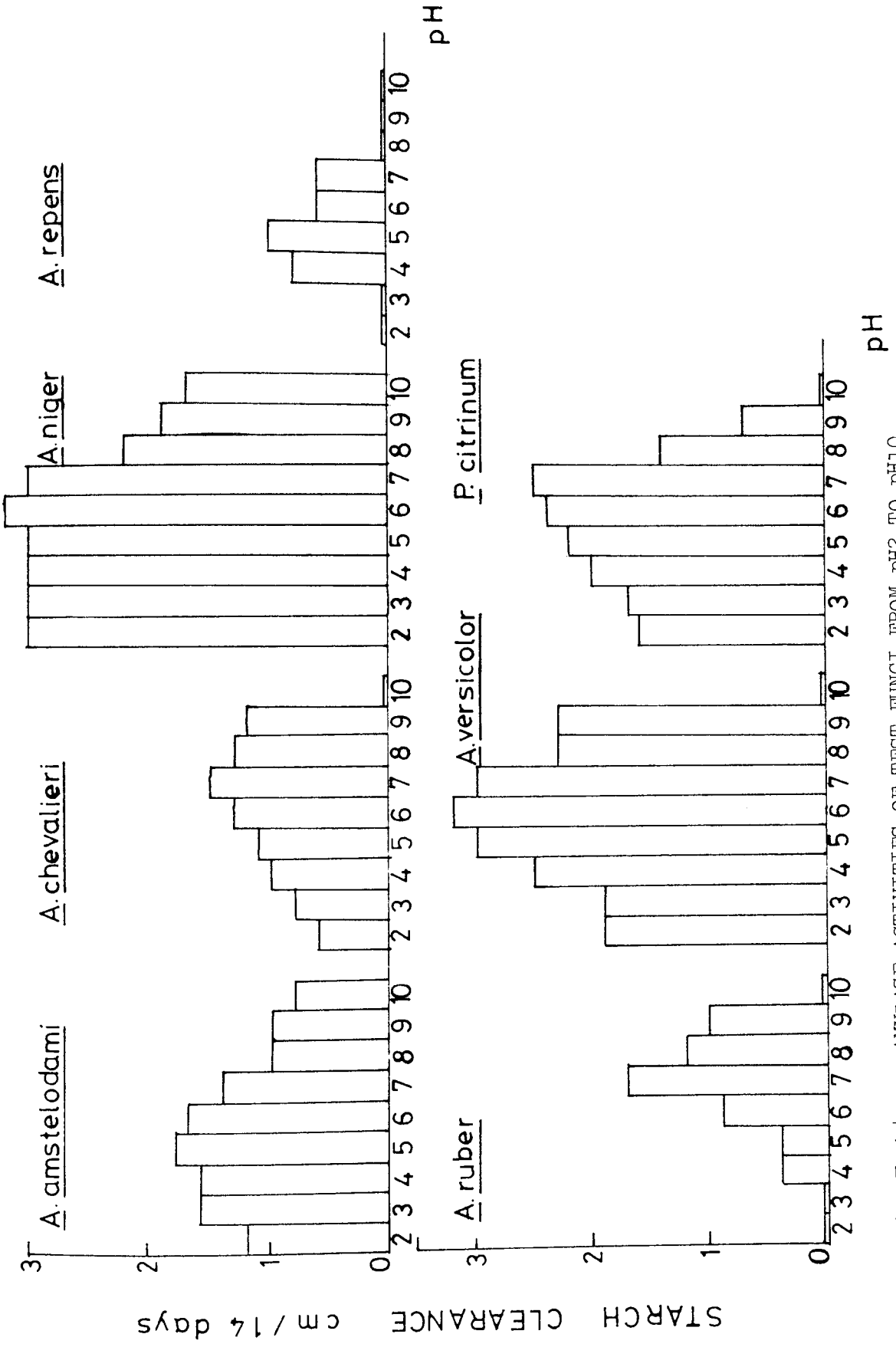


FIGURE 5.4 : AMYLASE ACTIVITIES OF TEST FUNGI FROM pH2 TO pH10

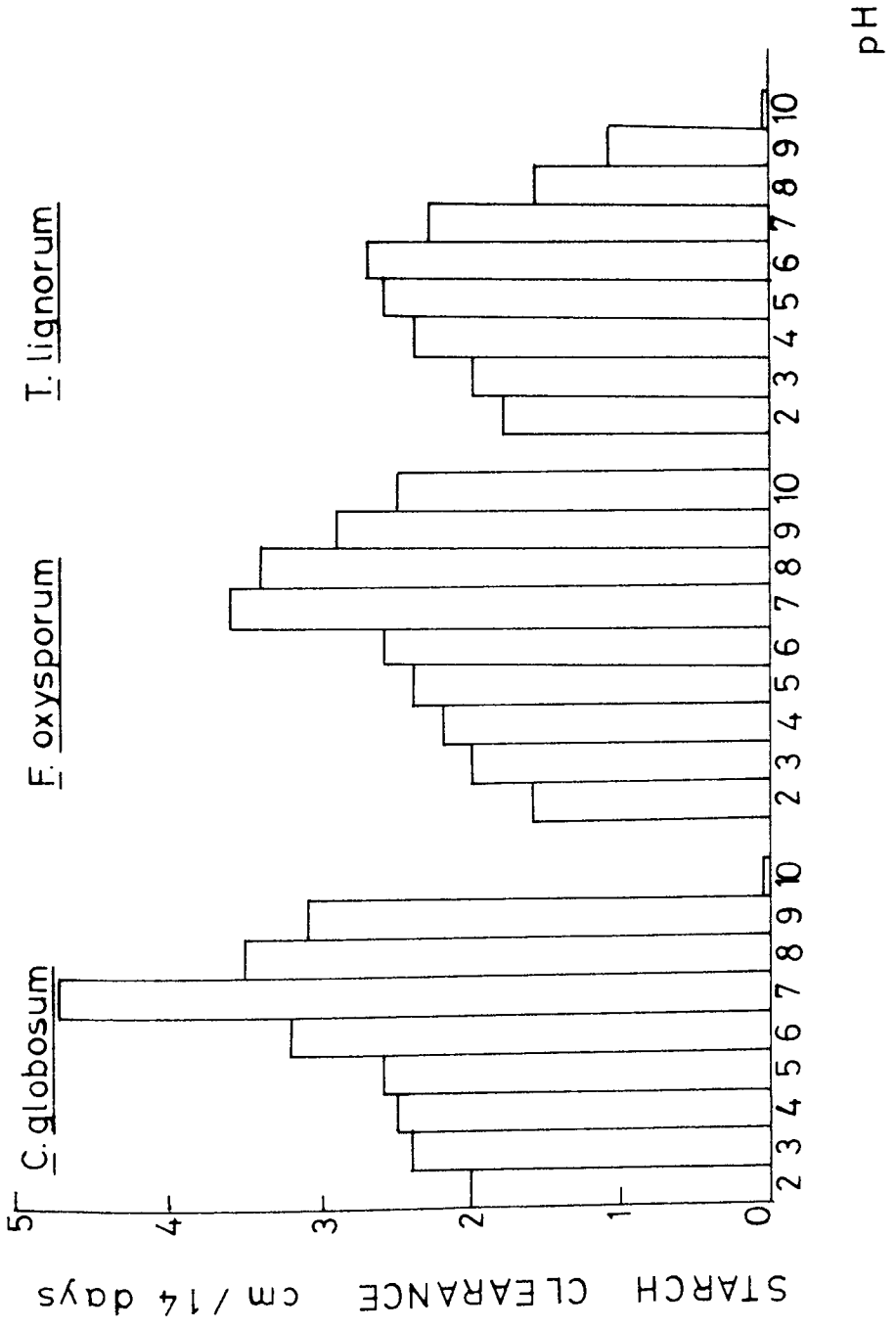


FIGURE 5.4 : AMYLASE ACTIVITIES OF TEST FUNGI FROM pH2 TO pH10

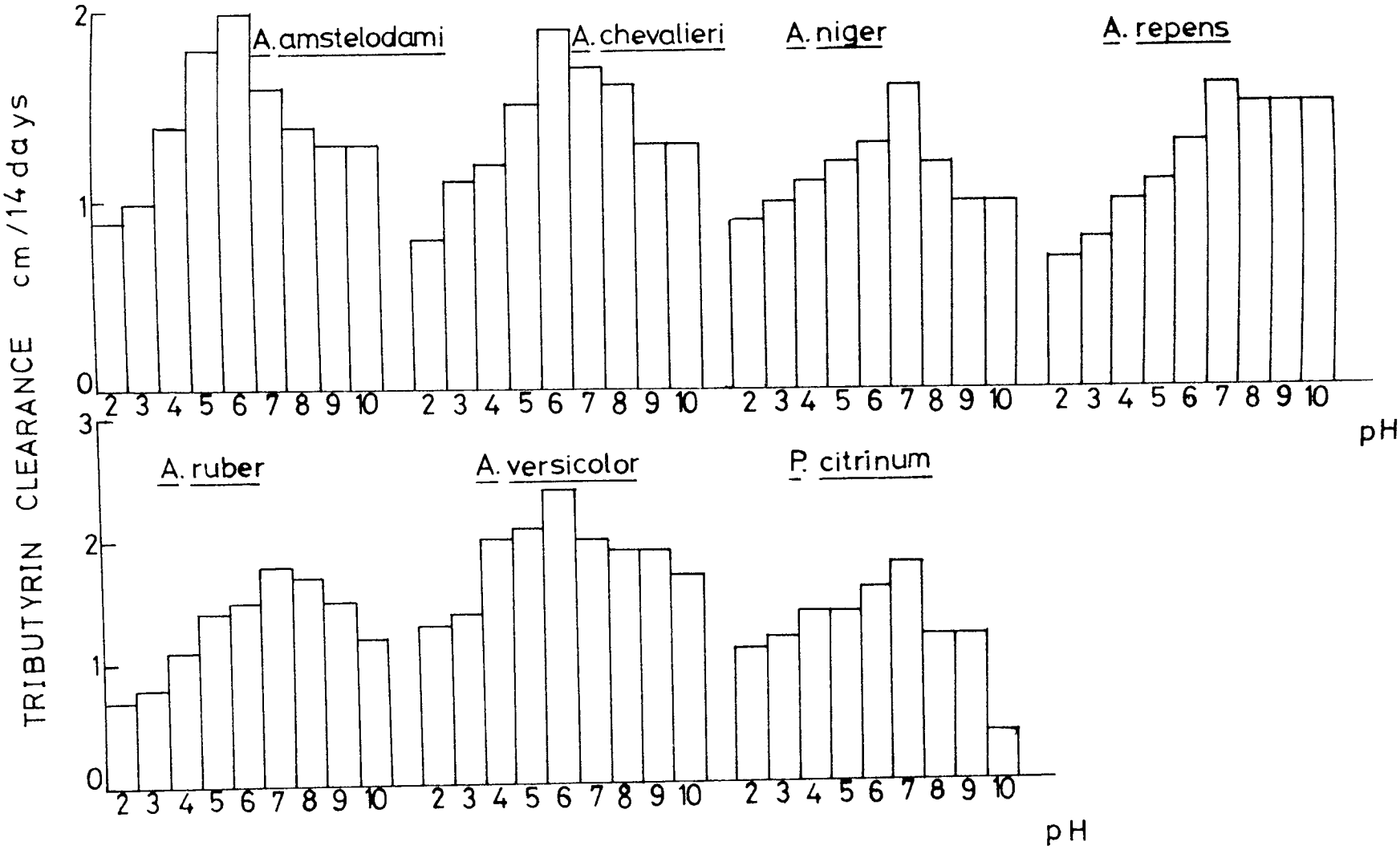


FIGURE 5.5 : LIPASE ACTIVITIES OF TEST FUNGI FROM pH2 TO pH10

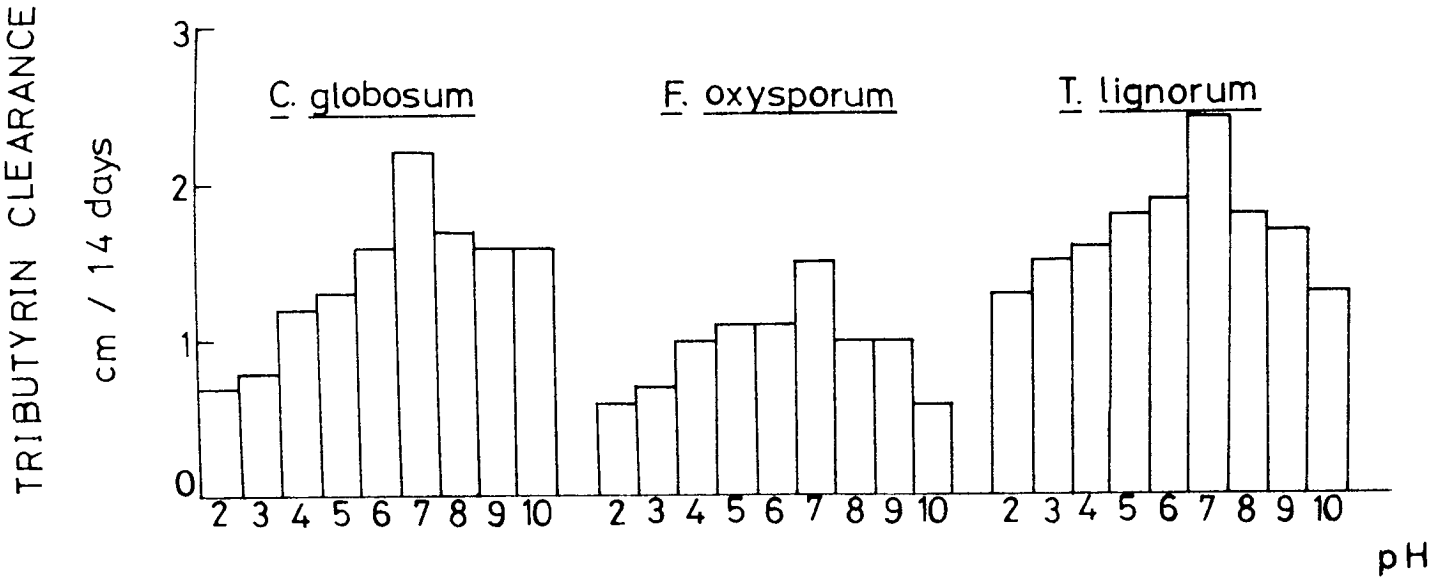


FIGURE 5.5 : LIPASE ACTIVITIES OF TEST FUNGI FROM pH2 TO pH10

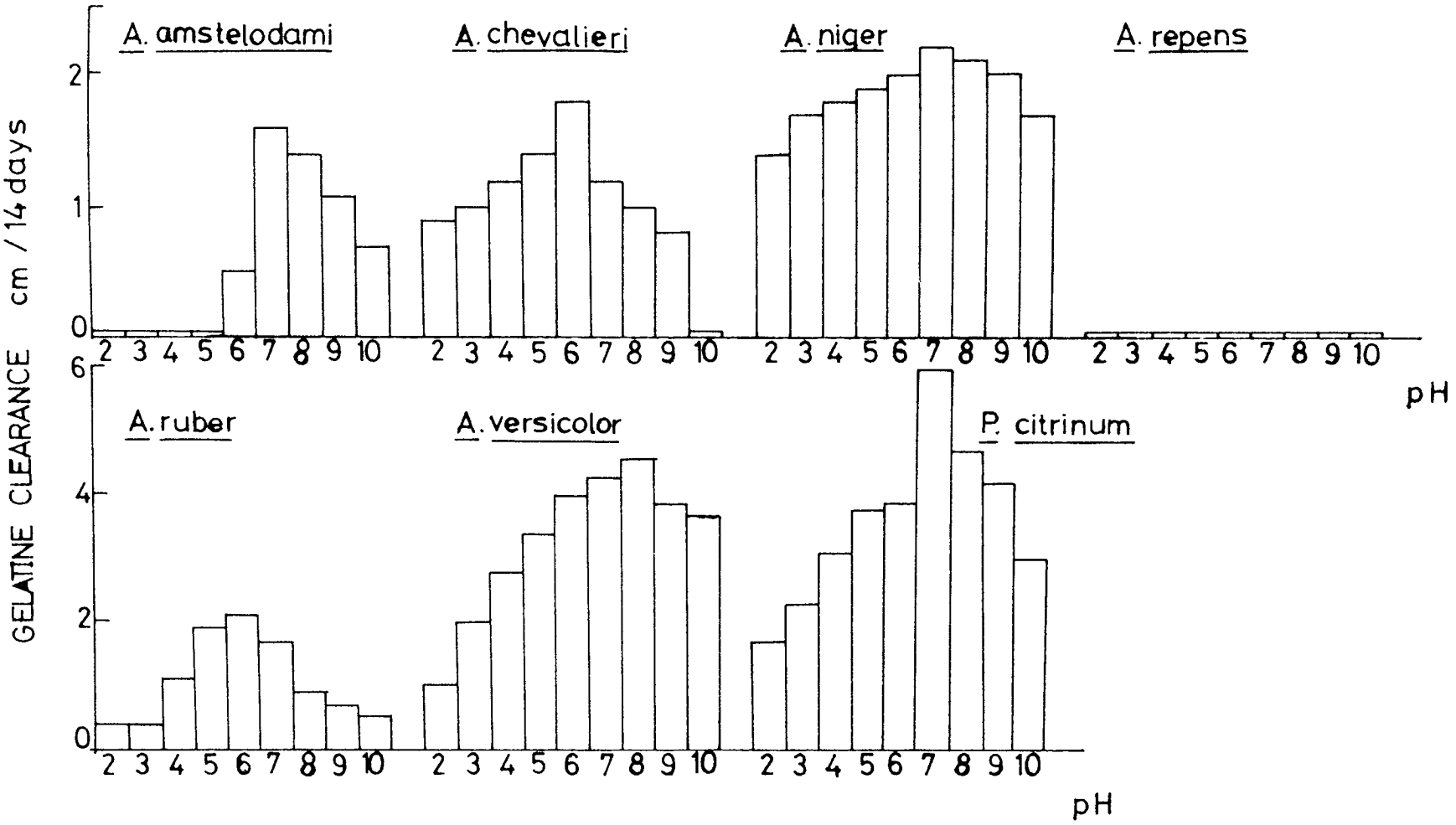


FIGURE 5.6 : PROTEASE ACTIVITIES OF TEST FUNGI FROM pH2 TO pH10

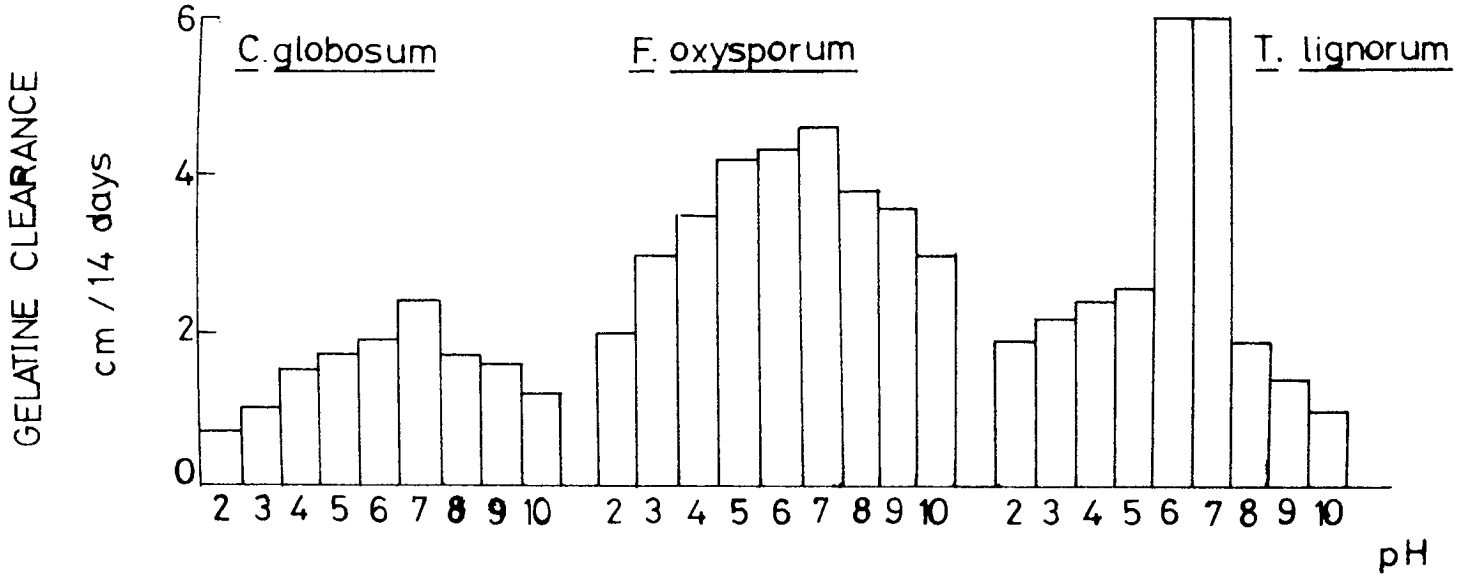


FIGURE 5.6 : PROTEASE ACTIVITIES OF TEST FUNGI FROM pH2 TO pH10

respectively. A. repens produced a pink to brown pigment in cellulose medium; a brown-black pigment in gelatine media (pH3 to pH9) and a blackish pigment in tributyrin medium at pH7. A pale brown pigment was produced by A. ruber at pH7 in a tributyrin medium. In cellulose medium at pH6 and tributyrin media from pH5 to pH10, A. versicolor produced a reddish-mauve pigment.

5.5.1 Effects of Solutes and a_w on Enzyme Activities

Enzyme activities of xerophilic test fungi were studied at a_w levels between 1.0 to 0.70 and non-xerophilic fungal activities studied at a_w levels between 1.0 to 0.90, using glycerol and NaCl as the controlling solutes. Molal concentrations of glycerol and NaCl were added to the PLA media to give a_w values of 0.97, 0.95, 0.93, 0.90, 0.87, 0.85, 0.83, 0.80, 0.77, 0.75, 0.73, 0.70 and a_w 0.97, 0.95, 0.93, 0.90, 0.87, 0.85, 0.83 and 0.80 respectively. Media were prepared as in Chapter 3.

The amylase activities were measured at pH5; lipase and protease activities were measured at pH7, the pH being adjusted with citrate-phosphate buffer (Gomori, 1965). The method of determining the enzyme activities by measuring the depth of clearance was similar to that used in section 5.3.1, with some modifications. To enable comparisons between enzyme activities at various a_w levels, readings were taken after one month. The pH and solute modified media were added to sterile 1.5cm diameter x 15cm length test tubes provided with suitable oxoid caps. After inoculation with plugs of fungal mycelium, the test tubes were placed in a_w controlled aquariaas shown in

figure 5.7 and incubated at 25°C. Each sample was done in triplicate.

Cellulase activity measurements at reduced a_w were omitted because most of the test fungi did not produce cellulases in cellulose agar and cellulose agar modified with either glycerol or NaCl at a_w 0.95.

5.5.2 Results

Figure 5.8 shows the amylase activities of the test fungi at reduced a_w. Amylases were produced between a_w levels of 0.90 to 0.83. Amylase activities in glycerol modified media were higher than in NaCl modified media, except for A. chevalieri and A. repens, where NaCl media showed higher activities above a_w 0.97 and C. globosum, where NaCl media showed higher activities above a_w 0.90. Amylase activities were recorded in A. chevalieri, A. repens and A. ruber at low a_w only in glycerol modified media.

Production of lipases decreased with decreasing a_w (figure 5.9). At high a_w, fungi growing on NaCl modified media produced higher levels oflipases than glycerol modified media, except for F. oxysporum. Between a_w levels of 0.93 to 0.80, lipase activities in NaCl media fell below those in glycerol media. Lipase activities in NaCl media in some species increased with decreasing a_w i.e. A. amstelodami maximum activity was at a_w 0.95; in A. chevalieri, A. ruber, A. versicolor, C. globosum and T. lignorum maximum activities were at a_w 0.97. A. amstelodami, A. chevalieri, A. repens, A. ruber and A. versicolor produce lipases in glycerol modified media up to a_w 0.70

FIGURE 5.7 : TEST TUBES CONTAINING ENZYME SUBSTRATE MEDIA IN a_w CONTROLLED AQUARIUM

Protease activities are shown in figure 5.10. A. glaucus group fungi (A. amstelodami, A. chevalieri, A. repens, A. ruber) did not shown enzyme activities in control media (PLA, a_w approximately 0.997) and A. chevalieri, and A. repens show no enzyme activities up to 0.97 a_w. The enzyme activities increased with decreasing a_w, and maximum activities were recorded between a_w 0.95 to 0.93 and a_w 0.97 to 0.95 in glycerol and NaCl media. No proteases were produced by A. chevalieri and A. repens at any a_w level in NaCl modified media. Protease activities of the A. glaucus group test fungi and A. versicolor were detected up to a_w 0.70.

In some instances, although high enzyme activities were recorded, no visible growths were seen from the inoculation plugs. It is possible that some microscopically detectable growth could have occurred, because exoenzyme production decreased with decreasing a_w.

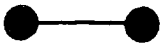
Diffusible pigments as recorded in section 5.4 .2., were produced by A. chevalieri, A. repens, A. ruber and A. versicolor in most of the gelatine and tributyrin media.

5.6 Discussion

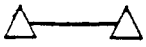
Both MEA and PLA proved satisfactory as basal media for the growth of all test fungi - growths of the test xerophilic species enhanced by glycerol or NaCl in the media to lower the a_w.

FIGURE 5.8 : AMYLASE ACTIVITIES OF TEST FUNGI AT REDUCED a_w LEVELS

GLYCEROL MEDIUM

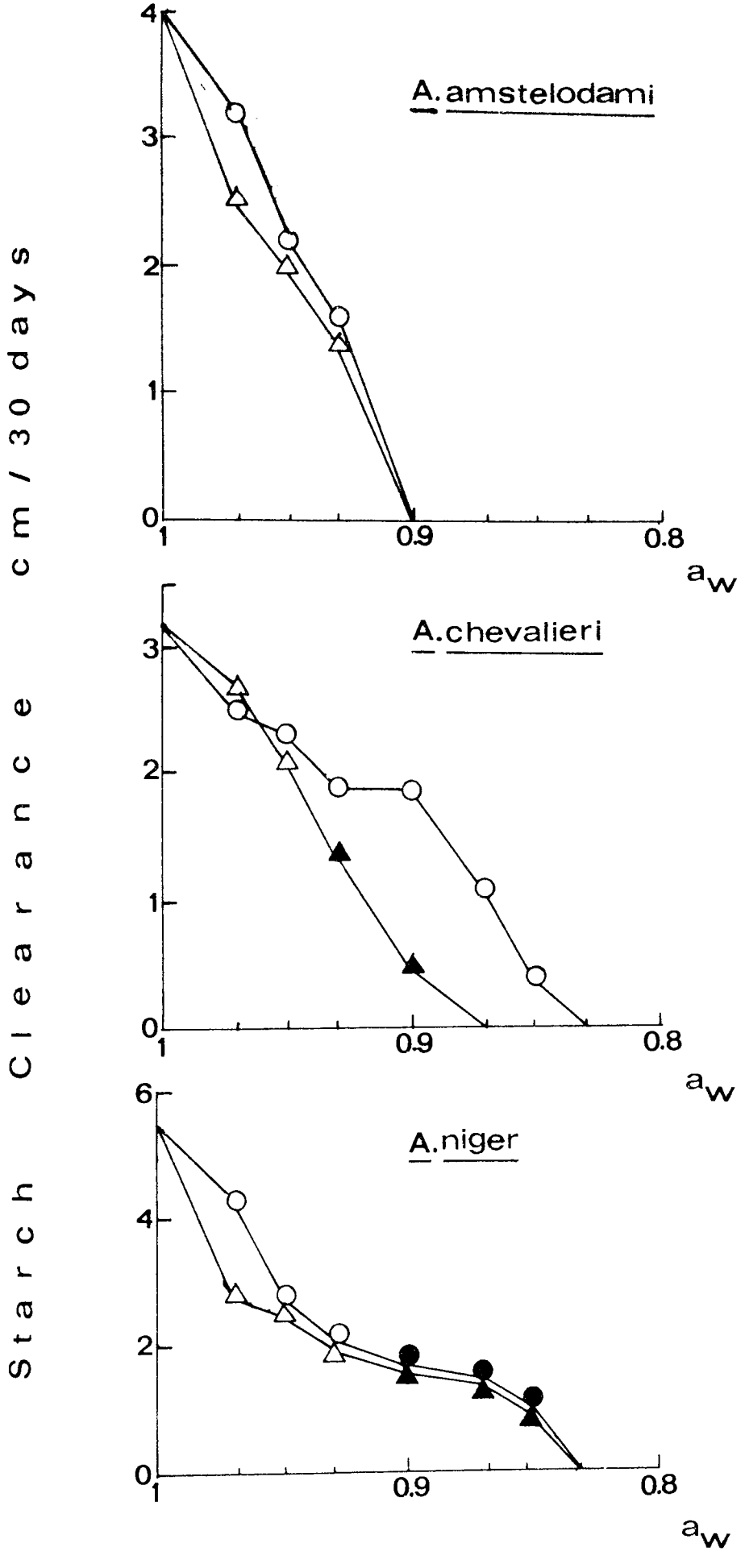


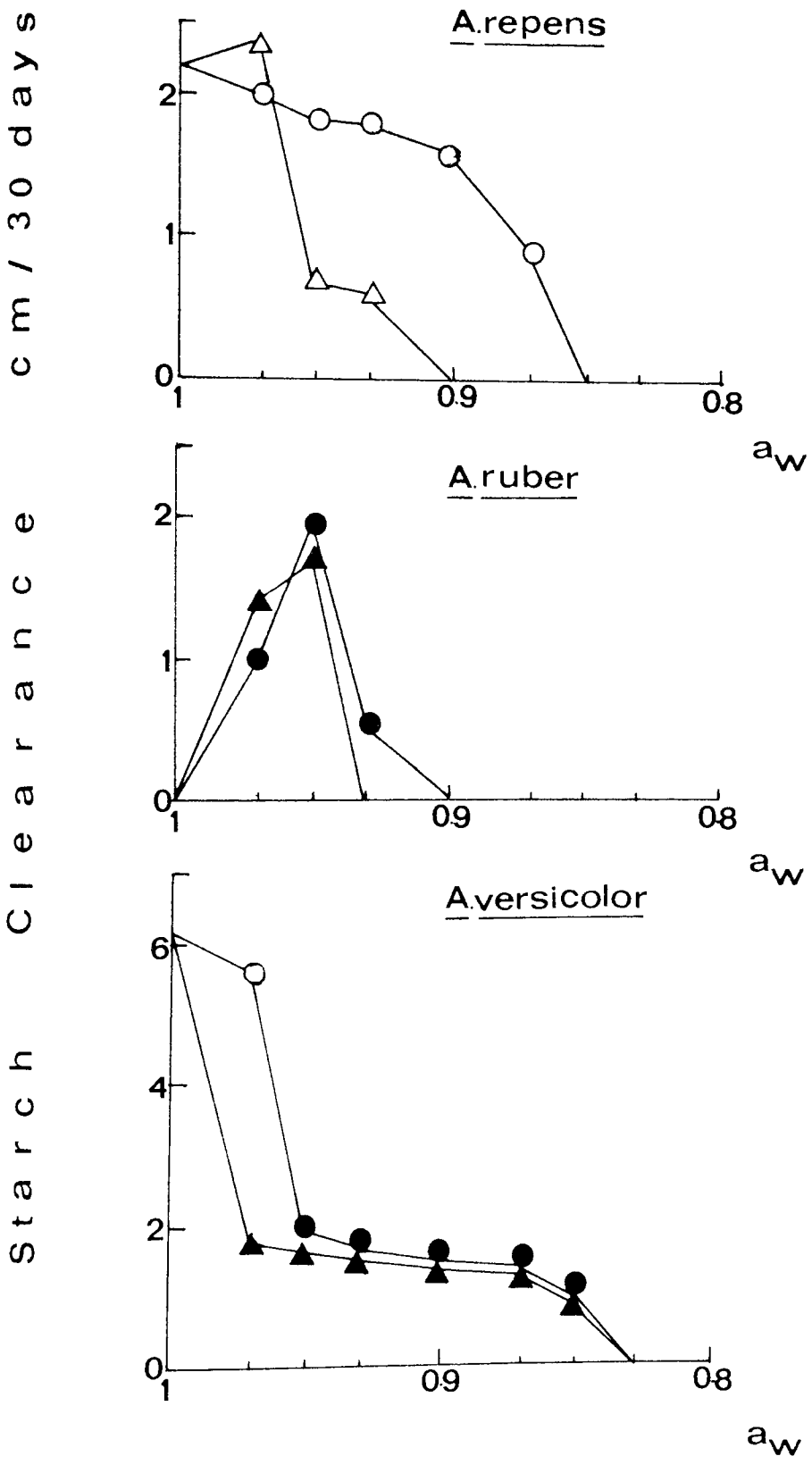
GLYCEROL MEDIUM. NO VISIBLE FUNGAL GROWTH

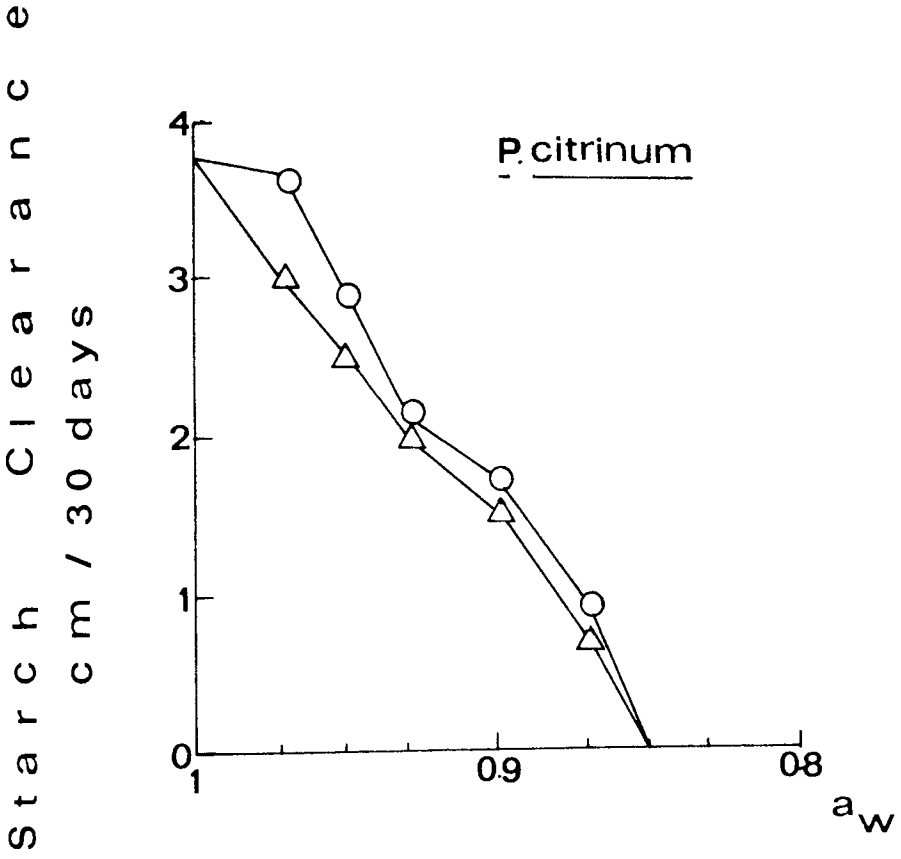


NaCl MEDIUM

NaCl MEDIUM. NO VISIBLE FUNGAL GROWTH


Starch Clearance cm / 30 days
A. amstelodami
A. chevalieri
A. niger
4
3
2
1
0
6
1
0.9
0.8
a_w


Starch Clearance cm / 30 days
A.repens
2
1
0
1
0.9
0.8
a_w
A.ruber
2
1
0
1
0.9
0.8
a_w
A.versicolor
6
4
2
0
1
0.9
0.8
a_w


Starch Clearance
cm / 30 days
4
3
2
1
0
1
0.9
0.8
a_w
P. citrinum

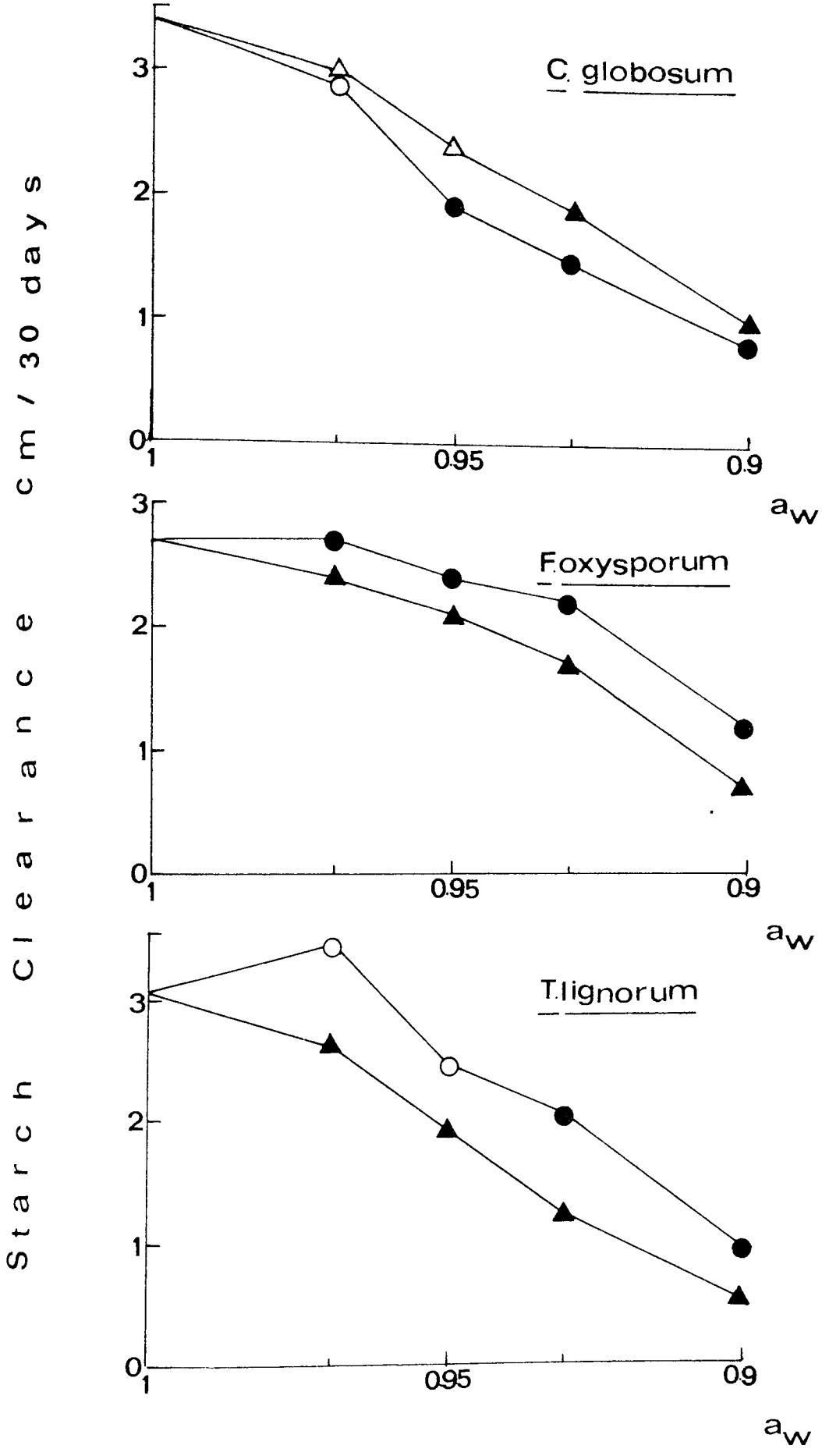
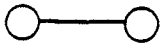
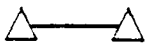

Starch Clearance cm / 30 days
C. globosum
F.oxysporum
T.lignorum
3
2
1
0
1
0.95
0.9
a_w

FIGURE 5.9 : LIPASE ACTIVITIES OF TEST FUNGI AT REDUCED a_w LEVELS



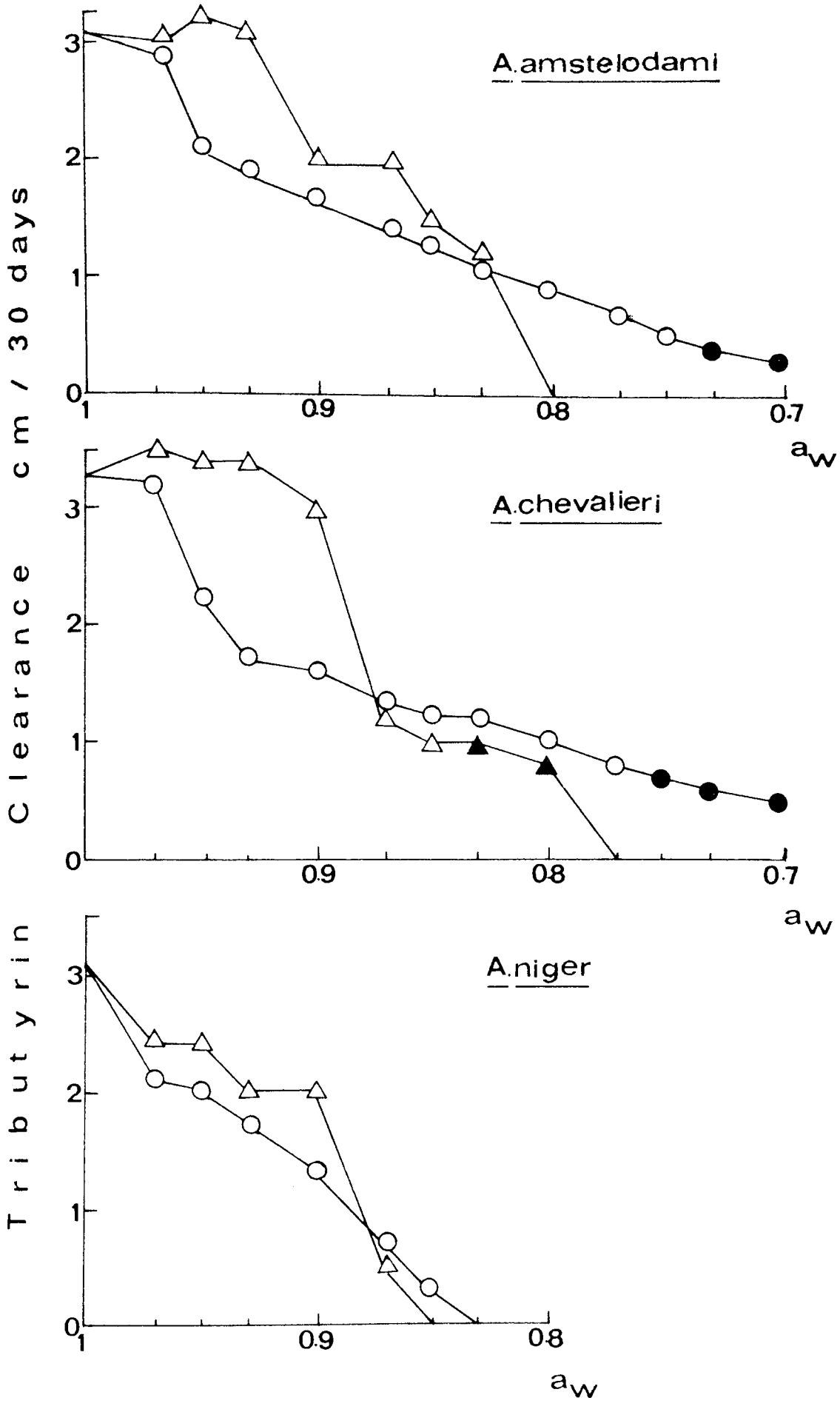
GLYCEROL MEDIUM

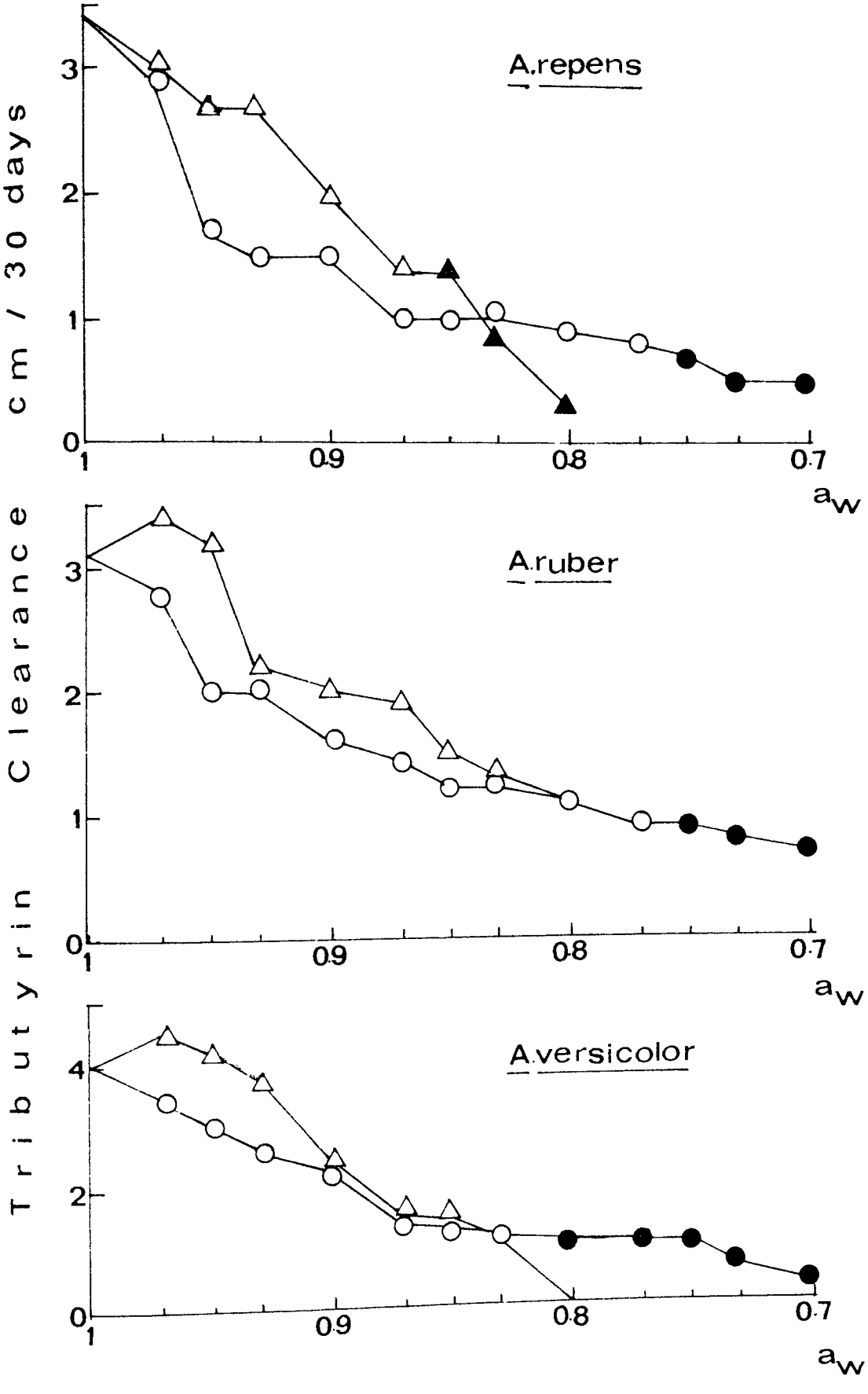
GLYCEROL MEDIUM. NO VISIBLE FUNGAL GROWTH

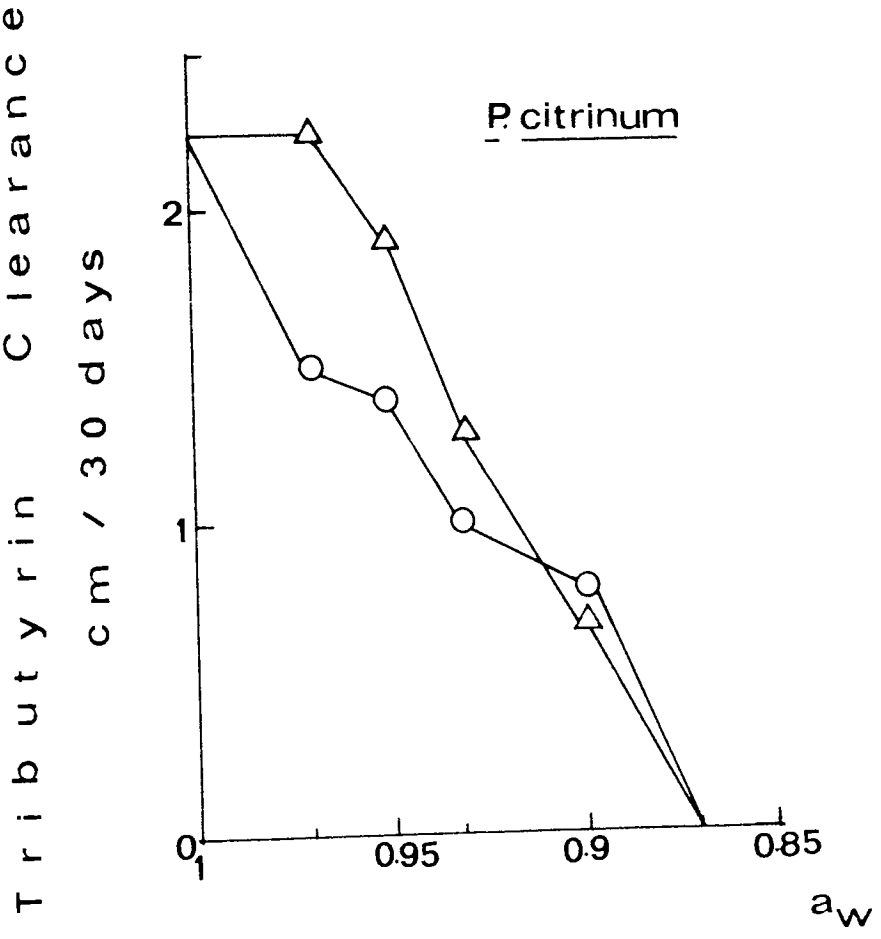


NaCl MEDIUM

NaCl MEDIUM. NO VISIBLE FUNGAL GROWTH


A.amstelodami
A.chevalieri
A.niger
Clearance cm / 30 days
Tributyrin
0
1
2
3
1
0.9
0.8
0.7
a_w


A.repens
cm / 30 days
3
2
1
0
1
0.9
0.8
0.7
a_w
A.ruber
Clearance
Tributyrin
3
2
1
0
1
0.9
0.8
0.7
a_w
A.versicolor
4
2
0
1
0.9
0.8
0.7
a_w


Tributyrin Clearance
cm / 30 days
P. citrinum
2
1
0
1
0.95
0.9
0.85
a_w

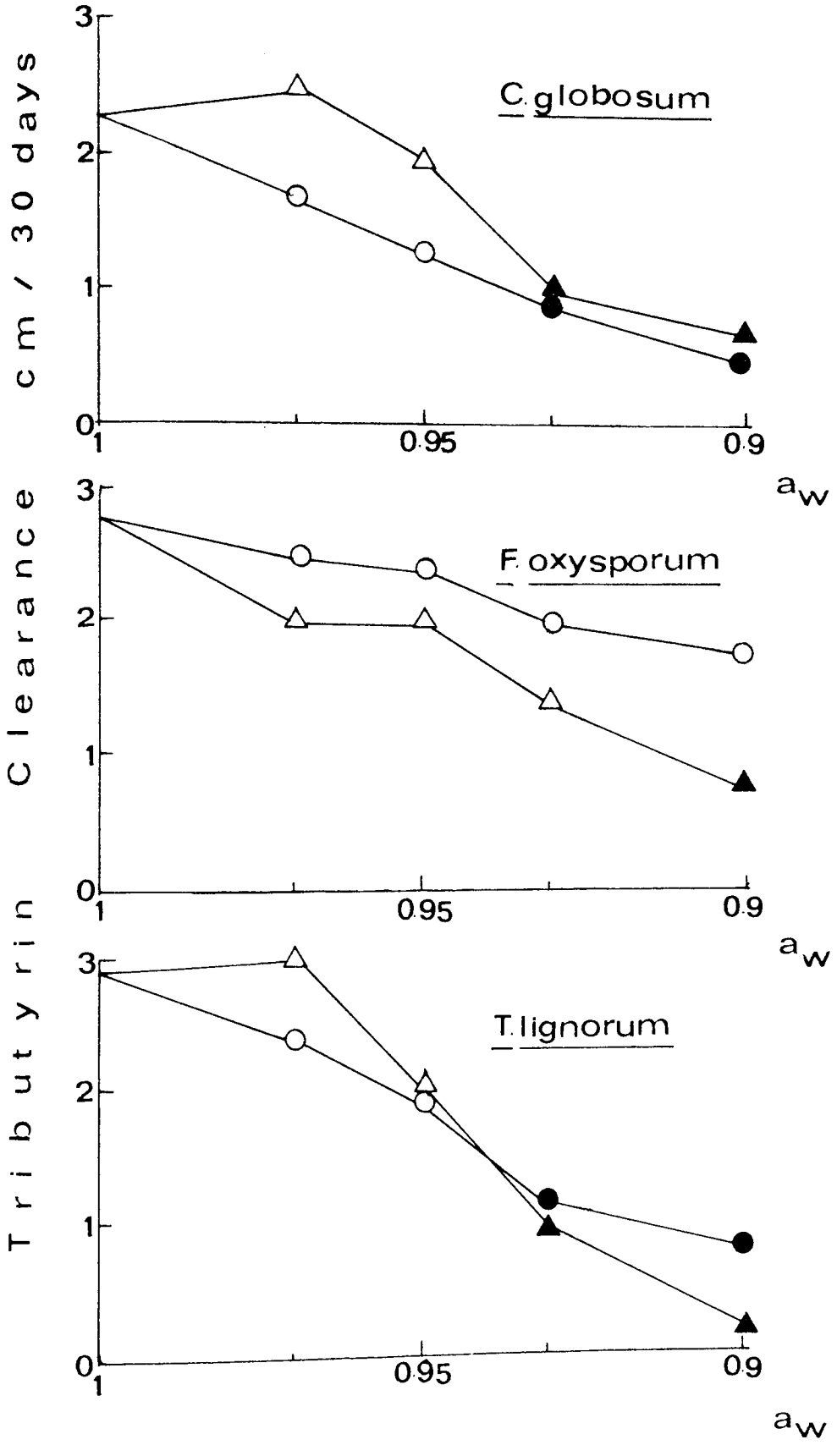
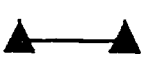

Tributyrin Clearance cm / 30 days
C. globosum
F. oxysporum
T. lignorum
3
2
1
0
1
0.95
0.9
a_w

FIGURE 5.10 : PROTEASE ACTIVITIES OF TEST FUNGI AT REDUCED a_w LEVELS

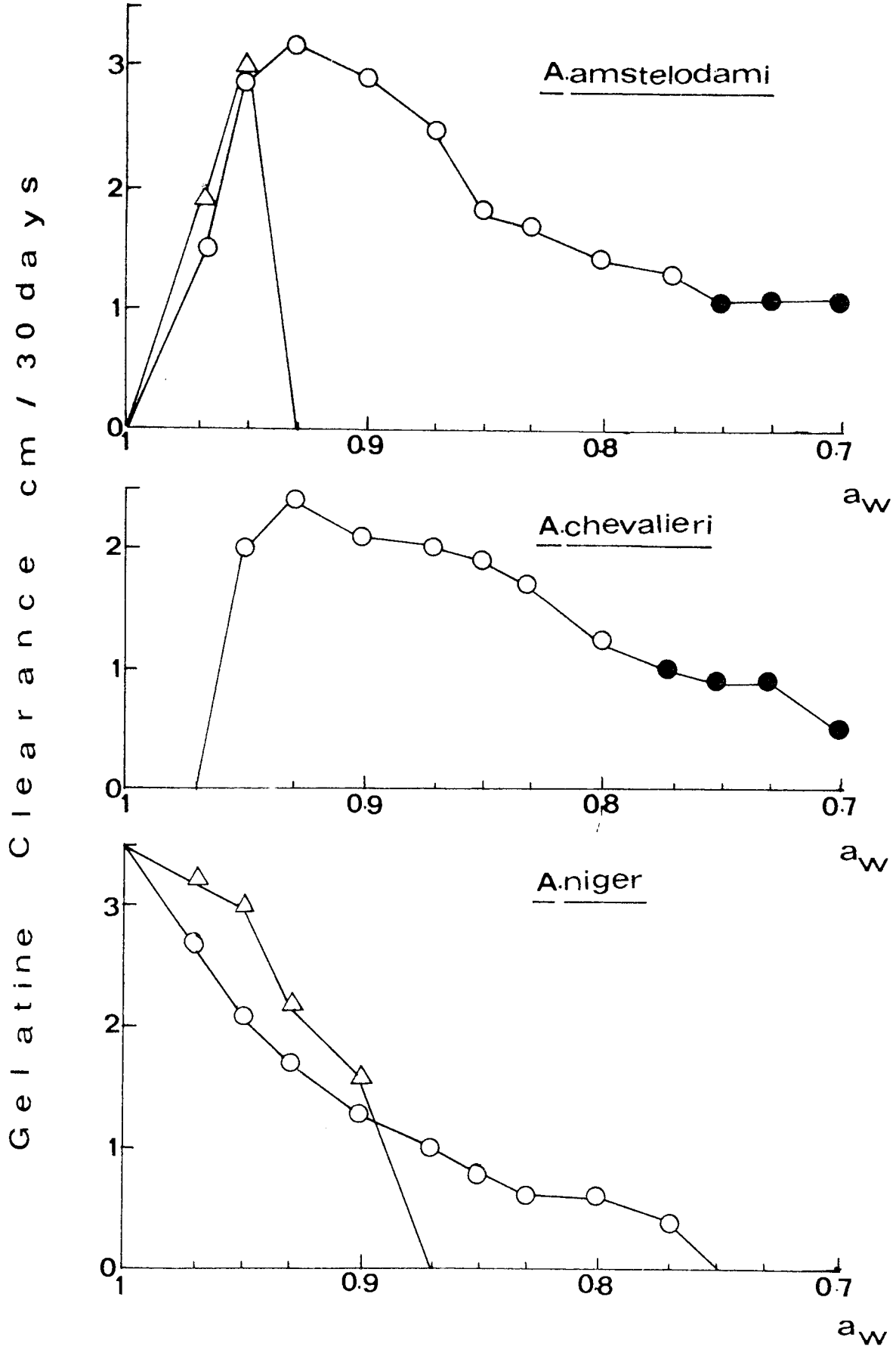
GLYCEROL MEDIUM

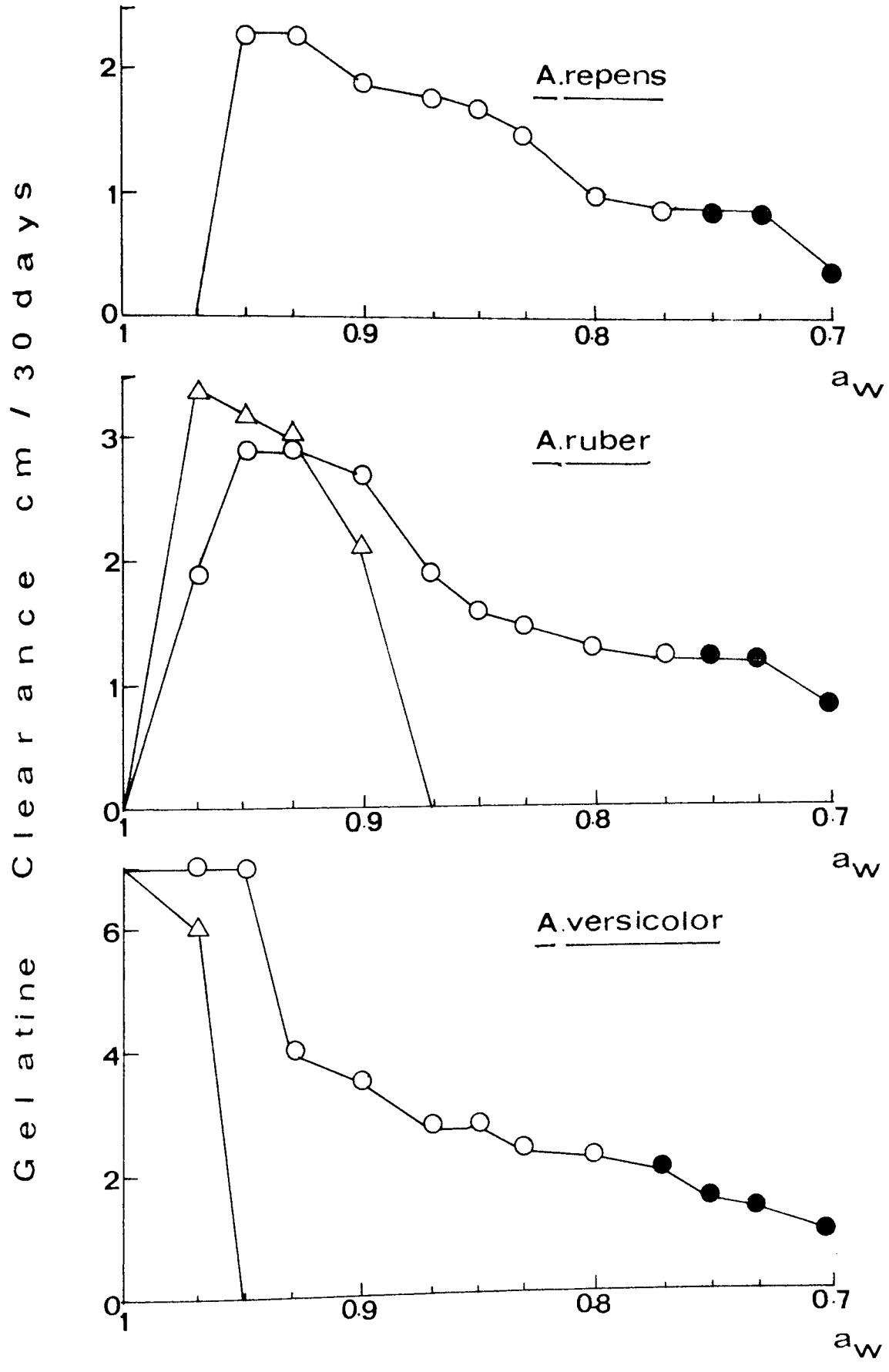
GLYCEROL MEDIUM. NO VISIBLE FUNGAL GROWTH

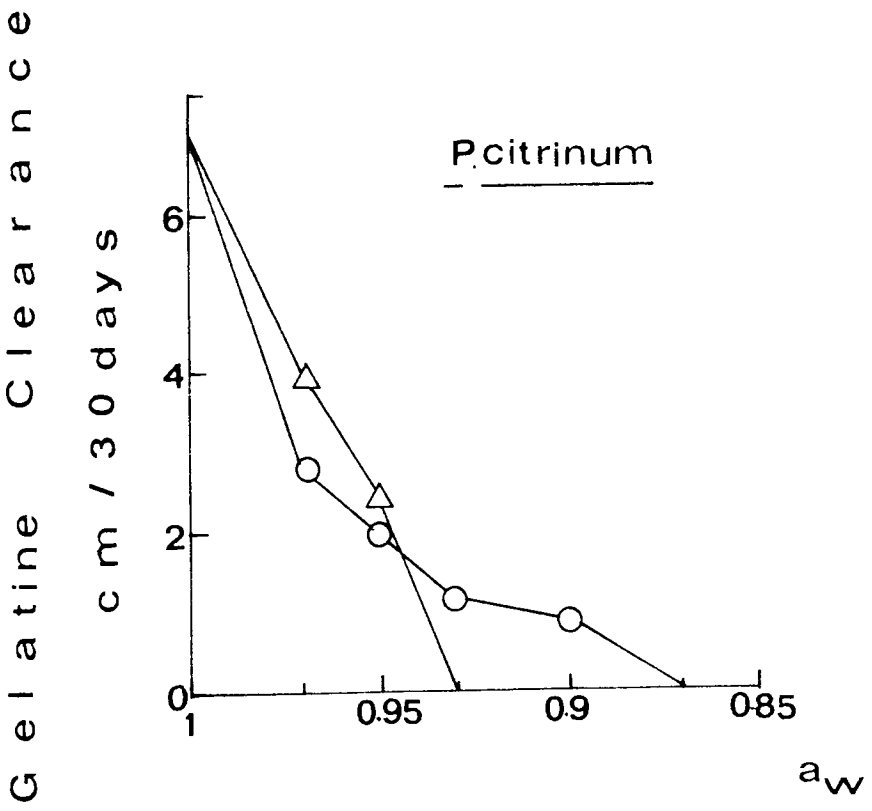
NaCl MEDIUM

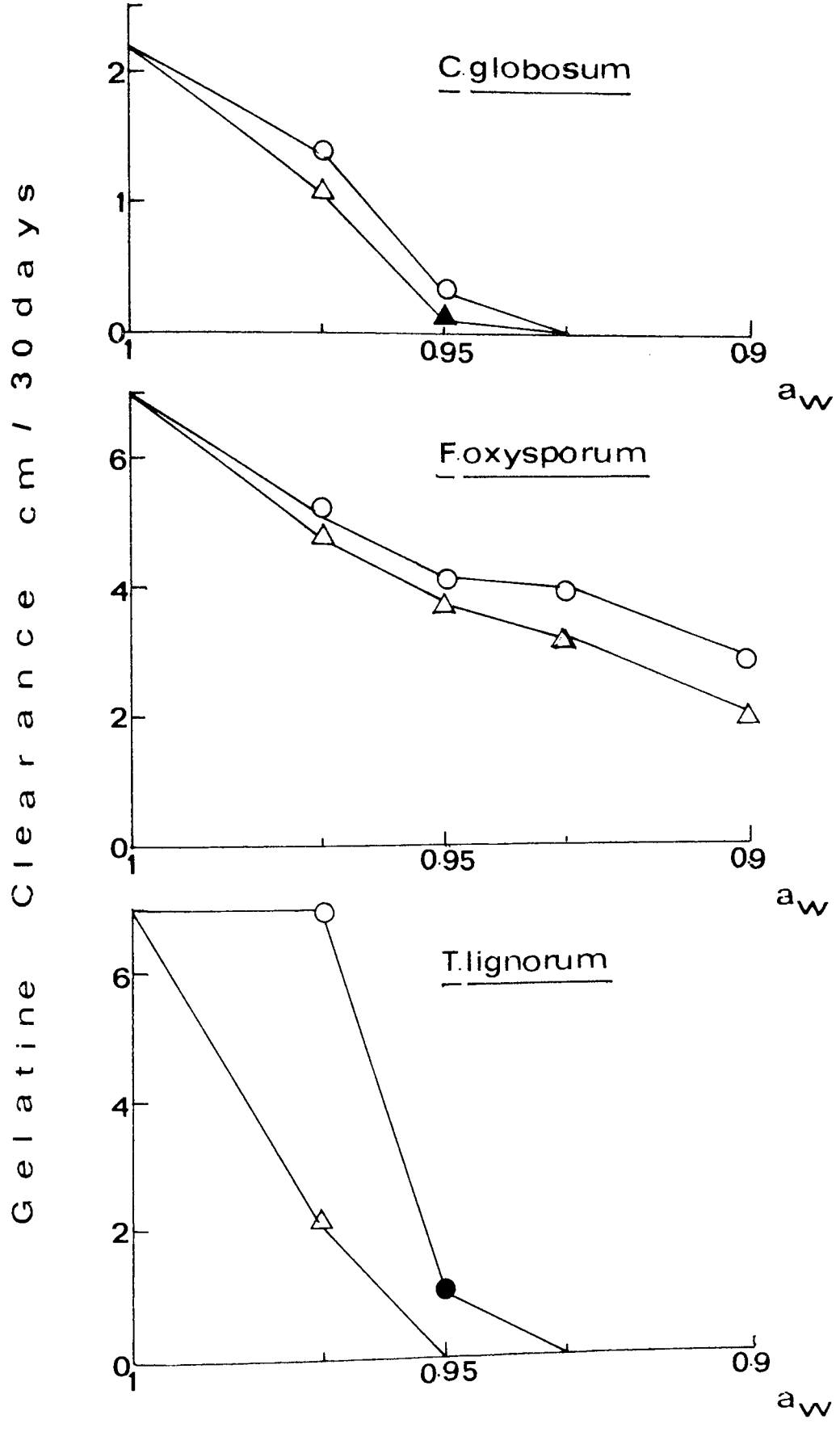


NaCl MEDIUM. NO VISIBLE FUNGAL GROWTH


Gelatine Clearance cm / 30 days
A.amstelodami
A.chevalieri
A.niger
0
1
2
3
1
0.9
0.8
0.7
a_w


Gelatine Clearance cm / 30 days
A.repens
A.ruber
A.versicolor
2
1
0
3
6
4
1
0.9
0.8
0.7
a_w


Gelatine Clearance
cm / 30 days
P.citrinum
6
4
2
0
1
0.95
0.9
0.85
a_w


Gelatine Clearance cm / 30 days
C.globosum
F.oxysporum
T.lignorum
2
1
0
6
4
2
0
1
0.95
0.9
a_w

The hydrolysis of the soluble starch (amylose) by amylases to glucose can be determined when the agar column was submerged into iodine solution. The starch formsa dark blue complex with iodine, whereas, glucose shows no such reaction and remains colourless.

The opaque tributyrin agar contained glycerol tributyrate, a neutral fat (ester of glycerol), as the fat substrate, which was hydrolysed by fungal lipases to produce fatty acid and glycerol.

Fungal proteases hydrolysed gelatine, a mixture of polypeptides into peptide and amino acid units. Unhydrolysed gelatineprecipitated, turning the medium opaque, while the peptide and amino acid units remained unprecipitated, leaving a clear medium when submerged in saturated $(NH_4)_2SO_4$ solution.

Colour changes were taken as semiquantitative measures of amylase activities and clearance zones were taken as semiquantitative measures of lipase and portease activities.

The use of PLA and not MEA as the basal medium was justified, because althouth all the fungi grew actively on them, enzyme activities of the fungi were higher on PLA. The effect of increased production of enzymes on a nutritionally limiting medium (PLA) than on an additional nutrient medium (MEA) is known as 'catabolic repression' (Staadtman, 1970). Catabolic enzymes in a substrate induced synthesis can be repressed when the carbon and energy requirements of fungal growth are supplied by a different catabolic

process. The synthesis of inducible enzymes is repressed by catabolites produced by one of several substrates that support rapid fungal growth, e.g. one or more catabolites produced during glucose dissimilation or glucose, when added to a substrate whose catabolism is under induced enzyme control, will be preferentially utilized as long as they are present and the enzyme required for catabolism of the second substrate will not be formed. From the results, the synthesis of amylases, lipases and proteases to various degrees appears to be substrate induced.

The effects of pH on amylase activities showed a maximum activity around pH6 to pH7. Only A. niger had a peak at pH5 and high activities in the acidic media. This could be due to the maximum diametric growth rate of this fungus observed in the acidic range.

The maximum lipase and protease activities were around neutral pH. A. repens did not produce any proteases at any pH value. However, protease activities at lowered a_w were recorded for this fungus, which suggests that the growth and enzyme activity are a_w dependant.

The results of enzyme activities at reduced a_w indicate that the activities are strongly influenced by the water availability and the nature of the solute. Enzyme activities of some test fungi between a_w 0.97 to 0.90 were enhanced in NaCl media. These results correlate with results obtained from diametric growth rates at reduced a_w.

The enzyme activities of the non-xerophilic fungi gradually decreased with decreasing a_w. No protease activities were detected in C. globosum and T. lignorum below a_w 0.93, but amylase and lipase activities were recorded up to a_w 0.90.

In the present studies, the amylase activities were restricted to a_w levels of about 0.90. None of the A. glaucus species showed any cellulase activity and no protease activities were seen in A. chevalieri and A. repens with NaCl as the controlling Solute. Flannigan and Bana (1978), working with the A. glaucus species found no amylase, cellulase or protease activities, except trace amylase activities in A. amstelodami and A. chevalieri. More strains of each species should be examined before definite conclusions are drawn.

All the test fungi produce lipases and proteases over a wide range of a_w levels. Lipases catalyse the hydrolytic split of triglycerides (tributyrin), producing non-esterified fatty acids, mono and diglycerides and glycerol. Glycerol is also used to control the a_w of the media, therefore, it may enhance lipase activity or may inhibit hydrolysis by mass effect. The overall manifestation of glycerol effects may then be a summation of the two actions.

The storage stability of products or enzyme solutions can be effected by microbial growth or denaturation of the enzyme protein. Basically, there are three ways whereby microbial growth can be repressed, 1) addition of chemical preservatives 2) pasteurization 3) addition of humectants. The mixing of chemical preservatives to foods and

medicines is undesirable as most of these are poisonous and pasteurization will denature the enzyme protein. Consequently, the most suitable method to improve storagibility is to decrease the water availability by decreasing the moisture content or dissolving the enzyme in high concentrations of humectant solutions, e.g. polyhedric alcohols, NaCl and sugars.

A 70% solution of sorbitol at a storage temperature of 33°C was found to stabilize amylases and proteases and also decrease the viable counts of A. niger, Mucor spinescens and P. citrinum (Yasumatsu et al., 1965).

Guardia and Hass (1967), found lipase of fungal origin capable of hydrolyzing olive oil at a low water concentration of 0.06%. By increasing the water content to 2%, the lipase activity increased greatly. Lipase activity of A. niger have been found to be present in the BET region of the sorption isotherm by Purr (1966), and concluded that lipase activity was independant of the availability of free water.

The removal of water from materials arrests the microbial growth and inhibits enzyme activity. The microbial and enzymic susceptibility due to low moisture content appears to be directly related to the physical and chemical availability of water, rather than to its absolute concentration. Most enzyme studies have been carried out on natural foods or dry model substrates. This system however lacks the homogenous distribution of water, i.e. the interaction of the enzyme and substrate in dry materials is mainly dependant on free capillary water (Acker, 1962). The use of liquid model systems or agar columns have the

advantage of having a homogenous water distribution.

From these experiments, it is seen that the test fungi, more importantly the xerophilic fungi, produce amylases, lipases and proteases over a wide a_w range. The efficiency of two fungicides at decreased a_w against three xerophilic fungi was investigated in the following chapter.

CHAPTER SIX

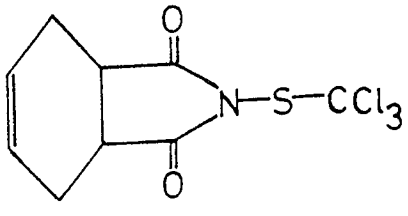
EFFECTS OF FUNGICIDES ON THE GROWTH OF XEROPHILIC FUNGI

6.1 Introduction

Efforts to control biodeterioration have resulted in a number of fungicides being used to protect materials. Hueck van der Plas (1966) has listed a number of fungicides representing different chemicals or conbination of chemicals marketed by various companies. The nature of the material will determine the type of fungicide which can be used, and the potential use of a fungicide will vary from one product to another.

When investigating the effects of biocides, it is essential to compare between biocidal (test) and non-biocidal (control) systems. In the following studies, test xerophilic fungi were grown at a_w of approximately 0.997 (MEA) and a_w of approximately optimum growth, 0.95 (MEA modified with glycerol - MEAG). Both these represent the two control, non-biocidal systems. To these were added increasing concentrations of fungicides.

Two fungicides were selected, captan and verdasan. The active ingredient of captan is N-trichloromethylthio-4-cyclohexane-1,2,dicarboximide (50% w/w) with the following formula :

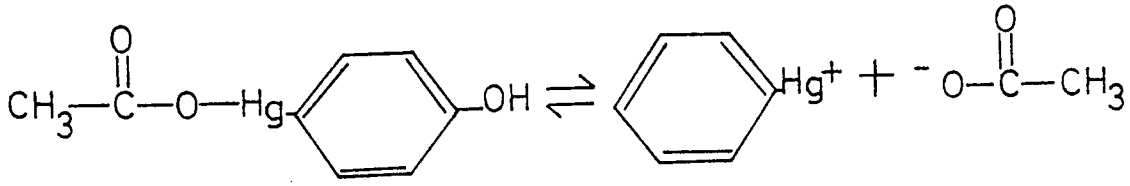


It is a broad spectrum fungicide and is used as a bird repellent on seeds, foliar protectant and in seed treatment. Richmond and Somers (1966), found that its activity is associated with the destruction of the thiol groups of matrix proteins in sensitive species.

Verdasan is an organo-mercury fungicide with phenyl-mercury acetate (PMA) as the active ingredient (2.5% w/w). Besides being used as a dormant spray on fruit trees, mildewicide, seed dressing and against turf diseases, it is an industrial fungicide. PMA is an active ingredient in fungicides which have applications in building materials, glue, paints, paper, rubber and textiles.

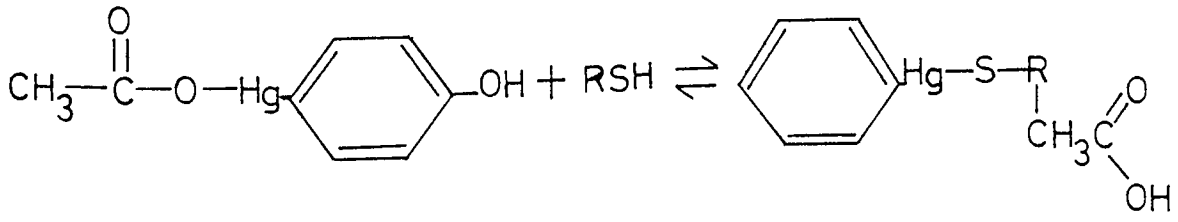
Its biological activity has been ascribed to either of two ways:

1) Formation of ionic bonds between the PMA cation and anions present within the living system :-



PMA PMA Cation

2) Formation of the mercaptide with the thiol groups of living tissue :-



PMA Thiol group Mercaptide

In the following series of experiments, the effects of the test fungicides were studied on the growth on three fungi. Two of these, A. amstelodami and A. chevalieri var. intermedius are often sited in situations of decreased water content (a_w), while A. niger plays an important role in the decomposition processes. All the fungi have been shown to display xerophilic tendency.

Three methods were employed to study biocide efficacy, 1(i)) linear growth rate, 1(ii)) dry weight production, 2) exo-enzyme production and 3) potassium ion (K^+) leakage.

6.2.1 Effect of Fungicides on the Growth Rates of Fungi

Appropriate amounts of captan and verdasan were suspended in sterile distilled water to give stock solutions containing 2000ppm (mg/l) of captan and 100ppm of verdasan. From the fungicide stock solution, known volumes were added to known volumes of sterile, cooled, liquid MEA and MEA modified with glycerol to give a_w 0.95 (MEAG), to give final concentrations of 25, 50, 150, 300, 400, 500ppm of captan and 0.1, 0.5, 1.0, 2.5, 5.0, 10ppm of verdasan.

Aliquots of 15ml of the sterile fungicide incorporated medium were dispensed in 9cm petri dishes with media without fungicide used as control. The plates were inoculated and diametric growth measurements taken up to a month in a similar manner to that shown in Chapter 3. Plates were incubated at $30^{\circ}C \pm 1.0^{\circ}C$. Five replicates were used for each sample.

6.2.2 Effects of Fungicides on the Dry Weight Production

Asceptically, known volumes of fungicides from stock solutions were added to Erlenmeyer flasks of 100ml capacity, each containing 30ml of sterile, cooled malt extract broth (MEB) or malt extract broth modified with glycerol to give a_w 0.95 (MEBG), to give final concentrations similar to those used in growth rate studies (section 6.2.1). Flasks without the fungicides were used as control. The flasks were inoculated with plugs of 1cm diameter of the test fungi and incubated at $30^{\circ}C \pm 1.0^{\circ}C$. Five replicates were used for each sample.

In order to avoid the formation of a layer of fruiting bodies over the fungal colony, which would result in a cessation of the colony growth, the flasks were gently stirred twice a day for 15 seconds. For dry weight analysis, the flasks were removed after 10 days, the fungal mass harvested onto a preweighed, oven-dried Whatman No.1 filter paper by suction filtration and dried at $90^{\circ}C$ to constant weight. The fungal dry weight was calculated as :

(Weight of dry fungal mass) - (Weight of dry fungal plug).

6.2.3 Results

Figure 6.1 shows the growth rates and mycelial dry weights of the test fungi at increasing concentrations of fungicides.

There was an increase in the growth rate at 25ppm of captan over the control in A. amstelodami and A. niger. Growth was obtained in all the test fungi on MEA, MEAG and MEBG up to 500ppm of captan, but it was markedly reduced from the control. No fungal mass was produced in MEB containing 150ppm of captan in A. amstelodami and A. chevalieri and MEB containing 500ppm of captan in A. niger.

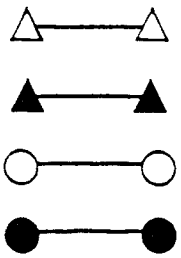
There was an increase in the growth rate of A. amstelodami and A. chevalieri at 0.1ppm of verdasan and of A. niger up to and including 2.5ppm of verdasan over the control with MEA as the basal medium. Mycelial production was found to be higher than the growth rate with increasing concentrations of verdasan in A. amstelodami and A. chevalieri with MEAG and MEBG as the basal media, but with MEA and MEB as the basal media, the reverse was true.

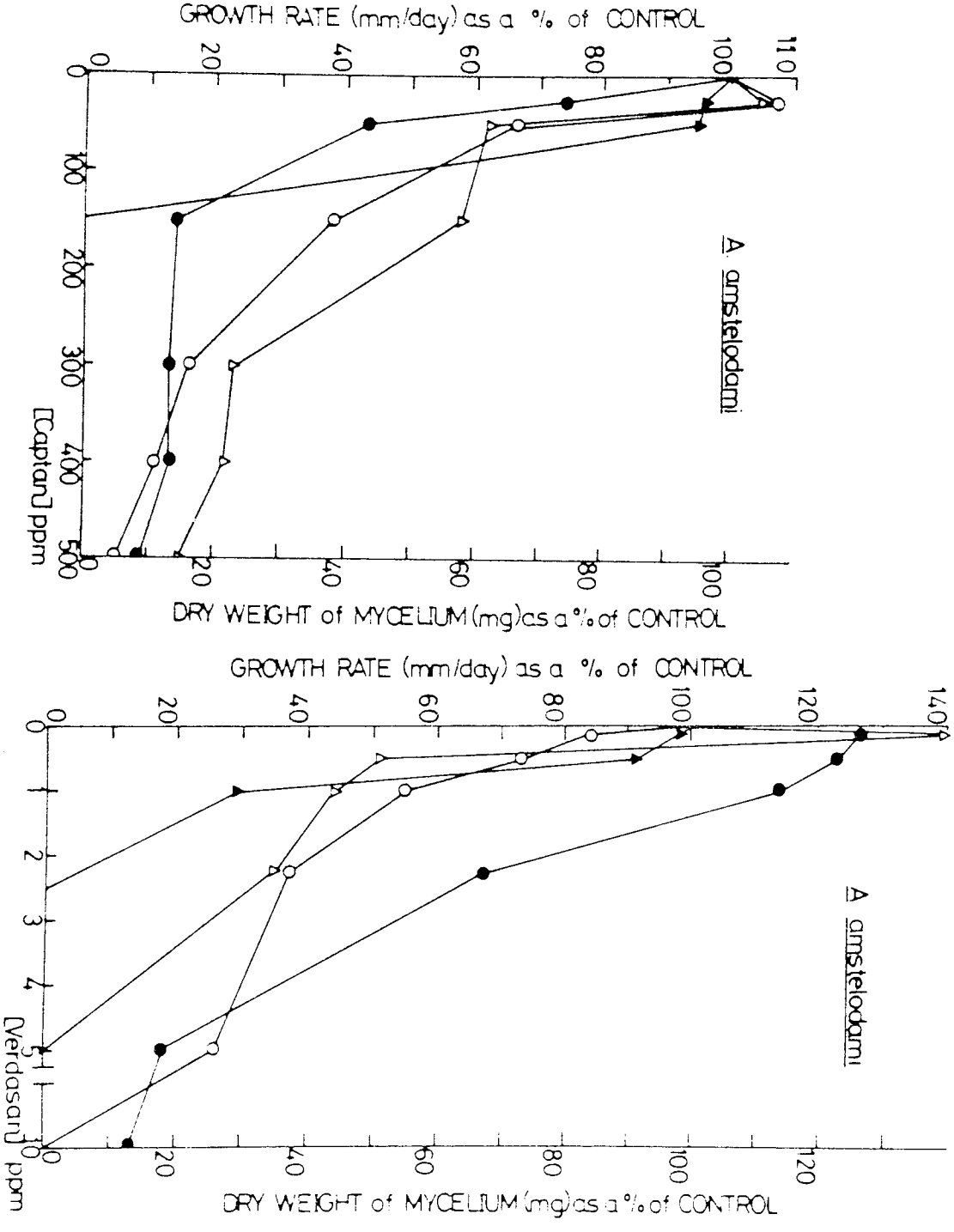
6.3.1 Effect of Fungicides on the Exo-enzyme Production

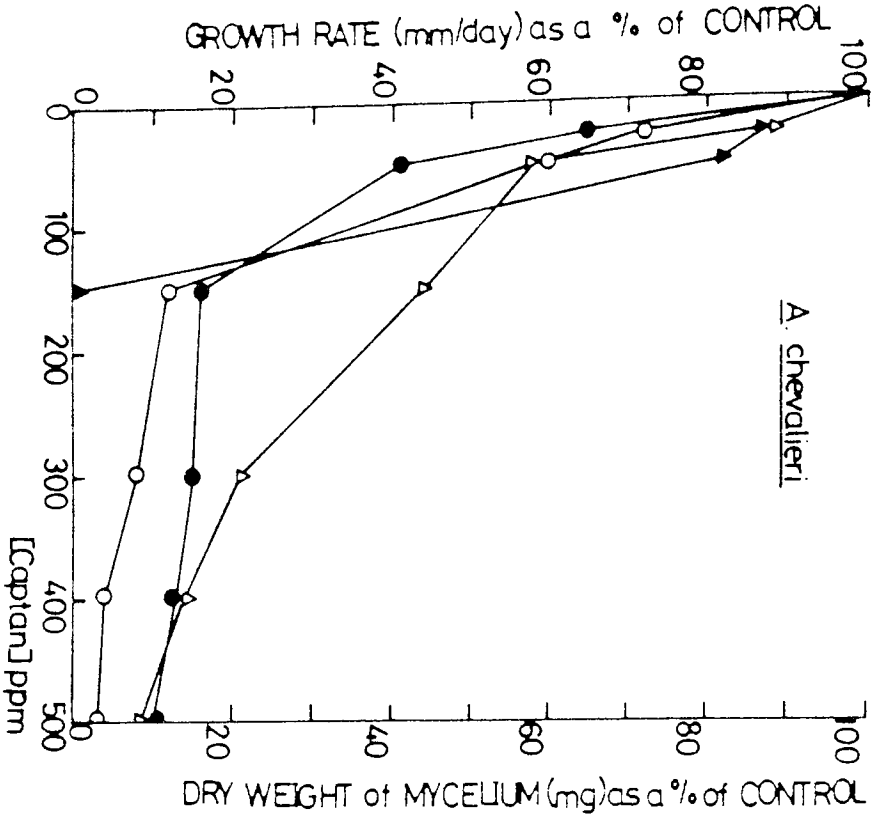
The technique used in the detection of exo-amylase, lipase and protease production by test fungi was similar to that used in Chapter 5.

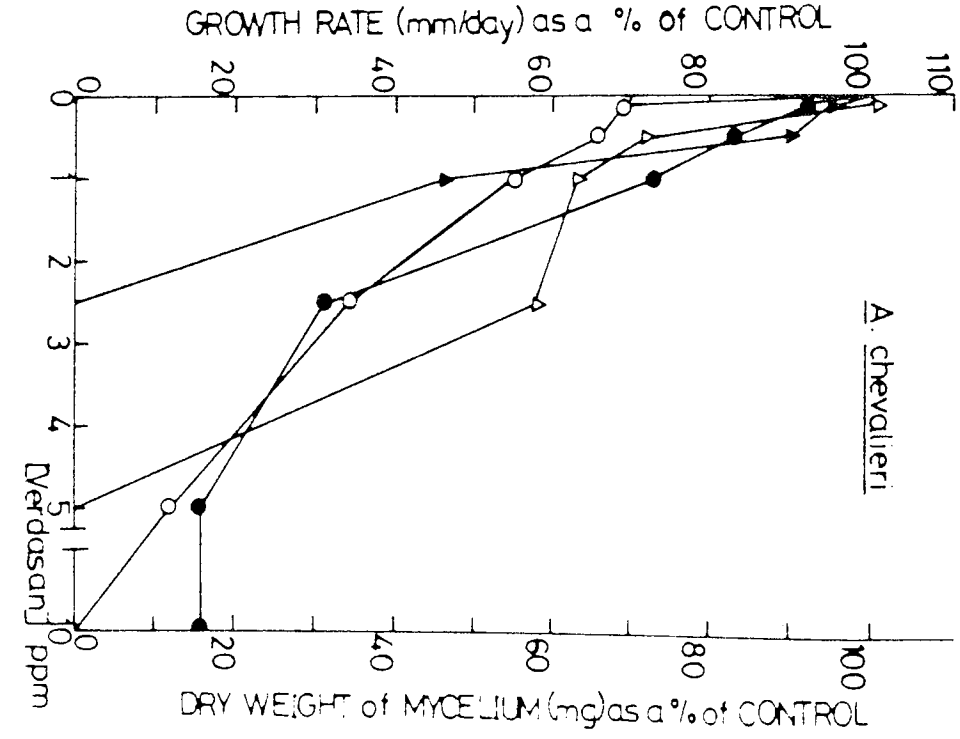
To known volumes of sterile, cooled, liquid peptone lab-lemco agar (PLA) and PLA + glycerol to give a_w 0.95 (PLAG) containing the enzyme substrate, appropriate volumes of fungicide from stock solutions were added to give final concentrations of 50 and 500ppm of captan and 0.5 and 10ppm verdasan. Media without the fungicide were used as control.

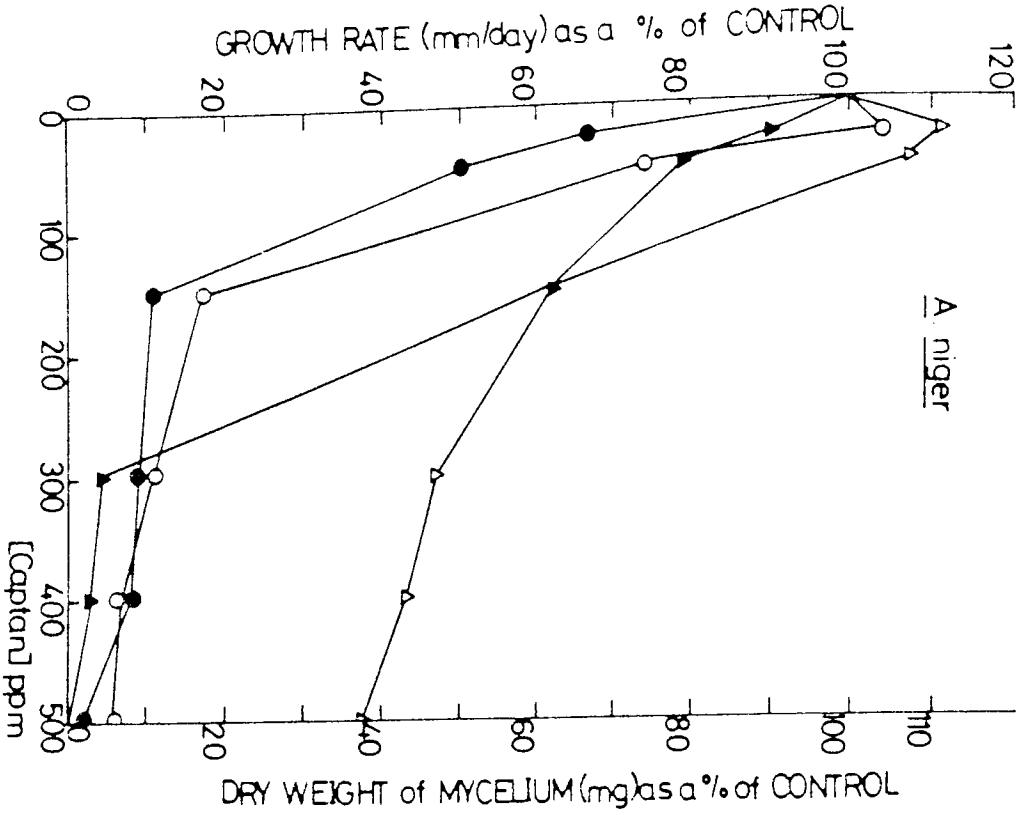
FIGURE 6.1 : THE EFFECT OF INCREASING CONCENTRATIONS OF FUNGICIDES ON THE GROWTH OF TEST FUNGI USING DIAMETRIC GROWTH RATES AND MYCELIAL DRY WEIGHTS

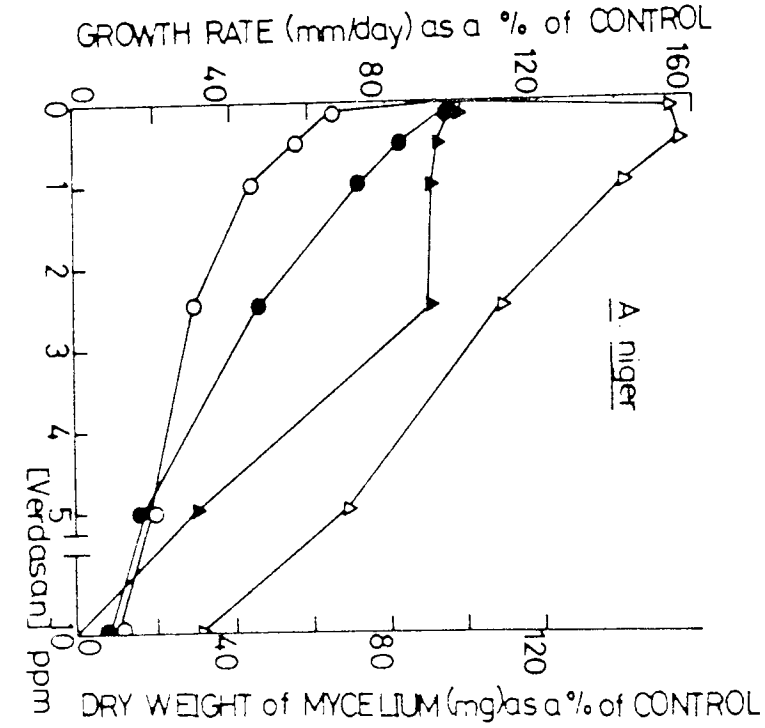



GROWTH RATE (mm/day) as a % of CONTROL
0
20
40
60
80
100
110
0
100
200
300
400
500
[Captan] ppm
A. arrhenatheri
DRY WEIGHT of MYCELIUM (mg) as a % of CONTROL
GROWTH RATE (mm/day) as a % of CONTROL
0
20
40
60
80
100
120
140
0
1
2
3
4
5
[Verdasan] ppm
A. arrhenatheri
DRY WEIGHT of MYCELIUM (mg) as a % of CONTROL


GROWTH RATE (mm/day) as a % of CONTROL
0
20
40
60
80
100
A. chevalieri
[Captan] ppm
0
100
200
300
400
500
DRY WEIGHT of MYCELIUM (mg) as a % of CONTROL


GROWTH RATE (mm/day) as a % of CONTROL
0
20
40
60
80
100
110
A. chevalieri
[Verdasan] ppm
0
1
2
3
4
5
10
DRY WEIGHT of MYCELIUM (mg) as a % of CONTROL


GROWTH RATE (mm/day) as a % of CONTROL
0
20
40
60
80
100
120
A. niger
[Captan] ppm
0
100
200
300
400
500
0
20
40
60
80
100
110
DRY WEIGHT of MYCELIUM (mg) as a % of CONTROL


GROWTH RATE (mm/day) as a % of CONTROL
0
40
80
120
160
A. niger
[Verdasan] ppm
0
1
2
3
4
5
10
0
40
80
120
DRY WEIGHT of MYCELIUM (mg) as a % of CONTROL

Using a sterile, automatic pipette-syringe, 12ml of the modified medium was added to sterile 1.5cm diameter x 15cm length test tubes, provided with suitable oxoid caps. After inoculation with plugs of fungal mycelium, the tubes were placed in a_w controlled aquari and incubated at $30^{\circ}C \pm 1.0^{\circ}C$. Eight replicates were used for each sample - two replicates were sacrificed at weekly intervals for four weeks. Methods of measuring the various enzyme activities were similar to those used in Chapter 5.

6.3.2 Results

Figure 6.2 shows the amylase activities of the test fungi at weekly intervals for four weeks at increasing concentrations of captan and verdasan with PLA and PLAG as basal media. Amylase activity was not detected at 500ppm of captan and 10ppm of verdasan in A. amstelodami and at 10ppm of verdasan in A. chevalieri. In the latter, amylases were only detected in the fourth week at 500ppm of captan.

In all the test fungi, amylase activity was equal to or higher with PLA as the basal medium than with PLAG, containing either fungicide, except in A. amstelodami, where at 50ppm of captan, the amylase activity from the second to the fourth week was slightly higher in PLAG as the basal medium than PLA.

The production of lipases at weekly intervals with increasing concentrations of captan and verdasan are shown in figure 6.3. Lipases were produced by the test fungi at all fungicide concentrations.

There was a decrease in lipase activity from the control tubes (PLA or PLAG) with increasing concentrations of fungicides, except in A. niger where the final lipase production was higher at 50ppm of captan than the controls.

Lipase production after four weeks was higher at any concentration with PLA as the basal medium than PLAG.

Protease activities of A. amstelodami and A. chevalieri are shown in figure 6.4. Except in PLAG control tubes, A. chevalieri did not show any protease activity.

The enzyme activity was inhibited in both fungi at 500ppm of captan. In A. amstelodami, protease production was enhanced at a particular concentration with PLAG as the basal medium than PLA and vice versa in A. niger, where proteases were not produced in PLAG medium at 10ppm of verdasan.

6.4.1 Effects of Fungicide on Potassium Ion Leakage

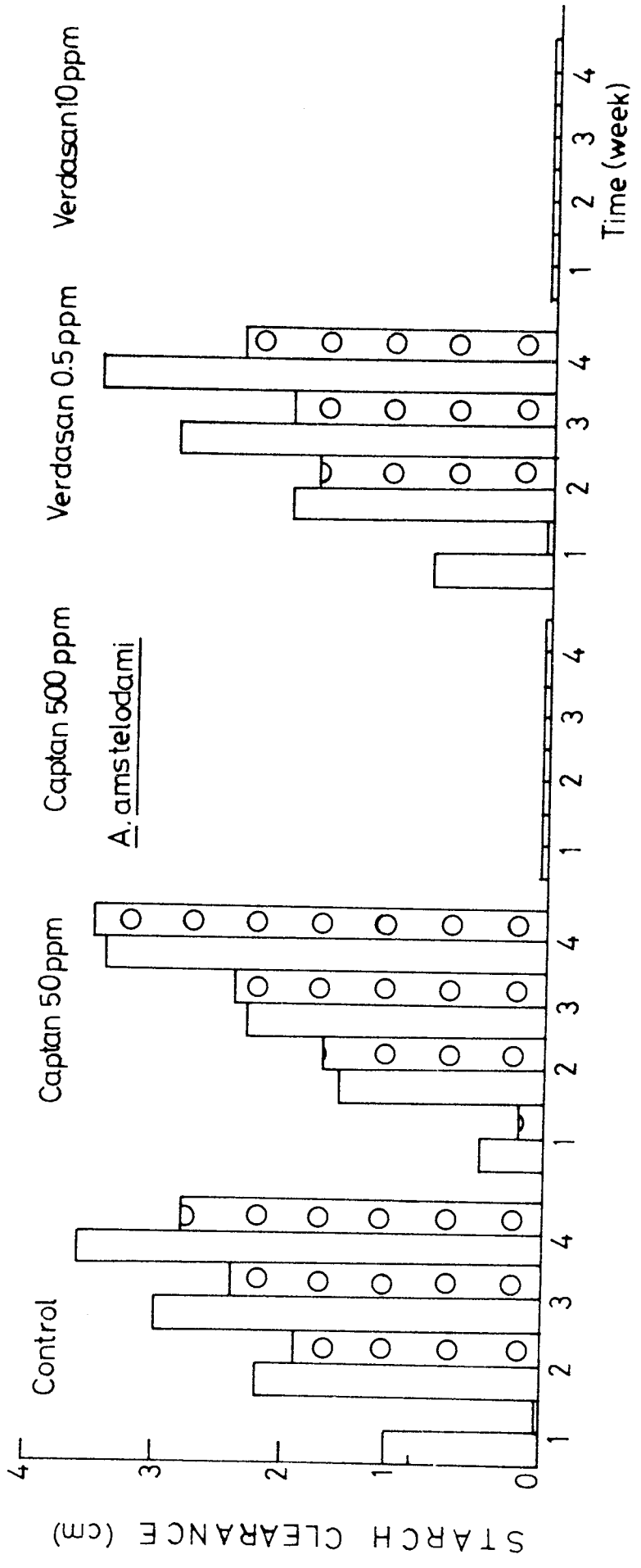
The measurement of metabolite leakage as an indication of membrane disruption in fungi has been used by several workers including MacDonald (1980). Viability of plant and fungal propagules have been determined by the extent of metabolite loss from the cell membrane.

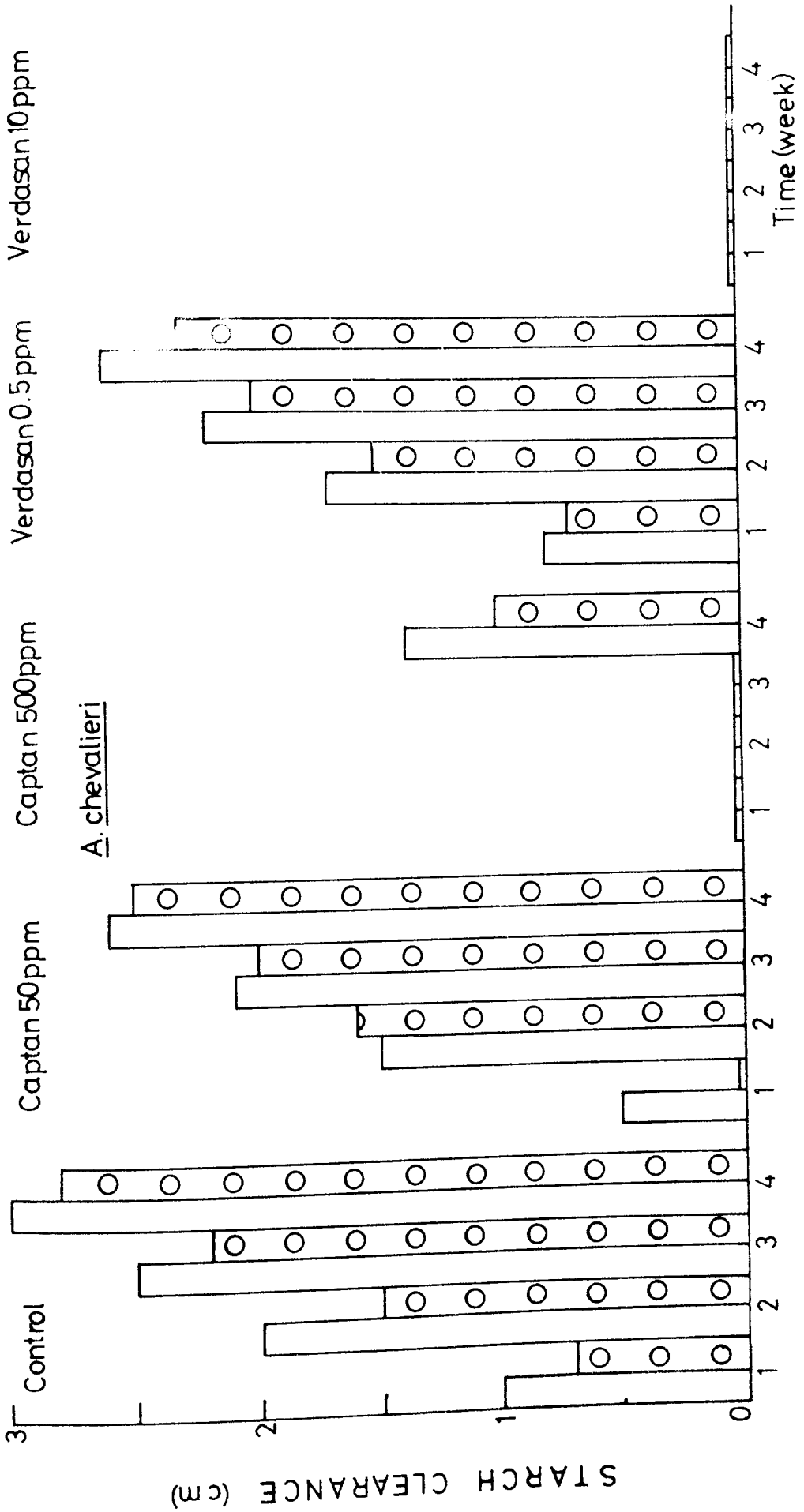
The effect of increasing concentrations of captan and verdasan on the permeability of the cell membranes of the test fungi to K^+ was

FIGURE 6.2 : AMYLASE ACTIVITIES OF TEST FUNGI AT INCREASING CONCENTRATIONS OF FUNGICIDES

PLA

PLAG


STARCH CLEARANCE (cm)
0
1
2
3
4
Control
Captan 50 ppm
Captan 500 ppm
Verdasan 0.5 ppm
Verdasan 10 ppm
A. amstelodami
Time (week)
1
2
3
4


A. chevalieri
Control
Captan 50ppm
Captan 500ppm
Verdasan 0.5ppm
Verdasan 10ppm
Time (week)
STARCH CLEARANCE (cm)
0
1
2
3
4

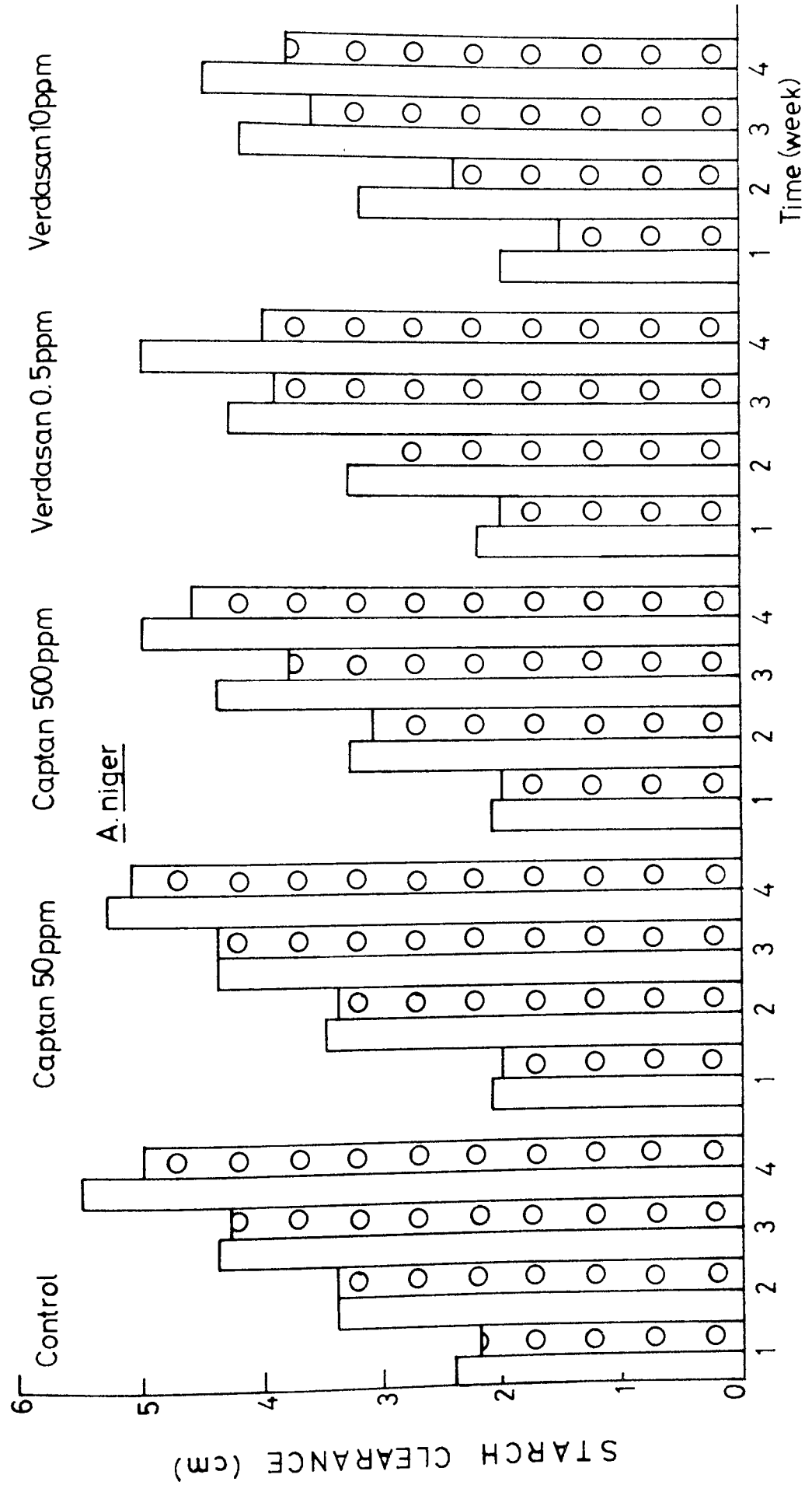
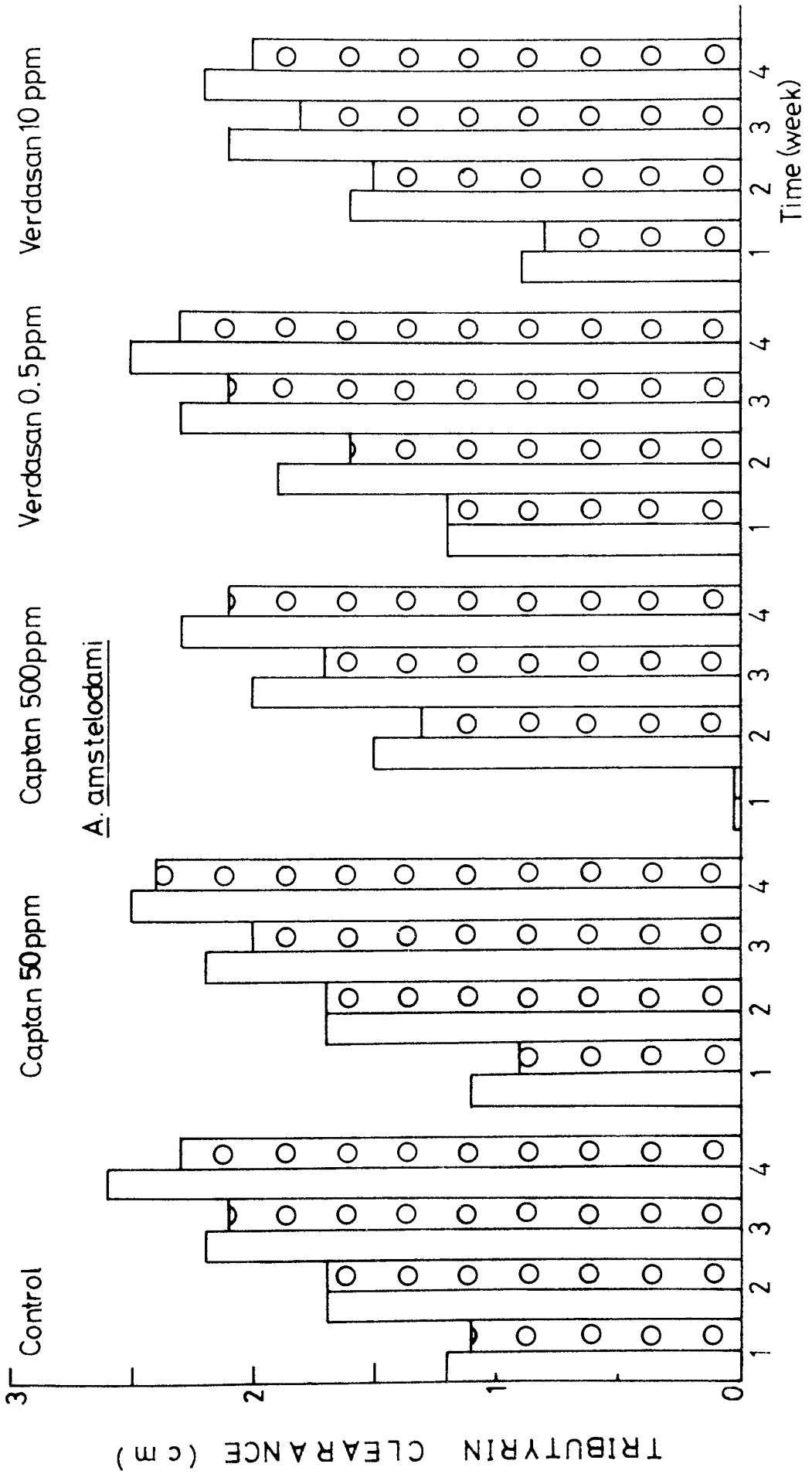
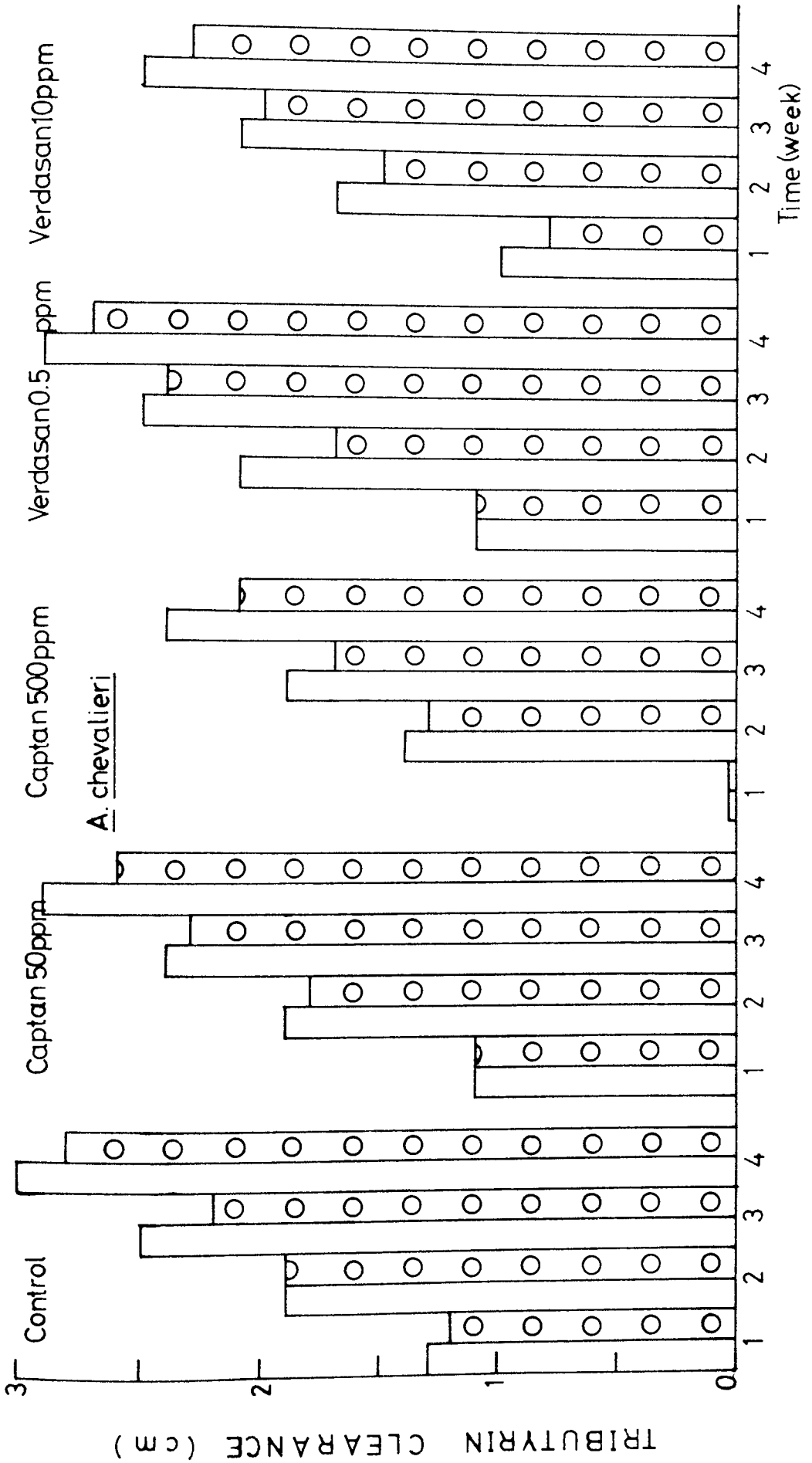

STARCH CLEARANCE (cm)
0
1
2
3
4
5
6
Control
Captan 50ppm
Captan 500ppm
Verdasan 0.5ppm
Verdasan 10ppm
A. niger
1 2 3 4
Time (week)

FIGURE 6.3 : LIPASE ACTIVITIES OF TEST FUNGI AT INCREASING CONCENTRATIONS OF FUNGICIDES

PLA

PLAG


TRIBUTYRIN CLEARANCE (cm)
0
1
2
3
Control
Captan 50ppm
Captan 500ppm
Verdasan 0.5ppm
Verdasan 10 ppm
A. amstelodami
1 2 3 4
Time (week)


TRIBUTYRIN CLEARANCE (cm)
0
1
2
3
Control
Captan 50ppm
Captan 500ppm
A. chevalieri
Verdasan0.5 ppm
Verdasan10ppm
1
2
3
4
Time (week)

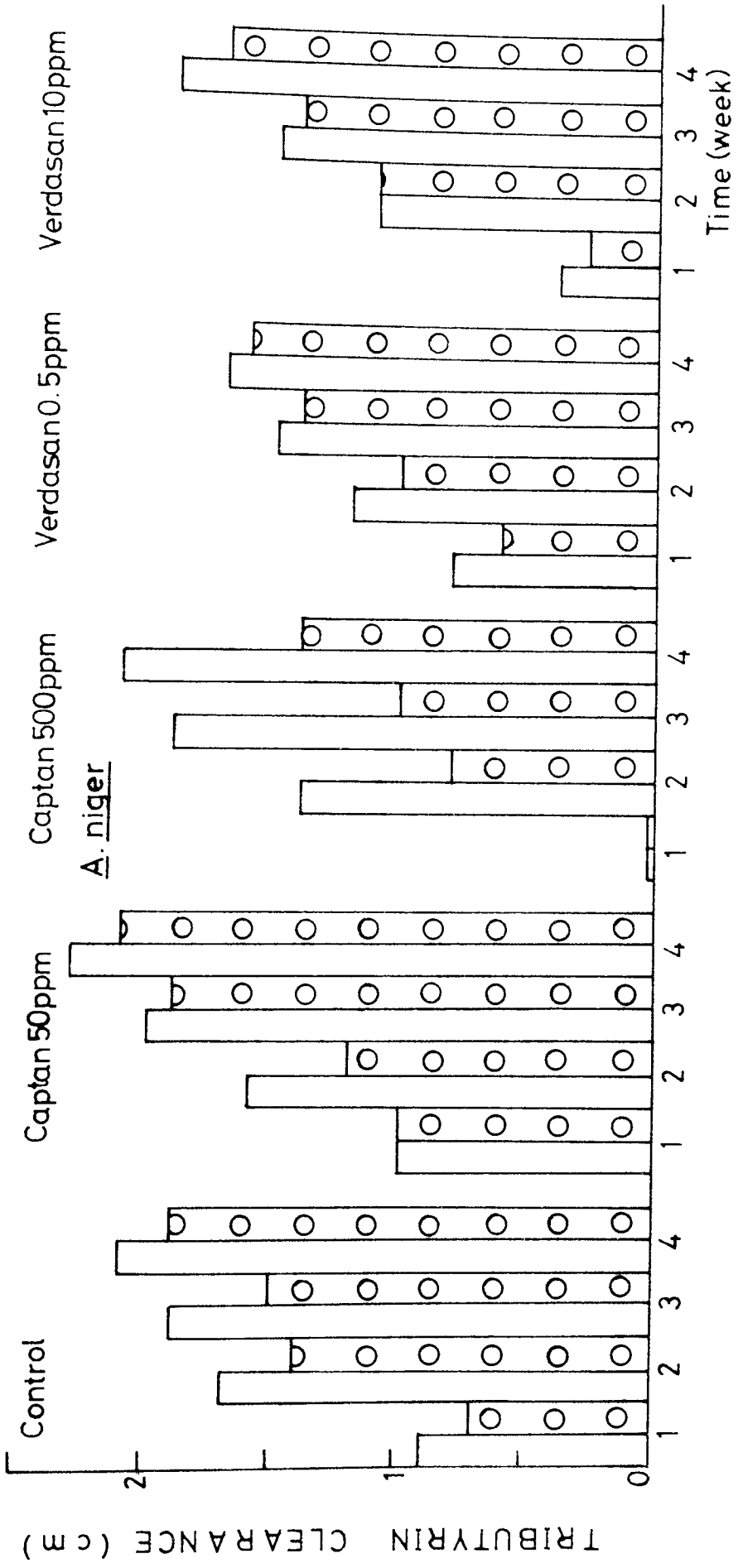
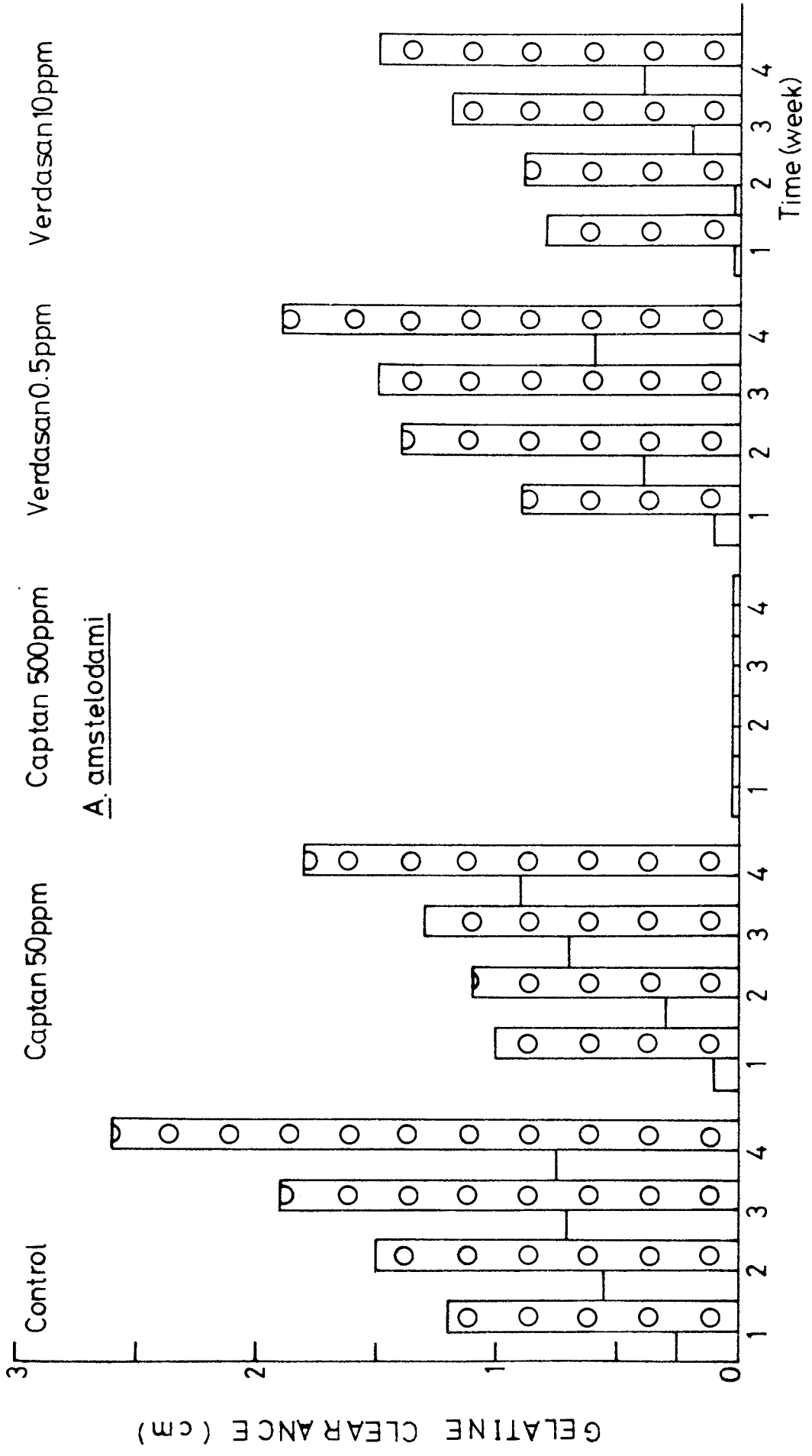
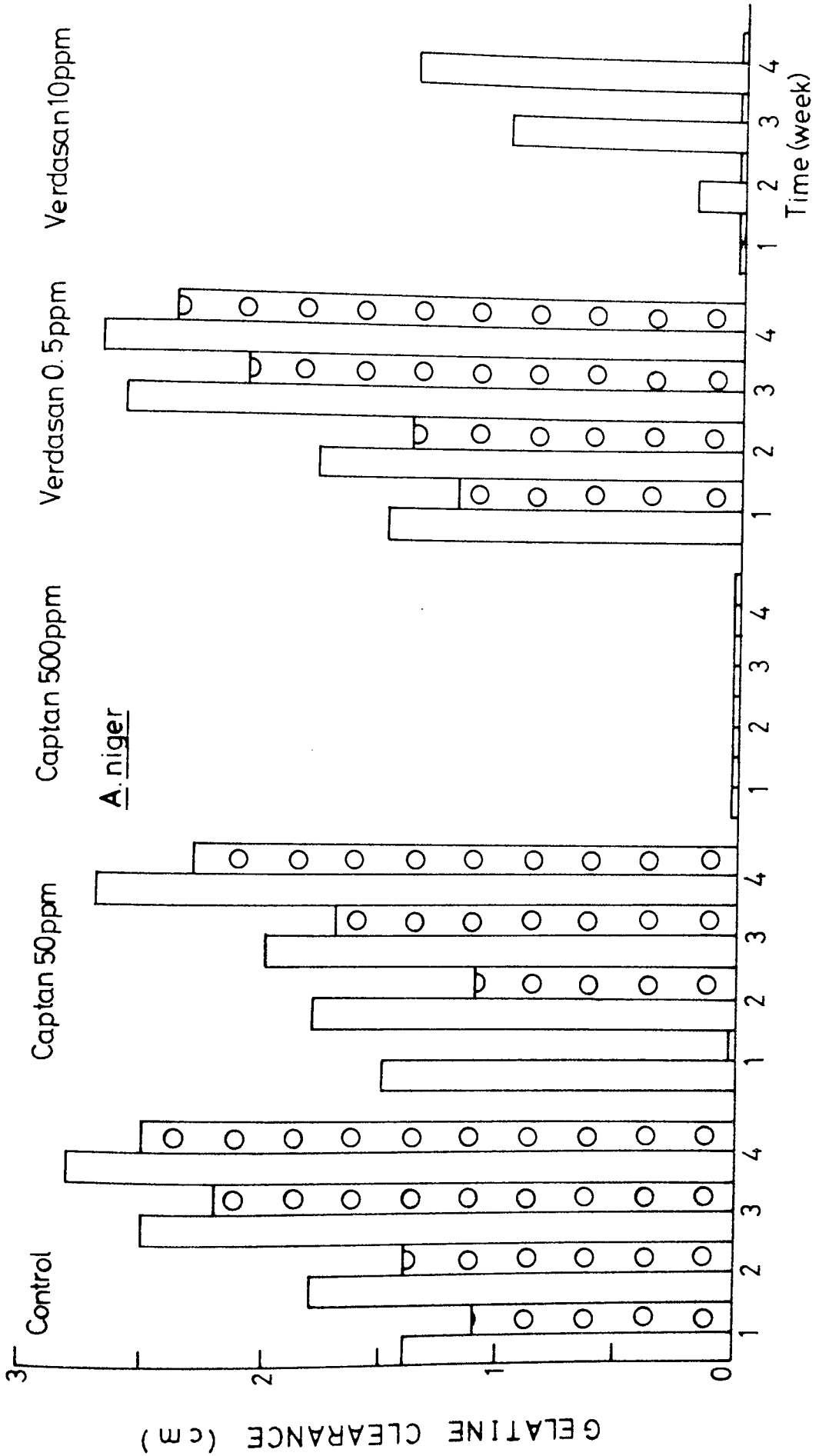

TRIBUTYRIN CLEARANCE (cm)
0
1
2
Control
Captan 50ppm
Captan 500ppm
Verdasan 0.5ppm
Verdasan 10ppm
A. niger
1 2 3 4
Time (week)

FIGURE 6.4 : PROTEASE ACTIVITIES OF TEST FUNGI AT INCREASING CONCENTRATIONS OF FUNGICIDES

PLA

PLAG


A. amstelodami
Control
Captan 50ppm
Captan 500ppm
Verdasan0.5ppm
Verdasan10ppm
Time (week)
GELATINE CLEARANCE (cm)
0
1
2
3
4


Control
Captan 50ppm
Captan 500ppm
Verdasan 0.5ppm
Verdasan 10ppm
A. niger
Time (week)
GELATINE CLEARANCE (cm)
0
1
2
3
4

investigated. Erlenmeyer flasks of 100ml capacity containing sterile, cooled MEB or MEBG were inoculated with plugs of fungal mycelium and incubated at 30°C in an orbital incubator rotating at 100 rpm. For each sample, five replicates were used. After five days, the resulting colonies were removed by suction filtration and gently washed with excess of distilled water which was kept at 30°C. Within 30 seconds of the removal of the colonies from their growth medium, they were transferred to fresh 100ml Erlenmeyer flasks, each containing 30ml of sterile solutions of captan (50 and 500ppm) and verdasan (0.5 and 10ppm) in distilled water or distilled water modified with glycerol to give a_w 0.95. Colonies cultured in MEB were transferred to flasks containing the various concentrations of fungicides in distilled water, while colonies cultured in MEBG were transferred to flasks containing the various concentrations of fungicides in glycerol modified distilled water. This was to avoid osmotic shock to the fungal mycelium. Flasks without the fungicides were used as control. The flasks were incubated at 30°C for 24 hours in an orbital incubator rotating at 60rpm. The reduced speed of rotation was to ensure that the fungal mycelium was not damaged.

After the incubation period, the colonies were harvested by suction filtration onto an oven dried, preweighed Whatman No.1 filter paper. They were dried at 90°C to constant weight.

The levels of K^+ in the filtrate were assayed with an EEL flame photometer. Reading of K^+ content in mg/l of the filtrate were read off a previously constructed standard curve (Appendix 6.6)

6.4.2 Results

Figure 6.5 shows the K^+ leakage from fungal mycelium with increasing concentrations of fungicides. The values of K^+ leakage have been presented after subtraction from the controls, i.e. without fungicides.

From the results, two points emerge:

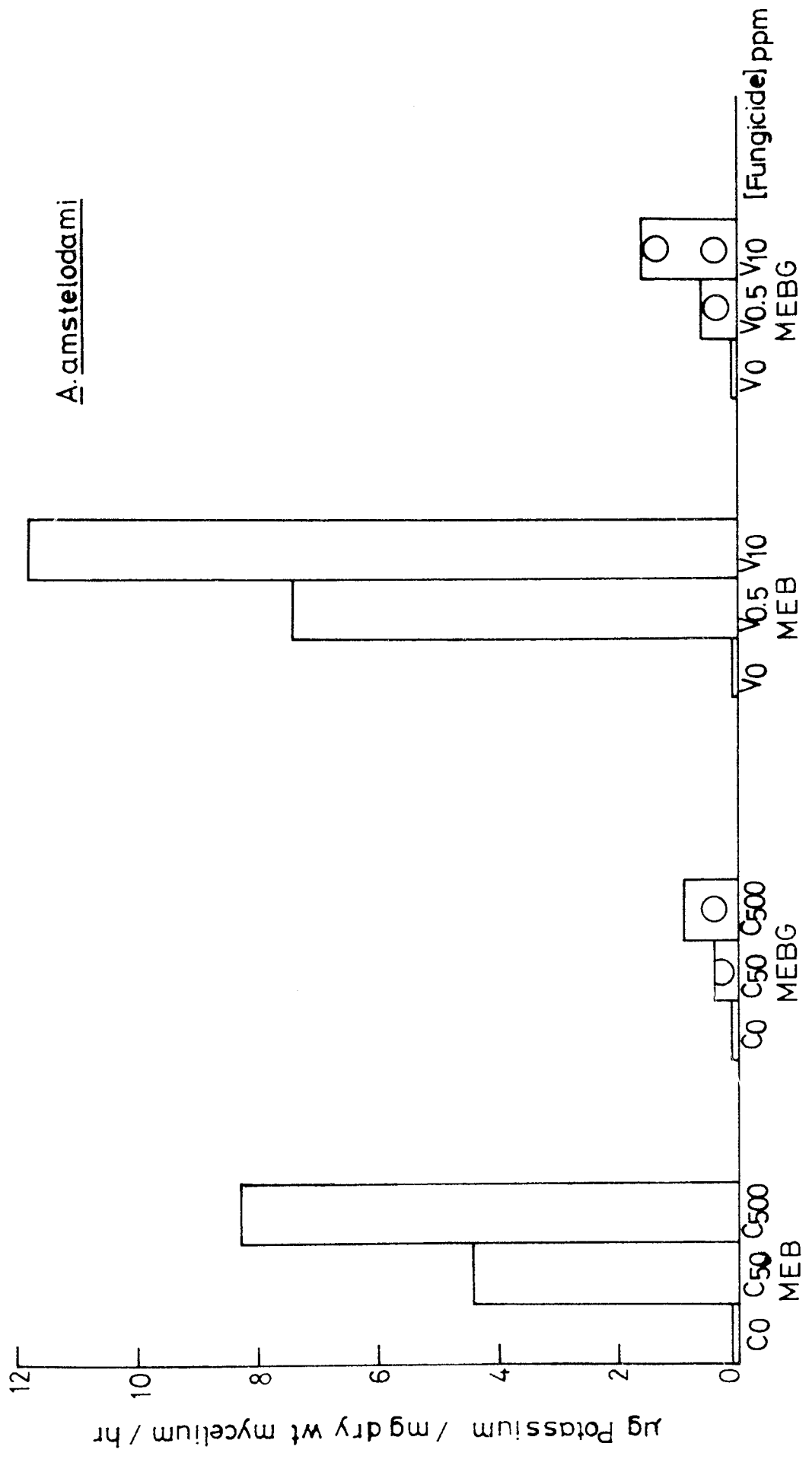
1) K^+ leakage was significantly reduced at both concentrations of either fungicide when the mycelium was grown in MEBG and incubated in distilled water modified with glycerol than in distilled water.

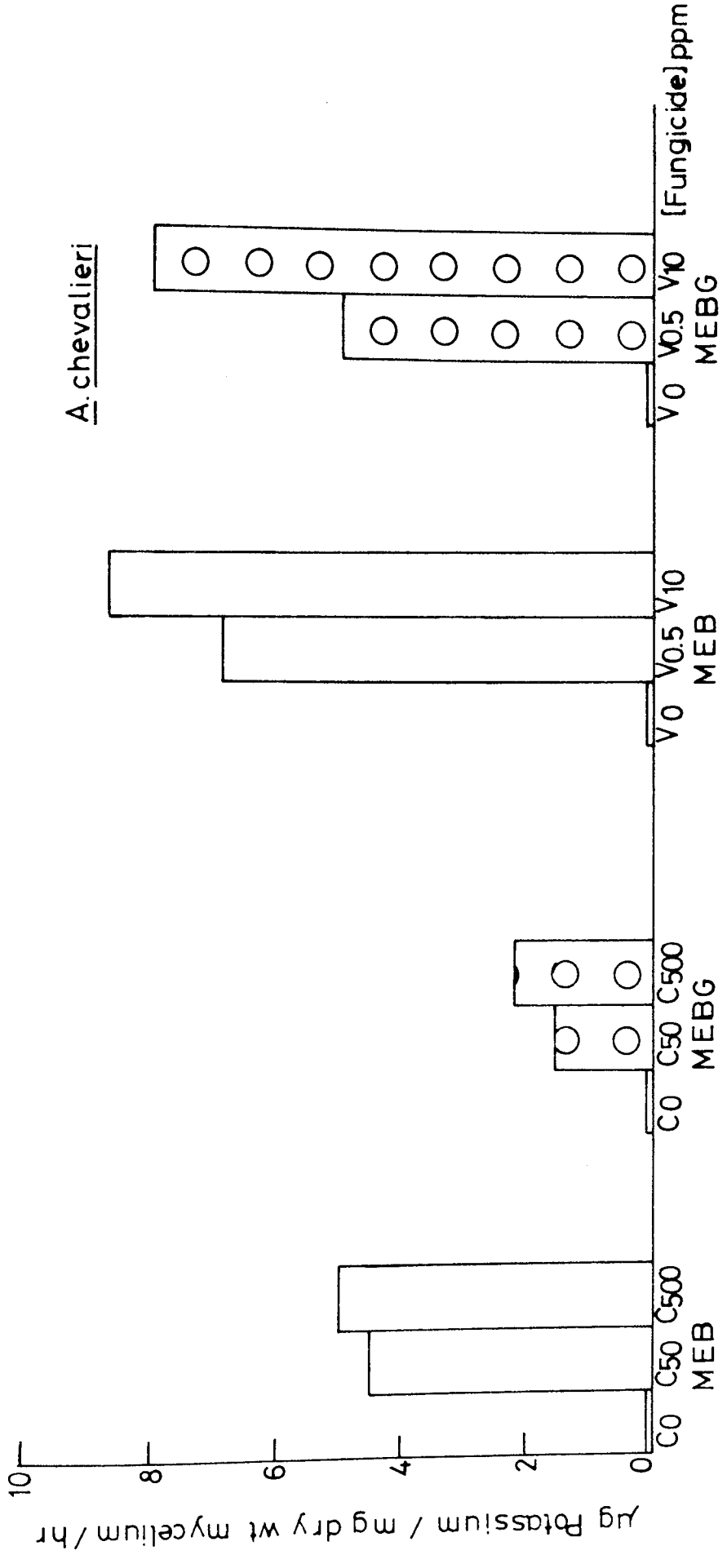
2) K^+ leakage from fungal mycelium is significantly increased in the presence of verdasan than captan.

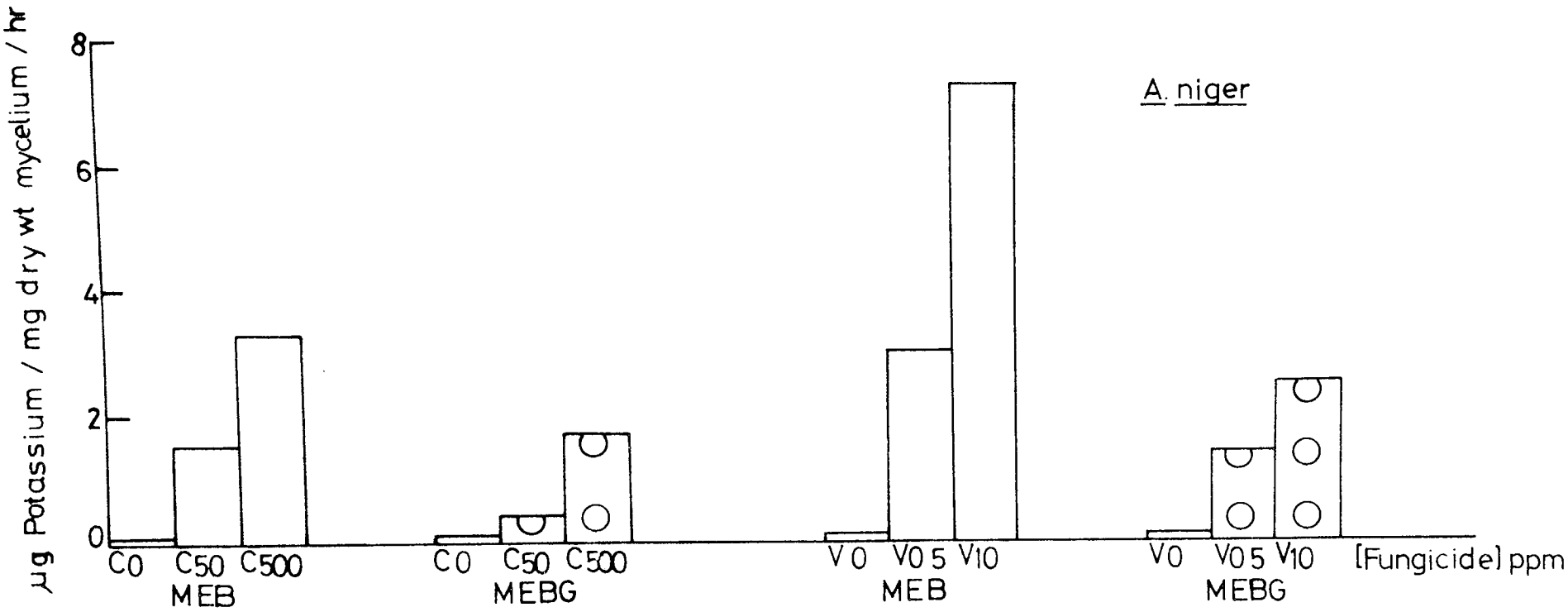
6.5 Discussion

Captan and verdasan were generally found to reduce the growth rate and mycelial production in the test fungi. The growth rates of *A. amstelodami* and *A. niger* on MEA increased at low concentrations of captan and verdasan. Increasing concentrations of either fungicide into MEB decreased mycelial production in the fungal species. This may be due to the chelation of the fungicides to the thiol groups (SH) in the agar medium. Greenaway (1973), studying the in vitro toxicity of mercury compounds attributed this to the presence of mercury-chelating thiol groups in the yeast extract and amino groups in asparagine present in the agar medium.

FIGURE 6.5 : POTASSIUM LEAKAGE FROM TEST FUNGI AT INCREASING CONCENTRATIONS OF FUNGICIDES IN MEB AND MEBG MEDIA


A. amstelodami
µg Potassium / mg dry wt mycelium / hr
0
2
4
6
8
10
12
C0 C50 C500
MEB
C0 C50 C500
MEBG
V0 V0.5 V10
MEB
V0 V0.5 V10
MEBG
[Fungicide] ppm


A. chevalieri
µg Potassium / mg dry wt mycelium / hr
0
2
4
6
8
10
C0
C50
C500
MEB
C0
C50
C500
MEBG
V0
V0.5
V10
MEB
V0
V0.5
V10
MEBG
[Fungicide] ppm


µg Potassium / mg dry wt mycelium / hr
8
6
4
2
0
A. niger
C0 C50 C500
MEB
C0 C50 C500
MEBG
V0 V0 5 V10
MEB
V0 V0 5 V10
MEBG
[Fungicide] ppm

Generally, the amylase, lipase and protease activities decreased with increasing concentrations of either fungicide. Amylase production was inhibited at 500ppm of captan and 10ppm of verdasan in A. amstelodami; 10ppm of verdasan in A. chevalieri and protease production was inhibited at all concentrations of fungicides in A. chevalieri. In T. viride (Reese and Mandels, 1957) and Basidiomycete 235M (MacDonald, 1980), mercury ions were found to generally reduce cellulase activity.

Secretions of exo-enzymes at increasing concentrations of fungicides suggest that the fungi are able to detoxify the fungicides to some degree. Richmond and Somers (1966), studying captan uptake by conidia of Neurospora crassa reported that captan reacted with the soluble thiol pool and the insoluble thiol groups of the proteins. They suggested that while the former is a detoxification process, the latter reaction may be the reason of its toxic action. The mercury tolerance of A. niger mycelium was attributed to the complexing of the mercury with a pool of non-protein thiol groups, thus protecting essential enzyme systems (Ashworth and Amin, 1964).

Metabolite leakage from organisms is normally a passive process, which can be related to either of two reasons, i.e. alteration of membrane structure (Simon, 1974) or combination of an alteration in membrane structure and interference with the uptake mechanisms of membranes (Sahid et al., 1981).

From the results, it was found that there was an increase in K^+ leakage with increasing concentrations of fungicides. Slayman (1970), reported that the uptake of potassium was effected by the potassium - sodium - hydrogen pump in Neurospora. Slayman and Tatum (1965), suggested that the pump was driven by a metabolic process, the reason being that the membrane potential is rapidly reduced by metabolic inhibitors.

There was an overall, significantly higher K^+ leakage from the mycelium in verdasan than captan. Reduced leakage in captan may be due to the nature of its toxicity. It has been reported that captan may not effect the lipoprotein structure of the fungal membrane. On the other hand, Rothstein (1959), reported that mercury based compounds inhibited metabolic phosphorylation and the metal interacted with fungal membrane ligands. Reaction of mercury with any sulphydryl group in the membrane structure would probably lead to a formation of S-Hg-S bridges, resulting in a generalised breakdown of all membranes.

An interesting observation and one that would warrant further investigation is that decreased K^+ leakage in both fungicides at corresponding concentrations in MEBG than MEB. Solutes controlling a_w can be divided into those which will penetrate the cell and those which do not, the exact permeability characteristics varying according to the organism and the selectivity of its cytoplasmic membrane. Scheie (1969), reported that in Escherichia coli B/r, non-penetrating solutes such as calcium chloride, sodium chloride and sucrose caused

plasmolysis and Alemohammad and Knowles (1974), found that in E. coli, penetrating solutes such as glycerol or urea cause short term plasmolysis followed by deplasmolysis. The stress of stretching or contracting of the cytoplasmic membrane was found to effect the pore size, hence the permeability to different agents (Macleod et al., 1978).

Kroll and Anagnostopoulus (1980), reported that the bactericidal activity of phenol was reduced by the penetrating solute, glycerol and enhanced by the non-penetrating solute, sucrose. Glycerol may have similar effects on the growth and leakage of K^+ from the test fungi in the presence of the fungicides.

CHAPTER SEVEN

DISCUSSION, CONCLUSIONS AND PROPOSALS FOR FUTURE WORK

Discussion, Conclusions and Proposals for Future Work

The role of microorganisms, their ability to survive extreme environments and the utilization of almost any substrate provides a fascinating and still not fully understood topic of study. The use of a_w as a parameter to describe water relations in microbial ecology and physiology has received a major impetus from microbiologists.

The microbial ecology of organisms that can tolerate low substrate a_w by the addition of solutes to the substrate can be broadly divided into two groups. Firstly, in foods, where to prevent spoilage, high concentrations of salt and sugars were added long before the existance of microorganisms was recognised. Secondly, in nature, there exist many salt and sugar-rich areas, e.g. lakes, oceans, salt marshes, concentrated plant saps and floral nectaries.

The research programme was undertaken to study the effects of substrate a_w on fungal growth limits and its interactions with other growth factors such as pH, temperature and the effects of a_w on exo-enzyme production and biocide efficiency. Although the project was approached from a theoretical angle, the applied implications of a_w were also considered. The reasons for such an approach were these - firstly, the effects of a_w were studied in a homogenous system; secondly, pure culture studies produced data on the relative abundance and behaviour of different fungal species which we found in nature and cause biodeterioration at decreasing a_w, and thirdly, techniques were devised or adapted to study a_w effects on particular growth aspects, e.g. enzyme production and thermal resistance of spores. Studies of such kind would eventually

lead to the understanding of the physiological basis of tolerance of reduced substrate water availability.

Addition of solutes to the substrate, i.e. agar medium gave accurate and easily controllable a_w values, the merits of this method having been dicussed already in Chapter 1. Glycerol and NaCl were used as controlling solutes because they are commonly used in food preparations and NaCl is frequently encountered in natural habitats.

The series of isolations from five ecologically different soil types at decreasing a_w did produce a range of fungal species. The incidence of Aspergillus and Penicillium species isolations at decreasing a_w were increased, a trend observed by Griffin (1963), Chen(1964) and Chen and Griffin (1966). It is interesting to note that isolation trends and fungal species obtained by decreasing the soil moisture stress in either of two methods, i.e. equilibration of the soil sample at controlled R H (Griffin, 1963; Chen and Griffin, 1966) or the addition of solute/s to control the a_w (Chen, 1964 and from present studies), presented similar patterns. This would indicate that the solute type does play an important but a subordinate role to a_w in the microbial growth. This was later verified in pure culture studies.

Fungal species were isolated from all soil types at decreasing a_w, suggesting that xerophytic species were present within all the ecologically different soils used. Griffin (1972), found no evidence for the selection of a xerophytic population in arid zones and reported that fungi were equally capable in soils from an English pasture and an

Australian rainforest as from a desert at corresponding low soil water potential.

Growth of the A. glaucus group test fungi, A. amstelodami, A. chevalieri, A. repens and A. ruber, on high a_w media (MEA) was greatly reduced. Optimum growth was obtained at decreased a_w levels around 0.95, with either glucose, glycerol or NaCl as the controlling solutes. It is therefore implied by the results that growth was enhanced by lowering the a_w than by specific solute action. Extremely xerophilic fungi, X. bisporus (Scott, 1957; Pitt and Christian, 1968) and S. sebi (Ormerod, 1967) have shown similar requirements for reduced substrate moisture for growth. Having said that, the solute type in general doeshave influence on the fungal growth within a_w limits of growth. The effects of glycerol and NaCl at corresponding a_w varied from one isolate to another. At optimum a_w of growth, there was an increased tolerance to pH.

Hyphal susceptibility to heat was higher than that of the spores in A. amstelodami, A. flavus, A. fumigatus, A. versicolor and P. citrinum. The addition of glycerol or NaCl, i.e. decreasing the a_w, to the heating menstruum failed to protect the ascospores/spores at corresponding a_w to an equivalent degree. Doyle and Marth (1975B), reported that at a_w values above 0.95, NaCl was less protective than glucose and sucrose but, at a_w values below 0.95, the protective action of NaCl was greater than glucose and sucrose in spores of A. flavus and A. parasiticus strains. Ascospores of A. amstelodami were thermally more resistant

than the spores. Clamydospores of Humicola fuscoatra and ascospores of Byssochlamys fulva were found to be more resistant to dry heat than spores of A. niger (von-Schelhorn and Heiss, 1975).

Only from results obtained from bacteria and yeasts can the protective mechanism to moist heat in fungi at low a_w be speculated. The leakage of intracellular substances from heat-treated cells of Candida utilis and E. coli was not proportional to the loss in viability of cells (Shibasaki and Tsuchido, 1973). They concluded that there was no quantitative relationship between cell membrane damage caused by exposure to high temperature and leakage, which was due to a secondary effect as a result of cellular degradation or membrane damage. It is possible that the maximum heat resistance of these fungi may occur in the region of the water monolayer zone of cell hydration, where the cell water is firmly bound and this may correspond at the optimum water content for heat stability of the proteins of cell component/s whose destruction would cause thermal death.

Enzymes are produced by fungi capable of growth on particular substrates. These include natural and stored products, e.g. oils, grains etc. On the other hand, enzymes are added to food manufacture processes such as meat tenderization, wine clarification and often the consumer will use enzyme preparations on an empirical basis. It is however up to the food manufacturer to acquire knowledge of the biochemistry of the enzyme action as the storagibility of such enzyme preparations is very important.

The a_w of the environment is known to influence the metabolism of microbial cells through effects on enzyme and nucleic acid systems (Potthast et al., 1975) available water within the substrate provides an important carrier function i.e. the transportation of the substrate to the enzyme. In this investigation, four commonly encountered substrates, starch, cellulose, tributyrin and gelatinewere used to study the amalyase, cellulase, lipase and protease activities of the test xerophilic and non-xerophilic fungi at decreasing a_w levels. The use of test tubes provided a simple and accurate method for the semi-quantitative measure of enzyme production.

At pH of about optimum enzyme production, the enzyme activities were strongly influenced by the available substrate water (a_w) and the nature of the controlling solute. Enzyme production in non-xerophiles was limited to a_w levels above and up to 0.90, but xerophiles, especially A. glaucus group test fungi produced amylases, lipases and proteases below 0.90 a_w. In general, lipases were produced at very low substrate moisture values. Potthast et al. (1975), found that lipid substrates were hydrolysed at a R.H. as low as 10%. Acker and Wiese (1972 A, B), postulated that the mobility of unsaturated lipids was sufficient to permit the formation of enzyme-substrate complexes. If so, the water molecules function as a substrate for lipolytic processes and a_w is important for the velocity of hydrolysis and reaction equilibrium.

The formulation and use of fungicides are complicated procedures, requiring expertise to evaluate the requirements of different situations.

Where the osmotic state of the microbial cell is effected, the uptake and activity of the antimicrobial agent is of particular significance.

Captan and verdasan were found to decrease the growth rate, exo-enzyme production and increase in K^+ leakage in A. amstelodami, A. chevalieri and A. niger. K^+ leakage was found to be significantly higher from the mycelium in verdasan than captan, indicating that the latter may not cause as much cell membrane damage as the former. The detoxification of both fungicides has been attributed to the reaction with the soluble, non-protein thiol pool within the microbial cells (Ashworth and Amin, 1964; Richmond and Somers, 1966).

The addition of glycerol to decrease the a_w produced a dramatic effect on the K^+ leakage. Leakage was reduced in either fungicide at corresponding concentrations in glycerol than in water. The penetration of glycerol into the fungal cells may have brought about plasmolysis followed by deplasmolysis. The consequent stress on the cytoplasmic membrane could have effected the permeability of the fungicides into the cell.

Finally, it can be concluded that certain fungal species are adapted to survival and growth in conditions of low a_w. Results obtained during this project indicate that more work has to be done for a further understanding of the tolerance by fungi to decreased substrate water availability. In the light of these observations, the following areas of research can be recommended for future work :

1) The role of internal solutes as compatible solutes in xerophilic fungi.

2) Increased thermal resistance at decreased a_w.

3) The effects of various solutes, including polyols on cell-free enzyme preparations.

4) Effects of various a_w controlling solutes on fungal cell membrane permeability and fungicide efficacy in their presence.

APPENDICES

Appendix 1

Media used in experimental work

Appendix 2.1

Fungi isolated from all soils

Appendix 2.2

Total number of fungi and their first appearance

Appendix 3.1

Effect of pH on the growth rates of test xerophilic and non-xerophilic fungi

Appendix 3.2

Combined effects of pH, solutes and a_w on the growth rates of test xerophilic and non-xerophilic fungi

Appendix 4.1

Effect of temperature on the growth rate of test fungi

Appendix 4.2

Effect of increasing temperature on the survival of ascospores/spores at decreasing water activity levels

Appendix 5.1

Enzyme substrate media

Appendix 5.2

Diametric growth of test xerophilic and non-xerophilic fungi on various media at decreasing a_w

Appendix 5.3

Enzyme activities of test xerophilic and non-xerophilic fungi on MEA and PLA media

Appendix 5.4

Enzyme activities from pH2 to pH10

Appendix 5.5

Amylase activities of test fungi at decreasing a_w - pH5

Appendix 5.6

Lipase activities of test fungi at decreasing a_w - pH7

Appendix 5.7

Protease activities of test fungi at decreasing a_w - pH7

Appendix 6.1

Effect of increasing concentrations of fungicides on the growth rates of test fungi. Growth rate also expressed as a % of control

Appendix 6.2

Effect of increasing concentrations of fungicides on the dry weight production of test fungi. Dry weight production also expressed as a % of control

Appendix 6.3

Effect of increasing concentrations of fungicides on the amylase activities of test fungi

Appendix 6.4

Effect of increasing concentrations of fungicides on the lipase activities of test fungi

Appendix 6.5

Effect of increasing concentrations of fungicides on the protease activities of test fungi

Appendix 6.6

Standard curve of potassium assay using potassium chloride

Appendix 6.7

Effects of increasing concentrations of fungicides on potassium leakage from test fungi

APPENDIX 1

MEDIA

1.1 Cellulose agar (Eggins and Pugh, 1962) (CA)

$KH_2 PO_4$	1.0g
$(NH_4)_2 SO_4$	0.5g
KCl	0.5g
L-Asparagine	0.5g
Oxoid Yeast Extract	0.5g
$MgSO_4 \cdot 7H_2O$	0.2g
$CaCl_2$	0.1g
Oxoid Agar No.1	20.0g
4% Ball-milled cellulose	250ml
Distilled water	750ml

pH 6.2

Sterilized by autoclaving at 121°C for 15 minutes

1.2 Czapek Dox agar with 20% Sucrose (C20S)

K_2HPO_4	1.0g
$NaNO_3$	2.0g
$MgSO_4\ 7H_2O$	0.5g
KCl	0.5g
$FeSO_4\ 7H_2O$	0.01g
Sucrose	200.0g
Oxoid Agar No.1	15.0g
Distilled water	up to 1 litre

pH 6.8

Sterilized by autoclaving at 115°C for 20 minutes

1.3.1 Distilled water agar (DWA)

Oxoid Agar No.1	15.0g
Distilled water	1 litre

pH 6.9

Sterilized by autoclaving at 121°C for 15 minutes

1.3.2 Distilled water agar + glycerol (a_w 0.95)

Same ingredients as 1.3.1 with glycerol (Analar grade) at 3.0 molal concentration

1.3.3 Distilled water agar + NaCl (a_w 0.95)

Same ingredients as 1.3.1 with NaCl (Analar grade) at 1.4 molal concentration

Sterilization in 1.3.2 and 1.3.3 was similar to that in 1.3.1

1.4.1 Malt extract agar (Oxoid CM59) (MEA)

Malt extract	30g
Mycological peptone	5g
Agar No.1	15g
Distilled water	1 litre

pH 5.4

Sterilized by autoclaving at 115°C for 10 minutes

1.4.2 Malt extract agar + glycerol (a_w 0.97 to 0.70)

Same ingredients as 1.4.1 with glycerol (Analar grade) at the following molal concentrations

a_w	Molality+
0.97	0.60
0.95	3.00
0.93	4.00
0.90	5.57
0.87	7.50

a_w	Molality[+]
0.85	8.50
0.83	9.50
0.80	11.50
0.77	13.00
0.75	14.80
0.73	16.00
0.70	18.30

[+]Molality = moles of solute/kg of solvent

Media above and including a_w 0.95 were sterilized by autoclaving at 115°C for 10 minutes

Media below a_w 0.95 were sterilized by steaming for 30 minutes

1.4.3 Malt extract agar + NaCl (a_w 0.97 to 0.80)

Same ingredients at 1.4.1 with NaCl (Analar grade) at the following molal concentrations

a_w	Molality
0.97	0.90
0.95	1.40
0.93	2.00
0.90	2.83
0.87	3.60
0.85	4.03
0.83	4.40
0.80	5.15

Sterilization of media of various a_w was similar to in 1.4.2

1.5.1 Malt extract broth (Oxoid CM 57) (MEB)

Malt extract	17g
Mycological peptone	3g
Distilled water	1 litre

pH 5.4

Sterilized by autoclaving at 115°C for 10 minutes

1.5.2 Malt extract broth + glycerol (a_w 0.95) (MEBG)

Same ingredients as 1.5.1 with glycerol (Analar grade) at 3.0 molal concentration

Sterilization similar to 1.5.1

1.6.1 Malt extract yeast extract 40% glucose agar (Pitt, 1975) (MY40G)

Malt extract	12g
Yeast extract	3g
Glucose	400g
Oxoid Agar No.1	12g
Distilled water	600g

pH 5.5 a_w 0.92

Sterilized by steaming for 30 minutes

1.6.2 Malt extract yeast extract 60% Glucose agar (Pitt, 1975) (MY60G)

Malt extract	8g
Yeast extract	2g
Glucose	600g
Oxoid Agar No.1	8g
Distilled water	400g

pH 5.3 a_w 0.85

Sterilized by steaming for 30 minutes

1.7.1 Mineral salts medium agar (Berk et al., 1957) (MSA)

$NH_4 NO_3$	1.0g
$KH_2 PO_4$	0.7g
K_2HPO_4	0.7g
$MgSO_4 \cdot 7H_2O$	0.7g
NaCl	0.005g
$FeSO_4 \cdot 7H_2O$	0.002g
$ZnsO_4 \cdot 7H_2O$	0.002g
$MnSO_4 \cdot 4H_2O$	0.001g
Oxoid agar No.1	20.0g
Distilled water	1 litre

pH 6.5

Sterilized by autoclaving at 121°C for 15 minutes

1.7.2 Mineral salts medium agar + glycerol (a_w 0.95)

Same ingredients as 1.7.1 with glycerol (Analar grade) at 3.0 molal concentration

1.7.3 Mineral salts medium agar + NaCl (a_w 0.95)

Same ingredients as 1.7.1 with NaCl (Analar grade) at 1.4 molal concentration

Sterilization in 1.7.2 and 1.7.3 was similar to that in 1.7.1

1.8.1 Peptone, Lab-lemco agar (PLA)

Mycological peptone - Oxoid L40	5g
Lab-lemco powder - Oxoid L29	3g
Oxoid agar No.1	15g
Distilled water	1 litre

pH 5.7

Sterilized by autoclaving at 121°C for 15 minutes

1.8.2 Peptone, Lab-lemco agar + glycerol (a_w 0.95)

Same ingredients as 1.8.1 with glycerol (analar grade) at 3.0 molal concentration

1.8.3 Peptone, Lab-lemco agar + NaCl (a_w 0.95)

Same ingredients as 1.8.1 with NaCl (Analar agar) at 1.4 molal concentration

Sterilisation in 1.8.2 and 1.8.3 was similar to that in 1.8.1

APPENDIX 2.1

FUNGI ISOLATED FROM ALL SOILS

Aspergillus amstelodami	Thom and Church
A. chevalieri var. *intermedius*	" " "
A. flavus (45°C; N.95)	Link ex. Fries
A. fumigatus (45°C; N.95)	Fresenius
A. niger	Van Tiegham
A. penicilloides	Spegazzini
A. repens	(Corda) Saccardo
A. restrictus	Smith
A. ruber	Thom and Church
A. versicolor	(Vuillemin) Tiraboschi
Cephalosporium curtipes	Saccardo
Chaetomium globosum	Kunze
Cladosporium herbarum	(Pers.) Link ex S.F. Gray
C. sphaerospermum	Penz
Eupenicillium pinetorum	
Fusarium sp.	
F. moniliforme	Sheldon
F. oxysporum	Schlecht
Gliocladium roseum	(Link) Bainier
Mucor plumbeus	Bonorden
Mycelia sterilia	
Mycogone nigra	(Morgan) Jensen
Penicillium aculeatum	Raper and Fennell

P. brevi-compactum	Dierckx
P. canescens	Sopp.
P. chrysogenum	Thom
P. citrinum	Thom
P. corylophilum	Dierckx
P. frequentans	Westling
P. nigricans	Bainier
P. nigricans (unusual isolate)	Bainier
P. restrictum	Gilman and Abbott
Phycomycete	
Scopulariopsis brevicaulis	(Sacc.) Bainier
Trichoderma glaucum	Abbott
T. koningi	Oudemans
T. lignorum	(Tode) Harz
T. viride	Persoon ex. S.F. Gray
Zygorhynchus moelleri	Vuillemin

APPENDIX 2.2

TOTAL NUMBER OF FUNGI AND THEIR FIRST APPEARANCE

Fungal Isolate	SOIL TYPES				
	Cactus Garden Soil	St Peter's G'den Soil	Sutton Park Soil	Swansea Beach I Soil	Swansea Beach II Soil
C. herbarum				(2)C*	
F. oxysporum		(1)C		(1)C	
Fusarium sp.				(1)C	
G. roseum		(2)C			
M. plumbeus		(1)C			
M. sterila				(1)C	
M. nigra			(2)C		
T. glaucum					(1)C
T. koningi	(1) C				
T. viride		(1)C			
Z. moelleri		(2)C			
C. globosum			(2)C/(3)N.97		
T. lignorum	(1)C/(2)N.97				
F. moniliforme	(1)C/(1)N.97/(2)N.97			(1)C/(2)N.97	
Phycomycete	(2)N.95	(2)N.95	(2)N.95	(1)C/(1)N.97/(2)N.95	(2)N.95

Fungal Isolate	S O I L T Y P E S				
	Cactus Garden Soil	St Peter's G'den Soil	Sutton Park Soil	Swansea Beach I Soil	Swansea Beach II Soil
C. curtipes	(2)N.97/(2)N.95/(3)N.93		(2)N.97/(2)N.95		(1)C/(2)N.97
P. canescens		(12)MY60G	(8)N.87	(2)C	(4)N.87/(8)N.85/(4)MY60G
P. chrysogenum	(2)N.95/(3)N.93	(2)N.97/(2)N.95/(4)N.90/(4)N.87	(2)N.95	(2)C/(2)N.97/(2)N.95/(3)N.93/(4)N.90/(4)MY60G	(2)N.97/(2)N.95/(4)N.85/(4)N.90
P. frequentans	(3)N.93/(12)MY60G	(2)N.95/(3)N.93/(4)N.90	(2)C/(2)N.97/(2)N.95/(3)N.93	(2)N.97/(2)N.95/(3)N.93	(2)N.95/(3)N.93
P. nigricans	(2)N.95/(3)N.93	(2)N.97/(2)N.95/(3)N.93/(12)MY60G	(2)N.97/(2)N.95/(3)N.93/(8)N.87	(2)C/(2)N.95/(3)N.93	(2)N.95/(3)N.93/(4)N.90/(4)N.87/(8)N.85/(4)MY60G
A. chevalieri var. intermedius	(12)MY60G	(3)N.95	(4)N.87		(2)N.93/(3)N.90/(4)MY60G/(8)N.83/(12)N.80
A. niger	(2)N.95/(3)N.93/(3)N.90/(4)N.87	(3)N.93			
A. ruber			(3)N.95/(8)N.85/(4)MY60G/(12)N.83/(12)N.80	(2)N.93	
A. versicolor		(3)N.93/(4)N.90/(12)MY60G	(3)N.95	(3)N.93	(4)N.90/(4)MY60G

Fungal Isolate	S O I L T Y P E S				
	Cactus Garden Soil	St Peter's G'den Soil	Sutton Park Soil	Swansea Beach I Soil	Swansea Beach IISoil
P. brevi-compactum	(2)N.95/(3)N.93/(8) N.87/(12)MY60G	(2)N.95	(4)N.87	(2)N.95/(4)N.90/(8) MY60G	(2)N.95/(12)N.85
C. sphaerospermum	(2)N.93/(4)N.90/(4) N.87/(12)MY60G				
P. citrinum			(4)N.93		
A. amstelodami		(3)N.90/(4)N.87	(3)N.90/(4)N.87/(4) N.85	(4)MY60G	(4)N.85/(8)N.83/(8) N.80
A. repens				(3)N.90/(4)N.97	(3)N.90/(4)N.87/(8) N.85/(12)N.83/(12) N.80
A. aculeatum				(4)N.90	(4)N.90
P. corylophilum		(4)N.90	(4)N.90		
P. restrictum			(4)N.90		
S. brevicaulis		(8)N.87			
E. pinetorum		(8)MY60G		(8)MY60G	(8)MY60G
A. penicilloides				(4)N.85/(8)N.83	
A. restrictus					(4)N.85/(12)N.83

* (2)C = Time in weeks and the Media on which they were Isolated

APPENDIX 3.1

EFFECT OF pH ON THE GROWTH RATES OF TEST XEROPHILIC AND NON-XEROPHILIC FUNGI

(mm/day - mean of 5 replicates)

pH	*A. amstelodami*			*A. chevalieri*		
	MEA	*GO.93	+NO.93	MEA	GO.93	NO.93
2	0.78	1.33	3.78	0.67	2.00	3.45
3	0.89	1.44	4.00	1.00	2.22	3.89
4	1.39	2.56	5.78	1.33	2.22	4.22
5	1.67	3.67	6.33	1.56	3.78	4.44
6	2.45	3.78	6.00	2.00	4.33	5.61
7	3.33	5.11	5.89	2.28	4.11	4.67
8	1.56	2.89	5.44	1.78	3.00	4.67
9	1.22	2.44	5.11	1.67	2.67	4.57
10	1.22	2.11	4.33	1.66	2.33	3.56

pH	*A. repens*			*A. ruber*		
	MEA	GO.93	NO.93	MEA	GO.93	NO.93
2	0.89	3.34	4.56	0.17	2.00	4.11
3	0.44	3.33	4.89	0.17	3.00	4.00
4	1.44	4.45	6.78	0.33	3.78	5.11
5	2.00	4.67	6.17	0.39	3.44	5.11
6	2.67	3.78	4.89	0.44	3.45	5.30
7	2.33	3.55	4.34	1.11	5.00	5.11
8	1.11	2.78	3.89	0.56	2.89	4.11
9	1.0	1.89	2.67	0.33	1.67	3.00
10	0	1.78	2.56	0	0.67	2.67

* GO.93 = MEA + glycerol (a_w 0.93)

\+ NO.93 = MEA + NaCl (a_w 0.93)

pH	A. niger			A. penicilloides		
	MEA	GO.95	NO.95	MEA	GO.95	NO.95
2	11.67	14.50	12.33	0	1.46	2.17
3	11.67	15.00	12.00	0	1.75	2.17
4	12.00	15.00	11.67	0.29	1.96	2.25
5	13.00	15.00	10.67	0.72	2.83	2.25
6	13.33	14.00	9.67	0.81	3.08	2.25
7	14.00	12.00	8.33	0.98	2.71	2.08
8	12.67	12.00	8.00	0.92	2.50	1.88
9	9.67	10.33	7.00	0.88	2.16	1.67
10	9.33	8.33	6.45	0.67	1.58	1.31

pH	A. restrictus			A. versicolor		
	MEA	GO.95	NO.95	MEA	GO.95	NO.95
2	1.60	1.44	1.94	1.67	1.52	1.67
3	1.61	2.87	2.00	2.00	1.89	2.67
4	1.61	3.17	2.33	2.78	1.90	3.11
5	1.78	3.28	3.67	2.80	3.33	3.22
6	2.11	3.39	3.89	2.55	3.44	3.56
7	3.67	4.00	5.28	3.00	3.67	3.89
8	2.33	3.22	4.33	2.33	2.33	3.67
9	2.22	2.56	3.89	2.22	2.11	3.22
10	1.83	2.11	2.94	1.44	2.11	2.67

pH	C. sphaerospermum			E. pinetorum		
	MEA	GO.95	NO.95	MEA	GO.95	NO.95
2	1.54	1.54	1.13	3.33	2.40	2.23
3	1.71	1.54	2.25	3.56	2.50	2.83
4	2.13	1.75	4.08	3.67	2.93	2.70
5	2.42	2.33	4.46	3.96	3.20	3.33
6	2.46	2.63	4.46	3.70	3.00	3.23
7	2.63	2.71	5.38	3.23	2.60	2.67
8	2.17	2.00	4.96	3.23	2.47	2.67
9	2.04	1.79	3.92	2.53	2.17	2.47
10	1.92	1.70	3.50	2.40	1.80	1.40

pH	P. brevi-compactum			P. chrysogenum		
	MEA	GO.95	NO.95	MEA	GO.95	NO.95
2	2.00	2.39	2.33	4.00	3.75	2.83
3	2.22	2.61	2.56	4.25	3.75	3.42
4	2.50	2.67	2.56	4.30	4.88	3.42
5	2.50	2.71	3.00	4.40	4.88	4.75
6	3.33	3.22	3.56	4.58	4.88	5.42
7	2.10	2.67	3.94	3.75	5.00	5.55
8	1.78	2.67	3.78	3.04	4.42	4.25
9	1.78	2.56	3.11	2.88	4.33	4.21
10	1.67	2.00	2.83	2.67	3.92	4.21

pH	P. citrinum			P. frequentans		
	MEA	GO.95	NO.95	MEA	GO.95	NO.95
2	1.99	2.25	2.50	5.30	5.65	6.00
3	2.57	2.65	2.95	5.50	6.01	6.53
4	3.28	3.79	4.00	5.73	6.25	6.75
5	4.51	5.75	5.10	6.35	5.80	6.89
6	5.25	5.00	6.90	5.85	5.60	6.01
7	4.00	4.75	5.62	5.24	4.75	5.40
8	3.65	4.20	5.00	5.00	4.10	4.80
9	2.37	3.27	4.00	3.55	3.08	4.00
10	2.00	3.10	2.67	3.30	3.00	2.31

pH	P. nigricans			S. brevicaulis		
	MEA	GO.95	NO.95	MEA	GO.95	NO.95
2	3.00	2.93	2.20	0	0	0
3	3.27	3.03	2.37	0	0	0
4	2.87	3.60	2.90	2.63	2.03	2.87
5	2.76	3.53	2.90	4.83	3.73	5.43
6	2.60	3.53	2.70	7.60	4.07	7.33
7	2.23	2.60	2.60	7.57	5.67	8.27
8	2.27	2.50	2.50	6.70	5.57	10.87
9	2.00	2.40	2.03	6.70	5.23	9.73
10	1.87	2.27	1.67	6.67	5.20	6.93

pH	C. globosum			C. herbarum		
	MEA	GO.97	NO.97	MEA	GO.97	NO.97
2	1.70	2.15	3.25	4.50	4.75	3.00
3	1.80	3.61	4.10	4.80	4.90	3.25
4	19.80	26.50	29.00	5.00	5.30	3.65
5	34.50	25.00	37.00	5.20	5.40	4.00
6	40.10	24.00	40.25	5.30	6.00	4.75
7	39.50	19.00	42.00	5.60	5.70	4.00
8	39.00	19.00	37.00	5.50	5.63	3.60
9	37.50	16.00	36.00	5.00	5.40	3.20
10	37.00	15.00	34.10	4.70	5.10	2.90

pH	F. moniliforme			F. oxysporum		
	MEA	GO.97	NO.97	MEA	GO.97	NO.97
2	4.20	3.00	2.60	18.80	17.15	16.00
3	4.20	3.15	2.75	18.80	17.35	16.10
4	7.20	5.15	3.15	19.80	18.55	17.30
5	7.70	5.40	3.45	19.80	19.00	17.00
6	7.70	6.20	4.30	17.80	15.00	14.10
7	9.70	6.00	4.58	17.00	14.75	13.85
8	8.80	5.15	4.00	16.80	14.00	12.60
9	8.70	5.00	3.83	16.80	13.60	12.00
10	8.70	4.90	3.50	16.00	13.50	11.20

pH	M. plumbeus		
	MEA	GO.97	NO.97
2	17.00	9.85	0
3	17.81	10.10	2.10
4	18.85	13.20	2.90
5	19.86	14.20	3.20
6	21.87	13.85	5.15
7	19.87	13.26	5.60
8	19.00	13.00	4.10
9	18.50	12.56	3.11
10	18.30	12.50	2.50

pH	T. glaucum			T. lignorum		
	MEA	GO.97	NO.97	MEA	GO.97	NO.97
2	30.00	12.10	5.00	16.80	12.10	7.20
3	30.20	15.20	7.15	18.80	13.65	7.50
4	29.95	17.00	7.00	19.82	14.50	8.20
5	21.55	16.85	6.15	17.83	15.00	6.90
6	18.80	11.50	4.85	12.85	10.00	6.50
7	13.20	7.31	3.15	10.88	9.15	4.00
8	13.20	6.90	0	10.53	7.85	4.00
9	12.51	6.00	0	9.00	5.10	3.00
10	12.20	5.90	0	7.11	3.00	1.15

APPENDIX 3.2

COMBINED EFFECTS OF pH, SOLUTES AND a_w ON THE GROWTH RATE OF TEST XEROPHILIC AND NON-XEROPHILIC FUNGI

(mm/day - mean of 5 replicates)

a_w	*A. amstelodami*				*A. chevalieri*			
	○G4.0	G6.5	△N4.0	N6.5	G4.0	G6.5	N4.0	N6.5
Control	0.83	2.15	0.83	2.15	1.02	1.66	1.02	1.66
0.97	2.02	3.30	5.51	5.63	1.67	3.52	4.72	5.03
0.95	4.28	5.81	6.03	6.37	4.64	4.74	4.03	4.78
0.93	4.69	6.22	6.25	7.02	4.35	4.89	2.82	4.57
0.90	5.22	5.77	4.93	6.02	4.02	5.02	0.83	4.27
0.87	4.20	3.57	2.83	6.12	2.53	3.58	0.53	2.30
0.85	3.22	3.34	1.82	3.30	1.94	2.61	0.25	0.30
0.83	2.95	2.92	1.10	1.63	1.64	1.88	0.10	0.17
0.80	1.25	1.07	0.23	0.17	0.83	1.07	0	0
0.77	1.00	0.93			0.73	0.77		
0.75	0.37	0.40			0.30	0.47		
0.73	0	0.23			0	0.13		
0.70	0	0			0	0		

a_w	*A. repens*				*A. ruber*			
	G4.0	G6.5	N4.0	N6.5	G4.0	G6.5	N4.0	N6.5
Control	2.90	2.12	2.90	2.12	0.27	1.08	0.27	1.08
0.97	3.77	2.97	4.52	4.93	1.20	1.53	4.20	4.55
0.95	4.90	4.70	5.40	6.86	5.65	6.16	4.23	5.86
0.93	5.22	4.67	4.59	6.08	5.93	6.34	4.40	5.89
0.90	5.50	3.76	1.12	3.35	5.48	6.63	0.95	5.15
0.87	5.07	3.77	0.25	1.83	4.00	3.83	0.70	4.17
0.85	4.60	3.54	0	0.40	3.35	2.81	0.65	1.32
0.83	3.33	2.96	0	0.03	2.62	2.37	0.33	0.78
0.80	1.24	1.20	0	0	1.06	1.00	0	0
0.77	0.57	0.50			0.33	0.40		
0.75	0.10	0.17			0.17	0.20		
0.73	0	0.03			0	0.07		
0.70	0	0			0	0		

○ G4.0 = MEA + glycerol at pH4.0

△ N4.0 = MEA + NaCl at pH4.0

a_w	A. niger				A. penicilloides			
	G4.0	G6.5	N4.0	N6.5	G4.0	G6.5	N4.0	N6.5
Control	12.14	10.00	12.14	10.00	0	0.66	0	0.66
0.97	12.13	9.80	10.17	8.93	0.20	0.57	0.70	1.28
0.95	11.53	8.39	8.30	7.58	1.10	1.69	0.93	1.71
0.93	8.83	8.03	6.85	6.13	1.41	2.81	1.60	1.98
0.90	5.55	4.52	5.00	3.71	1.43	2.50	1.19	1.73
0.87	4.58	4.32	3.85	1.51	1.31	2.05	0.64	1.65
0.85	3.81	2.90	0.85	0.33	0.64	1.84	0.26	0.62
0.83	1.91	1.56	0.56	0.20	0.50	1.24	0.15	0.45
0.80	0.10	0.03	0	0	0.20	0.77	0	0
0.77	0	0			0.20	0.63		
0.75	0	0			0.03	0.33		
0.73	0	0			0.03	0.27		
0.70	0	0			0	0		

a_w	A. restrictus				A. versicolor			
	G4.0	G6.5	N4.0	N6.5	G4.0	G6.5	N4.0	N6.5
Control	1.62	1.87	1.62	1.87	2.33	2.83	2.33	2.83
0.97	1.33	2.52	1.68	2.00	2.09	2.50	2.59	3.33
0.95	2.17	2.51	2.50	2.83	1.92	2.29	2.08	2.83
0.93	1.08	1.40	1.83	2.17	1.67	1.67	2.00	2.50
0.90	1.01	1.17	1.53	1.67	1.25	1.09	1.25	1.71
0.87	0.73	0.49	0.49	0.38	0.65	0.46	0.87	0.96
0.85	0.54	0.41	0.19	0.37	0.51	0.35	0.53	0.53
0.83	0.33	0.20	0.08	0.18	0.32	0.30	0.27	0.46
0.80	0.27	0.20	0	0	0.17	0.10	0	0
0.77	0	0			0.01	0.01		

a_w	C. sphaerospermum				E. pinetorum			
	G4.0	G6.5	N4.0	N6.5	G4.0	G6.5	N4.0	N6.5
Control	1.14	1.57	1.14	1.57	2.93	3.67	2.93	3.67
0.97	1.92	2.25	3.50	3.75	3.75	4.09	2.92	2.92
0.95	1.50	2.58	3.25	4.59	2.42	2.34	2.34	2.84
0.93	1.21	1.83	2.58	3.82	2.12	1.58	1.33	2.17
0.90	0.99	1.17	1.53	1.66	1.37	1.25	0.92	1.12
0.87	0.73	0.59	0.49	0.63	0.49	0.56	0.40	0.48
0.85	0.54	0.53	0.19	0.37	0.48	0.54	0.33	0.46
0.83	0.33	0.30	0.08	0.18	0.44	0.54	0	0.32
0.80	0.10	0.10	0	0	0.17	0.15	0	0

a_w	P. brevi-compactum				P. chrysogenum			
	G4.0	G6.5	N4.0	N6.5	G4.0	G6.5	N4.0	N6.5
Control	1.34	1.48	1.34	1.48	5.11	5.60	5.11	5.60
0.97	1.67	1.92	2.00	2.08	4.45	5.87	3.11	4.22
0.95	1.08	1.35	1.60	1.84	3.33	3.98	2.29	3.17
0.93	0.92	1.34	1.42	1.67	2.59	3.33	2.00	2.78
0.90	0.81	0.75	1.05	1.43	1.63	1.76	1.05	1.64
0.87	0.33	0.48	0.37	0.50	0.67	0.77	0.49	0.76
0.85	0.34	0.39	0.30	0.32	0.43	0.54	0.32	0.50
0.83	0.29	0.33	0.23	0.31	0.36	0.39	0.16	0.32
0.80	0.17	0.17	0	0	0.07	0.10	0	0

a_w	P. citrinum				P. frequentans			
	G4.0	G6.5	N4.0	N6.5	G4.0	G6.5	N4.0	N6.5
Control	2.80	2.31	2.80	2.31	3.21	5.35	3.21	5.35
0.97	5.10	4.73	4.20	3.25	4.70	7.11	3.20	4.22
0.95	5.35	5.00	4.00	3.00	4.00	3.51	2.95	3.00
0.93	3.60	3.00	2.80	2.10	2.81	2.10	2.74	2.78
0.90	3.00	2.65	1.95	1.75	1.75	1.40	1.55	1.56
0.87	2.10	1.37	0.88	0.67	0.82	0.76	0.61	0.58
0.85	1.30	0.77	0.40	0.36	0.41	0.38	0.20	0.25
0.83	0.76	0.22	0.11	0.09	0.10	0.12	0	0
0.80	0.30	0.10	0	0	0	0	0	0

a_w	P. nigricans				S. brevicaulis			
	G4.0	G6.5	N4.0	N6.5	G4.0	G6.5	N4.0	N6.5
Control	2.29	2.33	2.29	2.33	0.83	7.22	0.83	7.22
0.97	3.17	2.83	1.92	2.25	1.17	7.78	1.34	8.55
0.95	2.59	2.82	1.67	2.19	0.51	5.08	1.02	5.92
0.93	2.50	2.57	1.50	2.18	0	3.42	0.67	2.58
0.90	1.93	1.89	0.76	1.21	0	1.65	0.22	1.32
0.87	0.80	0.69	0.61	0.49	0	0.54	0	0.36
0.85	0.71	0.70	0.26	0.36	0	0.26	0	0.19
0.83	0.53	0.45	0	0.19	0	0.13	0	0.11
0.80	0.10	0.20	0	0	0	0	0	0

a_w	C. globosum				C. herbarum			
	G4.0	G6.5	N4.0	N6.5	G4.0	G6.5	N4.0	N6.5
Control	30.00	39.11	30.00	39.11	3.45	3.81	3.45	3.81
0.97	19.82	33.55	17.70	19.80	4.05	4.10	3.00	3.15
0.95	6.62	8.17	0	9.65	2.50	2.65	1.57	2.22
0.93	0	0	0	0	1.11	1.23	0.61	0.88
0.90	0	0	0	0	0	0.32	0	0

a_w	F. moniliforme				F. oxysporum			
	G4.0	G6.5	N4.0	N6.5	G4.0	G6.5	N4.0	N6.5
Control	8.00	8.00	8.00	8.00	19.89	19.80	19.89	19.80
0.97	6.82	7.13	5.01	5.00	19.31	16.81	17.50	13.57
0.95	2.11	2.33	3.14	3.00	12.54	9.96	8.45	7.75
0.93	0.95	1.15	1.11	1.10	5.93	5.00	3.30	3.41
0.90	0.20	0.34	0	0	0.75	0.33	0	0

a_w	T. glaucum				T. lignorum			
	G4.0	G6.5	N4.0	N6.5	G4.0	G6.5	N4.0	N6.5
Control	30.50	19.31	30.50	19.31	19.85	18.10	19.85	18.10
0.97	20.00	13.25	7.75	4.50	18.00	8.99	4.85	4.90
0.95	1.85	3.00	3.57	0	17.50	0.85	2.00	1.15
0.93	0.51	0.25	0.32	0	0.56	0	0.57	0
0.90	0	0	0	0	0	0	0	0

a_w	M. plumbeus			
	G4.0	G6.5	N4.0	N6.5
Control	26.31	27.51	26.31	27.50
0.97	21.00	24.00	4.75	7.99
0.95	10.11	15.51	2.51	3.31
0.93	3.85	5.69	0	0
0.90	0	0	0	0

APPENDIX 4.1

EFFECT OF TEMPERATURE ON THE GROWTH RATE OF

TEST FUNGI

(mm/day - mean of 5 replicates)

A. amstelodami TEMP °C	CONTROL	○ GO.95	GO.90	GO.85	GO.80	△ NO.95	NO.90	NO.85	NO.80
20°	1.79	6.00	5.56	3.06	1.21	6.22	6.06	2.40	0.10
25°	2.25	6.00	6.00	3.14	1.55	8.67	6.22	3.25	0.14
30°	3.17	6.83	7.11	3.38	2.34	8.33	7.33	3.23	0.17
35°	0	9.00	7.78	5.00	2.59	9.67	8.56	3.00	0.34
40°	0	7.33	6.67	3.40	1.52	10.17	8.00	1.69	0
45°	0	1.00	0.75	0.15	0	3.80	1.80	0	0
50°	0	0.14	0	0	0	0.21	0	0	0

○ GO.95 = MEA + glycerol (a_w 0.95) △ NO.95 = MEA + NaCl (a_w 0.95)

A. flavus Temp °C	CONTROL	GO.95	GO.90	GO.85	GO.80	NO.95	NO.90	NO.85	NO.80
20°	8.00	2.67	0.71	0.48	0	2.33	0.45	0.20	0
25°	9.00	11.00	1.04	0.58	0	4.67	0.51	0.26	0
30°	10.00	14.33	1.68	1.22	0	8.00	0.75	0.54	0
35°	12.33	16.67	4.89	1.46	0	10.33	4.33	0.56	0
40°	7.00	14.67	3.96	0.26	0	7.00	2.69	0	0
45°	4.80	9.80	1.00	0	0	3.60	0.15	0	0
50°	0.20	1.25	0	0	0	0.12	0	0	0

A. fumigatus TEMP °C	CONTROL	GO.95	GO.90	GO.85	GO.80	NO.95	NO.90	NO.85	NO.80
20°	2.04	3.94	1.12	0.38	0	0.83	0.82	0	0
25°	10.33	4.33	1.35	0.52	0	1.69	0.96	0.31	0
30°	16.00	7.33	1.84	0.72	0	3.00	1.42	0.41	0
35°	20.00	9.00	2.56	1.08	0	5.00	2.33	0.48	0
40°	24.00	17.33	3.04	1.31	0	1.92	1.01	0	0
45°	20.50	12.30	0.90	0.34	0	0.41	0	0	0
50°	10.00	3.60	0	0	0	0	0	0	0

A. versicolor TEMP °C	CONTROL	GO.95	GO.90	GO.85	GO.80	NO.95	NO.90	NO.85	NO.80
20°	3.02	2.40	1.04	0.14	0	2.48	1.69	0.29	0
25°	4.11	2.85	1.78	0.24	0	3.38	1.92	0.45	0
30°	3.19	2.33	0.57	0.17	0	4.44	2.40	0.58	0
35°	1.11	1.69	0.36	0	0	2.61	1.40	0	0
40°	0	0	0	0	0	0	0	0	0
45°	0	0	0	0	0	0	0	0	0
50°	0	0	0	0	0	0	0	0	0

P. citrinum TEMP °C	CONTROL	GO.95	GO.90	GO.85	GO.80	NO.95	NO.90	NO.85	NO.80
20°	2.89	2.22	1.37	0.58	0.24	3.11	1.57	0.94	0
25°	3.22	3.22	1.60	0.87	0.31	4.11	1.97	1.02	0.17
30°	3.56	5.00	1.96	1.12	0.41	4.83	2.56	1.20	0.31
35°	2.00	2.00	0.98	0.32	0	3.00	1.68	0.69	0
40°	0	0.23	0	0	0	0.69	0	0	0
45°	0	0	0	0	0	0	0	0	0
50°	0	0	0	0	0	0	0	0	0

APPENDIX 4.2

EFFECT OF INCREASING TEMPERATURE ON THE SURVIVAL OF ASCOSPORES/SPORES AT DECREASING WATER ACTIVITY LEVELS

(mean of 3 replicates)

A. amstelodami (spores)

Initial spore count : 45°C 9×10^4. 55°C 7.2×10^4. 65°C 4.7×10^4.

MEDIUM	TEMP °C	TIME (minutes)				
		10	30	60	120	180
CONTROL (Water)	45°	9.0×10^3	1.0×10^3	1.2×10^2	9.0×10	1.1×10
	55°	1.5×10^2	1.0×10^2	6.3×10	1.0×10	0.6×10
	65°	1.3×10	0.8×10	0.3×10	0.1×10	0
○ G0.95	45°	1.0×10^4	1.5×10^3	5.0×10^2	1.2×10^2	8.0×10
	55°	2.0×10^2	1.1×10^2	9.0×10	6.0×10	0.1×10
	65°	2.8×10	0.9×10	0.5×10	0.4×10	0
G0.90	45°	1.9×10^4	5.2×10^3	2.0×10^3	1.0×10^3	9.6×10^2
	55°	1.0×10^4	1.0×10^3	1.1×10^2	1.0×10^2	6.0×10
	65°	4.5×10	1.1×10	1.0×10	0.6×10	0.1×10
G0.85	55°	2.7×10^4	2.3×10^4	3.2×10^3	2.6×10^3	1.7×10^3
G0.80	55°	1.5×10^4	2.3×10^3	8.6×10^2	3.2×10^2	2.3×10^2
G0.75	55°	1.4×10^3	2.0×10^2	1.4×10^2	7.8×10	2.9×10
G0.70	55°	2.6×10^2	1.4×10^2	1.2×10^2	4.3×10	2.6×10
△ N0.95	45°	1.5×10^4	2.5×10^3	1.0×10^3	8.1×10^2	4.6×10^2
	55°	3.1×10^3	8.0×10^2	1.0×10^2	9.0×10	1.3×10
	65°	3.5×10	0.1×10	0.6×10	0.5×10	0
N0.90	45°	4.1×10^4	1.2×10^4	4.5×10^3	3.1×10^3	2.5×10^3
	55°	2.0×10^4	8.0×10^3	6.2×10^2	2.0×10^2	1.0×10^2
	65°	1.8×10^2	1.2×10^2	4.0×10	0.1×10	0.8×10

○ $_{G0.95}$ = Water + glycerol (a_w 0.95)

△ $_{N0.95}$ = Water + NaCl (a_w 0.95)

A. amstelodami (ascospores)

Initial spore count : 55°C 1.1×10^4

MEDIUM	TEMP °C	TIME (minutes)				
		10	30	60	120	180
CONTROL (Water)	55°	1.0×10^3	2.5×10^2	6.0×10	1.0×10	0.6×10
GO.95	55°	3.5×10^3	6.0×10^2	8.5×10	3.0×10	1.5×10
GO.90	55°	8.5×10^3	3.5×10^3	7.0×10^2	1.0×10^2	8.0×10
GO.85	55°	1.0×10^4	7.5×10^3	2.5×10^3	4.5×10^2	2.0×10^2
GO.80	55°	7.5×10^3	2.6×10^3	4.5×10^2	7.0×10	4.6×10
GO.75	55°	2.4×10^3	3.4×10^2	7.0×10	2.0×10	0.1×10
GO.70	55°	1.6×10^3	3.0×10^2	7.0×10	1.2×10	0.7×10
NO.95	55°	6.5×10^3	1.0×10^3	2.5×10^2	5.0×10	3.5×10
NO.90	55°	9.0×10^3	5.0×10^3	1.5×10^3	2.4×10^2	1.5×10^2

A. flavus

Initial spore count : 45°C 9.5×10^4. 55°C 7.5×10^4. 65°C 1.9×10^4.

MEDIUM	TEMP °C	TIME (minutes)				
		10	30	60	120	180
CONTROL (Water)	45°	6.0×10^2	4.5×10	2.3×10	2.0×10	1.0×10
	55°	2.1×10	1.5×10	1.0×10	0.7×10	0
	65°	0	0	0	0	0
GO.95	45°	1.0×10^4	6.1×10^3	5.0×10^2	6.0×10	3.0×10
	55°	4.0×10^3	5.1×10^2	1.0×10^2	4.0×10	1.0×10
	65°	0	0	0	0	0
GO.90	45°	8.0×10^4	7.0×10^4	3.0×10^4	2.2×10^4	7.9×10^3
	55°	4.0×10^4	2.0×10^4	9.0×10^3	1.9×10^3	8.0×10^3
	65°	1.0×10^2	4.6×10	2.5×10	3.8×10	0
GO.85	55°	7.0×10^4	5.4×10^4	3.0×10^4	9.5×10^3	6.1×10^3
GO.80	55°	4.8×10^4	2.6×10^4	1.3×10^4	2.5×10^3	1.1×10^3
GO.75	55°	8.5×10^3	2.5×10^3	5.0×10^2	1.6×10^2	5.4×10
GO.70	55°	4.0×10^3	7.5×10^2	1.8×10^2	5.2×10	1.9×10
NO.95	45°	6.0×10^3	2.0×10^3	1.8×10^2	3.6×10	1.5×10
	55°	6.0×10^2	3.3×10^2	9.2×10	2.0×10	0.71×10
	65°	0	0	0	0	0
NO.90	45°	6.3×10^4	2.8×10^4	1.0×10^4	9.0×10^3	4.0×10^3
	55°	3.2×10^4	1.4×10^4	6.0×10^3	1.0×10^3	6.0×10^2
	65°	4.9×10	1.9×10	1.1×10	0	0

A. fumigatus

Initial spore count : 45°C 2.5×10^4. 55°C 8.8×10^4. 65°C 12 $\times 10^4$.

MEDIUM	TEMP °C	TIME (minutes)				
		10	30	60	120	180
CONTROL (Water)	45°	1.0×10^3	3.0×10^2	9.0×10	1.5×10	0.42×10
	55°	2.3×10^2	9.5×10	4.0×10	2.1×10	0.6×10
	65°	2.0×10	0.95×10	0.4×10	0.195×10	0
GO.95	45°	2.0×10^3	9.3×10^2	2.0×10^2	6.0×10	2.0×10
	55°	5.0×10^3	6.0×10^2	3.8×10^2	9.0×10	6.0×10
	65°	1.8×10^2	1.0×10	5.0×10	2.5×10	1.2×10
GO.90	45°	9.4×10^3	7.0×10^3	2.2×10^3	7.0×10^2	1.9×10^2
	55°	6.0×10^4	3.4×10^4	1.1×10^4	8.0×10^2	3.0×10^2
	65°	1.4×10^3	5.0×10^2	2.0×10^2	1.3×10^2	7.0×10
GO.85	55°	8.0×10^4	6.5×10^4	4.2×10^4	5.6×10^3	2.0×10^3
GO.80	55°	7.0×10^4	3.8×10^4	1.5×10^4	1.6×10^3	6.1×10^2
GO.75	55°	1.7×10^4	2.1×10^3	7.3×10^2	2.5×10^2	1.3×10^2
GO.70	55°	6.0×10^3	6.5×10^2	3.6×10^2	1.0×10^2	7.1×10
NO.95	45°	6.2×10^3	1.1×10^3	4.0×10^2	8.0×10	6.0×10
	55°	1.0×10^3	1.1×10^2	1.0×10^2	6.0×10	4.0×10
	65°	8.1×10	6.0×10	3.9×10	1.0×10	0.5×10
NO.90	45°	2.4×10^4	1.0×10^4	8.0×10^3	3.0×10^3	1.0×10^3
	55°	4.1×10^4	1.2×10^4	2.5×10^2	5.6×10^2	2.0×10^2
	65	6.0×10^2	1.3×10^2	1.0×10^2	6.0×10	2.0×10

A. versicolor

Initial spore count : 45°C 4.8×10^4. 55°C 6.5×10^4. 65°C 4.3×10^4.

MEDIUM	TEMP °C	TIME (minutes)				
		10	30	60	120	180
CONTROL (Water)	45°	2.6×10^2	6.0×10	1.5×10	1.1×10	1.0×10
	55°	1.1×10	0	0	0	0
	65°	0	0	0	0	0
GO.95	45°	8.0×10^2	4.0×10^2	3.0×10^2	1.0×10^2	7.0×10
	55°	5.0×10^2	1.5×10^2	8.0×10	2.0×10	0
	65°	0	0	0	0	0
GO.90	45°	9.8×10^3	8.2×10^3	9.4×10^2	6.0×10^2	4.6×10^2
	55°	3.5×10^3	2.0×10^3	6.1×10^2	9.0×10	0.38×10
	65°	0	0	0	0	0
GO.85	55°	1.3×10^3	0.1×10	0	0	0
GO.80	55°	2.6×10^2	0	0	0	0
GO.75	55°	4.2×10	0	0	0	0
GO.70	55°	1.1×10	0	0	0	0
NO.95	45°	6.0×10^3	1.8×10^3	5.0×10^2	1.7×10^2	1.3×10^2
	55°	1.0×10^3	6.0×10^2	1.6×10^2	4.0×10	0.19×10
	65°	0	0	0	0	0
NO.90	45°	3.0×10^4	1.0×10^4	4.0×10^3	1.5×10^3	9.0×10^2
	55°	1.2×10^4	6.0×10^3	2.0×10^3	5.1×10^2	4.8×10
	65°	0	0	0	0	0

P. citrinum

Initial spore count : 45°C 1.2×10^4. 55°C 1.1×10^4. 65°C 7.4×10^4.

MEDIUM	TEMP °C	TIME (minutes)				
		10	30	60	120	180
CONTROL (Water)	45°	9.0×10^2	2.2×10^2	5.0×10	0.7×10	0
	55°	6.1×10^2	1.0×10^2	1.0×10	0	0
	65°	0	0	0	0	0
GO.95	45°	1.5×10^3	7.9×10^2	3.1×10^2	8.0×10	0.1×10
	55°	1.6×10^3	2.3×10^2	9.2×10	0.91×10	0
	65°	0	0	0	0	0
GO.90	45°	8.2×10^3	6.2×10^3	8.6×10^2	1.4×10^2	9.0×10
	55°	4.4×10^3	1.9×10^3	5.1×10^2	2.2×10^2	8.0×10
	65°	1.0×10	0	0	0	0
GO.85	55°	3.0×10^3	5.3×10^2	2.0×10^2	5.7×10	2.1×10
GO.80	55°	9.0×10^2	1.2×10^2	1.5×10	0.3×10	0
GO.75	55°	0.71×10	0.53×10	0.36×10	0	0
GO.70	55°	0.5×10	0.26×10	0	0	0
NO.95	45°	5.0×10^3	1.0×10^3	3.8×10^2	1.0×10^2	4.0×10
	55°	2.0×10^3	5.2×10^2	1.0×10^2	1.4×10	0.6×10
	65°	0	0	0	0	0
NO.90	45°	1.0×10^4	8.9×10^3	5.2×10^3	6.2×10^2	3.0×10^2
	55°	8.0×10^3	2.8×10^3	7.4×10^2	3.0×10^2	1.4×10^2
	65°	1.8×10	0.9×10	0.3×10	0	0

APPENDIX 5.1

ENZYME SUBSTRATE MEDIA

5.1.1 Starch agar for amylase activity

Oxoid Mycological peptone	5.0g
Oxoid Lab-lemco powder	3.0g
Soluble starch	2.0g
Oxoid agar No. 1	15.0g
Distilled water	1 litre

pH 5.8

5.1.2 Tributyrin agar for lipase activity

Oxoid Mycological peptone	5.0g
Oxoid yeast extract	3.0g
Glycerol tributyrate	10.0g
Oxoid agar No. 1	15.0g
Distilled water	1 litre

pH 5.5

5.1.3 Gelatine agar for protease activity

Oxoid Mycological peptone	5.0g
Oxoid Lab-lemco powder	3.0g
Gelatine	4.0g
Oxoid agar No. 1	15.0g
Distilled water	1 litre

pH 5.4

An 8% gelatine solution in water was separately sterilized and added to the rest of the sterilized, cooled liquid medium at the rate of 5ml/100ml medium.

Molal concentrations of analar grade glycerol and NaCl were added to the above three enzyme media to produce a range of a_w from 0.97 to 0.70 and a_w from 0.97 to 0.80 respectively - Appendices 1.4.2 and 1.4.3.

Media up to and including a_w 0.95 were sterilized by autoclaving at 121°C for 15 minutes. Media below a_w 0.95 were sterilized by steaming for 30 minutes.

APPENDIX 5.2

DIAMETRIC GROWTH OF TEST XEROPHILIC AND NON-XEROPHILIC FUNGI ON VARIOUS MEDIA AT DECREASING a_w

(cm/3 or 7 days - mean of 5 replicates)

DIAMETRIC GROWTH CM/*3 OR 7 DAYS / FUNGI	MEDIA											
	I			II			III			IV		
	DWA	°GDWA	△NDWA	MEA	GMEA	NMEA	NSA	GNSA	NNSA	PLA	GPIA	NPLA
A. amstelodami	0	0.2	1.5	1.1	4.3	5.0	1.4	2.7	3.5	1.2	3.6	3.7
A. chevalieri	0.2	0.7	0.3	1.0	4.2	3.9	1.2	2.0	1.4	2.0	3.0	1.8
A. niger	2.4	3.0	0	2.8	5.0	4.8	2.4	3.7	1.0	4.2	5.6	3.0
A. repens	0	1.0	0	1.8	5.8	5.2	1.4	2.0	0.8	1.4	4.4	4.0
A. ruber	0	1.4	1.4	0.2	4.8	4.5	0.2	3.0	2.5	0.2	4.6	2.7
A. versicolor	0.5	0.3	0.4	1.6	0.9	1.2	0.8	1.0	0.1	1.5	0.7	1.1
P. citrinum	1.6	1.5	0.3	1.8	1.7	1.2	1.6	1.5	0.9	1.6	1.4	0.8
*C. globosum	2.5	0	0	7.9	0.9	0.5	5.7	0.8	0	7.9	1.1	1.3
*F. oxysporum	0.2	0.1	0	4.5	2.8	2.0	1.6	1.5	0.5	3.7	2.7	1.8
*T. lignorum	4.5	0	0	7.9	0.2	0.1	6.9	0.2	0	6.0	0.4	0

°GDWA = DWA + glycerol (a_w 0.95) △NDWA = DWA + NaCl (a_w 0.95)

APPENDIX 5.3

ENZYME ACTIVITIES OF TEST XEROPHILIC AND NON-XEROPHILIC FUNGI ON MEA AND PLA MEDIA

(cm/14 days - mean of 5 replicates)

FUNGI	AMYLASE *pH 5		LIPASE *pH 7		PROTEASE *pH 7	
	PLA	MEA	PLA	MEA	PLA	MEA
A. amstelodami	1.9	0	1.7	1.5	0	0
A. chevalieri	1.4	0	1.9	1.6	0	0
A. niger	3.1	2.3	2.0	1.5	1.5	0.5
A. repens	1.5	0	1.7	1.7	0	0
A. ruber	1.1	0	1.5	1.0	1.6	0.8
A. versicolor	3.2	2.9	2.5	1.4	3.2	1.0
P. citrinum	2.3	2.1	1.3	0.8	2.5	0.5
C. globosum	2.5	2.4	1.0	0.8	1.3	0.5
F. oxysporum	3.4	3.2	1.1	1.0	3.2	0.6
T. lignorum	2.6	2.5	1.3	1.1	3.5	0

*pH5, pH7 - Media buffered with citrate-phosphate buffer

APPENDIX 5.4

ENZYME ACTIVITIES FROM pH2 TO pH10

(cm/14 days - mean 5 replicates)

pH	*A. amstelodami*				*A. chevalieri*			
	†A	†C	†L	†P	A	C	L	P
2	1.2	0	0.9	0	0.6	0	0.8	0.9
3	1.6	0	1.0	0	0.8	0	1.1	1.0
4	1.6	0	1.4	0	1.0	0	1.2	1.2
5	1.8	0	1.8	0	1.1	0	1.5	1.4
6	1.7	0	2.0	0.5	1.3	0	1.9	1.8
7	1.4	0	1.6	1.6	1.5	0	1.7	1.2
8	1.0	0	1.4	1.4	1.3	0	1.6	1.0
9	1.0	0	1.3	1.1	1.2	0	1.3	0.8
10	0.8	0	1.3	0.7	0	0	1.3	0

pH	*A. niger*				*A. repens*			
	A	C	L	P	A	C	L	P
2	3.0	0.8	0.9	1.4	0	0	*0.7	0
3	3.0	0.8	1.0	1.7	0	0	*0.8	0
4	3.0	0.9	1.1	1.8	0.8	0	*1.0	0
5	3.0	0.5	1.2	1.9	1.0	0	*1.1	0
6	3.2	0	1.3	2.0	0.6	0	*1.3	0
7	3.0	0	1.6	2.2	0.6	0	1.6	0
8	2.2	0	1.2	2.1	*0	0	1.5	0
9	1.9	0	1.0	2.0	*0	0	1.5	0
10	1.7	0	1.0	1.7	*0	0	1.5	0

†A = amylase †C = cellulase

†L = lipase †P = protease

* = no visible growth

pH	A. ruber				A. versicolor			
	A	C	L	P	A	C	L	P
2	0	0	*0.7	0.4	1.9	0	1.3	1.0
3	0	0	*0.8	0.4	1.9	0	1.4	2.0
4	0.4	0	*1.1	1.1	2.5	0	2.0	2.8
5	0.4	0	*1.4	1.9	3.0	0	2.1	3.4
6	0.9	0	*1.5	2.1	3.2	0	2.4	4.0
7	1.7	0	1.8	1.7	3.0	0	2.0	4.3
8	1.2	0	1.7	0.9	2.3	0	1.9	4.6
9	1.0	0	1.5	0.7	2.3	0	1.9	3.9
10	0	0	1.2	0.5	0	0	1.7	3.7

pH	P. citrinum			
	A	C	L	P
2	1.6	*0	1.1	1.7
3	1.7	0	1.2	2.3
4	2.0	0	1.4	3.1
5	2.2	0	1.4	3.8
6	2.4	0	1.6	3.9
7	2.5	0	1.8	6.0
8	1.4	0	1.2	4.7
9	0.7	*0	1.2	4.2
10	0	*0	0.4	3.0

pH	*C. globosum*				*F. oxysporum*			
	A	C	L	P	A	C	L	P
2	2.0	*0	*0.7	0.7	1.6	0	0.6	2.0
3	2.4	*0	*0.8	1.0	2.0	0	0.7	3.0
4	2.5	0.4	1.2	1.5	2.2	0	1.0	3.5
5	2.6	0.4	1.3	1.7	2.4	0	1.1	4.2
6	3.2	0.6	1.6	1.9	2.6	0	1.1	4.3
7	4.7	0.1	2.2	2.4	3.6	0.2	1.5	4.6
8	3.5	*0	1.7	1.7	3.4	0.4	1.0	3.8
9	3.1	*0	1.6	1.6	2.9	0	1.0	3.6
10	0	*0	1.6	1.2	2.5	0	0.6	3.0

pH	*T. lignorum*			
	A	C	L	P
2	1.8	0.3	1.3	1.9
3	2.0	1.2	1.5	2.2
4	2.4	0.6	1.6	2.4
5	2.6	0.6	1.8	2.6
6	2.7	0.4	1.9	6.0
7	2.3	0.4	2.4	6.0
8	1.6	0.4	1.8	1.9
9	1.2	*0	1.7	1.4
10	0	*0	1.3	1.0

APPENDIX 5.5

AMYLASE ACTIVITIES OF TEST FUNGI AT DECREASING a_w - pH5

(cm/30 day - mean of 3 replicates)

a_w	A. amstelodami		A. chevalieri	
	GLYCEROL	NaCl	GLYCEROL	NaCl
CONTROL	4.0	4.0	3.2	3.2
0.97	3.2	2.5	2.5	2.7
0.95	2.2	2.0	2.3	2.1
0.93	1.6	1.4	1.9	*1.4
0.90	*0	*0	1.9	*0.5
0.87	*0	*0	1.1	* 0
0.85	*0	*0	*0.4	* 0

a_w	A. niger		A. repens	
	GLYCEROL	NaCl	GLYCEROL	NaCl
CONTROL	5.5	5.5	2.2	2.2
0.97	4.3	2.8	2.0	2.4
0.95	2.8	2.5	1.8	0.7
0.93	2.1	2.0	1.8	0.6
0.90	*1.7	*1.6	1.6	* 0
0.87	*1.5	*1.4	1.9	* 0
0.85	*1.0	*0.9	* 0	* 0

a_w	A. ruber		A. versicolor	
	GLYCEROL	NaCl	GLYCEROL	NaCl
CONTROL	* 0	* 0	6.2	6.2
0.97	*1.0	*1.4	5.6	*1.8
0.95	*2.0	*1.7	*2.0	*1.7
0.93	*0.5	* 0	*1.7	*1.5
0.90	* 0	* 0	*1.5	*1.3
0.87	* 0	* 0	*1.4	*1.2
0.85	* 0	* 0	*1.0	*0.9

*No visible growth

a_w	*P. citrinum*		*C. globosum*	
	GLYCEROL	NaCl	GLYCEROL	NaCl
CONTROL	3.7	3.7	3.4	3.4
0.97	3.6	3.0	2.9	3.0
0.95	2.9	2.5	*1.9	2.4
0.93	2.1	2.0	*1.5	*1.9
0.90	1.7	1.5	*0.8	*1.0
0.87	0.9	0.7	* 0	* 0
0.85	0	0	* 0	* 0

a_w	*F. oxysporum*		*T. lignorum*	
	GLYCEROL	NaCl	GLYCEROL	NaCl
CONTROL	*2.7	*2.7	3.0	3.0
0.97	*2.7	*2.4	3.4	*2.6
0.95	*2.4	*2.1	2.4	*1.9
0.93	*2.2	*1.7	*2.0	*1.2
0.90	*1.2	*0.7	*0.9	*0.5
0.87	* 0	* 0	* 0	* 0
0.85	* 0	* 0	* 0	* 0

APPENDIX 5.6

LIPASE ACTIVITIES OF TEST FUNGI AT DECREASING a_w - pH7

(cm/30 days - mean of 3 replicates)

a_w	A. amstelodami		A. chevalieri	
	GLYCEROL	NaCl	GLYCEROL	NaCl
CONTROL	3.1	3.1	3.3	3.3
0.97	2.9	3.0	3.2	3.5
0.95	2.1	3.2	2.2	3.4
0.93	1.9	3.1	1.7	3.4
0.90	1.7	2.0	1.6	3.0
0.87	1.4	2.0	1.3	1.2
0.85	1.3	1.5	1.2	1.0
0.83	1.1	1.2	1.2	*1.0
0.80	0.9	0	1.0	*0.8
0.77	0.7		0.8	
0.75	0.5		*0.7	
0.73	*0.4		*0.6	
0.70	*0.3		*0.5	

a_w	A. niger		A. repens	
	GLYCEROL	NaCl	GLYCEROL	NaCl
CONTROL	3.1	3.1	3.4	3.4
0.97	2.1	2.4	2.9	3.0
0.95	2.0	2.4	1.7	2.7
0.93	1.7	2.0	1.5	2.7
0.90	1.3	2.0	1.5	2.0
0.87	0.7	0.5	1.0	1.4
0.85	0.3	* 0	1.0	*1.4
0.83	* 0	* 0	1.0	*0.9
0.80	* 0	* 0	0.9	*0.3
0.77	* 0		0.8	
0.75	* 0		*0.7	
0.73	* 0		*0.5	
0.70	* 0		*0.5	

a_w	A. ruber		A. versicolor	
	GLYCEROL	NaCl	GLYCEROL	NaCl
CONTROL	3.1	3.1	4.0	4.0
0.97	2.8	3.4	3.4	4.5
0.95	2.0	3.2	3.0	4.2
0.93	2.0	2.2	2.6	3.7
0.90	1.6	2.0	2.2	2.4
0.87	1.4	1.9	1.4	1.5
0.85	1.2	1.5	1.3	1.4
0.83	1.2	1.3	1.1	*1.1
0.80	1.1	1.1	*1.0	* 0
0.77	0.9		*1.0	
0.75	0.9		*1.0	
0.73	*0.8		*0.6	
0.70	*0.7		*0.3	

a_w	P. citrinum	
	GLYCEROL	NaCl
CONTROL	2.3	2.3
0.97	1.5	2.3
0.95	1.4	1.9
0.93	1.0	1.3
0.90	0.8	*0.7
0.87	0	* 0

a_w	*C. globosum*		*F. oxysporum*	
	GLYCEROL	NaCl	GLYCEROL	NaCl
CONTROL	2.3	2.3	2.8	2.8
0.97	1.7	2.5	2.5	2.0
0.95	1.3	2.0	2.4	2.0
0.93	*0.9	*1.0	2.0	1.4
0.90	*0.5	*0.7	1.8	*0.8

a_w	*T. lignorum*	
	GLYCEROL	NaCl
CONTROL	2.9	2.9
0.97	2.4	3.0
0.95	1.9	2.0
0.93	*1.1	*1.0
0.90	*0.8	*0.2

APPENDIX 5.7

PROTEASE ACTIVITIES OF TEST FUNGI AT DECREASING a_w - pH7

(cm/30 days - mean of 3 replicates)

a_w	A. amstelodami		A. chevalieri	
	GLYCEROL	NaCl	GLYCEROL	NaCl
CONTROL	0	0	0	0
0.97	1.5	1.9	0	0
0.95	2.9	3.0	2.0	0
0.93	3.2	0	2.4	0
0.90	2.9	0	2.1	0
0.87	2.5	0	2.0	0
0.85	1.8	0	1.9	* 0
0.83	1.7	0	1.7	* 0
0.80	1.4	0	1.2	* 0
0.77	1.3		*1.0	
0.75	*1.1		*0.9	
0.73	*1.1		*0.9	
0.70	*1.1		*0.5	

a_w	A. niger		A. repens	
	GLYCEROL	NaCl	GLYCEROL	NaCl
CONTROL	3.5	3.5	0	0
0.97	2.7	3.2	0	0
0.95	2.1	3.0	2.3	0
0.93	1.7	2.2	2.3	0
0.90	1.3	1.6	1.9	0
0.87	1.0	0	1.8	0
0.85	0.8	0	1.7	* 0
0.83	0.6	* 0	1.5	* 0
0.80	0.6	* 0	1.0	* 0
0.77	0.4		0.9	
0.75	* 0		*0.9	
0.73	* 0		*0.9	
0.70	* 0		*0.4	

a_w	*A. ruber*		*A. versicolor*	
	GLYCEROL	NaCl	GLYCEROL	NaCl
CONTROL	0	0	7.0	7.0
0.97	1.9	3.4	7.0	6.0
0.95	2.9	3.2	7.0	0
0.93	2.9	3.0	4.0	0
0.90	2.7	2.1	3.5	0
0.87	1.9	0	2.7	0
0.85	1.6	0	2.7	0
0.83	1.5	* 0	2.3	* 0
0.80	1.3	* 0	2.2	* 0
0.77	1.2		*2.0	
0.75	*1.2		*1.5	
0.73	*1.2		*1.4	
0.70	*0.8		*1.0	

a_w	*P. citrinum*	
	GLYCEROL	NaCl
CONTROL	7.0	7.0
0.97	2.9	4.0
0.95	2.0	2.5
0.93	1.2	0
0.90	0.9	0
0.87	0	0

a_w	*C. globosum*		*F. oxysporum*	
	GLYCEROL	NaCl	GLYCEROL	NaCl
CONTROL	2.2	2.2	7.0	7.0
0.97	1.4	1.1	5.1	4.8
0.95	0.3	*0.1	4.2	3.8
0.93	0	* 0	4.0	3.2
0.90	0	* 0	2.9	2.0

a_w	*T. lignorum*	
	GLYCEROL	NaCl
CONTROL	7.0	7.0
0.97	7.0	2.2
0.95	*1.1	* 0
0.93	* 0	* 0
0.90	* 0	* 0

APPENDIX 6.1

C[illegible] OF INCREASING CONCENTRATIONS OF FUNGICIDES ON THE GROWTH RATES

C[illegible] TEST FUNGI. GROWTH RATE ALSO EXPRESSED AS A % OF CONTROL

(mm/day - mean of 5 replicates)

FUNGICIDE CONCENTRATION ppm	GROWTH MEDIUM	A. amstelodami		A. chevalieri		A. niger	
		GROWTH RATE mm/day	% OF CONTROL	GROWTH RATE mm/day	% OF CONTROL	GROWTH RATE mm/day	% OF CONTROL
- CONTROL	MEA	3.25	100	2.24	100	7.63	100
CAPTAN 25	MEA	3.42	105.23	1.99	88.34	8.50	111.40
50	MEA	2.02	62.15	1.29	57.59	8.20	107.47
150	MEA	1.90	58.46	0.98	43.75	4.76	62.39
300	MEA	0.76	23.38	0.48	21.43	3.56	46.66
400	MEA	0.71	21.85	0.31	13.84	3.31	43.38
500	MEA	0.50	15.38	0.21	9.38	2.93	38.40
- CONTROL	*MEAG	9.21	100	12.38	100	17.17	100
CAPTAN 25	MEAG	9.90	107.49	8.90	71.89	17.83	103.84
50	MEAG	6.08	66.02	7.35	59.37	12.75	74.26
150	MEAG	3.52	38.22	1.52	12.38	3.00	17.47
300	MEAG	1.45	15.74	0.93	7.51	1.89	11.01
400	MEAG	1.00	10.86	0.52	4.20	1.20	6.99
500	MEAG	0.45	4.89	0.31	2.50	1.07	6.23
VERDASAN 0.1	MEA	4.56	140.31	2.25	100.45	11.75	154.00
0.5	MEA	1.67	51.38	1.62	72.32	11.88	155.70
1.0	MEA	1.44	44.31	1.42	63.39	10.80	141.55
2.5	MEA	1.14	35.08	1.29	57.59	8.40	110.09
5.0	MEA	0	0	0	0	5.29	69.33
10.0	MEA	0	0	0	0	2.37	31.06
VERDASAN 0.1	MEAG	7.70	83.60	8.50	68.22	11.38	66.28
0.5	MEAG	6.68	72.53	8.20	66.24	9.85	57.37
1.0	MEAG	5.06	54.94	6.85	55.33	7.70	44.85
2.5	MEAG	3.44	37.35	4.27	34.49	5.17	30.11
5.0	MEAG	2.43	26.38	1.43	11.55	3.19	18.58
10.0	MEAG	0	0	0	0	1.67	9.73

*MEAG = MEA + glycerol (a_w 0.95)

FUNGICIDE	CONCENTRATION ppm	GROWTH MEDIUM	A. amstelodami		A. chevalieri		A. niger	
			GROWTH RATE mm/day	% OF CONTROL	GROWTH RATE mm/day	% OF CONTROL	GROWTH RATE mm/day	% OF CONTROL
-	CONTROL	MEA	3.25	100	2.24	100	7.63	100
CAPTAN	25	MEA	3.42	105.23	1.99	88.34	8.50	111.40
	50	MEA	2.02	62.15	1.29	57.59	8.20	107.47
	150	MEA	1.90	58.46	0.98	43.75	4.76	62.39
	300	MEA	0.76	23.38	0.48	21.43	3.56	46.66
	400	MEA	0.71	21.85	0.31	13.84	3.31	43.38
	500	MEA	0.50	15.38	0.21	9.38	2.93	38.40
-	CONTROL	*MEAG	9.21	100	12.38	100	17.17	100
CAPTAN	25	MEAG	9.90	107.49	8.90	71.89	17.83	103.84
	50	MEAG	6.08	66.02	7.35	59.37	12.75	74.26
	150	MEAG	3.52	38.22	1.52	12.38	3.00	17.47
	300	MEAG	1.45	15.74	0.93	7.51	1.89	11.01
	400	MEAG	1.00	10.86	0.52	4.20	1.20	6.99
	500	MEAG	0.45	4.89	0.31	2.50	1.07	6.23
VERDASAN	0.1	MEA	4.56	140.31	2.25	100.45	11.75	154.00
	0.5	MEA	1.67	51.38	1.62	72.32	11.88	155.70
	1.0	MEA	1.44	44.31	1.42	63.39	10.80	141.55
	2.5	MEA	1.14	35.08	1.29	57.59	8.40	110.09
	5.0	MEA	0	0	0	0	5.29	69.33
	10.0	MEA	0	0	0	0	2.37	31.06
VERDASAN	0.1	MEAG	7.70	83.60	8.50	68.22	11.38	66.28
	0.5	MEAG	6.68	72.53	8.20	66.24	9.85	57.37
	1.0	MEAG	5.06	54.94	6.85	55.33	7.70	44.85
	2.5	MEAG	3.44	37.35	4.27	34.49	5.17	30.11
	5.0	MEAG	2.43	26.38	1.43	11.55	3.19	18.58
	10.0	MEAG	0	0	0	0	1.67	9.73

*MEAG = MEA + glycerol (a_w 0.95)

APPENDIX 6.2

EFFECT OF INCREASING CONCENTRATIONS OF FUNGICIDES ON THE DRY WEIGHT PRODUCTION OF TEST FUNGI. DRY WEIGHT PRODUCTION ALSO EXPRESSED AS A % OF CONTROL

(mg/day - mean of 5 replicates)

FUNGICIDE	CONCENTRATION ppm	GROWTH MEDIUM	A. amstelodami		A. chevalieri		A. niger	
			DRY WEIGHT mg	% OF CONTROL	DRY WEIGHT mg	% OF CONTROL	DRY WEIGHT mg	% OF CONTROL
-	CONTROL	MEB	27.36	100	33.06	100	34.09	100
CAPTAN	25	MEB	26.35	96.31	28.78	87.05	30.72	90.11
	50	MEB	26.19	95.72	27.10	81.97	26.97	79.11
	150	MEB	0	0	0.17	0.51	21.13	61.98
	300	MEB	0	0	0	0	1.34	3.93
	400	MEB	0	0	0	0	1.05	3.08
	500	MEB	0	0	0	0	0	0
-	CONTROL	MEBG	101.42	100	108.69	100	184.94	100
CAPTAN	25	MEBG	74.58	73.54	70.71	65.06	123.54	66.80
	50	MEBG	43.78	43.17	44.56	41.00	91.83	49.65
	150	MEBG	14.53	14.33	17.26	15.88	20.45	11.06
	300	MEBG	13.23	13.04	16.04	14.76	15.74	8.51
	400	MEBG	13.08	12.90	14.40	13.25	15.29	8.27
	500	MEBG	9.25	9.12	10.96	10.08	4.34	2.35
VERDASAN	0.1	MEB	26.87	98.21	31.46	95.16	33.02	98.86
	0.5	MEB	24.96	91.23	29.73	89.93	32.08	94.10
	1.0	MEB	7.86	28.73	15.21	46.01	31.31	91.85
	2.5	MEB	0	0	0	0	30.96	90.82
	5.0	MEB	0	0	0	0	10.23	30.00
	10.0	MEB	0	0	0	0	0	0
VERDASAN	0.1	MEBG	128.24	126.44	100.38	92.35	176.62	95.50
	0.5	MEBG	124.89	123.14	90.99	83.72	155.32	83.98
	1.0	MEBG	116.08	114.45	79.59	73.23	134.22	72.57
	2.5	MEBG	68.22	67.26	33.91	31.20	87.35	47.23
	5.0	MEBG	17.93	17.68	17.06	15.70	32.43	17.54
	10.0	MEBG	12.76	12.58	16.85	15.50	16.43	8.88

APPENDIX 6.3

EFFECT OF INCREASING CONCENTRATIONS OF FUNGICIDES ON THE AMYLASE ACTIVITIES OF TEST FUNGI

(cm/7days - mean of 2 replicates/7 days)

FUNGI	TIME (WEEKS)	CONTROL		CAPTAN 50 ppm		CAPTAN 500 ppm		VERDASAN 0.5 ppm		VERDASAN 10 ppm	
		PLA	°PLAG	PLA	PLAG	PLA	PLAG	PLA	PLAG	PLA	PLAG
A. amstelodami	1	1.2	0	0.5	0.2	*0	* 0	0.9	* 0	*0	* 0
	2	2.2	1.9	1.6	1.7	*0	* 0	2.0	1.8	*0	* 0
	3	3.0	2.4	2.3	2.4	*0	* 0	2.9	2.0	*0	* 0
	4	3.6	2.8	3.4	3.5	*0	* 0	3.5	2.4	*0	* 0
A. chevalieri	1	1.0	0.7	0.5	0	*0	* 0	0.8	0.7	*0	* 0
	2	2.0	1.5	1.5	1.6	*0	* 0	1.7	1.5	*0	* 0
	3	2.5	2.2	2.1	2.0	*0	0	2.2	2.0	*0	* 0
	4	3.0	2.8	2.6	2.5	1.5	1.0	2.6	2.3	*0	* 0
A. niger	1	2.4	2.2	2.1	2.0	2.1	2.0	2.2	2.0	*2.0	1.5
	2	3.4	3.4	3.5	3.4	3.3	3.1	3.3	3.1	*3.2	2.4
	3	4.4	4.3	4.4	4.4	4.4	3.8	4.3	3.9	*4.2	3.6
	4	5.5	5.0	5.3	5.1	5.0	4.6	5.0	4.0	*4.5	3.8

°PLAG = PLA + glycerol (a_w 0.95) *no visible growth

APPENDIX 6.4

EFFECT OF INCREASING CONCENTRATIONS OF FUNGICIDES

ON THE LIPASE ACTIVITIES OF TEST FUNGI

(cm/7 days - mean of 2 replicates/7 days)

FUNGI	TIME (WEEKS)	CONTROL		CAPTAN 50 ppm		CAPTAN 500 ppm		VERDASAN 0.5 ppm		VERDASAN 10 ppm	
		PLA	PLAG	PLA	PLAG	PLA	PLAG	PLA	PLAG	PLA	PLAG
A. amstelodami	1	1.2	1.1	1.1	0.9	0	0	1.2	1.2	0.9	0.8
	2	1.7	1.7	1.7	1.7	1.5	1.3	1.9	1.6	1.6	1.5
	3	2.2	2.1	2.2	2.0	2.0	1.7	2.3	2.1	2.1	1.8
	4	2.6	2.3	2.5	2.4	2.3	2.1	2.5	2.3	2.2	2.0
A. chevalieri	1	1.3	1.2	1.1	1.1	0	0	1.1	1.1	1.0	*0.8
	2	1.9	1.9	1.9	1.8	1.4	1.3	2.1	1.7	1.7	1.5
	3	2.5	2.2	2.4	2.3	1.9	1.7	2.5	2.4	2.1	2.0
	4	3.0	2.8	2.9	2.6	2.4	2.1	2.9	2.7	2.5	2.3
A. niger	1	0.9	0.7	1.0	1.0	0	0	0.8	0.6	0.4	0.3
	2	1.7	1.4	1.6	1.2	1.4	0.8	1.2	1.0	1.1	1.1
	3	1.9	1.5	2.0	1.9	1.9	1.0	1.5	1.4	1.5	1.4
	4	2.1	1.9	2.3	2.1	2.1	1.4	1.7	1.6	1.9	1.7

APPENDIX 6.5

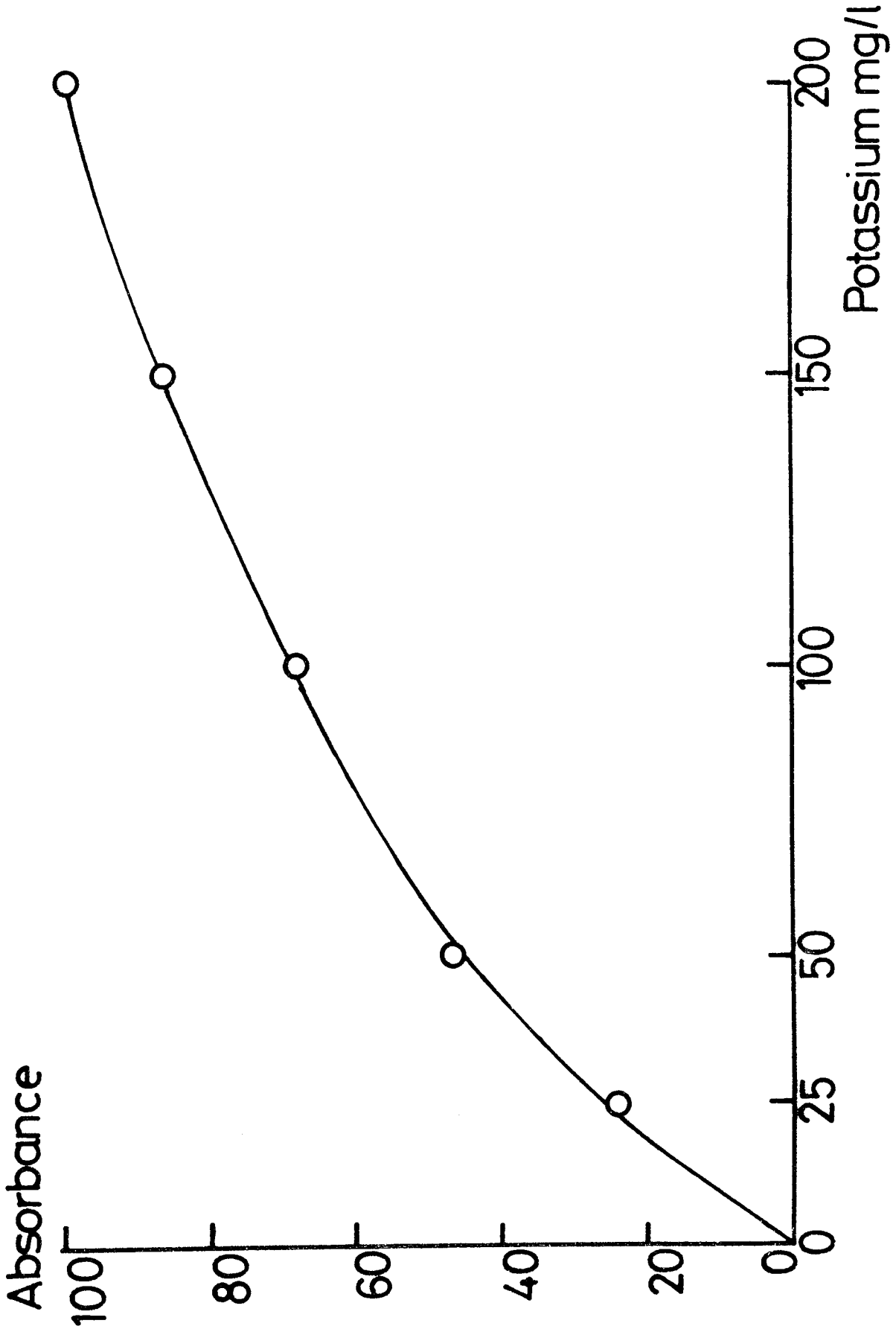
EFFECTS OF INCREASING CONCENTRATIONS OF FUNGICIDES ON PROTEASE ACTIVITIES OF TEST FUNGI

(cm/7 days - mean of 2 replicates/7 days)

FUNGI	TIME (WEEKS)	CONTROL		CAPTAN 50 ppm		CAPTAN 500 ppm		VERDASAN 0.5 ppm		VERDASAN 1.0 ppm	
		PLA	PLAG	PLA	PLAG	PLA	PLAG	PLA	PLAG	PLA	PLAG
A. amstelodami	1	0.25	1.2	0.1	1.0	0	0	0.1	0.9	*0	*0.8
	2	0.55	1.5	0.3	1.0	0	0	0.4	1.4	*0	*0.9
	3	0.7	1.9	0.7	1.3	0	0	0.5	1.5	*0.2	1.2
	4	0.75	2.6	0.9	1.8	0	0	0.6	1.9	*0.4	1.5
A. chevalieri	1	0	0	0	0	0	0	0	0	*0	*0
	2	0	0	0	0	0	0	0	0	*0	*0
	3	0	0	0	0	0	0	0	0	*0	*0
	4	0	0	0	0	0	0	0	0	*0	0
A. niger	1	1.4	1.1	1.5	0	0	0	1.5	1.2	*0	0
	2	1.8	1.4	1.8	1.1	0	0	1.8	1.4	*0.2	0
	3	2.5	2.2	2.0	1.7	0	0	2.6	2.1	*1.0	0
	4	2.8	2.5	2.7	2.3	0	0	2.7	2.4	*1.4	0

APPENDIX 6.6

STANDARD CURVE OF POTASSIUM ASSAY USING POTASSIUM CHLORIDE


Absorbance
100
80
60
40
20
0
0
25
50
100
150
200
Potassium mg/l

APPENDIX 6.7

EFFECTS OF INCREASING CONCENTRATIONS OF FUNGICIDES ON POTASSIUM LEAKAGE FROM TEST FUNGI

(ug potassium/mg dry wt. of mycelium/hr - mean of 5 replicates)

FUNGICIDE CONCENTRATION ppm	GROWTH MEDIUM	ug Potassium/mg dry wt. of mycelium/hr.		
		A. amstelodami	A. chevalieri	A. niger
- CONTROL	MEB	*0 (7.58)	0 (7.10)	0 (5.10)
- CONTROL	MEBG	0 (1.74)	0 (3.58)	0 (3.70)
CAPTAN 50	MEB	4.47	4.48	1.54
500	MEB	8.33	4.99	3.34
CAPTAN 50	MEBG	0.40	1.48	0.38
500	MEBG	0.93	2.20	1.74
VERDASAN 0.5	MEB	7.37	6.88	3.01
10.0	MEB	11.81	8.73	7.34
VERDASAN 0.5	MEBG	0.55	4.99	1.36
10.0	MEBG	1.61	7.96	2.49

*0 (7.58) = values of control are substracted from values obtained with increasing concentrations of fungicides.

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