STUDIES ON THE STORAGE ROT OF SWEET POTATO (*IPOMOEA BATATAS* L & LAM) BY *BOTRYODIPLODIA THEOBROMAE* PAT. AND OTHER FUNGI

By

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A B S T R A C T

The storage rot of sweet potato (s.p.) (Ipomoea batatas) tuberous roots by Botryodiplodia theobromae (B.t.), Botrytis cinerea (B.c.) and Cladosporium cucumerinum (C.c.) was studied. The tuber was susceptible to rot by B. theobromae but was colonized to a limited extent by B. cinerea and C. cucumerinum.

The role of pectic enzymes in the successful rotting of s.p. by B.t. was investigated. B.t. produced four PG isoenzymes in vitro one of which was recovered from rotted sweet potato tissue. The properties of these isoenzymes were studied. The possible interaction between the host's metabolites (phenols and oxidative enzymes) and the pectic enzymes of B.t. was discussed in relation to the successful rotting of the tuber by the fungus.

Comparatively little pectic enzyme (PG) was recovered from tissues inoculated with B.c. and no pectic enzyme was found in tissues inoculated with C.c.

Low temperature treatment (0-7°C) of the tuber induced chilling injury rendering the tissues more susceptible to rot by the fungi.

The accumulation of antifungal compounds by s.p. inoculated with B.t., B.c. and C.c. was examined. All the fungi induced the formation of four terpenoid phytoalexins. The terpenes were present at lower concentrations in spreading B.t. lesions than in the very restricted lesions formed by the other fungi. B.t. was insensitive to the phytoalexins at concentrations at which they occur in the tissue whereas B.c. and C.c. were sensitive in varying degrees to these compounds. In culture, B.t. degraded the compounds within 24h while B.c. and C.c. degraded them little or not at all.

The roles of pectic enzymes and phytoalexins in the susceptibility and resistance of s.p. to rot is discussed.

ABBREVIATIONS

B.c.	Botrytis cinerea
B.t.	Botryodiplodia theobromae
°C	degree(s) Centigrade
C.c.	Cladosporium cucumerinum
cm	centimeter(s)
Fig	figure
F.s.	Fusarium solani
g	gram(s)
h	hour(s)
l	litre(s)
LANS	Long Ashton Nutrient Solution
LSA	Leonian Solution Agar
NaPP	Sodium polypectate
NaDSS	Sodium dioctylsulphosuccinate
N	Normal
nm	nanometer(s)
No.	number(s)
mg	milligram(s)
ml	millilitre(s)
mm	millimetre(s)
min	minute(s)
M	Molar
p., pp	page, pages

P	Pectin
P.e.	*Phoma exigua*
P.G.	polygalacturonase
p.p.m.	parts per million
p.s.i.	pounds per square inch
PTE	Pectate trans-eliminase
PVP	Polyvinylpyrollidone
R.H.	relative humidity
s	second(s)
SCA	Sucrose-casamino acid
TLC	Thin layer chromatography
T.B.A.	Thiobarbituric acid
uv	ultra violet
$v/_v$	volume/volume
$w/_v$	weight/volume
µg	microgram(s)
µl	microlitre(s)
µ	micron(s)
%	percent

C O N T E N T S

INTRODUCTION

The sweet potato (tuberous) root in storage is subject to decay by a number of microorganisms. In Nigeria, one of the most important agents of decay is the fungus Botryodiplodia theobromae, an ubiquitous parasite in the tropics, attacking a large number of storage crops (Arinze et al. 1975).

The fungus is particularly successful in invading parenchymatous tissues which have suffered bruises or cuts. In doing so, it produces, like many phytopathogenic organisms several enzymes which degrade the complex polysaccharides of the plant's cell wall. Reports had shown that the fungus produced pectic enzymes in culture (Ogundana et al., 1971, Arinze et al., 1976). These reports speculated on the possible involvement of the enzymes in the rot of yams and sweet potato. The enzymes obtained in those reports were however not purified. The purification and characterization of these pectic enzymes is one of the aims of the work presented in this thesis.

The possible role of the pectic enzymes in causing rot of sweet potato and the systems of the host (metabolites) which may inactivate or inhibit these enzymes have been studied. The interplay of the host's metabolites and the fungal enzymes which result in successful colonization of the tubers by the fungus have been considered.

Sweet potato is known to form antifungal substances (phytoalexins) in response to infection by fungi. These

substances inhibit the growth of fungi in the tissue and thus induce resistance. However, observation shows that despite these phytoalexins, B. theobromae rots the tubers successfully. During this investigation, it was found that under similar storage conditions Botrytis cinerea and Cladosporium cucumerinum form only limited lesion or none at all. This work aims to investigate the accumulation of the phytoalexins by sweet potato when challenged with these fungi and to explain why despite the presence of phytoalexins, B. theobromae successfully invades the tissues while B. cinerea and C. cucumerinum do not.

REVIEW OF LITERATURE

1. ENZYME STUDIES

I The pathogen

Botryodiplodia theobromae, Pat. is a member of the Fungi Imperfecti, of the Order Sphaeropsidales, Family Sphaeropsidaceae.

The nutritional requirements of the fungus are wide and varied. In addition to simple sugars, it utilises lactose, oligosaccharides and the polysaccharides, starch and dextrin (Srivastava and Tandon, 1968, 1968, 1970) and a wide variety of N_2 sources in culture (Arinze, 1974). For growth, B. theobromae tolerates a pH range of 2-12 with optimum at pH 5.0; requires a temperature of 20-35°C with optimum at 25-30° and growth is not affected by light duration or intensity.

Reproduction is by means of dark coloured, typically one-septate conidia (pycnospores) produced in semi-closed dark, rounded pycnidia. The fungus produces abundant pycnidia on several media with best results on natural media but only under continuous fluorescent light (Ekundayo & Haskins, 1969). Pycnospore germination is however not affected by light.

B. theobromae parasitizes a wide variety of tropical crops e.g. cocoa (from which it derives its name), banana (Ogawa, 1970), guava, oranges and mango (Srivastava & Tandon,

1969, 1970), yams (Adeniji, 1970) cotton (Cauquil, Follin & Goffete, 1968-1969) cassava (Ekundayo & Daniels, 1973) and sweet potato (Arinze et al., 1975).

Typically the fungus is a wound parasite and is highly successful in invading tissues which have suffered bruises or cuts during cultivation or storage.

II Storage rots of sweet potato

B. theobromae causes soft rot of sweet potato when stored under high temperature and humidity. There are, however, a number of other fungi which also cause rots of one form or the other in stored sweet potato tubers. A brief mention of these is worthwhile here.

By far the most widely reported is the "soft rot" caused by Rhizopus stolonifer (Daines, 1942; Srivastara & Walker, 1959). The "black rot" caused by Ceratocystis fimbriata is widely known in Japan and USA and the phytopathological biochemistry of sweet potato with respect to the disease has been widely treated by Uritani and his team in Japan. Fusarium oxysporum f. batatas also causes "surface rot" of the tubers. Here, the infected tissues are sub-epidermal and lesions generally circular as tissue invasion radiates from a wound (Martin & Person, 1951). Other less well known diseases of sweet potato are the "circular spot" caused by soil-borne Sclerotium rolfsii (Martin 1953) and the "soil

rot" caused by Streptomyces ipomoae (Person & Martin, 1940).

Like B. theobromae, most of these pathogens enter the tuber either in the soil or during storage through wounds caused by nematode attack (Birchfield & Martin, 1965; Kreis, 1937; Burk & Tennyson, 1941) or by insect attack (Cuthbert 1965, 1967; and Marcovitch, 1946) and mostly through mechanical wounds (bruises and cuts) caused during harvesting, packing and transportation.

III The production of pectic enzymes by plant pathogens

(A) Historical background

De Bary (1886) was perhaps one of the earliest workers to demonstrate that a cell-wall dissolving principle was contained in a fungus extract. He showed that juice extracted from rotted carrot roots infected with Sclerotinia libertiana contained a thermolabile principle capable of dissolving cell walls of plants. He was however not able to show whether this action was due to one or more substances. The findings of De Bary were substantiated by Ward (1888) who concluded that the entrance of a hypha into the tissue depended upon excretion of a "ferment" which dissolved cellulose.

Jones (1905, 1909, 1910) later described a pectinase produced by Bacillus carotovorus (Erwinia carotivora) which he

demonstrated was capable of dissolving the middle lamella of plant cells and hence he concluded that this was responsible for the soft rot of vegetables. Brown (1915) went a step further to demonstrate that treatment of parenchyma with extracts of young hyphae from Botrytis cinerea led to a loss of coherence between cells, collapse of cell walls and even death of the protoplast. These findings gave impetus to the numerous literature available today on the involvement of pectic enzymes in many host-parasite interactions. These pectic enzymes occur in diverse types of diseases - soft rots, dry rots, wilts, leaf spots etc. (Bateman and Miller, 1966).

(B) Classification of pectic enzymes

Pectic enzymes are classified according to the following criteria:

(1) The mechanism by which the α-1,4 glycosidic bond in a substrate is split (i.e. hydrolytic or lytic),

(2) Enzyme specificity for the substrate (i.e. pectin or pectic acid),

and (3) The position in the pectic chain at which cleavage occurs (i.e. random or terminal).

At first, it was thought that the hydrolytic polygalacturonases were the only type of chain-splitting enzymes. The work of Albersheim

Neukom & Deuel (1960a) showed the existence of an enzyme which ruptured the ∝-1, 4 linkages by transelimination reaction. The product is an unsaturated compound with double bond between carbon 4 and 5. The enzymes are called transeliminases or lyases. The hydrolytic and transeliminative reaction products are distinguishable by their reaction with thiobarbituric acid (TBA) (Neukom 1960; Ayers, Papavizas & Diem, 1966).

Hydrolases which exhibit a distinct specificity for pectin rather than pectic acid as substrate are called pectin-methylgalacturonases whereas those that exhibit preference for pectic acid are polygalacturonases. The pectinmethylesterases show high specificity towards methyl ester groups of pectinic acids, saponifying these to yield methyl alcohol and pectinic acids of lower methoxyl content. The polygalacturonases on the other hand rupture the uronide chains hydrolytically at the glycosidic linkages to give shorter chains and at the same time liberate reducing groups.

Pectic enzymes are also classified into exo-enzymes which attack their substrate in a terminal manner, splitting off only terminal monomeric products (galacturonic acid residues) and endo-enzymes which attack randomly along the chain to release oligomers (Demain and Phaff, 1957).

(C) In vitro production of pectic enzymes by plant pathogens

Many plant pathogens are known to produce pectic enzymes in vitro and the cultural conditions which affect the secretion of these enzymes has been of interest. In most instances, the enzymes are produced ~~inductively~~ inducibly rather than constitutively. Pectic substances are the main inducers of pectic enzymes in culture media (Gäumann, 1947; Fergus & Wharton, 1957; Singh & Wood, 1956). Kamal & Wood (1956) however showed that Verticillium dahliae produced pectic enzymes in the absence of pectic substances.

The effect of various cultural conditions on the secretion of pectic enzymes have been reported. Damle (1952) demonstrated that B. cinerea is capable of producing different pectic enzymes depending on the pH of the culture medium. When grown in an acid culture the fungus produced enzyme with optimum activity at pH 5.6 whereas on an alkaline medium, the enzyme produced had optimum activity at pH 8.0. Later work by Bateman (1966) with Fusarium solani f. phaseoli and by Hancock (1965) with Colletotrichum trifolii showed that the pH of the medium determined the type of activity expressed by the enzyme. Consequently, an advantage of in vitro studies is that the properties of the enzymes could be modified by varying the cultural conditions. The possibility of exploiting this in disease development and control is an obvious significance of in vitro experiments.

It appears from recent finding that the more important determinative factors in regulating pectic enzyme synthesis are induction and catabolite repression. Cell-wall degrading enzymes are usually induced by a monomer or a dimer of the polysaccharide substrate of the enzyme. Thus galacturonic acid and its structural derivatives induce synthesis of pectic enzymes (Keen and Horton, 1966; Pardee, 1962) cellobiose induces synthesis of cellulases (Mandels and Reese, 1960) and galactose induces synthesis of ∝-galactosidase (English, Jurale and Albersheim, 1971).

However synthesis of these enzymes can be repressed when the inducers are present in slight excess of the requirement for growth. Cooper (1974) demonstrated that synthesis of endo PG and PTE by Verticillium albo-atrum increased with inducer supply until the inducer began to accumulate, then decreased sharply and was almost completely repressed at supply rates three times the optimal level. This catabolite repression was first described by Magasanik (1961) and recently reviewed by Paigen and Williams (1970). Induction and catabolite repression have so far been described in in vitro experiments and are thought to be of particular significance in pathogenesis (Cooper and Wood 1975).

(D) In vivo production of pectic enzymes by plant pathogens

Wood (1960) has argued that the fact that a pathogen produces a pectic enzyme in culture may not be significant in pathogenesis considering the fact that highly degraded substrates are used to measure the enzyme activity. It appears that the only satisfactory proof that a pectic enzyme plays a part in disease development is a demonstration that the enzyme is present in the plant tissue in an active form. This view is held by several workers (Bateman, 1963; Bateman and Beer, 1965; Hancock, Millar and Lorbeer, 1964, Spalding, Bruehl and Foster, 1961). Several pathogens are now known to produce polygalacturonase in infected tissues, Rhizopus stolonifer (Spalding, 1963) Rhizoctonia solani (Bateman, 1963a), Ceratocystis fimbriata (Uritani and Stahmann, 1961a) Penicillium expansum (Cole and Wood, 1961b); Cladosporium cucumerinum (Strider and Winstead, 1961) etc. Hancock and Millar (1965b) demonstrated that Colletotrichum trifolii produced both PG and a pectic transeliminase in alfalfa stem.

However, the failure to locate a pectic enzyme in diseased tissue may not necessarily indicate a non-involvement of the enzyme in pathogenesis. A study of the relationship between phenolase of bean (Vicia faba) and the pectic enzymes of Botrytis fabae and B. cinerea has led Deverall and Wood (1961) to suggest that the enzymes may have been functional at the early

phases of pathogenesis but became inactivated later. They suggest that assay of pectic enzymes at various stages of disease development would establish whether or not inactivation had occurred.

(E) Pectic enzymes and tissue maceration

The significance of pectic enzymes in disease development has been well known but the exact mechanism of action on living tissue to cause disease has been a subject of immense concern and continued investigation. Perhaps Brown (1915) first used the term "maceration" to describe the loss in coherence of parenchyma cells when treated with extracts of young hyphae of B. cinerea.

The cell wall of higher plants is known to consist of three main regions, the middle lamella, the primary cell wall and the secondary cell wall. The middle lamella consists essentially of pectates and both the primary and seondary walls consist of a matrix containing cellulose microfibrils. The microfibrils are not containedin the middle lamella though this zone is continuous with the primary cell wall. Northcote (1958, 1972) has shown that the microfibrils are composed of α-cellulose consisting of chains of β-1, 4 linked glucose residues. He demonstrated that the pectic substances are localised in the middle lamella and the primary wall and are absent from the

secondary wall. The secondary wall has more cellulose but less hemicellulose than the primary wall. It is now known that in parenchyma and similar tissues the secondary cell wall is often poorly developed (Wood 1967).

When parenchyma cells are attacked by a "macerating" enzyme the cells separate along the line of the middle lamella. Since the middle lamella consists essentially of pectates the macerating enzymes must be capable of dissolving them. Albersheim, Jones and English (1969) stated that most plant pathogens produce enzymes which can degrade all components of the primary cell wall. However most enzymatic preparations used to demonstrate maceration have been in a crude form. Bateman and Millar (1966) suggested that the term "macerating enzyme" be used where a crude preparation is involved. Where crude preparations of pectic enzymes contain some cellulase, there is evidence that the latter does not contribute significantly (Spalding, 1963; Cole and Bateman, 1969; Bateman, 1963; Kuć, 1962). So far there has been no evidence of involvement of cellulase in maceration.

A correlation of macerating ability of diseased tissue extract with macerating ability of pectic enzymes present in such extracts has been considered evidence of the involvement of pectic enzymes in pathogenesis (Bateman and Millar, 1966). Also Ca++ which is known to inhibit

degradation of pectic acid by endo-PG(Corden, 1965) has been shown to inhibit tissue maceration by the same enzyme (Bateman, 1964). On the other hand, Ca++ both activates endo lyases and stimulates macerating ability of diseased tissue extract containing endo-lyases (Hancock and Millar, 1965; Stephens, 1974).

Based on the information that the middle lamella consists essentially of pectates, several workers have concluded that degradation of the middle lamella could result from action of polygalacturonases alone. Furthermore, because maceration is often relatively rapid, an endo rather than an exopolygalacturonase is considered more likely to be involved. However, the ability of culture filtrates of Pythium debaryanum to macerate potato discs and their inability to degrade soluble pectates in vitro has led Wood and Gupta (1958) to reconsider the hypothesis. Since the filtrates can degrade high methoxylated pectinates in solution, it appears that the middle lamella also has pectins as important constituent. Based on the findings of Orgell (1955) and others Wood (1967) concluded that "maceration involves no more than solubilisation of insoluble pectic substances of the middle lamella and the primary cell wall by polygalacturonases alone or with pectin methyl galacturonases".

(F) Pectic enzymes and cell death

Brown (1915) showed that in addition to the cell-wall dissolving ability, the culture filtrates of B. cinerea possessed toxic properties. He recognised a close link between cell wall dissolution and the death of the protoplast. Spalding (1969) showed also that purified PG from Rhizopus stolonifer, free from phosphatidase and with negligible proteinase activity caused maceration and death of sweet potato tissue. It is now widely accepted that pectic enzymes of the endo type can cause maceration and cell death of tissues. There are many views on the exact mechanism of causation of cell death.

Perhaps Brown was first to attempt a separation of the two phenomena, maceration and death of cells. He found that the response of both activities to heat, dialysis, pH and mechanical shaking were essentially similar and concluded that both maceration and toxicity were as a result of the action of one and the same substance or group of substances. Since 1915, several attempts have been made to separate the toxic factor from the pectic enzymes from a number of pathogens (Deshponde, 1959; Fushtey, 1957). Kamal and Wood (1956) showed that heat treatment of Verticillium dahliae culture filtrate at 60-100° for 5 min decreased toxicity more than maceration. This difference in response has been explained on the basis of different kinetics for two processes caused by a common factor (Mount et al.1970).

It is still difficult to understand how pectic enzymes induce cell death unless the cellular membranes contain pectic substances or the products released as a result of enzyme action are in themselves injurious. There is so far no conclusive evidence to suggest that pectic substances are a constituent of these membranes and it is unlikely that the products of hydrolysis of cell wall are toxic considering the low concentration of the products formed (Fushtey, 1957).

An attempt to explain the mechanism of cell death was made by Wood (1967). He suggested the following possibilities:

1. That the protoplasts are killed by toxins which are not cell wall degrading enzymes and which act independently of them
2. That toxins are present which cannot act until the cells are conditioned by the action of the macerating enzymes
3. That there are no very active toxins present and that it is the macerating enzymes which kill cells
4. That maceration involves or is associated with activity of proteolytic enzymes which also kill protoplasts.

The current view on how pectic enzymes cause death is from plasmolysis effects. Tribe (1955), Kamal and Wood (1956),

Fushtey (1957), and Hall (1963) all show that different cells which have been plasmolysed with electrolytes and non electrolytes are more readily macerated than killed. Recent evidence support these earlier reports and show that there is a close connection between factors which cause maceration and death of protoplasts. Hall and Wood (1970) showed that in the absence of plasmolysis, PTE from Erwinia carotovora and PG from Corticium practicola macerated potato tissue and killed the protoplasts. When plasmolysis occurred however, maceration occurred as usual but death of protoplast was delayed. As a result of their findings, Stephen and Wood (1975) concluded that the death of protoplasts in normal unplasmolysed tissue caused by PTE could be a purely physical event. Based on recent information on the macromolecular structure of cell wall (Keegstra et al. 1973; Talmadge, 1973) they suggest that cell death could depend on alteration of the cell wall structure resulting in the rupture of the protoplast under turgor pressure. This is still a field of current investigation.

(G) Inhibitors of pectic enzymes

Evidence for the production of pectic enzymes in vitro has been complicated by the difficulty of extracting these enzymes from the killed tissue. Inhibitors of PG have been found in extracts of various plants - in apples (Byrde, 1957),

in sweet potato (Uritani and Stahmann, 1961), in broad been leaves (Deverall and Wood, 1961b). Prominent among these inhibitors are the phenols or the oxidation products of the host's phenolic compounds (Balasubramaniam, Deverall and Murphy, 1971; Cole, 1956; Byrde, [illegible], 1957, [illegible]). Among the phenols the following are known to inactivate pectic enzymes: catechin and chlorogenic acid (Pollard et al., 1959) catechol and hydroxyphenylalanine (Deverall and Wood, 1961b) [illegible] tannic acid or tannins (Hathway and Seakins, 1958). Williams (1963) elaborated on the inhibition of PG and macerating enzymes of Sclerotinia fructigena by a number of phenolic compounds. In their study on the black rot of sweet potato caused by Ceratocystis fimbriata, Uritani and Akazawa (1955, 1959) showed that the host produced polyphenols and coumarins in healthy tissue adjacent to rotted area.

The significance of phenolic oxidation is demonstrated from the report that catechol, chlorogenic acid, catechin and epicatechin which had little or no effect before oxidation can cause substantial inhibition of Sclerotinia fructigena PG after oxidation has occurred at very low concentrations. (Byrde et al 1960) The role of oxidation is further demonstrated by comparing inhibition caused by unoxidised and oxidised products and by adding reducing or chelating agents which prevent oxidation (Cole, 1956; Deverall and Wood, 1961b; Williams, 1963).

In order to reduce enzyme inhibition during extraction

procedures several phenol-binding compounds have been considered (Anderson et al., 1968; Jones et al., 1961; Loomies and Battaile, 1966; Van Sumere et al., 1973). Phenols combine with proteins reversibly by hydrogen bonding and irreversibly by oxidation followed by covalent condensation. They are effectively removed from H-bonded complexes with proteins by adding large amounts of substances which contain groups similar to the peptide bond. Polyvinylpyrrolidone (PVP) has been used essentially to achieve this result. Apart from its ability to absorb phenols, it is used to inhibit phenolase of host tissue directly (Jones and Hulme 1961). This is evident from the observation that darkening of extracts was prevented by PVP. Furthermore, the use of antioxidants in the extraction procedure is aimed at achieving the same result (Byrde, [illegible], 1957; Byrde et al., 1960; Cooper, 1974; Spence, 1961).

Specifically, the importance of changes in polyphenol oxidase and peroxidase content of tissues following fungal infection has been reported (Stahmann & Demorest, 1971). Tomiyama and Stahmann (1964) recorded that the concentration of both peroxidase and polyphenol oxidase increased near the inoculated potato surface. The increase was greater and extended deeper into the tissue when incompatible race of potato was used. Similar reports have been made for bean leaves inoculated with Pseudomonas phaseolicola (Rudolph and Stahmann, 1966) and for

bean inoculated with rust (Staples and Stahmann, 1964). Increase of these oxidative enzymes in infected tissues has been implicated in disease resistance (Stahmann et al., 1966; Lovrekovich et al., 1968a).

The above review shows instances of production of pectic enzymes both in vivo and in vitro by various plant pathogens, the possible roles of these enzymes in disease development and factors in the host tissue which inhibit the activity of the enzymes in vivo. The rot of sweet potato by B. theobromae will be examined in this thesis, in relation to the above criteria.

When some plants are attacked by a pathogen, the host tissue produces a barrier (chemical or physical) which is used to ward off the invader. If the barrier is broken by the pathogen, successful infection occurs; if not, infection is checked and the plant remains healthy. Sweet potato produces a chemical barrier when attacked by a potential pathogen. The production of chemical barriers in most host tissues in response to attack by fungi will be reviewed in the following section. How B. theobromae may counter the chemical barrier formed by sweet potato resulting in successful infection will be reviewed in relation to other host/parasite situations.

2. DEFENCE MECHANISMS IN PLANTS

It is obvious from general observation that a plant is resistant to most potential pathogens and susceptible to only a few. Put in another way, most pathogens are not able to attack most plants, so that disease resistance is more common that susceptibility. The subject of disease resistance in plants and especially the factors which confer it have been of paramount importance to the phytopathologist mostly for the all-important reason of breeding for resistance. Four definite mechanisms of resistance have emerged from various studies reviewed by Chester (1933) Tarr (1972) and Wood (1972):-

(a) Physical barriers that retard growth of pathogen

(b) Lack of suitable nutrient in resistant host, hence growth is retarded

(c) Pre-existing substances which inactivate toxins or enzymes of the pathogen

(d) Substances inhibitory to the parasite which are actively produced by the host as a result of specific stimulation by the parasite.

Mechanism (a) deals with the physical basis for resistance and (b) with the nutritional. I shall in this review restrict myself to aspects of (c) and (d) which together form the chemical basis for resistance.

I Chemical basis for resistance

(A) Substances inhibitory to pathogens

Several types of inhibitory substances are produced by plants. Depending on the timing of their formation they may be classified into:

(a) Pre-existing or passively produced substances

(b) Substances present in one form but become converted to an inhibitory form upon infection

(c) Actively induced substances, i.e. substances absent in healthy tissue but which accumulate only upon stimulation by a pathogen - the phytoalexins.

Some workers have used the terms "inhibitins", "prohibitins" and "post-inhibitins" to describe one or the other of these substances (Ingham, 1973; Mahadevan, 1970; Schmidt, 1933) and there appears to be no consensus on the application of these terms. Because of possible confusion, these terms shall be avoided in this thesis. We shall regard here the pre-infectional inhibitory substances as the pre-existing or passively produced substances and the post-infectional inhibitory substances as the actively-induced substances or simply the "phytoalexins". The latter is the subject of interest in this review.

(B) The phytoalexins

Of the various forms of inhibitory substances found in plants the most widely reported are the phytoalexins. Perhaps Ward (1905) and Bernard (1911) were among the first to recognise that pathogenesis is a dynamic process and that host/parasite interaction was a complex phenomenon. To emphasize the point, Allen (1959) stated that "it is not a condition of the plant which constitutes resistance but a process of response."

The pioneer work of Müller and Börger (1939, 1940) on the symptom responses of cut curface of potato varieties inoculated with virulent and avirulent strains of Phytophthora infestans has served as the working basis for today's research on phytoalexins. In summary, they found that incompatible races induced the production of an anti-fungal compound on inoculated surface of potato resulting in the protection of this surface against infection by the compatible race. Based on this finding Müller and Börger (1940) postulated the phytoalexin theory and therein defined phytoalexins as "antibiotics which are produced as a result of the interaction of two metabolic systems, host and parasite and which inhibit the growth of micro-organisms pathogenic to plants". This original definition had to be modified in the light of subsequent experiments. Firstly work by Uritani et al. (1960) Cruickshank

& Perrin (1963), Perrin and Cruickshank (1965), Schwochau & Hadwiger (1968) demonstrated that phytoalexins could be induced in various tissues by non-biological stimulation. Secondly, it is now known that phytoalexins are not produced as a result of specific responses to specific pathogens. Carrot tissue is induced to produce isocoumarin by inoculation with either Fusarium oxysporium f. lycopersici, Thielaviopsis basicola, Ceratocystis ulmi or Helminthosporium carbonum (Condon and Kuć, 1962; Hampton, 1962) and ipomeamarone formation can be stimulated in sweet potato by several fungi (Suzuki, 1957). These findings have necessitated a redefinition of phytoalexins simply as compounds inhibitory to the pathogen produced by the host as a result of interaction between the host and pathogen.

(C) Distribution of phytoalexins

Since the pioneer work of Müller and Borger, phytoalexins have been detected, characterized and studied by various researchers in various plants mostly in the Leguminosae and Solanaceae. The reviews of Kuć (1972), Deverall (1972), Cruickshank (1963), Cruickshank, Biggs and Perrin (1971), Ingham (1972) should be consulted for their comprehensive treatment of the subject.

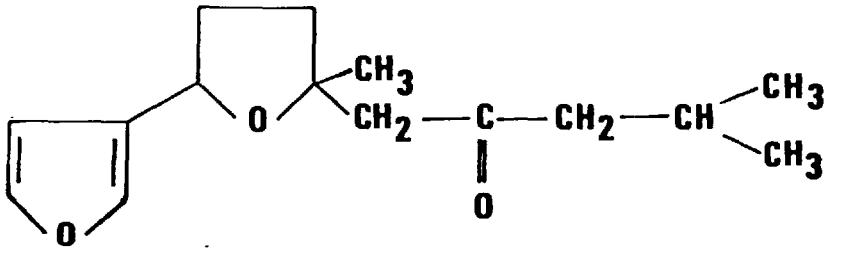
II Phytoalexins of sweet potato

Hiura (1943) first reported that sweet potato in response to infection by Ceratocystis fimbriata accumulates a "bitter principle" toxic to the fungus. Later, Kubota and his group (1952, 1953, 1956, 1957) and Akazawa (1960) isolated and characterized the bitter principle as Ipomeamarone - a sesquiterpene, furanoterpenoid (2-methyl-2-(4,methyl-2-oxypentyl) 5-(3-furyl tetrahydrofuran) (Fig. 1). Other two phytoalexins of sweet potato have been described, Ipomeanin (Kubota and Ichikawa, 1954) and Ipomeamaronol (14-hydroxy-ipomeamarone) (Kato et al., 1971). It is now known from thin-layer chromatography that sweet potato in response to attack by several pathogens or to treatment with such compounds as $HgCl_2$, mono-iodoacetic acid and 2,4 dinitrophenol accumulates several terpenoid compounds (Uritani et al., 1960). Presumably these compounds are formed as part of a general stimulation of terpenoid synthesis by sweet potato.

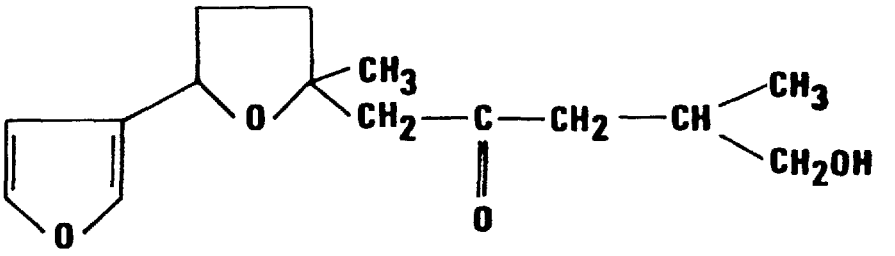
III Biological activity of phytoalexins

It is a known fact that despite the occurence of phytoalexins in infected tissues, fungi still penetrate further. Some reasons may account for this:-

Firstly, a fungus may not be capable of inducing an inhibitory level of phytoalexins in the host.



Ipomeamarone



Ipomeamaronol

Fig. 1. Two sweet potato phytoalexins

Secondly, the fungus may be insensitive to the phytoalexin and,

Thirdly, the fungus may be capable of degrading or detoxifying the phytoalexins. . . .

I shall discuss here the second and third reasons.

(A) Sensitivity of pathogens to phytoalexins

Although by definition phytoalexins are not specific in their action against fungi, fungi are differentially sensitive to them. The antifungal spectra of several phytoalexins have generally revealed that whereas non-pathogens are generally sensitive to them, pathogens are often insensitive to the phytoalexins they induce. Cruickshank & Perrin (1960) reported that Monilinia fructicola, a non-pathogen of pea was much more sensitive to pisatin induced in pea than Aschyta pisi, a pathogen of pea. Furthermore, Colletotrichum lindemuthianum, a pathogen of French beans was sensitive to pisatin but tolerant to phaseollin produced by French bean (Akazawa & Wada, 1961; Cruickshank, 1962). Working with various strains of C. fimbriata, Kojima and Uritani (1976) demonstrated that the fungal strain pathogenic to sweet potato was slightly inhibited in the infected tissue extract whereas spore germination and mycelial growth of the non-pathogenic strains were "severely inhibited".

These workers have speculated that this differential sensitivity might be important in host-parasite specificity between sweet potato and various strains of C. fimbriata.

Extracts of inoculated tissues of potato tuber, carrot root and sweet potato are generally highly fungitoxic to fungi incapable of parasitising the host (Kuc, 1961).

(B) Phytoalexin degradation by pathogens

The metabolic detoxification of phytoalexins has been demonstrated in many host parasite interactions and is considered to be critical in our understanding of pathogenecity. One of the earliest reports in this field was by Uehara (1964) who stated that pear pathogens are capable detoxifying pisatin. Later, De Wit Elshove (1968, 1969) reported that Fusarium solani and Ascochyta pisi which are pathogens of pea, detoxified pisatin while non-pathogens did not. The rate of pisatin degradation was influenced by sugar or sucrose concentration (De Wit Elshove & Fuchs, 1971).

In vitro studies of the metabolism of the pterocarpenoid phytoalexin, phaseollin, showed that different products of metabolism are formed depending on the fungus causing the breakdown. Thus while Stemphylium botryosum converts it to phaseollinisoflavan (Heath & Higgins, 1973; Higgins, Stoessl

and Heath, 1974), Fusarium solani f. sp. phaseoli hydroxylates it to hydroxyphaseollone (Heuvel & Van Etten, 1973; Heuvel et al., 1974) and Colletotrichum lindemuthianum hydroxylates it to hydroxyphaseollin (Burden, Bailey and Vincent, 1974). It was later reported that hydroxyphaseollin was further converted to another compound, 6a-7-dihydroxyphaseollin which disappeared from the extract within 24 hours. Thus conversion of phaseollin by C. lindemuthianum was by progressive hydroxylation. All the conversion products were less fungitoxic than phaseollin.

The ability of Nectria galligena to cause disease in unripe apple has been attributed to the ability of the fungus to metabolise the anti-fungal benzoic acid which accumulates in the tissue following infection (Brown and Swinburne, 1971). Benzoic acid is converted by the fungus to p-hydroxybenzoic acid and protocatechuic acid both of which are less anti-fungal and accumulate in advanced rots (Brown & Swinburne, 1973). Stoessl, Unwin Unwin & Ward, (1973) reported that the phytoalexin, capsidiol in infected pepper is oxidised to a less fungitoxic capsenone by isolates of Fusarium oxysporum. They suggested that the oxidation might be an important step in the colonization of pepper by the fungus. Another report which shows relationship between phytoalexin degradation and pathogenecity was made by Higgins and Miller (1968, 1969a, 1969b, 1970). They showed in their papers that Medicarpin, a phytoalexin of alfalfa was degraded by Stemphylium botryosum to vestitol which on

further incubation with the pathogen slowly disappeared probably due to degradation to CO_2 or assimilation by the fungus (Higgins, Stoessl and Heath, 1974). A non-pathogen of alfalfa, Helminthosporium turcicum did not degrade the phytoalexin at all.

MATERIALS AND METHODS

1. ENZYME STUDIES

I The culture and maintenance of fungi

The sweet potato isolate of Botryodiplodia theobromae Pat, used for this work was supplied by Professor J.A. Ekundayo, University of Lagos. Isolates of Botrytis cinerea Pers. and Cladosporium cucumerinum Ell & Arth. were supplied by Dr. I.M. Smith, Imperial College. The fungi were grown on agar slopes in McCartney bottles; B. theobromae was grown on malt extract, B. cinerea on X medium (see below) and C. cucumerinum on V_8 juice. Subcultures were made every three weeks and stored under sterile mineral oil at 25°C.

II Growth media

Two types of culture media were generally prepared (A) stationary culture media for production of inocula and (B) shake cultures for enzyme induction in vitro.

(A) Stationary cultures:

B. theobromae:

An inorganic salt solution (Leonian solution)

(Király et al., 1974) was used to obtain mycelia for inoculation and 2% agar was added for pycnidium production by the fungus. The medium contained the following in 1 litre of distilled water:

KH_2PO_4	1.25g
$MgSO_4\ 7H_2O$	0.625g
Maltose	6.25g
Mycological peptone	0.625g
Malt extract	6.25g

B. cinerea

To obtain conidia of B. cinerea, X medium was prepared as follows:

Dextrose	10g
$NaNO_3$	6g
KH_2PO_4	1.52g
$MgSO_4\ 7H_2O$	0.52g
Casein hydrolysate	3.0g
Yeastrel	0.5g
Mycological peptone	2.0g
KCl	0.5g
Agar	20.0g
Distilled water	1 litre

Where mycelial suspension of B. cinerea was required for inoculation, Sucrose Casamino acid medium (SCA) was prepared. The composition was:-

Casamino acids	4.6g
$KH_2 PO_4$	1.0g
$MgSO_4 7H_2O$	0.5g
Sucrose	15.0g
Glucose	5.0g
Minor elements solution	10.0ml made up to 1 litre with distilled water.

The minor elements solution contained the following in 1 litre of distilled water.

$FeSO_4 7H_2O$	20mg
$ZnSO_4 7H_2O$	100mg
$NaMoO_4 2H_2O$	2mg
$CaSO_4 5H_2O$	2mg
$MnCl_2 4H_2O$	2mg

C. cucumerinum

V_8 agar supported good sporulation of the fungus. The medium was prepared by dissolving 20g agar in 800ml water and adding 200ml V_8 vegetable juice. There was no need to adjust the pH.

Inoculation of stationary cultures

Agar plugs (obtained with 8mm diam. cork borer) from actively growing cultures of B. theobromae and B. cinerea were

used for inoculation of B.t. and B.c. media. Spore suspension preparation of C.c was used for inoculation of C.c. media.

(B) Shake cultures

Medium for shake cultures had the same constituents as SCA except that the carbon sources sucrose and glucose were was replaced with either 2% sodium polypectate (NaPP), pectin or plant cell wall (Section X).

Inoculation of shake cultures

The medium was dispensed in 30ml aliquots in 150ml Erlenmeyer flasks. Two plugs of agar obtained from the edge of an actively growing SCA culture by means of 8mm diam. cork borer were used to inoculate the medium and the culture was shaken in a rotary shaker (200rpm) at 25°C for the appropriate number of days.

(C) Preparation of spore suspensions:

(i) Spores of B t were obtained by growing the fungus on Leonian's solution agar (LSA) and incubating under fluorescent light for 14 days at 25°C. The pycnidia formed were dislodged with an inoculating loop and dispersed in sterile distilled water. The pycnidia were then crushed with a spatula to release the pycnospores. Fragments of pycnidial walls were filtered off through a double layer of muslin covered with

non-absorbent cotton wool. The spores were washed three times by centrifugation at 1500g for 1 min with changes of sterile distilled water and the concentration adjusted accordingly with a haemocytometer.

(ii) B.c. was grown on X medium under ~~blue~~ black light (supplied by Phillips TL 40W/08 RS F40 BLB) at 20-23°C for 12-14 days to prepare spore suspension. About 50ml sterile distilled water was added to the sporulating culture and the flask shaken vigorously to remove the spores from the mycelial mat. Fragments of mycelia were removed by filtering through muslin and cotton wool and washed by centrifugation as described above.

(iii) For C.c. V_8 juice agar was inoculated with a spore suspension of the fungus in order to obtain a uniform mycelial mat. This was incubated at 25°C for 8 days. The culture was then flooded with 30ml distilled water and the conidia dislodged gently with a camel hair brush. The suspension was filtered as described above for B.t.

(D) Culture filtrates for enzyme studies

Shake cultures after 4 days incubation were strained through several layers of muslin and filtrates were centrifuged

at 12000g for 30 min at 4°C. The filtrates were either subjected immediately to ammonium sulphate precipitation as preliminary step in enzyme purification or dialysed at 4°C for 24h using Visking cellulose tube (8" X 32" diameter) against distilled water (20ml filtrate : 500ml H_2O) at pH 7.0 before storing at -20°C.

III Chemical reagents

All chemical reagents used in this work were of the analytical grade. Where necessary, the sources of the special chemicals have been indicated in the text. The agar used in most media was the general purpose Davis Standard agar (Davis Gelatine Ltd., Leamington Spa). In others, Oxoid No.3 was used for special purposes. The Neutral Red stain was obtained from Bio-Rad Laboratories, Richmond, California, USA. Sodium dioctylsulphosuccinate (NaDSS) was obtained as "Manoxol OT" from Hardman and Holden Ltd., Manchester. Glass distilled water was used for all experimental purposes unless otherwise stated.

IV Pectic enzyme substrates

Sodium polypectate (NaPP) (Sunkist Growers Inc. California) was purified by washing before use. About 30g of powder was added to 150ml 70% ethanol containing 0.05 N HCl

mixed for 30 min in a magnetic stirrer and filtered under reduced pressure. The residue was washed several times with 70% ethanol until washings were free of chloride (tested with $AgNO_3$). It was finally washed with 95% ethanol followed by absolute ethanol and dried over an oven at 50°C in aluminium foil.

Pectin from citrus (Sunkist Growers Inc. California) with a high methoxyl content was also purified as above.

These substrates (NaPP and Pectin) were also used to induce pectic enzyme synthesis in vitro.

To overcome the practical difficulty of bringing these substances into solution, they were slowly added to water and stirred vigorously on a magnetic stirrer. Solutions were made up at double strength and then mixed with an equal volume of buffer to obtain the desired concentration.

(V) Buffers

Mixtures of appropriate volumes of the following were used for desired pH values as described by Dawson, Elliot, Elliot and Jones (1959).

Citric acid-sodium citrate	pH 3.0 - 6.2
Na_2HPO_4 - Na H_2PO_4	pH 5.7 - 8.0
Tris (hydroxymethyl) amino methane - HCl	pH 7.2 - 9.0
Glycine - NaOH	pH 8.6 - 10.6

All solutions were prepared on the day of use.

(VI) Sterilisation

Pipettes and petri dishes were sterilised in metal canisters in an oven at 400°C for 6hr.

Growth media and salt solutions were autoclaved at 121°C for 15min.

For enzyme induction, precautions were taken to retain the molecular identity of the pectic substances. They were therefore added aseptically to the mineral solution already sterilised at 121°C for 10min and then treated at 112°C for 10min.

Where plant cell walls were used as the carbon source for induction studies, the material was added to the sterilised mineral solution and treated at 110°C for 5min. This again was to prevent excessive solubilization of the hot water soluble part of the plant material by excessive high temperature treatment (Cooper, 1974).

(VII) Measurement of pH

pH measurements were carried out in a Pye Unicam model 292 meter (Pye Unicam Cambridge) or in a Beckman Zeromatic model.

(VIII) Spectrophotometry

All light absorption measurements were carried out with a Beckman model DB spectrophotometer attached to a Beckman potentiometric Recorder. All readings were taken in fused silica cuvettes of 1cm path length.

(IX) Preparation of rotted tissue for enzyme studies

(a) Tissue inoculation

Sweet potato tubers were surface sterilised by washing in 70% ethanol. Discs (5mm diameter) of young cultures of B. theobromae cut with a sterile cork borer were introduced aseptically into holes made in the tubers and sealed with vaseline. The inoculated tubers were placed in plastic boxes (cleaned with 90% ethanol and lined with filter paper moistened with water). The plastic boxes were closed and stored at $25 \pm 1^{o}C$ for seven days.

(b) Extraction of rotted tissue for enzyme assays

Rotted tissue was removed, mixed with 0.1M phosphate buffer pH7.0 containing 0.2M NaCl, 10^{-3}M dithio threitol and 5% insoluble polyvinyl pyrollidone (PVP) (Sigma) in the

proportion 1g tissue : 10ml buffer. NaCl was used to deabsorb protein from tissue (Blackhurst and Wood, 1963), PVP to absorb phenols, and dithiothreitol to prevent oxidation by oxidative enzymes present in the tissue (Anderson, 1968). The tissue was homogenised in a Sorvall Omnimixer for 5 min at full speed, strained through several layers of muslin and centrifuged at 15000g for 30 min. The sample was either ultra-filtered (See below) or dialysed against distilled water with Visking tubing for 24h at 4°C before storage at -20°C.

(X) Extraction of plant cell wall

One month old plants (Phaseolus vulgaris, var. Prince) grown under 17h photoperiod were used. Extraction method was loosely based on the method of Karr and Albersheim (1970). Stems were frozen in liquid nitrogen and ground thoroughly in a mortar. The resultant powder was suspended in 10 volumes of 0.1M sodium phosphate buffer pH7.0 and blended in a Sorval Omnimixer for 3 min at full speed. It was then filtered through a sintered glass funnel, resuspended in fresh buffer and the procedure repeated until the filtrate was clear. Final washing of the insoluble material was done with distilled water. The material was suspended in 10 volumes of chloroform: methanol (1:1), blended for 5 min, filtered, washed several times with chloroform: methanol (1:1) and finally with several

proportion 1g tissue : 10ml buffer. NaCl was used to deabsorb protein from tissue (Blackhurst and Wood, 1963), PVP to absorb phenols, and dithiothreitol to prevent oxidation by oxidative enzymes present in the tissue (Anderson, 1968). The tissue was homogenised in a Sorvall Omnimixer for 5 min at full speed, strained through several layers of muslin and centrifuged at 15000g for 30 min. The sample was either ultra-filtered (See below) or dialysed against distilled water with Visking tubing for 24h at 4°C before storage at -20°C.

(X) Extraction of plant cell wall

One month old plants (Phaseolus vulgaris, var. Prince) grown under 17h photoperiod were used. Extraction method was loosely based on the method of Karr and Albersheim (1970). Stems were frozen in liquid nitrogen and ground thoroughly in a mortar. The resultant powder was suspended in 10 volumes of 0.1M sodium phosphate buffer pH7.0 and blended in a Sorval Omnimixer for 3 min at full speed. It was then filtered through a sintered glass funnel, resuspended in fresh buffer and the procedure repeated until the filtrate was clear. Final washing of the insoluble material was done with distilled water. The material was suspended in 10 volumes of chloroform: methanol (1:1), blended for 5 min, filtered, washed several times with chloroform: methanol (1:1) and finally with several

volumes of acetone until the filtrate was clear. After acetone extraction, the cell walls were oven-dried on aluminium foil at 50°C for 24h and stored in a desiccator at -20°C.

(XI) Enzyme purification

The procedures described below apply to both culture filtrate and rot extract enzymes.

(A) Ultra filtration

An "Amicon" Ultrafiltration cell model 10-PA (Amicon Corp., Lexington, Massachusetts, USA) was used with "Diaflo" membrane filters. The membranes were first washed in changes of distilled water on a magnetic stirrer. These filters are made up of inert material having selective solute retention properties. This property was exploited for partial purification of enzyme solutions by removing lower molecular weight solutes and mostly for concentrating enzyme solutions when higher activities were required or when smaller volumes of enzyme preparations were required. After use, the membranes were floated on 10% ethanol and stored at 4°C.

An Amicon - P - 10 pressurised unit was the source of pressure for the set-up.

(B) $(NH_4)_2SO_4$ precipitation

Solid $(NH_4)_2SO_4$ was added to crude filtered culture filtrates and rot extracts as a first step in the fractionation of proteins present in the solutions. $(NH_4)_2SO_4$ saturation levels of 40, 60, 80, 90 and 100% were obtained. The amount of salt added to achieve the desired saturation level was calculated from Tables (Green and Hughes, 1955). Addition of salt was carried out at 25°C in a water bath. The precipitate obtained at each saturation level was removed (after leaving to stand for 30 min) by centrifugation at 12,000g for 30 min at 25°C. The supernatant was used for the next higher saturation. The precipitate at each level was dissolved in a small volume of water and dialysed against water at 4°C for 24h before storing at -20°C.

(C) Sephadex gel filtration

(i) Preparation of gel slurry

40g of Sephadex G75 Superfine (Pharmacia Fine Chemicals) was added to about 500ml water and heated on a bunsen burner while stirring slowly and gently for about 2h. The gel was left to cool and excess water decanted from the top.

(ii) Column set-up

A Sephadex K25/100 (Pharmacia Fine Chemicals Ltd.) glass column was used. The outlet tube was closed and the nylon net at the base of the columnwas covered with distilled water to exclude air bubbles.

The gel slurry was slowly poured into the column along a glass rod to obtain even sedimentation, care being taken to remove any trapped air. The column was filled to a height of about 25cm and was then connected to a reservoir containing 0.05M NaCl in 0.005 M citrate buffer pH 5.0. The column was flushed with salt solution (300ml) at a flow rate of 40ml/h in order to stabilise and equilibrate the gel bed. The gel finally settled to constant height of about 20cm and the eluant on top of gel adjusted to about 8cm height. To check the homogeneity of the bed, Blue dextran 2000 (Pharmacia Chemicals) at 2mg/ml was run through and collected in a 5ml sample. 0.02% sodium azide (NaN_3) was added to the reservoir to prevent the growth of microorganisms.

(iii) Sample application

The reservoir was dismantled from the column and the eluant pipetted off from the top of the gel leaving about 1cm liquid on top. A sample applicator consisting of a nylon

net was carefully placed on top of the gel to avoid trapping air bubbles. The protein sample was then evenly layered on the sample applicator. With the outlet tube of the column open, buffer was slowly and carefully pipetted on to the sample through the side of the column, care being taken to avoid violent mixing of sample and buffer. When the sample had drained into the bed, the column was refilled with buffer and the connection to the reservoir restored. The column was run at a flow rate of 15ml/h at 5°C and 5ml fractions were collected. These were stored at -20°.

(D) Isoelectro focusing

The principle of isoelectric focusing was utilized as the final step in enzyme purification. This method had two practical merits (1) Large dilute enzyme samples were readily concentrated (2) Different proteins could be separated and analysed differently. The principle of operation is based on the establishment of a pH gradient between two electrodes, so that the proteins contained in the sample migrate to positions on this gradient which correspond to their isoelectric points. At their isoelectric points, the proteins acquire a net charge of zero and thus cease to migrate. An L.K.B. carrier ampholyte, "Ampholine" (a mixture of a large number of different aliphatic polyaminopolycarboxylic acids) is used to provide the required pH gradient and a non-ionic

compound, sucrose, is incorporated as a density gradient to provide a convection-free system.

Electro focusing was done with the LKB 8101 (110ml) electro focusing equipment. The general procedure used is as described by Flatmark and Vesterberg (1966). For details of apparatus and set up, see LKB handbook for isoelectric focusing.

Before use, the protein sample was dialysed for 24h at $4^{o}C$ with several changes of 500vols distilled water to remove as much salt as possible (to prevent local overheating in the column). The sample was incorporated into the "light" electrode solution before mixing with the "dense" in a gradient mixer (LKB 8121 (110ml)). In all runs, the cathode solution was placed at the top of the column and the anode at the bottom. Both electrode solutions were added through a funnel by means of a Pasteur pipette.

An LKB power supply unit supplied 2-3W maintained throughout the run at $4^{o}C$. During the run voltage was adjusted to a value of about 2-300V (less than 10mA). The system was left to run for 48-72h until the current decreased to a constant minimum value.

At the end of the run, the samples were collected by gravity feed in 2 or 4ml fractions in test tubes. The tubes were quickly capped, transferred to an ice bucket and the pH taken at $4^{o}C$. Before analysis, the proteins were separated from the sucrose and the Ampholine by dialysis at 4^{o} against changes of distilled H_2O.

XII Enzyme assays

(A) Polygalacturonase

Polygalacturonase was assayed by various methods.

(i) Viscometry:

This was the method routinely used to determine the chain-splitting action of culture filtrates and rot extracts. The reaction mixture consisted of 4ml 1% pectic substrate in 0.1M citrate buffer pH5.0, 1ml water and 2ml enzyme sample. Enzyme activity was expressed in relative viscometric units defined as $\frac{1000}{t}$ where t = time in min for 50% loss in viscosity of the reaction mixture. Loss in activity was measured in a Cannon-Fenske viscometer size 200 at 26 $\pm$ 1°C in a water bath. Viscometers were calibrated against water, the flow time of which represented 100% viscosity reduction. Boiled enzyme was used as control.

(ii) Cup-plate assay

The technique (Dingle, Reid & Solomons, 1953) was used for rapid screening of fractions collected from the Sephadex and electro focusing columns. One gram of Napp was dissolved in 50ml water and the pH adjusted to 5.0. An

equal volume of 0.2M sodium citrate buffer pH5.0 was added to yield a solution of 1% NaPP in 0.1M citrate buffer. 1gm oxoid No.3 agar was added and the mixture autoclaved for 10min at 121°C. 20ml aliquots were transferred to sterile plastic petri-dishes and when set, wells were cut out of the agar with a sterile cork borer (4mm diameter) and 0.02ml of enzyme sample pipetted into each well under sterile conditions. Plates were incubated at 25°C for 18h after which the agar surface was flooded with 5N HCl. Where enzyme activity occurred, a white precipitate formed around the well within 5 min. The diameter of the precipitate around the well was used as a measure of enzyme activity.

(iii) Release of reducing group

The carbonyl group (a reducing group) at carbon 1 of galacturonic acid is normally involved in the formation of the α-1, 4 glycosiduronic linkage in pectic substances. When the linkage is split by hydrolysis or transelimination the reducing group is released and can be qualitatively and quantitatively analysed. The method of analysis here is based on the Nelson-Somogyi method (1944) which involves oxidation of the reducing group by means of cupric ions. The reaction mixture consists of enzyme sample, pectic substrate (1% in 0.1 M citrate buffer pH5.0) and water in the proportion

2 : 4 : 1. This was incubated in a water bath at 25 $\pm$ 1°C and at intervals of 5 min, 1ml samples were removed, diluted to 10ml with distilled water and 0.2ml samples taken from this and estimated for reducing group content. The following reagents were made up:

(a) Copper reagent A.

25g Na_2CO_3 (anhydrous)
25g Potassium sodium tartrate
20g $NaHCO_3$
200g Na_2SO_4 (anhydrous)

) dissolved in 800ml H_2O and made up to 1 litre with H_2O

(b) Copper reagent B.

15g $CuSO_4 \cdot 7H_2O$
2 drops of Conc H_2SO_4

) in 100ml H_2O

(c) Arsenomolybdate reagent

25g Ammonium molybdate dissolved in 450ml H_2O

21ml Conc H_2SO_4 added

3g sodium arsenate ($Na_2HAsO_4 \cdot 7H_2O$) in 25ml H_2O added

The solution was incubated at 37°C for 48h and stored at room temperature in brown glass bottle.

25 parts of copper reagent A and 1 part of reagent B were mixed to obtain "the Cu reagent" just before the test was carried out. To the test sample (0.2ml) was added 0.8ml water and 1.0ml

of "the reagent". The mixture was heated for 30min in a boiling water bath and cooled rapidly. 1ml arsenomolybdate reagent was added and mixed thoroughly on a whirlimixer until CO_2 effervescence ceased. The intensity of the blue colour which developed was estimated against a reagent blank made up with autoclaved enzyme at 660nm in a spectrophotometer. A standard curve was obtained with galacturonic acid (5-50µg/ml) for calibration.

(iv) T.B.A. reaction

This reaction was used to establish that the cleavage of the pectic chains was by hydrolysis and not by transelimination. Products of hydrolysis of pectic substances would react with thiobarbituric acid (TBA) to give a pinkish-orange complex which absorbs maximally at 515nm whereas the transeliminative complex would be cherry-pink in colour and absorb at 550nm.

The reaction mixtures were as described for viscometry in the same proportion. The mixture was incubated at 25°C in a water bath for 1h. 2.5ml samples were then removed and added to a mixture of 2.5ml 1NHCl and 5ml 0.04M thiobarbituric acid in a test tube and mixed thoroughly. The test tube was capped, boiled in a water bath for 30 min, cooled and the colour produced read both at 515nm for PG activity and at 550nm for transeliminase activity. Autoclaved enzyme sample was used for blank reading.

(B) Polyphenol oxidase

A colorimetric method was used to determine the activity of the enzyme. The assay is based on the formation of a dark coloured polymeric compound from catechol or dihydroxyphenylalanine (DOPA). Tissue extracts were prepared as described by Maxwell and Bateman (1966). Firstly, the oxidase in the extract was activated by adding sodium dioctylsulphosuccinate (NaDSS) (Kenten, 1957, 1958; Deveral and Wood, 1961) in the following mixture:

0.2ml dialysed rotted extract

0.6ml H_2O

0.14ml 4mM NaDSS

0.06ml 0.1M sodium citrate buffer pH 7.0

The mixture was left to stand for 20 min at 20°C after which 1ml 0.1M citrate buffer pH 7.0 and 1ml 10^{-3}M catechol or DOPA were successively added and the volume brought up to 6ml with water. The mixture was transferred quickly to a cuvette and increase in absorbance at 495nm over a 60s period followed in a spectrophotometer. Enzyme activity was expressed as change in absorbance (ΔA)/ml extract/min at 495nm at 25°C. Controls were taken using autoclaved extracts.

(C) Peroxidase

The peroxidase assay was based on the same principle as the phenoloxidase (Lück, 1963). After activation, 1ml of the enzyme mixture was diluted to 1000ml with water and to this was added successively the following:

1ml sodium citrate-phosphate buffer pH 4.1

0.4ml 0.1% H_2O_2

1ml 0.1M p-phenylenediamine diluted to 6ml with distilled water

The mixture was transferred to a spectrophotometer and peroxidase activity measured as change in absorbance per 0.001ml extract/min at 485nm.

XIII Activity of pectic enzymes on plant tissues

(A) Macerating activity

The ability of enzyme samples to separate the cells in tissue was determined using sweet potato and potato tissues. Long cores of tissues were cut from healthy tubers by means of a cork borer and discs (0.5 X 0.05cm) were cut out from the cores by means of a hand microtome. The discs were suspended in several changes of 0.05M citrate buffer pH 5.0 to wash off starch and other cytoplasmic materials from the surface of cut

cells. They were quickly dried on tissue paper and transferred to an enzyme reaction mixture (6 discs/ml) consisting of

2ml enzyme sample

1ml 0.05M citrate buffer pH 5.0

1ml water

This was incubated at 25°C in a water bath and at intervals of 20 min, 3 discs were removed, washed in citrate buffer pH 5.0 and the macerating activity of the enzyme tested. This was estimated by testing the loss in coherence of the discs, determined from the ease with which the discs were pulled apart with two dissecting needles. A macerating index (MI) with 0-5 linear scale was used to grade the results. A rating of 0 was for control tissue, with cells intact and 5 for complete cell separation.

(B) Cell death

A colorimetric method based on a modification of Tribe's (1955) method was used to estimate the death of tissues exposed to enzyme reaction mixture. Tissue discs (0.5 X 0.05cm) obtained as described above were incubated in the reaction mixture (6 discs/ml). At intervals, 1 disc was removed and transferred to a mixture of the following:

0.1ml 1% Neutral Red in 0.1M phosphate buffer
pH 7.5 (solution was freshly prepared
to avoid precipitating Neutral Red)
1ml phosphate buffer

After 20 min, the disc was transferred to 3 changes of $MKNO_3$ solution for 15 min. The disc became plasmolysed and the accumulated neutral red stain in the living cells could be clearly seen in the vacuoles. The KNO_3 was drained from the disc and 3ml methanol added to it. This was left for 1h until all the stain was extracted by the methanol. The O.D. of the methanol extract was determined against a methanol blank in a spectrophotometer at 465nm at 25°C. Controls used were a boiled disc representing 100% death and an untreated disc representing 0% death.

(C) Release of ions from tissues

The leakage of electrolytes from tissues treated with pectic enzymes was investigated. This leakage is due to increased membrane permeability to ions and is often associated with cell death. The permeability changes of potato and sweet potato tissues treated with the enzymes were measured in a conductivity meter Type MCI (Mark IV) (Wilbury Way, Hitchin, Herts).

The reaction mixtures were similar to those used for maceration and cell death. Mixture containing the discs was

incubated at 25°C and at intervals of 10 min, five discs were removed, rinsed in 10ml 0.4M sucrose (to remove the highly conducting reaction mixture from the disc surface) and then transferred to 15ml distilled water, shaken for 20s and the conductivity of the bathing solution taken. Conductivity was read in μmhos. Autoclaved enzyme samples (121°C for 30 min) were used as controls. Care was taken to wash the tubes used in the experiment thoroughly and rinse in distilled water to avoid contamination. [illegible] conducting.

XIV Enzyme adsorption by tissues

Purified enzyme, diluted to give a relative viscosity unit of 30 was used to test enzyme binding to tissues. (Cervone, Scala and Scala, 1977). Tissues used were thinly sliced (where tubers were used) or chopped up finely (where stems or fruits were used). One gram of the tissue was washed with 10ml citrate buffer pH5.0 (to remove excess ions released by cell damage) and transferred to 5 ml of the appropriately diluted enzyme solution incubated in a water bath at 25°C for about 3h. At 5 min intervals, 1 ml enzyme sample was removed, transferred to a viscometer containing 5 ml of 1% NaPP in 0.1M citrate buffer pH5.0 and 1 ml water in a water bath at 27°C. Loss in activity of the mixture over a 30 min period was determined.

The following precautions were observed:-

(1) Amount of enzyme solution added/g tissue was kept at 5 ml. Since the number of binding sites on the tissue is finite and limited, it would be easier to detect binding if the amount of enzyme corresponded with the binding capacity of the tissue.

(2) Enzyme activity was reduced to a relative viscometric unit of 30. This was critical since a too high activity would result in substantial maceration and release of ions (especially calcium ions) which would interfere with subsequent viscometry.

(3) The tissue was finely divided for accessibility of sites to enzyme and washed thoroughly with buffer to remove excess ions.

Two controls were set up:-

Control No.1: consisted of enzyme incubated in buffer over the same period of time (3h) from which aliquots were taken for test.

Control No.2: Aliquots of cell free liquid leaked from tissue was added to an aliquot of the enzyme and incubated for 3h. These were added at twice the strength used for experiment above to ensure ~~the least~~ any possible inhibition. This control was set up to see if there was any enzyme inhibition by substances leaking from the tissue. If the only proof of binding is a

loss in enzyme activity, it was important to show that the loss did not arise from other causes e.g. action of phenolic substances or the like or release of Ca^{++} ions from the tissue.

1 ml samples were removed from each of the controls and their viscosity reduction of the sodium polypectate determined as in experimental.

XV K_m determination

The K_m of the isoenzymes was determined by the Lineweaver–Burke plot using pectin and NaPP as substrates. Enzyme activity was determined from reducing sugar released from reaction mixture (See Section XII, A (III)).

XVI Determination of total phenols

The method used was an adaptation of Swain & Hillis (1955).

(A) Extraction of total phenols

An aqueous extract of tissue (rotted or healthy) was obtained by blending in a Sorval Ommimixer, 3 min at maximum speed, (1g fresh weight/30 ml water). Extract was strained through muslin and residue re-extracted with 10 volume water filtered

and washed till clear with several changes of distilled water. The pH of the extract was adjusted to 4.0 and the solution shaken vigorously with 3 volumes of diethyl ether in a separating funnel under a fume chamber. The ether was evaporated to 1 volume in a rotary evaporator and the residue shaken vigorously with 1 volume 0.2N NaOH in a separating funnel, and left to stand for 1h. When fully separated the phenols concentrated in the NaOH fraction. The mixture was acidified again with ConC HCl to pH 3.0 and the phenols finally extracted with 3 volumes of ether. The ether extract was shaken with analar anhydrous sodium sulphate in order to remove any water remaining. The ether was finally evaporated to dryness and the residue taken up in 10 ml ethanol before storage at $+4^{o}C$.

(B) Phenol estimation

The following reagents were used:

(i) Folin-Denis reagent (BDH, Poole, England)

(ii) Saturated Na_2CO_3 solution

An aliquot (0.5 ml) of the ethanol extract was diluted to 7 ml with water in a test tube and 0.5 ml Folin-Denis reagent was added, mixed and shaken. Three minutes later, 1 ml of saturated Na_2CO_3 was added and the mixture made up to 10 ml with distilled water. One hour later, the solution was centrifuged at 10,000g for 5 min to remove precipitates, filtered and the

extinction value read at 725nm in a spectrophotometer against a reagent blank prepared with ethanol.

A calibration curve was prepared using 10-100 µg catechol/ml and results expressed as µg catechol equivalent/g fresh weight.

XVII Thin layer electrofocusing (after Karlsson, Davies, Öhman & Andersson, 1973)

The thin layer electrofocusing technique was used as an analytical technique rather than as a preparative one in the pectic enzyme studies. This method of enzyme analysis has two special advantages over the other methods: The electrofocusing time is much faster and it was possible to analyse several enzyme samples simultaneously under identical conditions. The principle of operation is basically similar to the isoelectric focusing described earlier except that a thin layer system consisting of polyacrylamide gel is used here.

(A) Preparation of polyacrylamide gel (PAG) solution

Reagents:

(i) Acrylamide solution 29.1% (w/v)

The solution was prepared and filtered through 2 layers of muslin.

(ii) N, N^1 - Methylenebisacrylamide (Bis) solution 0.9% ($^{W}/_{V}$).
Filtered as above.

(iii) 1% ammonium persulphate

All the chemicals were obtained from BDH, Poole, England.

The following solutions were mixed in a 100 ml vacuum flask:

10 ml acrylamide solution, (i) above
10 ml (ii) above
37 ml H_2O in which 7.5g sucrose (analytical grade) is dissolved
3.0 ml (iii) above
0.04 ml N, N, N^-, N^- - tetramethylethylenediamine (TEMED)
3.0 ml Ampholine pH 3.5-6

The solutions were mixed thoroughly by rotating the vacuum flask and de-aerating with a vacuum pump for 1 min. This is the polyacrylamide gel solution.

(B) Gel polymerization

The PAG solution was introduced by means of a pipette into two mounted glass plates (LKB), (125X260/3mm and 125X260X1mm)

separated by a rubber gasket (care taken to avoid air bubbles in the solution). The gel was left sandwiched between the glass plates at room temperature to polymerize. Polymerization occurred within 1h. The gel was carefully removed from the plates by means of spatulas and stored at 4°C in a thin polythene sheet if not used immediately.

The LKB 2117 Multiphor was the electrofocusing equipment used. The anode and the cathode poles were provided by two electrofocusing strips (LKB) soaked in the electrode solutions for pH 2.0-6.0 range. These were placed on to the gel plate within the appropriately marked area on the template. The cooling plate of the Multiphor was washed with distilled water and about 7 ml water distributed evenly on the surface. The gel plate was slowly lowered on to the cooling plate (care taken to displace air bubbles) and the Multiphor cover mounted. Cold water from a tap was used to cool the system.

(C) Sample application and run

The protein sample was concentrated to about 1.5mg/ml by ultrafiltration. Two samples were applied across the width of the PAG (within the area marked for sample application on the template) by means of Whatman chromatography paper, 3MM (5X10mm pieces).

The LKB power unit was used with an initial voltage of

400 volts and by the end of the run (2.5h) adjusted to a maximum of 900V (less than 17mA). To avoid the risk of the proteins adsorbing to the filter paper during electrofocusing, the filter papers were removed after the first hour of run.

(D) Protein location

At the end of electrofocusing, the PAG plate was placed in a fixing solution consisting of

150 ml methanol

50 ml distilled water

17.25g sulphosalicyclic acid

57.5g trichloroacetic acid,

for 1h to precipitate the proteins and at the same time allow the Ampholine to diffuse out of the gel. The gel was then cut into strips corresponding to the two protein samples applied. One strip was stained for proteins at 60°C for 10 mins in 0.115% Coomassie Brilliant Blue R250 in a solvent of 8% ($^{v}/_{v}$) acetic acid and 25% ($^{v}/_{v}$) ethanol in water. The strip was destained in several changes of the solvent until the gel was clear of excess stain. Bands of proteins were assessed visually. Strips Discs of gel corresponding to the protein bands were cut out from the unstained strip and eluted with 1 ml citrate buffer (0.005M) pH 5.0 and tested for PG activity by the cup-plate assay method.

XVIII Protein measurement

The protein content of samples was determined conventionally by two methods, a colorimetric one described by Lowry, Rosebrough, Farr and Randall (1951) and ultraviolet absorption method (Layne (1957) after Warburg and Christian (1941)).

(A) Colorimetric method with Folin-Ciocalteu reagent

This method was used mostly for crude protein preparations.

Reagents:

A. 2% ($^{w}/_{v}$) Ha_2CO_3 in 0.1N NaOH

B. 1% ($^{w}/_{v}$) $CuSO_4$ $5H_2O$ solution 2% ($^{w}/_{v}$) Potassium tartrate. Equal proportions mixed on day of use.

C. 50 ml reagent A added to 1 ml of reagent B; mixed on day of use.

D. Folin-Ciocalteu reagent (BDH, Poole) (Folin & Ciocalteu (1927)).

To 5ml of reagent C was added 1ml of protein sample at room temperature and left for 30 min. 0.5ml reagent D was added and after a further 30 min the mixture was transferred

absorption (A)
to a cuvette and ~~O.D~~ at 750nm estimated in a spectrophotometer against a reagent blank. Preparations of crystalline bovine serum albumin (Koch-Light Ltd) containing between 10-100 µg/ml were used to obtain a calibration curve.

(B) Ultra violet absorption method

This was especially for colourless and partially purified protein samples. The technique is based on the maximal absorption of ultraviolet light of most proteins at 280nm. However since nucleic acids absorb at 280nm, correction for this interference is made by measuring ~~O.D~~ (A) of sample also at 260nm at which nucleic acids absorb more strongly. The ~~O.D~~ (A) of sample was thus read both at 280nm and 260nm against distilled water in a cuvette (1cm light path) and correction for nucleic acids calculated from the equation:

$$\frac{\text{~~O.D~~ (A) at 280nm}}{\text{~~O.D~~ (A) at 260nm}} = F$$

F was then substituted in the following equation:

$$\text{Protein conc (mg/ml)} = F \times \frac{1}{d} \times \text{~~OD~~ (A) at 280nm}$$

where d = length of light path in cuvette (in cm)

2. THE PHYTOALEXINS OF SWEET POTATO

I Preparation of furanoterpenoids from sweet potato tubers

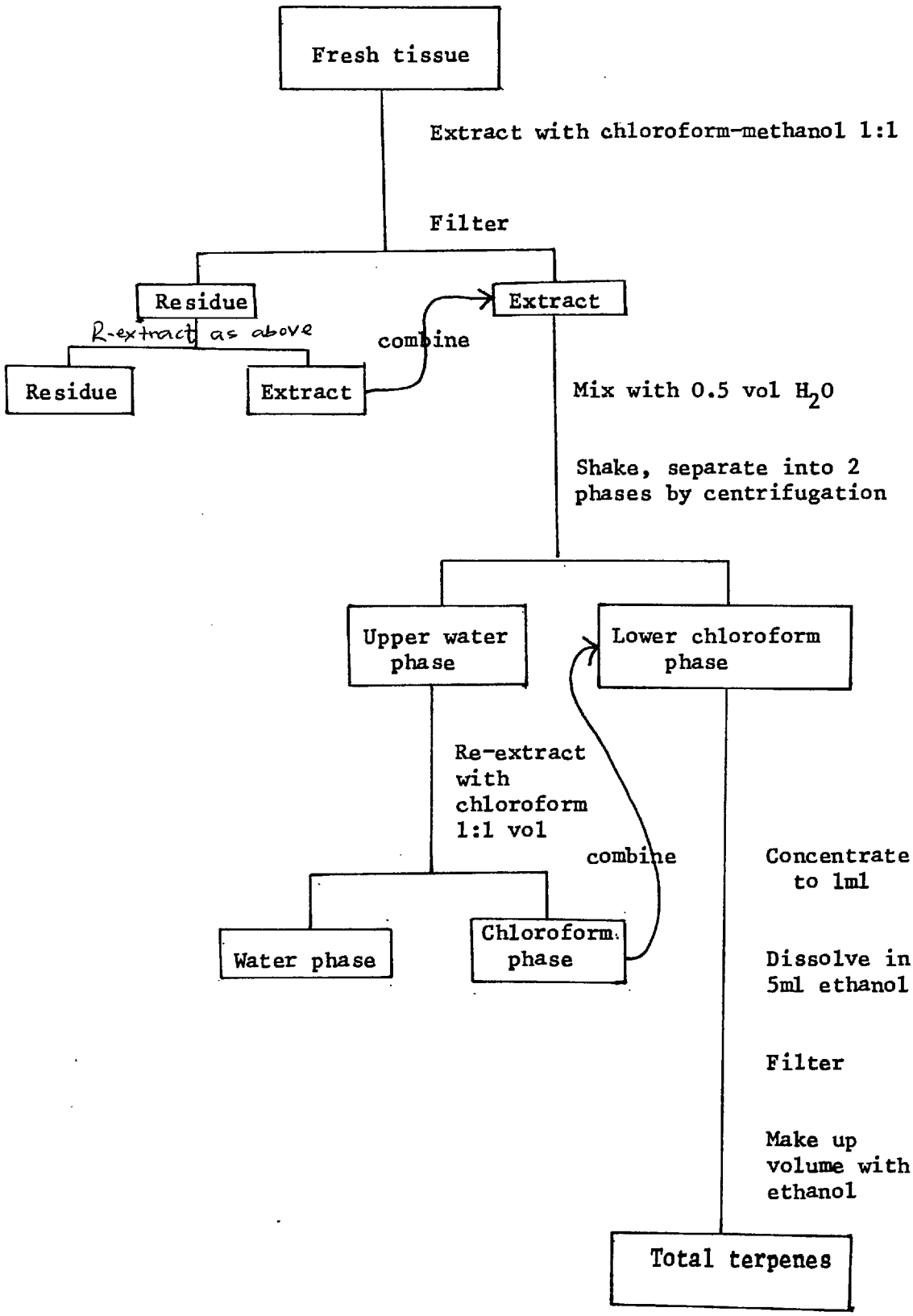
(A) Inoculation of tubers

Tubers were surface-sterilised by washing in 70% ethanol, cut into 3-4cm thick slices, inoculated with agar plugs of appropriate fungus (as described earlier) or with spore/mycelial suspension of the fungus. The slices were then placed in aerated desiccators at 25°C, R.H.80%.

(B) Extraction of terpenes from infected tissue

The rotted tissue was taken up in chloroform-methanol 1:1 (in the proportion 1g fresh weight tissue/4ml), broken down in a mortar and homogenised in a Sorvall Omnimixer. The material was filtered and the residue washed with several changes of chloroform/methanol 1:1. The filtrates were combined and mixed with 0.5 vol water, shaken vigorously and the mixture allowed to separate in two phases in a separating funnel. The upper phase was re-extracted with a further 1 vol chloroform. The chloroform phases were combined and concentrated to about 1ml in a rotary evaporator. The concentrate was dissolved in 5ml ethanol, filtered through 3 layers of muslin to remove undissolved material and made up with ethanol to desired volume (Figure 2).

FIGURE 2 Scheme for extraction of total terpenes



II Thin layer chromatography (TLC) of furanoterpenoids

(A) Set up of TLC

Ascending TLC was routinely used for the detection of the compounds in the extract. TLC was carried out on precoated plastic sheets (20 X 20cm, 0.25mm Silica gel with fluorescent indicator 254nm) (Camlab, Cambridge). Developing tanks (Shandon) containing the solvent were lined with blotting paper and left overnight to saturate the system.

Aliquots of the ethanol extract were applied as a band (2-4cm) at the origin of the silica gel plate, 2cm from margin. The plates were developed in a number of solvent systems as a preliminary check:

Chloroform in methanol 1-20%

Various proportions of n-hexane/ethylacetate

n-hexane : ether (1:1)

In subsequent experiments, n-hexane ethylacetate 3:2 system was used as a routine unless otherwise stated.

(B) Visualisation reagents

The separated compounds were visualised firstly under uv 254nm and then by means of reagents. Visualisation reagents were sprayed on to the plates in a fume chamber by means of a "Shandon" spray gun (Shandon Scientific Co. Ltd., Willesden, London).

The following reagents were used:

(i) 5% ethanolic solution of 95% H_2SO_4 (Anthony, 1964)

(ii) 5% solution of 95% H_2SO_4 in acetic anhydride (Anthony, 1964).

The chromatogram was sprayed, allowed to dry for 15 min in air and heated to 110°C in an oven until maximum colour development. Colour changes were noted.

(iii) Carr-Price reagent (Takeda, Hara, Wada & Matsumoto, 1963) 25g Antimony chloride was dissolved in 75ml chloroform to give a saturated solution of $SbCl_3$ in chloroform. Sprayed plates were heated for 10 min at 100°C and colour changed noted.

(iv) Dinitrophenylhydrazine (A)

0.4% solution of 2, 4-dinitrophenylhydrazine in 2N HCl. This reagent was used to distinguish free aldehydes, keto groups and ketoses. When sprayed, the phenols combine with the hydrazines to form 2-4 dinitrophenylhydrazones (DNPH). The plates were then sprayed with 0.2% solution of potassium hexacyanoferrate III in 2N HCl. Saturated keto DNPH would show blue colour immediately, saturated aldehyde DNPH would show olive green colour more slowly while unsaturated carbonyl derivatives would change only slowly or not at all.

(v) Ehrlich's reagent (Heacock and Mahon, 1965) as adapted by Akazawa (1960).

5.0g p-dimethylaminobenzaldehyde (Analar) was added to 50ml each of Conc HCl and absolute alcohol. This reagent was the most sensitive for terpenes.

III Bioassay of compds separated on chromatograms against Cladosporium cucumerinum

(A) Location of antifungal zones

The conidia of C. cucumerinum were obtained as described earlier. A dense suspension of the conidia was made in Sucrose-casamino acid medium Using a clean "Shandon" spray gun the dense spore preparation was sprayed uniformly over the surface of a TLC plate developed in n-hexane ethyl acetate 3:2. The spores were sprayed in a fume cupboard until the plate was just wet. The first spray was allowed to dry and the spraying repeated. Care was taken to prevent the liquid running about on the chromatogram surface. The plate was finally placed in a sterilized plastic box over wet blotting paper, sealed and incubated for 4 days in the dark. The plate was checked for location of antifungal zones.

(B) Elution of antifungal compounds

The bands corresponding to the inhibitory zones were scraped from similarly developed TLC plates with a spatula,

suspended in chloroform/methanol (1:1), mixed in a Whirlimixer for 1 min, centrifuged at 1,000g for 10 min and filtered through sintered filter tubes (B4T) containing discs of Whatman No.3 filter paper. The chloroform/methanol eluate was further concentrated under reduced pressure and purified further on TLC, run in different solvents:

n-hexane/ethyl acetate (3:2; 8:2; and 9:1)

n-hexane/ether (1:1)

This was to ensure that single compounds were eluted from the chromatogram for bioassay. The chromatogram, after development, was cut into two strips, one for locating the spots (uv 254nm and reagent spray) and the other for bioassay.

The compounds from the second TLC run were eluted with ethanol and uv absorption recorded again. The ethanol solution was finally evaporated and the compound taken up in volumes of either 2% ethanol or 0.5% dimethyl sulphoxide (DMSO) to give desired concentration (g fresh weight tissue/ml). This was used for further bioassay.

IV Quantitative evaluation of terpenes

(A) Total terpenes

The chloroform extract taken up in ethanol was adjusted to give a concentration of 5g fresh weight tissue/ml.

From this, an aliquot was taken and made up to 4ml with ethanol. The following were successively added to the solution:

2ml 10% ($^{W}/_{V}$) p-dimethylaminobenzaldehyde in ethanol

4ml 40% ($^{V}/_{V}$) aqueous H_2SO_4

The mixture was incubated at 30°C in a water bath for 15 min and the ~~O.D~~ A of the pink colour read immediately at 527nm (light path 1cm at 25°C). At this wavelength the ~~O.D~~ A was found to be proportional to terpene concentration. It was essential to read the ~~O.D~~ A within 5 minutes of colour development because the pink colour decreased with time.

The amount of total terpene was calculated from the equation,

$$\text{mg terpene (crude oil)} = \frac{A527 + 0.007}{0.820}$$

where A527 = ~~O.D~~ A at 527nm using 1cm light path at 25°C (Uritani, 1977)

(B) Ipomeamarone and other terpene components

These were assayed by eluting the compounds from developed but unsprayed chromatograms with 20ml chloroform/methanol (1:1). The mixture was centrifuged, filtered, evaporated to dryness, taken up in 5ml ethanol (95%) and aliquots of this used for assay. Reaction mixture and

technique for assay of terpene components was same as for total terpenes, above, except that the reaction mixture was incubated in a water bath at 50° for 30 min.

The equation for ipomeamarone was

$$\text{mg ipomeamarone} = \frac{A527 + 0.057}{1.9}$$

where A527 = as above.

The concentration of the other components were expressed in units of ipomeamarone equivalent.

(V) Sensitivity of fungi to phytoalexins of sweet potato

The sensitivity of the test fungi to the phytoalexins eluted from TLC were tested by spore germination assay and by further growth of pre-germinated spores (sporelings).

(A) Spore germination assay

The spores of B.t., B.c., and C.c., were obtained as described earlier. The concentrations were adjusted to 10^6 spore/ml in distilled water by means of a haemocytometer. Assays were performed on glass slides placed on glass rods over moist filter paper in plastic boxes (Purkayastha & Deverall, 1965). Three drops of the test solution (antifungal compound) containing about 20ml/drop were placed apart on a slide and a drop of the spore suspension in water containing approx 2,000 spores was added to

the centre of each drop. These were mixed with the tip of a sterile needle and incubated at 25°C for 24h after which % germination was recorded.

Three hundred spores randomly selected were examined in each case. Germination was recorded to have occurred when an emergent germ tube was perceptible.

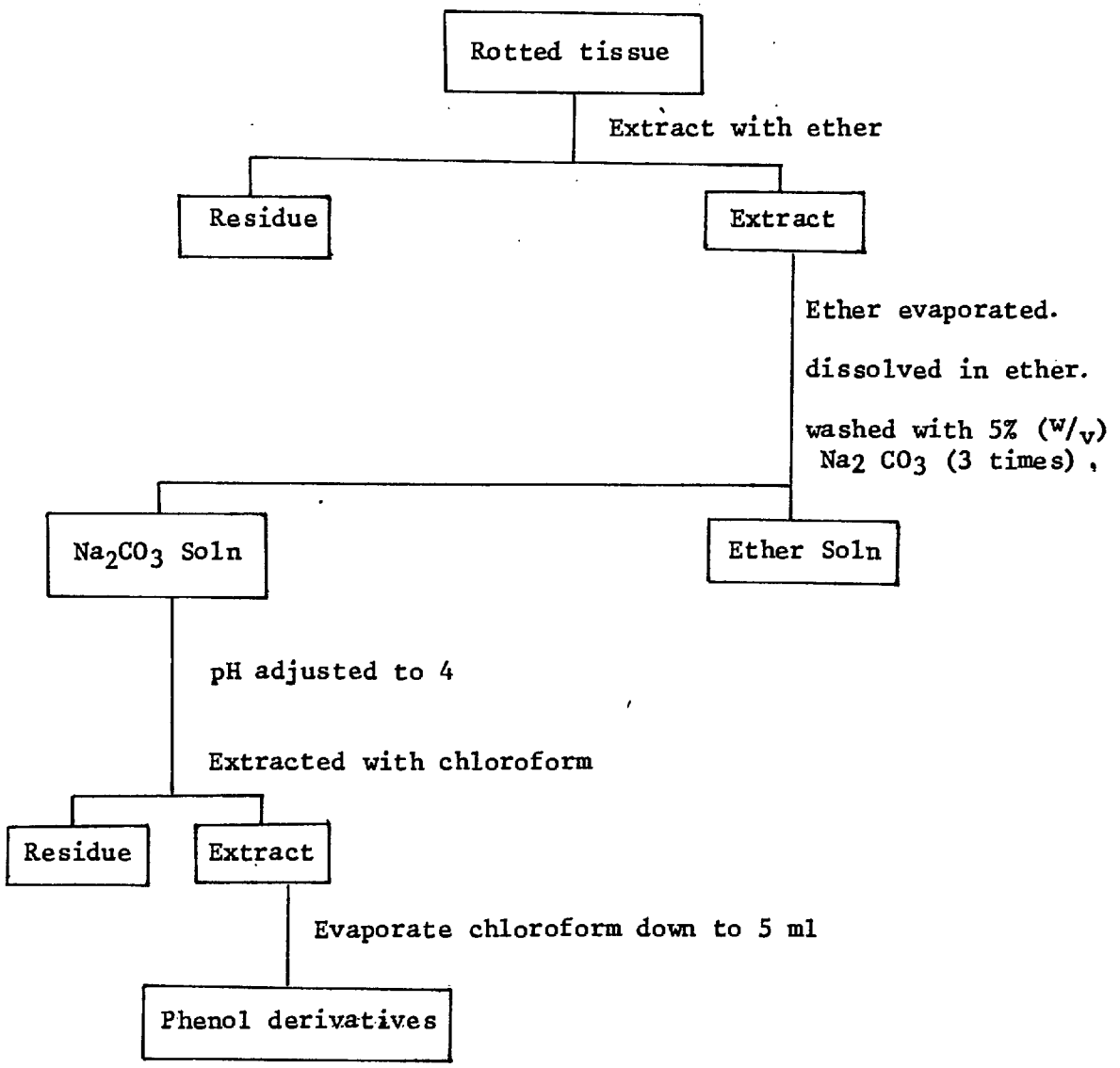
(B) Sporeling growth

Assays were similarly performed with 24h old sporelings grown on glass slides in drops of distilled water. The lengths of the sporelings were measured by means of a slide micrometer. The germination fluid was withdrwwn with a micropipette and 20 µl of the appropriate phytoalexin added and incubated again on glass rods over moist filter paper in plastic boxes. The length of the sporelings was taken every 24h for 5 days. The control solution consisted of eluted chromatogram taken up in 0.5% DMSO. (At this concentration, DMSO did not inhibit growth of the fungal spores.)

(VI) Isolation of other phenol derivatives from rotted tissue

The procedure for extraction of phenol derivatives was based on the method of Akazawa, Uritani and Kubota (1960). The procedure is schematically represented in Fig. 3.

FIGURE 3 Scheme for extraction of phenol derivatives from rotted tissue



The final chloroform extract was applied to TLC on silica gel and developed successively in two solvents:-

a) Ethanol-conc ammonia soln - H_2O (80:5:15)

b) n-butanol saturated with water

Solvent b improved the separation of the compounds on the chromatogram. The plate was developed for about 5h. after which the fluorescent spots were detected under u.v. (254nm). The plate was then sprayed with Benedict's reagent (Egger, 1969). The compounds were eluted with chloroform from unsprayed chromatograms, re-run on TLC using same solvents, eluted and taken up in Long Ashton Nutrient solution (LANS) (Hewitt, 1966) for bioassay.

VII Degradation of Ipomeamarone and total terpenes by fungi

(A) Preparation of mycelial suspension

Discs of actively growing mycelia were cut out from PDA culture for B.t., X medium for B.c. and V8 agar for C.c. These were separately grown on 150 ml liquid sucrose-casamino acid (SCA) for B.t. and B.c. and on liquid V8 juice medium for C.c. in 250 ml Erlenmeyer flasks, incubated at 25°C for 5 days. Since B.c. and C.c. produced less mycelium per unit time several flasks of these were set up to produce sufficient amounts of mycelium to work with. The cultures of each fungus were then combined and

homogenised in a Sorvall Omnimixer for 1 min at setting 1 followed by 20s at setting 5 and finally 30s at setting 1. 5 ml of approx equal weight of suspension was transferred into specimen tubes (7.5 X 2.5cm), capped and left for 24h for the mycelia to recover from homogenisation.

(B) Degradation arrangement

An appropriate concentration of ipomeamarone or total terpenes in DMSO was added to each tube. At intervals of ½, 1, 2, 4, 8, 12 and 24h three replicate tubes were harvested by centrifugation at 15,000g and the supernatant partitioned twice with equal volume of chloroform/methanol 1:1, concentrated to dryness and taken up in 5 ml ethanol. The ethanolic solution was scanned in a spectrophotometer and later run on a TLC in a solvent system of 3% methanol in chloroform. Chromatograms were sprayed with the following alternative reagents to locate products of degradation:

a) 5% ($^{v}/_{v}$) H_2SO_4 in methanol

b) 5% ($^{v}/_{v}$) solution of 95% ($^{v}/_{v}$) H_2SO_4 in acetic anhydride

c) Ehrlich's reagent.

Bands from similarly developed chromatograms were eluted with ethanol (95%) and tested for loss of compounds.

The following controls were set up:-

(i) Autoclaved mycelium was added to the phytoalexin incubated, harvested and extracted as experimental.

(ii) At intervals of 4, 12 and 24h the residue (mycelium) after centrifugation was washed with ethanol to ensure that there was no binding of compounds on mycelial cell wall. Washings were similarly scanned in a spectrophotometer, run on TLC and sprayed with the same location reagents.

EXPERIMENTAL WORK AND RESULTS

1. ENZYME STUDIES

I Preliminary experiments

1. Conditions for B. theobromae (B.t.) to produce pectic enzymes in culture.

There has been difficulty in getting B. theobromae to produce pectic enzymes in sufficiently large quantities for experimental work. This had led some workers to think that the fungus did not produce the enzymes in culture. The following preliminary experiments were aimed at establishing the best cultural conditions for pectic enzyme secretion by the fungus.

(A) Effect of various carbon sources on pectic enzyme secretion

The carbon sources used were glucose, sodium polypectate (NaPP, citrus, pectin and plant cell walls obtained from the bean plant (Phaseolus vulgaris). These were separately incorporated into sucrose-casamino-acid medium (SCA) at 2%. Thirty ml portions were dispensed into 150 ml Erlenmeyer flasks, autoclaved, inoculated and incubated (25°C) for 4 days and harvested as described under Materials and Methods. Enzyme assay was by viscometry. The reaction mixture consisted of the following:

2 ml dialysed culture filtrate

1 ml water

4 ml 1% ($^{W}/_{V}$) NaPP or Pectin in 0.1M citrate buffer pH 5.0 or in 0.1M Tris-HCl buffer pH 9.0.

In parallel runs, 1 ml 0.01M $CaCl_2$ was used in place of 1 ml water. The results are shown in Table 1. Results showed that pectic enzyme production by B.t. was inductive. No pectic enzyme was induced by glucose. NaPP, pectin and plant cell wall all induced pectic enzyme which reduced the viscosity of the reaction mixture at pH 5.0. The viscosity-reducing activity of the culture filtrate was very low or negligible at pH 9.0. The inclusion of Ca^{++} in the reaction mixture reduced the activity of the culture filtrate even at pH 5.0. Preference was shown for NaPP as substrate.

Since the viscosity reducing activity of the filtrate was negligible at pH 9.0 and pronounced at pH 5.0 in the absence of Ca^{++}, it was likely that no transeliminase enzyme was involved.

(B) Effect of age of culture on enzyme production

SCA was prepared in Erlenmeyer flasks using 2% NaPP as carbon source, and inoculated with B.t. At 24h interval,

Table 1. Viscosity-reducing activity of culture filtrates of B. theobromae grown on media of different carbon sources.

	NaPP				Pectin			
	pH 5.0		pH 9.0		pH 5.0		pH 9.0	
	$+Ca^{++}$	$-Ca^{++}$	$+Ca^{++}$	$-Ca^{++}$	$+Ca^{++}$	$-Ca^{++}$	$+Ca^{++}$	$-Ca^{++}$
Glucose	0	0	0	0	0	0	0	0
NaPP	8	200	<1	16	2	120	<1	<1
Pectin	<1	108	<1	7	6	110	<1	<1
Cell wall	4	75	<1	4	<1	60	<1	<1

Enzyme activity expressed in relative viscometric units was calculated from $\frac{1000}{t}$ where t = time (min) for 50% loss in viscosity of reaction mixture.

growth was arrested by filtering off mycelia from five flasks. After centrifuging, dialysing and ultra-filtering to one third volume, the culture filtrate was assayed for viscosity-reducing activity using the same reaction mixture as above but containing 4 ml 1% ($^{W}/_{V}$) NaPP in 0.1M citrate buffer pH 5.0.

Results (Fig. 4) showed that maximum viscosity reduction of the reaction mixture occurred on the 4th day of incubation. Beyond the 4th day, there was progressive decline in enzyme activity. On the 4th day of incubation, the culture filtrate was clear (transparent) indicating complete breakdown of the viscous pectic material.

Presumably, after the 4th day of incubation, other factors begin to operate in the culture which reduce the activity of the pectic enzyme produced. The age of the culture filtrate is therefore a critical factor in determining the activity of the culture filtrates.

2. Pectic enzyme activities of crude culture filtrates

The following properties of the culture filtrates of B.t. were determined as a preliminary step in characterising the pectic enzymes produced by the fungus.

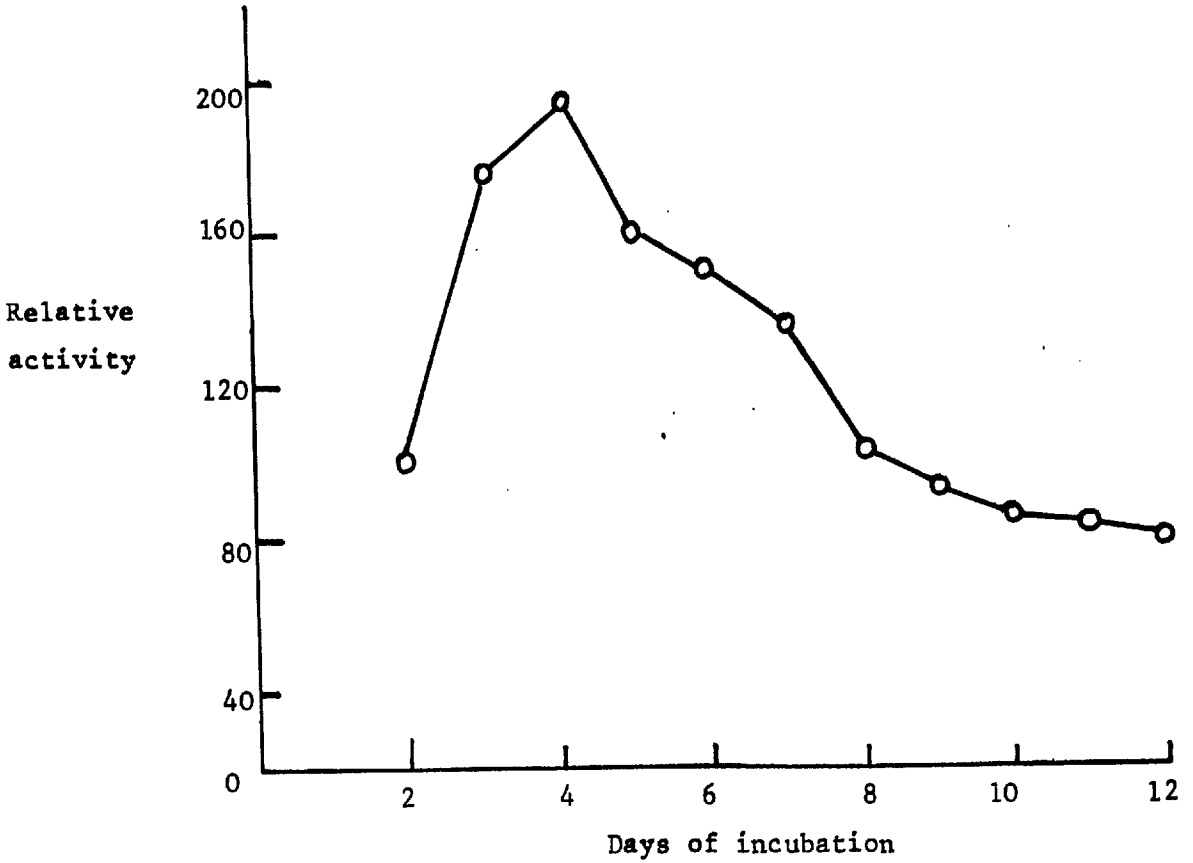


Fig. 4. Effect of age of culture on enzyme activity of culture filtrate of B. theobromae

Carbon source for growth of culture was 2% NaPP and NaPP was used as substrate. Reaction mixture was adjusted to pH 5.0. Enzyme activity, determined by viscometry.

(A) Release of reducing groups

The ability of the culture filtrate to release reducing groups from NaPP and pectin was determined. Reducing groups were estimated by the Nelson-Somogyi (1944) method against a reagent blank. The reaction mixture, the same as for viscometry, was incubated at 26 ± 1°. At intervals, 1 ml samples were removed, diluted to 10 ml and 0.2 ml samples taken for reducing sugar estimation. Table 2 shows a comparison of results obtained using NaPP and pectin as substrates.

The results show that more reducing groups were released when NaPP was used as substrate than when citrus pectin was used. It appears that pectin-methylesterase activity is not as important as polygalacturonase activity in the composition of the culture filtrate of the fungus.

(B) Effect of dilution of culture filtrate

The culture filtrate was diluted serially and the effect of the dilutions on the reducing groups released from NaPP also determined.

The results, Table 3, shows a near linearity between enzyme activity and dilution.

Table 2. Release of reducing groups (µg/ml) by culture filtrate of B. theobromae from NaPP and pectin substrates.

Incubation time (min)	NaPP	Pectin
5	18	< 0
10	35	<10
20	66	<10
30	74	-
40	82	10
50	87	22
60	94	38

Reducing groups released were in terms of galacturonic acid equivalent. This was read from a standard curve prepared with the acid.

Table 3. The effect of dilution on activity of culture filtrates of B.t.

Incubation time (min)	Dilutions				
	0.8	0.6	0.4	0.2	0.1
5	16	11	0	0	0
10	28	-	10	0	-
2o	39	20	18	0	0
30	41	-	26	-	-
40	56	42	34	15	0
50	64	54	44	28	0
60	72	60	-	40	10

Activity of enzyme was estimated as reducing sugars (galacturonic acid equivalent) released from reaction mixture.

(C) Thiobarbituric acid (TBA) test of culture filtrate

The TBA test was used to confirm the presence or absence of a transeliminase enzyme activity in the culture filtrate.

Reaction mixtures were same as described for viscometry with 1% ($^{W}/_{V}$) NaPP as susbtrate at pH 5.0 and 9.0 . When used at pH 9.0, 0.01 M $CaCl_2$ was substituted for 1 ml H_2O. 1 ml sample was removed from the reaction mixture after 1h incubation and tested for reaction with TBA. Figure 5 showed that with NaPP as substrate (pH 5.0) there was a peak of absorbance of the pinkish-orange colour produced at 515nm and no peak at 550 nm. With the reaction mixture at pH 9.0 in the presence of $CaCl_2$, there was no comparable absorbance at these wavelengths.

The above test confirmed that there was no transeliminase enzyme activity in the culture.

3. Extraction of rotted tissue from sweet potato

The extraction of pectic enzyme from rotted tissue of sweet potato was embarked upon to demonstrate the involvement of the enzymes in pathogenesis.

Rotted tissue was extracted firstly with 0.85% ($^{W}/v$) NaCl in buffer over a range of pH 4 - 9 and the pectic enzyme

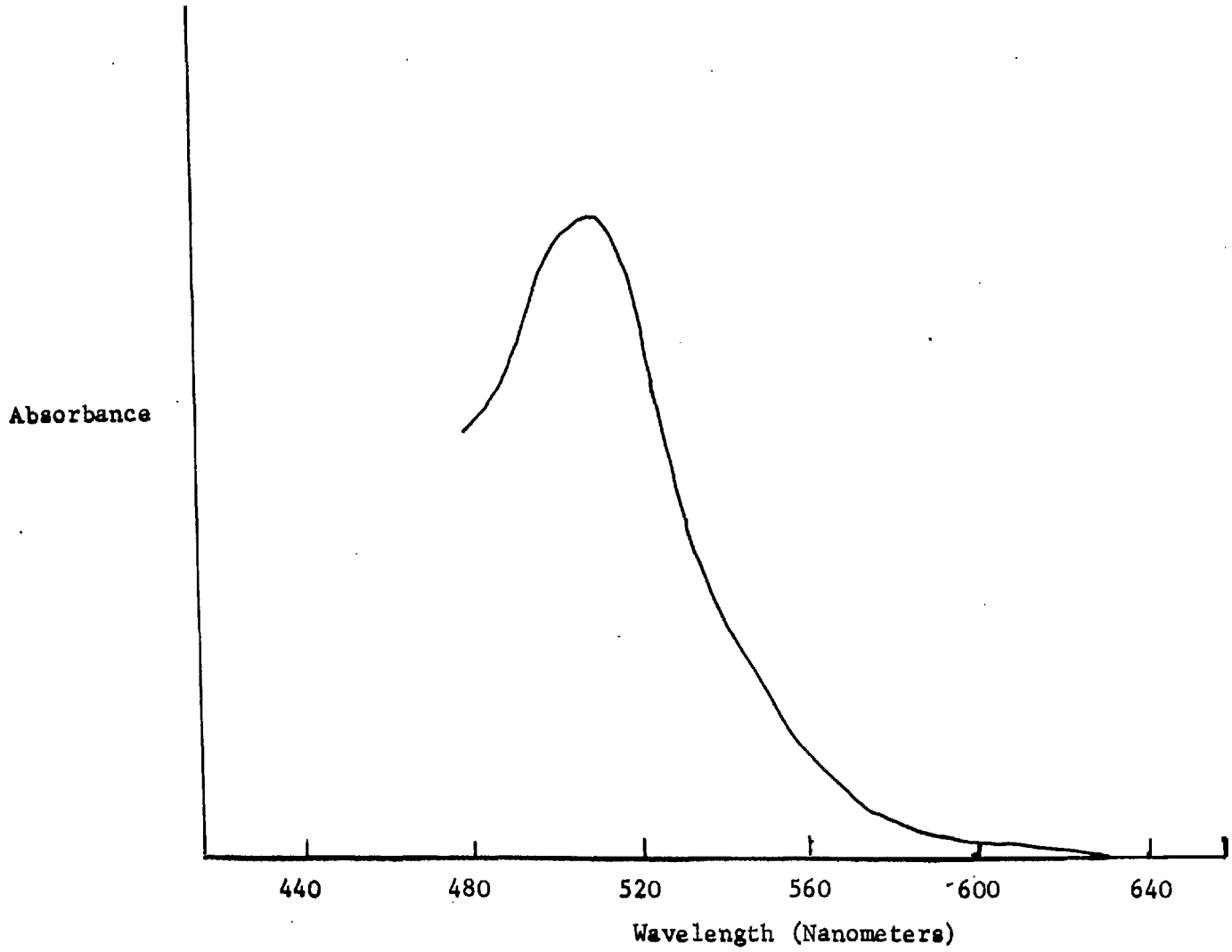


Fig. 5. Absorption spectrum of colour produced from reaction between products of culture filtrate enzyme reaction with NaPP and thiobarbituric acid.

activity of the extract tested by viscometry. It was found that activity was very low or negligible. Because the extract quickly discoloured, turning to dark brown, two antioxidants, ascorbic acid (Spalding, 1968) and dithiothreitol, (Cooper, 1974) were separately used as extractants over a range of pH 4 - 9.

Table 4 shows that there was a sixfold increase in activity by extracting with ascorbic acid and a seven fold increase with dithiothreitol in the presence of 5% P.V.P. at pH 5.0. There was negligible enzyme activity when the pH of the extractants was raised above 6.0. The optimum pH for enzyme extraction was 5.0.

Subsequent enzyme extractions were done in 0.01 M citrate buffer pH 5.0 in the presence of 10^{-3} M dithiothreitol and 5% ($^{W}/_{V}$) insoluble PVP.

4. Pectic enzyme activities of crude rot extracts

(A) Viscosity-reducing activity

The rot extract was firstly assayed by viscometry as described for crude culture filtrate using NaPP and pectin as substrates at pH 5.0 and 9.0 both in the presence and absence of Ca^{++} (0.01 M final).

Reaction mixture was of similar composition as for viscometry described earlier.

Table 4. Relative enzyme activity (by viscometry) of sweet potato rot extracts obtained with three different extractants and at different pH.

pH	NaCl	NaCl + AA	NaCl + DT + PVP
4	39	116	120
5	27	163	188
6	23	122	135
7	<1	48	47
8	<1	<1	28
9	<1	<1	<1

Units of enzyme activity = $\frac{1000}{t}$ where t = time (min) for 50% viscosity reduction of reaction mixture

NaCl - 0.85% sodium chloride; AA - 10^{-3} M ascorbic acid; DT - 10^{-3} M dithiothreitol; PVP - 5% insoluble polyvinylpyrrolidone.

The result is shown in Table 5. As with the culture filtrate, activity of the rot extract was higher with NaPP as the sole substrate than with pectin. Enzyme activity was inhibited when $CaCl_2$ was included in the reaction mixture. Enzyme activity was more than ten times greater at pH 5.0 than at pH 9.0.

It appears that, as with the culture filtrate, there is no transeliminase action in the rot extract.

(B) Release of reducing groups

For comparative purposes the reducing groups released by rot extract was determined using NaPP and pectin at pH 5.0. Reaction mixture was the same as for viscometry above without Ca^{++} ions.

The results, Table 6, showed that reducing groups were more readily released from NaPP than from pectin. The preference for NaPP over pectin is more strongly expressed by the rot extract than by the culture filtrate. These results also suggest that in the rot extract, polygalacturonase activity might be more important than pectinmethylesterase activity.

(C) TBA test of rot extract

The rot extract was further subjected to TBA test to investigate the secretion or non-secretion of a transeliminase

Table 5. Viscosity reducing activity of crude rot extract of sweet potato rotted by B.t.

Substrates		pH 5.0	pH 9.0
NaPP	$+Ca^{++}$	14	<1.0
	$-Ca^{++}$	160	12.0
Pectin	$+Ca^{++}$	<1	<1
	$-Ca^{++}$	59	<1

Enzyme activity is expressed in relative viscometric units.

Table 6. Release of reducing groups (µg/ml) by rot extracts of sweet potato from NaPP and pectin at pH 5.0

Incubation time (min)	NaPP	Pectin
5	14	0
10	25	0
20	47	0
30	75	10
40	80	17
50	-	28
60	91	34

Units are galacturonic acid equivalents.

in vivo by the same procedure as for the culture filtrate. The result is shown in Fig. 6.

There was no peak of absorbance at 550nm at either pH 5.0 or 9.0 A peak of absorbance showed at 515nm when reaction mixture was at pH 5.0. This was a further confirmation of the absence of any lyases in the rot extract.

II Purification of PG from culture filtrate of B. theobromae and sweet potato rot extract.

1. Ammonium sulphate fractional precipitation

The rot extracts and culture filtrates (200 ml each) were fractionally precipitated with solid $(NH_4)_2SO_4$ at room temperature. Precipitates were formed at 0-40%, 41-60%, 61-80% and 81-90% saturations. Each fraction was then estimated for protein content and PG activity. Protein was estimated by the colorimetric method with Folin-Ciocalteu reagent and PG by viscometry. The results of the estimations are summarized in Table 7.

There was some separation and concentration of proteins and PG activities in the 60-90% fractions. In all the cases, PG activity was highest where the proteins were concentrated. This initial step in PG purification did not significantly separate the PG from other proteins but served to concentrate the enzyme.

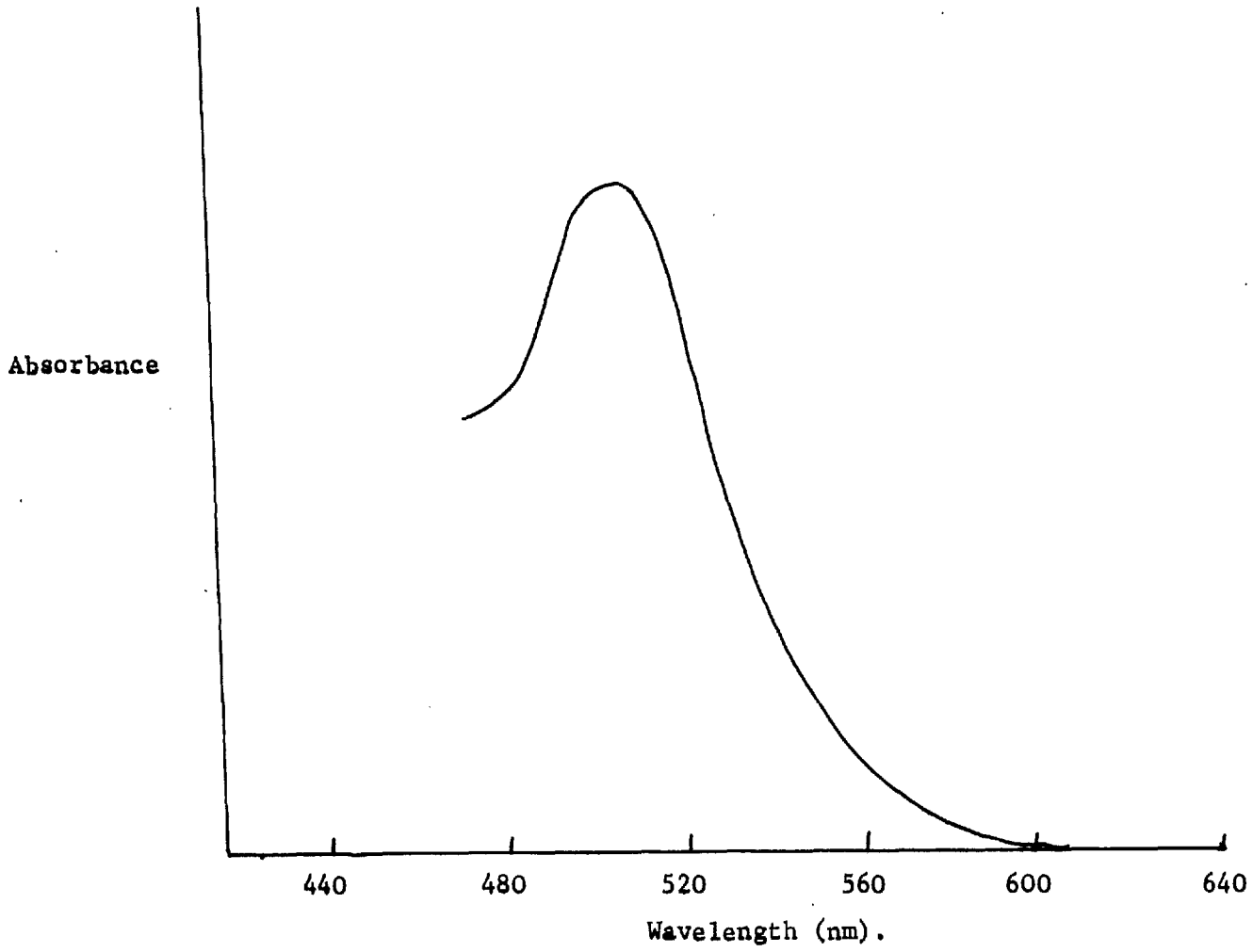


Fig. 6. Absorption spectrum of colour produced from reaction between products of rot extract enzyme reaction with NaPP and thiobarbituric acid.

Table 7. Protein content and PG activities in $(NH_4)2SO_4$ precipitates of B. theobromae culture filtrates and sweet potato rot extract.

		$(NH_4)_2SO_4$ fractions			
		0-40%	41-60%	61-80%	81-90%
Culture filtrate	Protein*	16	28	50	75
	PG+	<1	26	126	158
Rot extract	Protein	0	12	41	70
	PG	0	<1	52	172

* Protein was estimated with Folin-Ciocalteu reagent and units expressed as µg/ml

+ PG activity is expressed in relative viscometric units.

2. Gel filtration with Sephadex G75 superfine

The precipitates which showed enzyme activity (41-90% for culture filtrate and 61-90% for rot extract) were further fractionated by passing them through a column containing Sephadex G75 superfine gel. 5ml fractions were collected and their protein content immediately measured by μv absorption method (Layne, 1957) and PG assayed by the cup plate technique.

Fig. 7 shows the separation of the culture filtrate $(NH_4)_2SO_4$ precipitate fraction on the Sephadex gel. PG activity appeared in one band of fractions (11-20) coinciding with the main protein peaks. Some purification from other proteins was obtained at this stage.

3. Isoelectric focusing

This technique was used as the final step in enzyme purification. In some cases the gel filtration stage was omitted and fractions from $(NH_4)_2SO_4$ fractionation were subjected directly to isoelectric focusing after several steps of dialysis in several changes of water.

Samples were electrofocused firstly at pH 3 -10 and then at 3.5 - 6 for 72h. 4 ml sample fractions were collected for pH 3 -10 and 2 ml fractions for pH 3.5 - 6 focusing.

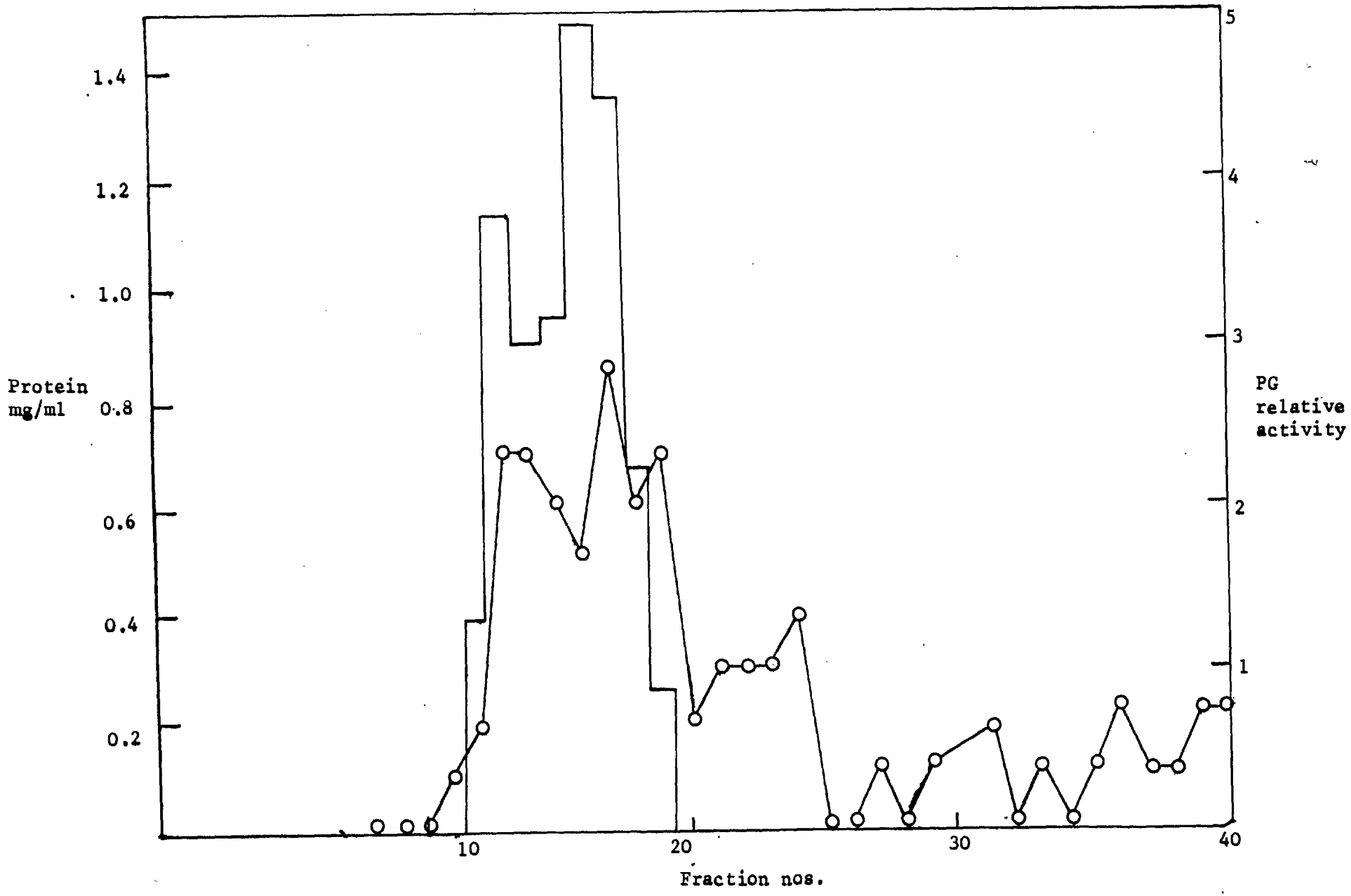


Fig. 7. Separation of the 80-90% Ammonium sulphate fraction of culture filtrate on Sephadex G75 superfine. PG assayed by the cup plate method and protein, by absorbance at 280 and 260 nm.

——— PG. o———o Protein.

(A) pH 3 - 10 focusing

At this range of pH, there was good separation of the proteins from the PG in both culture filtrate and rot extract. Culture filtrate PG focused in a single band (8 - 15 fractions) separate from most of the proteins which occurred at the earlier and later fractions. Fig. 8 shows this result.

Rot extract PG was also separated from most of the proteins at this pH range of focusing. The PG here occurred in only 5 fractions 12 - 16 (see Fig. 9).

(B) pH 3.5 - 6 focusing

Fractions which showed PG activity from the first step of focusing were refocussed at pH 3.5 - 6. At this range, the culture filtrate was resolved into four peaks, two major and two minor, with isoelectric points at pH 4.1 (PG 1), 4.3 (PG 2), 5.1 (PG3) and 5.8 (PG 4) (Fig.10).

When rot extract was subjected to similar purification only one peak was obtained corresponding to pH 5.1 (PG 3) (Fig.11). The same result was obtained , with both extracts, if the initial pH 3 - 10 electrofocusing stage was omitted.

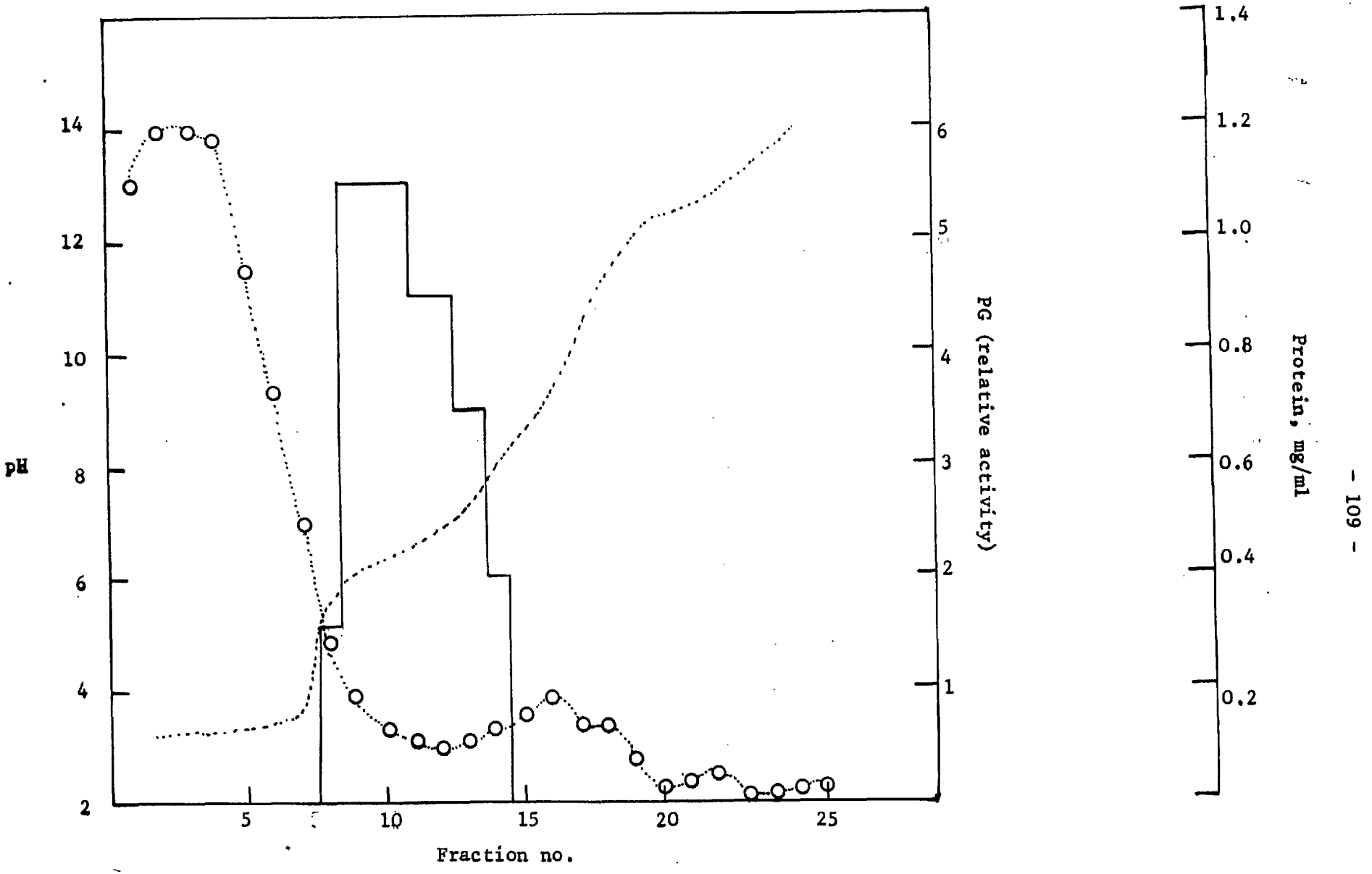


Fig. 8. Isoelectric focusing of culture filtrate PG (after Sephadex gel filtration) using Ampholine carriers pH 3-10. 4 ml fractions collected. PG assayed by cup-plate method; proteins by absorbance at 280 and 260 nm.
—— PG, o——o protein, ---- pH gradient.

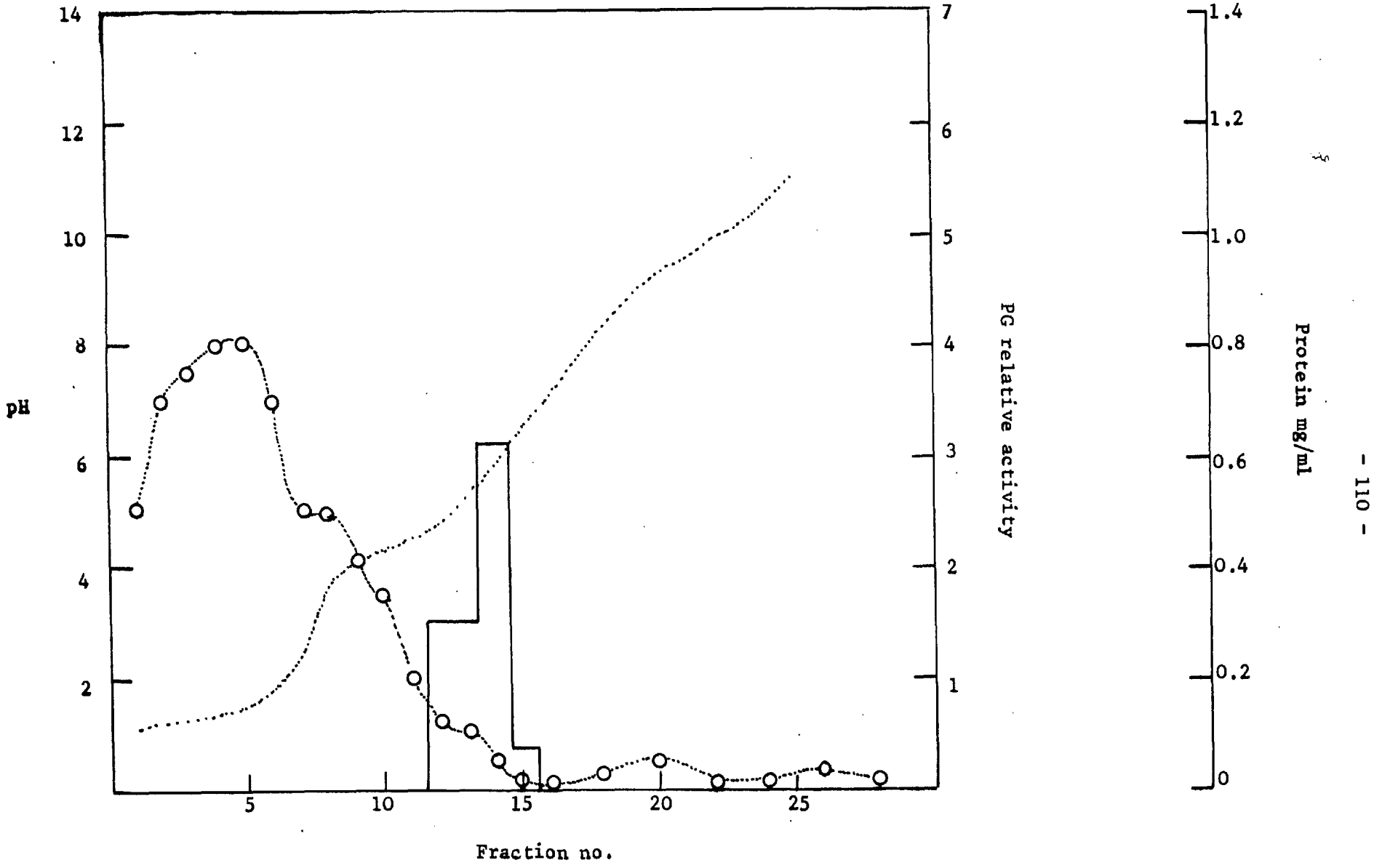


Fig. 9. Isoelectric focusing of rot extract PG (after Sephadex gel filtration) using Ampholine carriers pH 3-10. 4ml fractions collected. PG assayed by cup-plate method; proteins by absorbance at 280 nm and 260 nm. —— PG, o-----o Protein, ---- pH gradient.

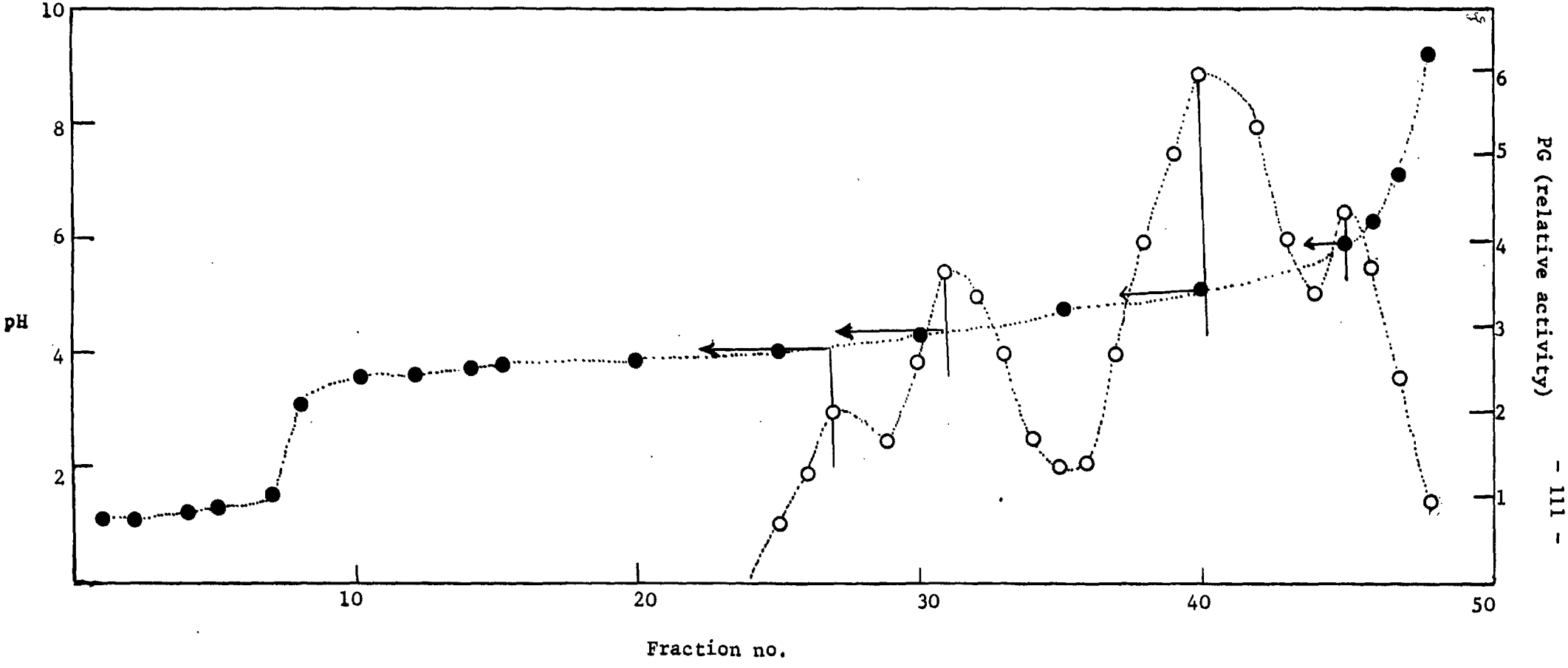


Fig.10. Isoelectric focusing of culture filtrate PG (fractions 7-14, Fig. 5) using Ampholine carriers pH 3.5-6.2ml fractions collected. PG assayed by the cup plate method.

●—● pH gradient, o——o PG.

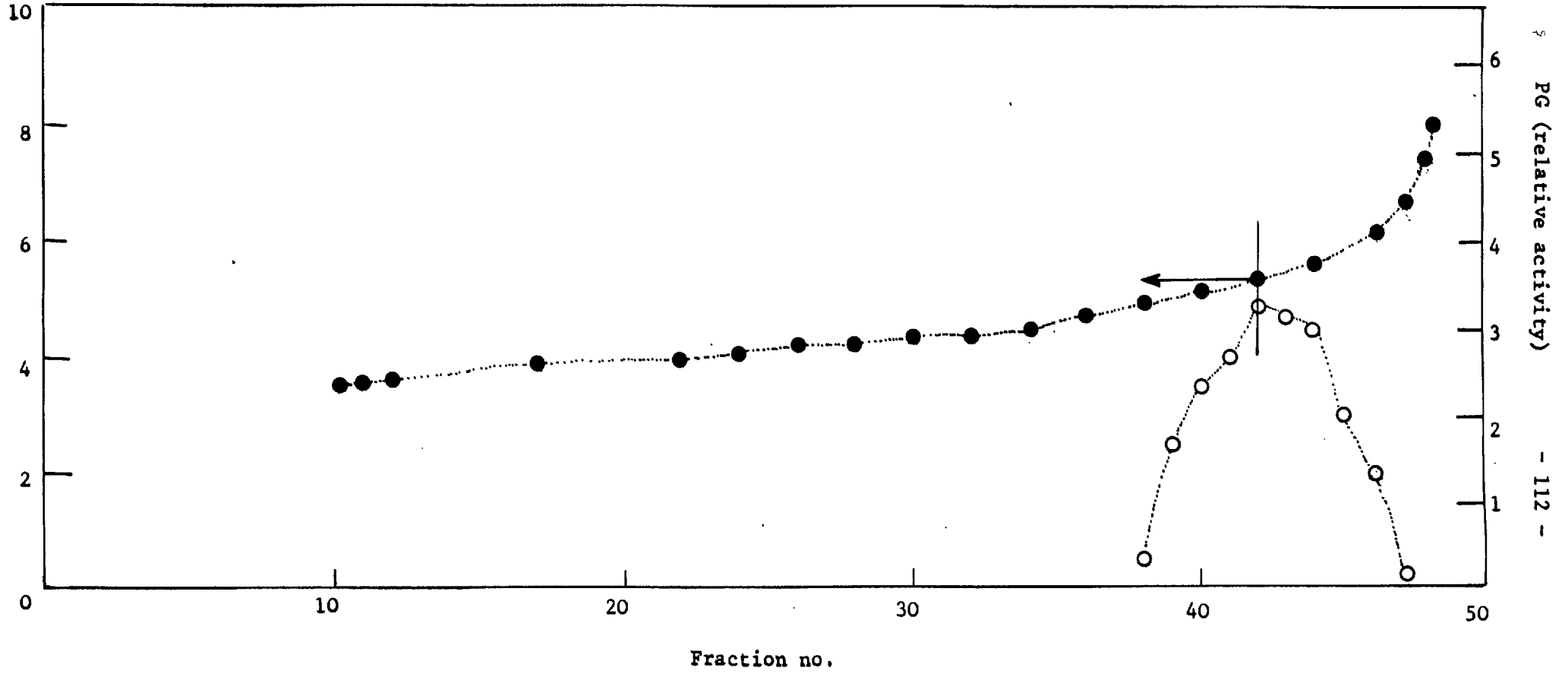


Fig. 11. Isoelectric focusing of rot extract PH (fractions 12-16, Fig.6) using Ampholine carriers pH 3.5-6. 2ml fractions collected. PG assayed by cup plate method.

●----● pH gradient, o——o PG

4. Thin-layer isoelectrofocusing

The thin layer electrofocusing technique was used as an analytical method to test whether the four peaks obtained with culture filtrate PG separated by column electrofocusing at pH 3.5 - 6 did correspond to distinct isoenzymes. A sample of the PG active eluate from the Sephadex column was firstly electrofocused in two strips. One strip was stained for proteins with Coomassie Brilliant Blue R.250 and discs of gel corresponding to the protein bands on the second unstained strip were cut out and eluted with 1 ml 0.005 M citrate buffer pH 5.0 and tested for PG activity by cup-plate assay and later by viscometry. Four such bands were obtained and the result of the cup-plate assay and viscometry is shown in Table 8.

Fractions corresponding to the four peaks (obtained from column focusing) were subjected to the thin layer electrofocusing together with a sample of the active eluate from the Sephadex column. Coomassie Blue staining showed that the four distinct isoenzymes of the active eluate corresponded in position with the main staining bands obtained for the four fractions. The four peaks from the isoelectric focusing column thus corresponded with four distinct isoenzymes of PG with the following PI values PG1 - 4.1, PG2 - 4.3, PG3 - 5.1 and PG4 - 5.8 (Fig. 12).

Table 8. Activity of PG isoenzymes eluted from thin layer electrofocusing gel.

	Enzyme activity *	
Band	RVu	Cup-plate assay
1	2.0	0.7
2	6.5	2.5
3	28	7.2
4	15.4	4.8

*Enzyme activity expressed in relative viscometric units (RVu) and cup-plate method (diameter (mm) of halo formed).

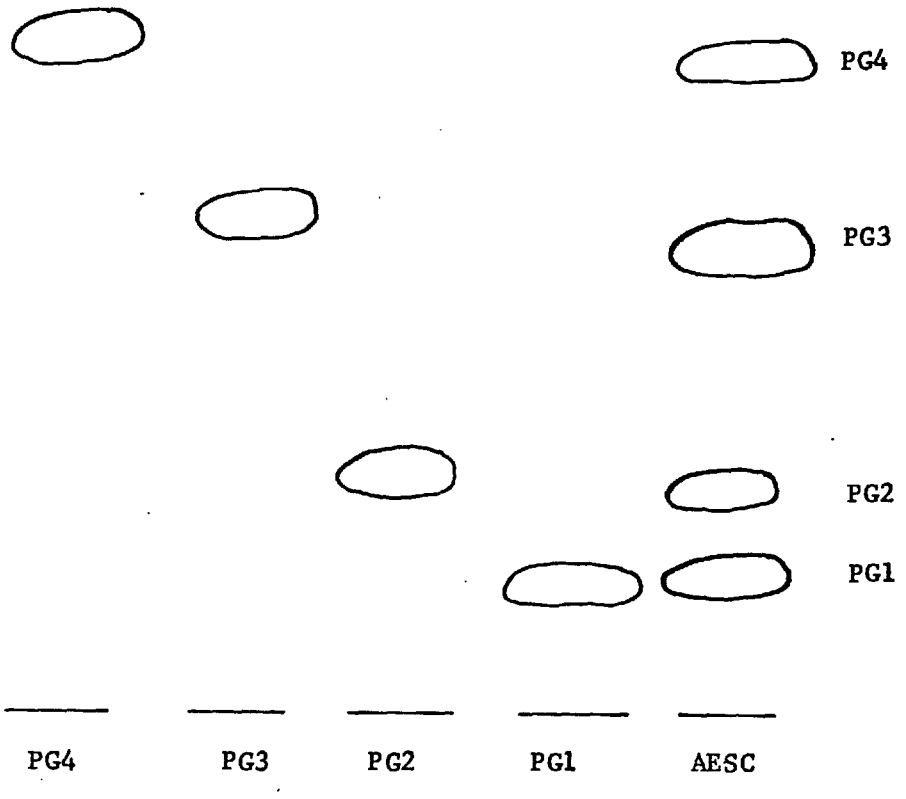


Fig. 12. Diagram of thin-layer electrofocusing of active eluate from Sephadex column (AESC) and fractions corresponding to the four peaks PG1, 2, 3 and 4 from Fig. 7. Gel stained with 0.115% Coomassie Brilliant Blue R250 in 8% acetic acid + 25% ethanol in H_2O.

III Properties of purified culture filtrate PG isoenzymes and rot extract PG.

1. Effect on tissues

When tested for their viscosity-reducing property, the culture filtrate PG isoenzymes 1, 2, 3 and 4, had relative activities (RVU) of 60, 70, 217 and 133 respectively. Rot extract PG had an activity of 200.

The isoenzymes were also tested for their ability to release reducing groups from NaPP. The amount of galacturonic acid equivalent released (µg/ml/h) were as follows: PG1 = 32.1, PG2 = 38.0, PG3 = 115.6 and PG4 = 70.2. The ratio of the reducing groups released was similar to the ratio for viscosity reducing activity of the isoenzymes. This indicates a similar pattern of hydrolysis of the substrate (NaPP) chain by all the enzymes.

When tested for PTE activity (TBA test) all the enzymes gave negative results.

The enzymes were adjusted in concentration to give a relative activity of 60 and then tested for their ability to macerate and kill cells of sweet potato and potato.

(A) Maceration

Maceration was estimated by the loss in coherence

test. Reaction mixture consisted of the following:-

2 ml enzyme

1 ml 0.05 M citrate buffer pH 5.0

1 ml water

Twenty-four discs of either sweet potato or potato were incubated in the mixture at 27 $\pm$ 1° and at intervals of 1h samples of 3 discs were removed and tested for maceration. The results are shown in Table 9.

Both PG1 and PG2 did not macerate either sweet potato or potato tissues after 5h incubation. Both isoenzymes PG3 and 4 and rot extract PG completely macerated both tissues after 5h.

(B) Cell death

Cell death was determined by a colorimetric method based on Tribe's 1955 Neutral Red Index technique (see Materials and Methods). Reaction mixtures and disc incubation were the same as described for maceration.

At intervals of 1h one disc was removed and stained with Neutral Red in phosphate buffer for 20 min. The disc was washed in $MKNO_3$ and the stain extracted with methanol. The extracted stain was read in a spectrophotometer and percent cell death calculated from optical density readings. Table 9 shows the results.

Table 9. Toxic and maceration effects of PG1, 2, 3 and 4* on sweet potato and potato tissues.

	Maceration Index				
Tissue maceration 5h	PG1	PG2	PG3	PG4	Rot extract
Sweet potato disc	0[a]	0	5	5	5
Potato disc	0	1	5	5	5

Cell death (5h)	Percent cell death				
Sweet potato disc	0[b]	21	97	97	99
Potato disc	0	38	99	99	99

* Enzyme activites standardised to a viscometric value of 60 before testing.

a Maceration index from 0 (no maceration) to 5 (complete maceration)

b Percent cell death calculated from optical density readings of neutral red stain extracted with methanol.

PG1 neither killed nor macerated either tissue. PG2 did not macerate sweet potato discs and had little effect on potato. Both isoenzymes PG3 and 4 and rot extract PG macerated and killed sweet potato and potato discs completely in 5h.

There appears to be a relation between tissue maceration and cell death caused by these isoenzymes.

(C) Release of electrolytes from tissue

The release of ions from cells treated with pectic enzymes is often associated with changes in the membrane physiology of the cells following the death of the cells.

The tissue discs were immersed in the reaction mixture as described for maceration and cell death and at 10 min intervals five discs were removed, rinsed in 0.4 M sucrose and then shaken in 15 ml water for 20s to allow the ions flow into the bathing solution. Conductivity was read in a meter, Type MC1 (Mark IV).

Discs treated with PG3, 4 and rot extract PG showed similar rapid release of ions. PG2 gave rather less and PG1 did not cause any ion release from the discs (Fig. 13).

2. K_m

The Michaelis-Menten constants (K_m) of culture filtrate

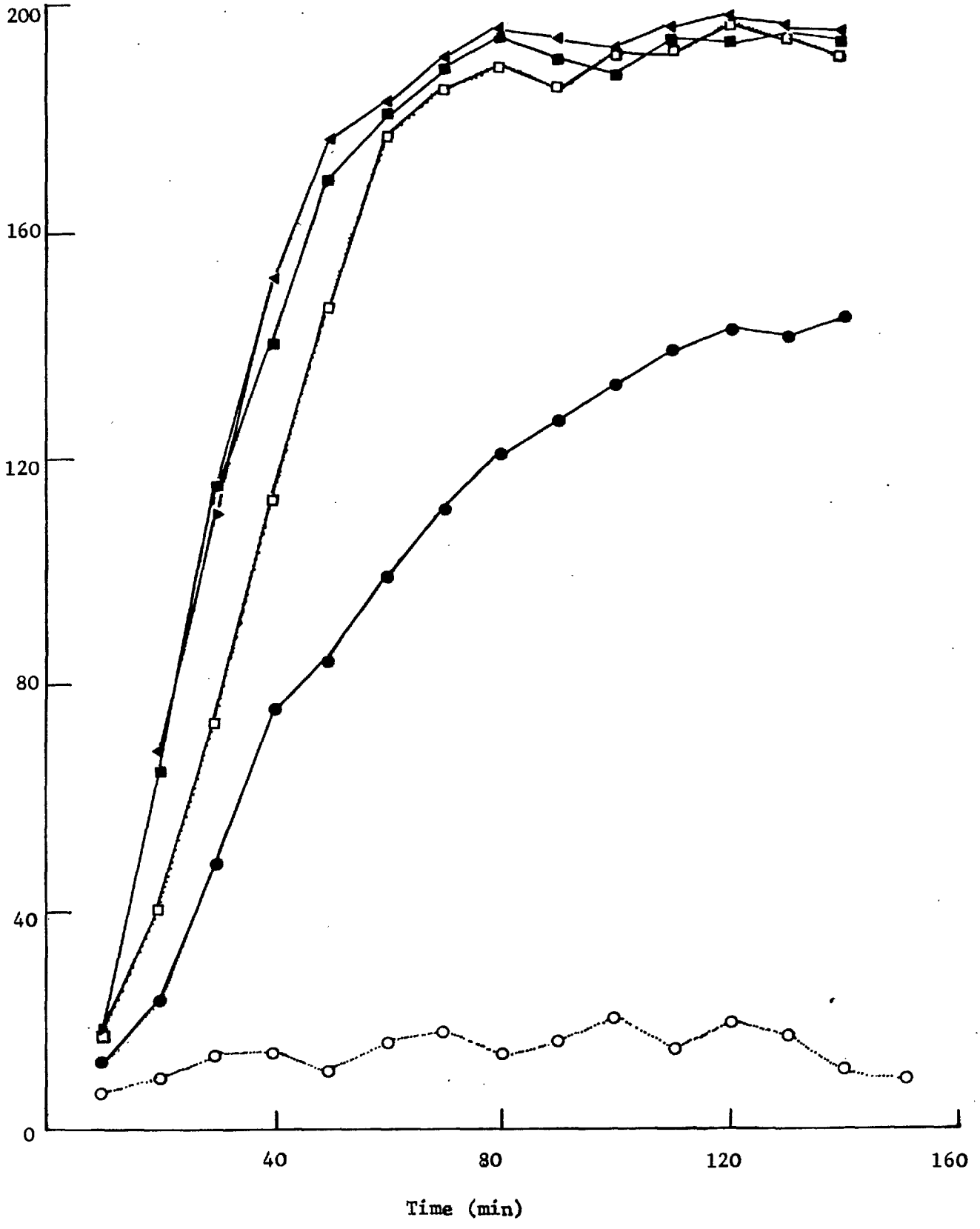


Fig. 13. Comparison of electrolyte leakage from sweet potato slices treated with PG1 (o-----o), PG2 (●——●), PG3 (▲——▲), PG4 (□——□), and rot extract PG3 (■——■). Conductivity readings taken on 15 ml bathing solution at room temperature.

and rot extract PG3 were determined from the release of reducing sugar (galacturonic acid equivalent) in reaction mixtures at different substrate concentration. Lineweaver-Burke plots gave Km values for culture filtrate PG3 and rot extract PG3 of 3.38 and 3.33 g/l respectively with NaPP as substrate and 5.4 and 5.3 g/l with citrus pectin (see Figs. 14, 15, 16 and 17).

The closeness of the values for these isoenzymes from different sources suggests that they are one and the same.

IV Binding of enzymes to tissues

The binding experiment was performed principally to test the hypothesis that enzymes of a pathogen bind onto tissues which are susceptible hosts. This hypothesis derives from known properties of enzymes as proteins and from the high selectivity and sensitivity involved in biological reaction. Most physiological responses are highly selective, sensitive and occur rapidly. They are also reversible in most cases. This being the case it will be expected that the union between the ligand binding sites and receptors, though of high affinity will be non-covalent, weak bonds. Recent studies on the physico-chemical properties of enzyme/substrate interactions as shown from x-ray crystallography confirm these properties (Poljak, 1975). The configurational properties of the enzyme/

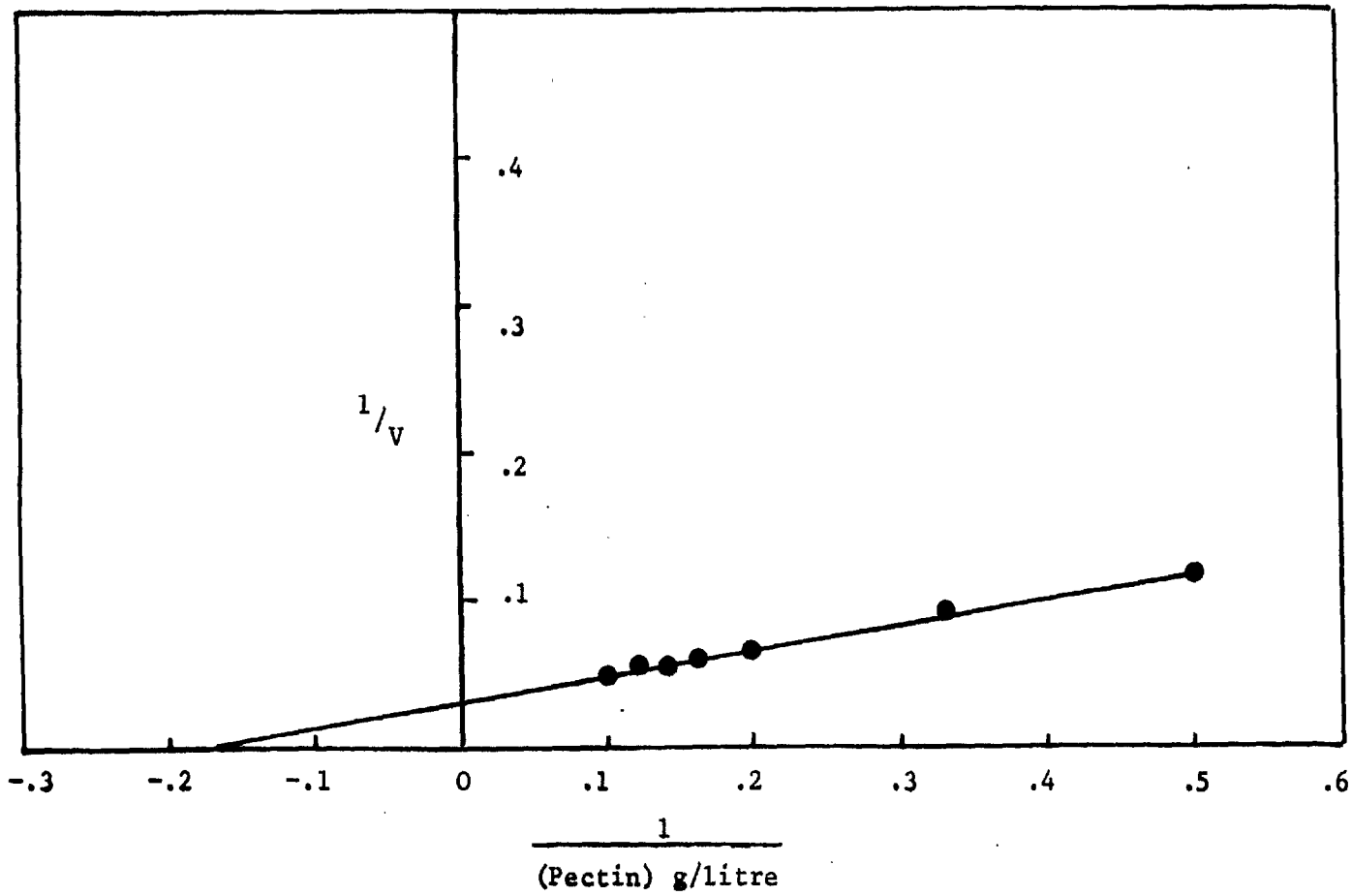


Fig. 14. Determination of K_m of culture filtrate PG3 using Citrus pectin as substrate. Km was 5.4 g/litre. Activity, V is expressed as µg equivalent of galacturonic acid released from reaction mixture in 5 min.

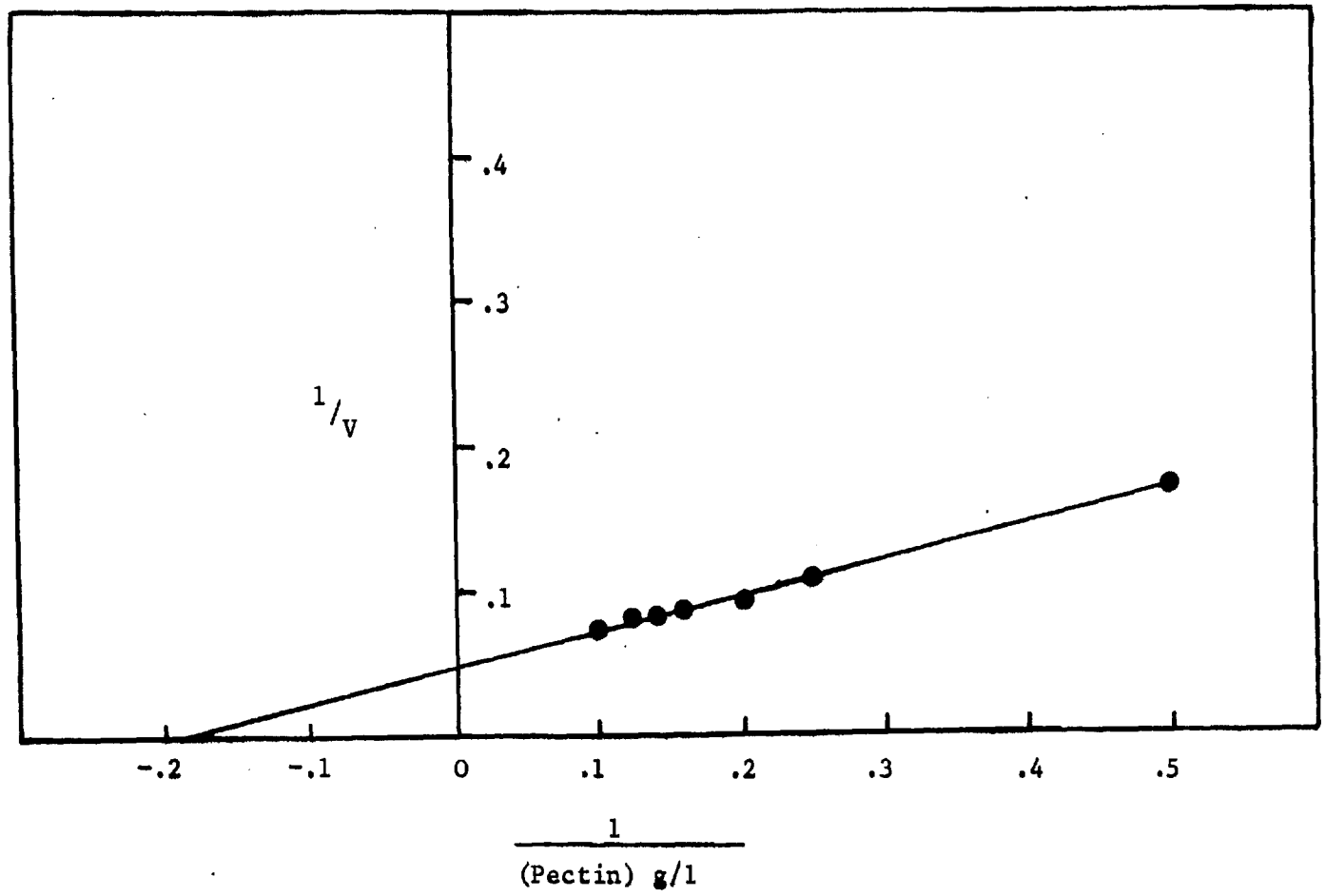


Fig. 15. Determination of K_m of rot extract PG using citrus pectin as substrate. K_m was 5.3 g/litre. Activity, V, is expressed as µg equivalent of galacturonic acid released from reaction mixture.

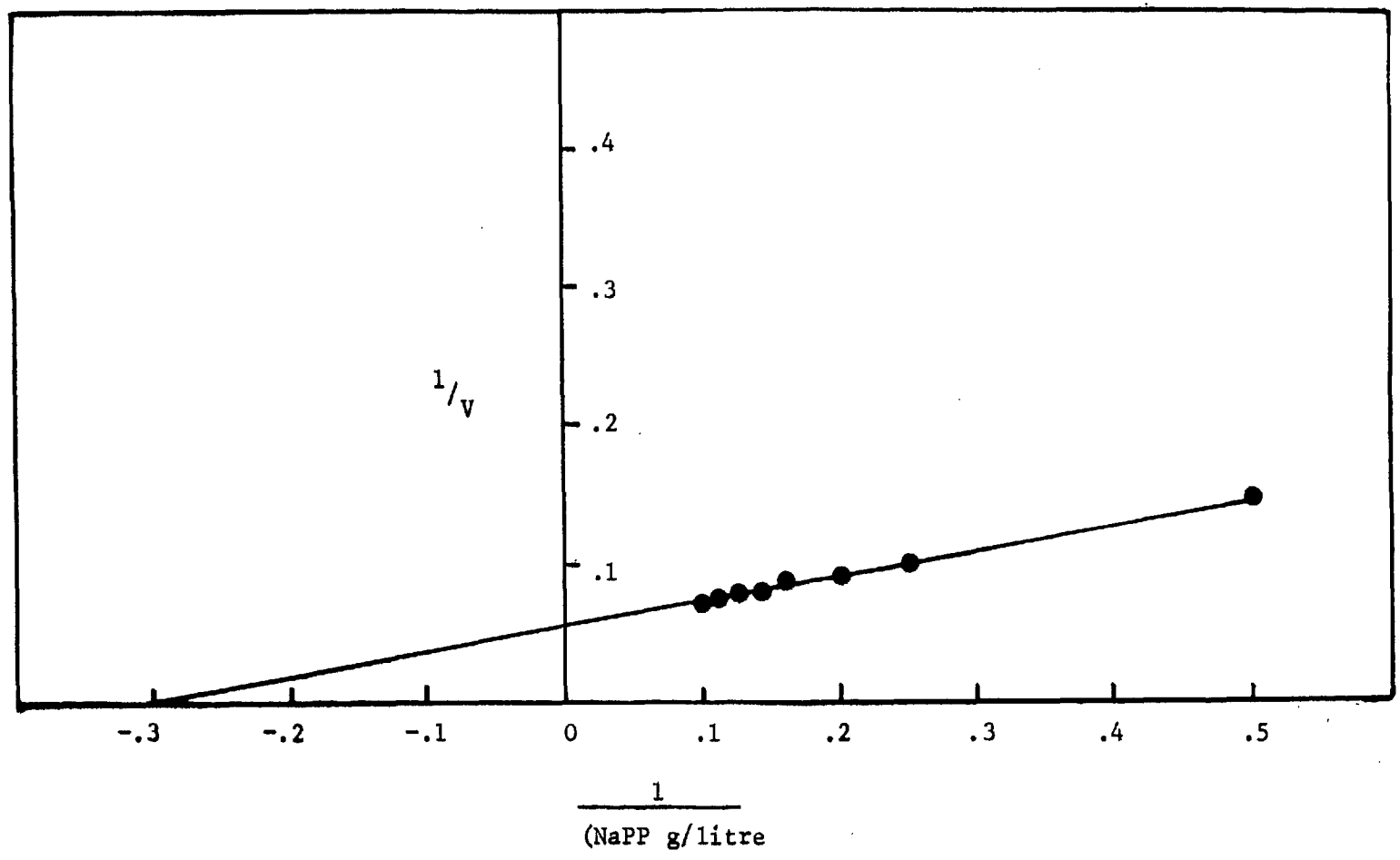


Fig. 16. Determination of K_m of rot extract PG using sodium polypectate as substrate. Km was 3.33 g/litre. Activity, V, is expressed as µg equivalent of galacturonic acid released from reaction mixture in 5 min.

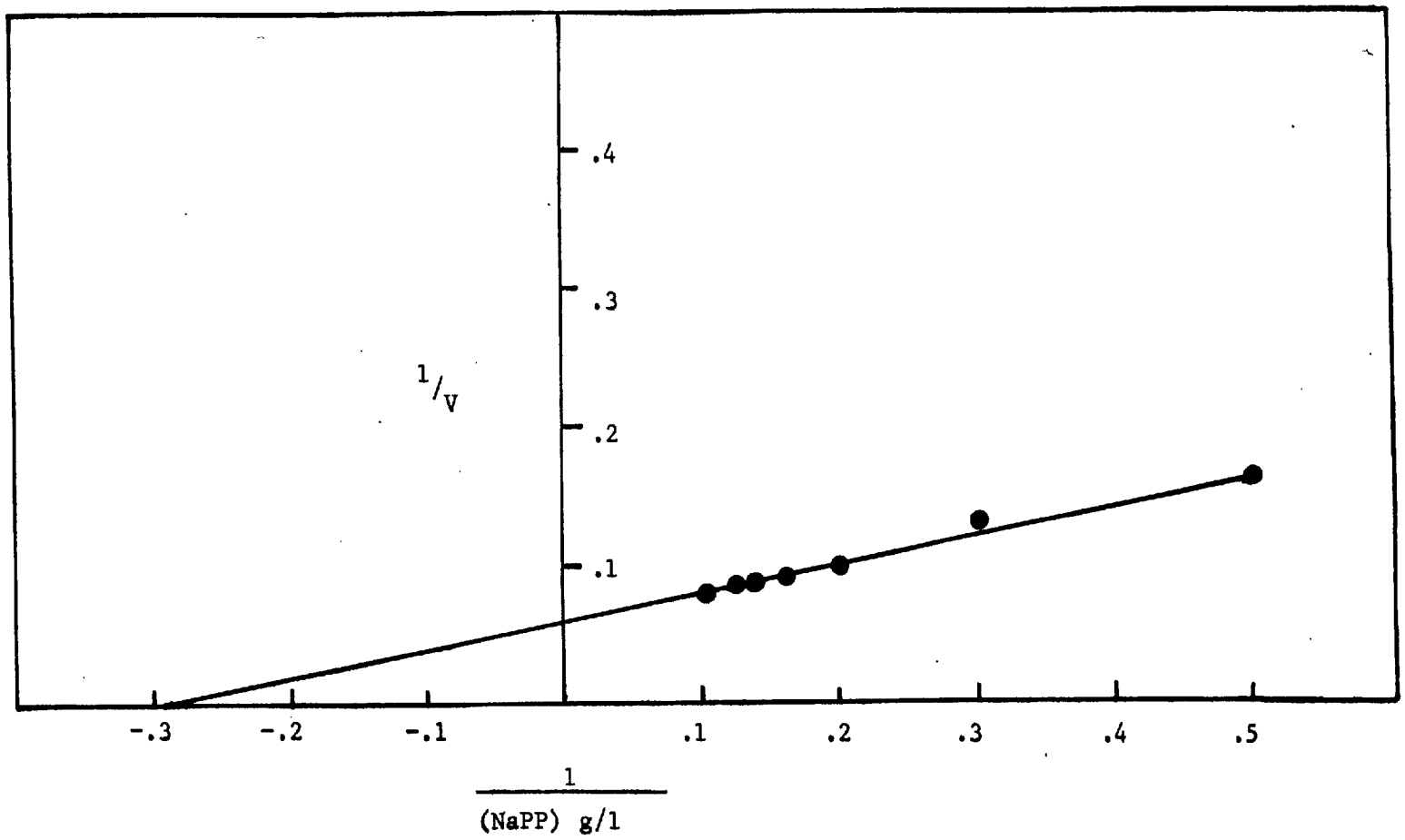


Fig. 17. Determination of K_m of culture filtrate PG3 using sodium polypectate as substrate. Km was 3.38. Activity, V, is expressed as µg equivalent of galacturonic acid released from reaction mixture in 5 min.

substrate interactions support the view that recognition of receptors by enzymes is of a discriminatory nature and hence specific (Burgen et al. 1975). This recognition and binding of enzymes to substrates has been suggested as a basis for specificity in pathogenesis in host/parasite situations (Cervone et al., 1977). The experiment reported here was designed to test the capacity of the isoenzyme PG3 of B. theobromae to bind on to several plant tissues.

Tissues of various plant materials were either sliced or chopped up, then introduced into enzyme solution with activity adjusted to RVU 30 and incubated at 25° for 3h in a water bath. One ml samples were removed at 5 min intervals and activity determined by viscometry. Controls were set up as described in Materials and Methods. The plant materials tested were sweet potato, carrots, onions, bean stem, tomato fruit and potato.

A preliminary experiment using the culture filtrate isoenzymes showed that leakage from the plant materials caused inhibition of enzyme activity, so that loss in activity of enzymes in which tissues were incubated could not be attributed to binding alone. The rot extract PG3 was therefore used for binding tests being the only isoenzyme that is not inhibited during the rotting process.

The results are shown in Fig. 18.

There was a decrease in enzyme activity when all the tissues were incubated with the enzyme. The decrease was however

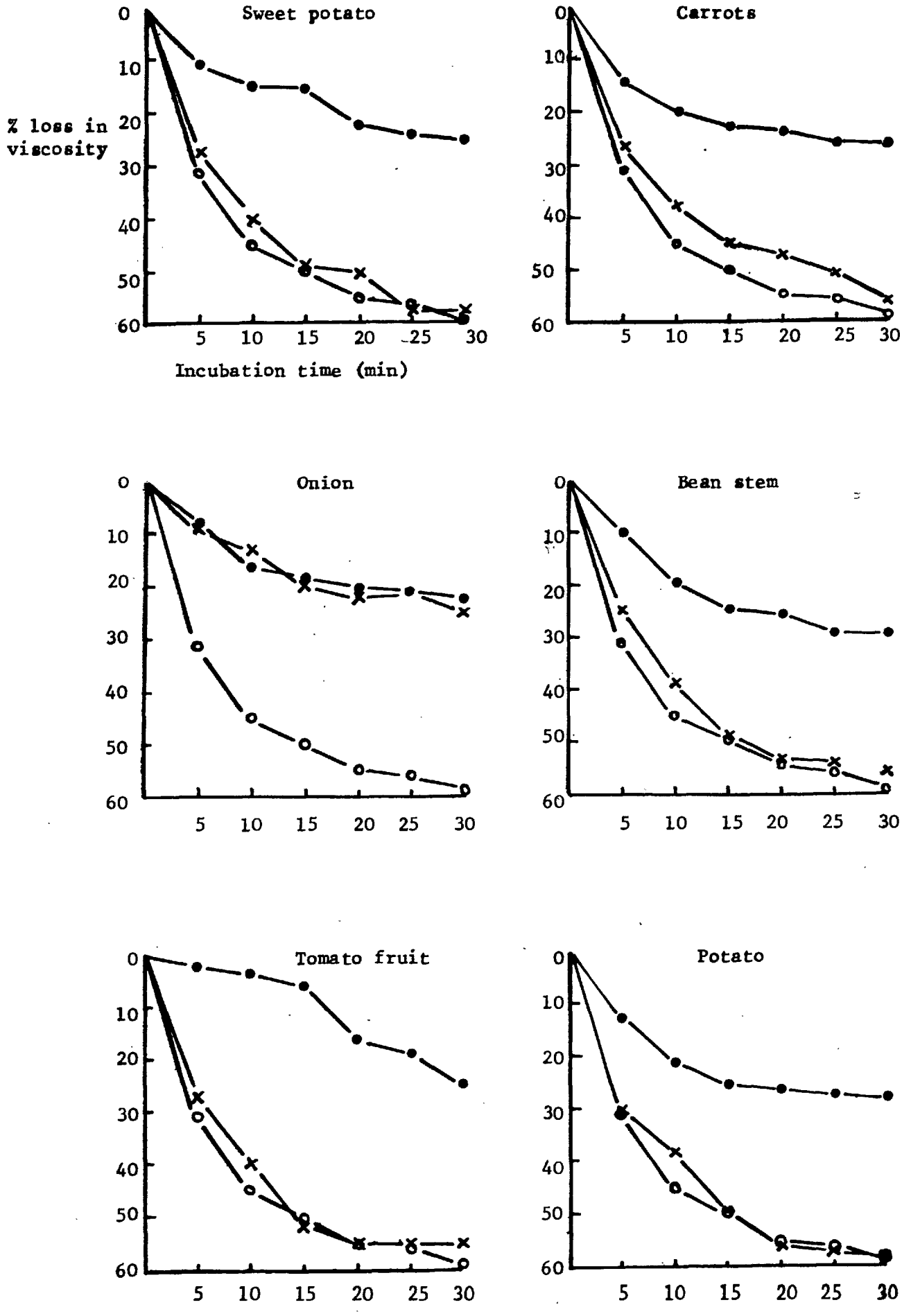


Fig. 18. Effect of incubation of different tissues with enzyme (PG3) on ability of the enzyme to reduce the viscosity of 1% NaPP.
●——● , tissue slices, x——x leakage control,
o——o , Pure enzyme control.

more with sweet potato and tomato fruit tissues. Carrot, bean stem and potato tissues caused more or less the same amount of decrease in enzyme activity. With the exception of onion, leakage control from all the tissues did not inhibit enzyme activity.

It was later found that given suitable conditions (25°C, RH, 80%), spore/mycelial suspension of B.t. infected everyone of the tissues tested. Perhaps this enzyme binding phenomenon can account for the wide host range of B.t.

Evidently, the success of a pathogen on host depends on a variety of complex interacting forces between the host and pathogen. Several factors have been known to limit the activity of cell-wall degrading enzymes in vivo and their study is essential in understanding some aspects of resistance of plant tissues to infection.

V Enzyme inhibition studies

As demonstrated earlier, of the four isoenzymes of B. theobromae, only one, PG3, was detected in rotted sweet potato. Perhaps the other isoenzymes were inhibited or inactivated during infection. Phenols and their oxidation products are known to inhibit PG activity in tissues and their accumulation during infection has been a subject of interest to

the phytopathologist. Different extracts of sweet potato were tested for their inhibitory action on PG and the accumulation of phenols and some oxidases during infection were investigated.

1. Effect of aqueous extracts of sweet potato on activity of purified PG

Earlier result showed that the extraction of rotted sweet potato tissue in the presence of an antioxidant increased PG activity six times at pH 5.0. This suggests that there is some oxidative system which the antioxidant is preventing. We can measure the degree of inhibition of PG by determining PG activity in the presence and absence of the inhibitor.

40g healthy and rotted tissues were extracted in the presence and absence of 10^{-3}M dithiothreitol + PVP + NaCl. The extracts were concentrated under reduced pressure and their effect on purified rot extract PG (RVU 833) determined by viscometry.

The reaction mixture consisted of the following:

4 ml 1% ($^{w}/_{v}$) NaPP in 0.05 M citrate buffer pH 5.0

1 ml enzyme

1 ml aqueous extract.

The extract was first added to the enzyme and stood for 10 min at 25^{o} in a water bath before adding to the substrate. A control was set up with one ml water in place of 1 ml extract.

The results (Fig. 19) showed that extract of rotted tissue in the absence of antioxidant caused 68% inhibition of PG whereas rotted tissue extracted with the antioxidant caused 30% inhibition. Healthy tissue extracted with antioxidant also caused more than twice as much inhibition as that extracted with the antioxidant. It is noteworthy here that there is much greater inhibition by rot extract than by healthy tissue.

The results suggest that there is some role for oxidative enzymes in inhibiting PG activity. Further work on the role of inhibitors were looked at from point of view of well defined zones distinguishable in the lesion caused by B. theobromae.

2. Sweet potato rot zones caused by B. theobromae

Seven days after inoculation of sweet potato with B. theobromae definite zones of rot were distinguishable by their colour and texture (Fig. 20). The central zone (zone 1, the oldest) was a black mass of crumbled tissue while the middle zone (zone 2) was soft and creamy. The outermost zone (zone 3, peripheral) quickly turned dark brown on exposure to air. It did not contain any fungal hyphae and the cells were still living. Zone 4 extended 2 cm beyond zone 3 and was apparently unaffected by the rot.

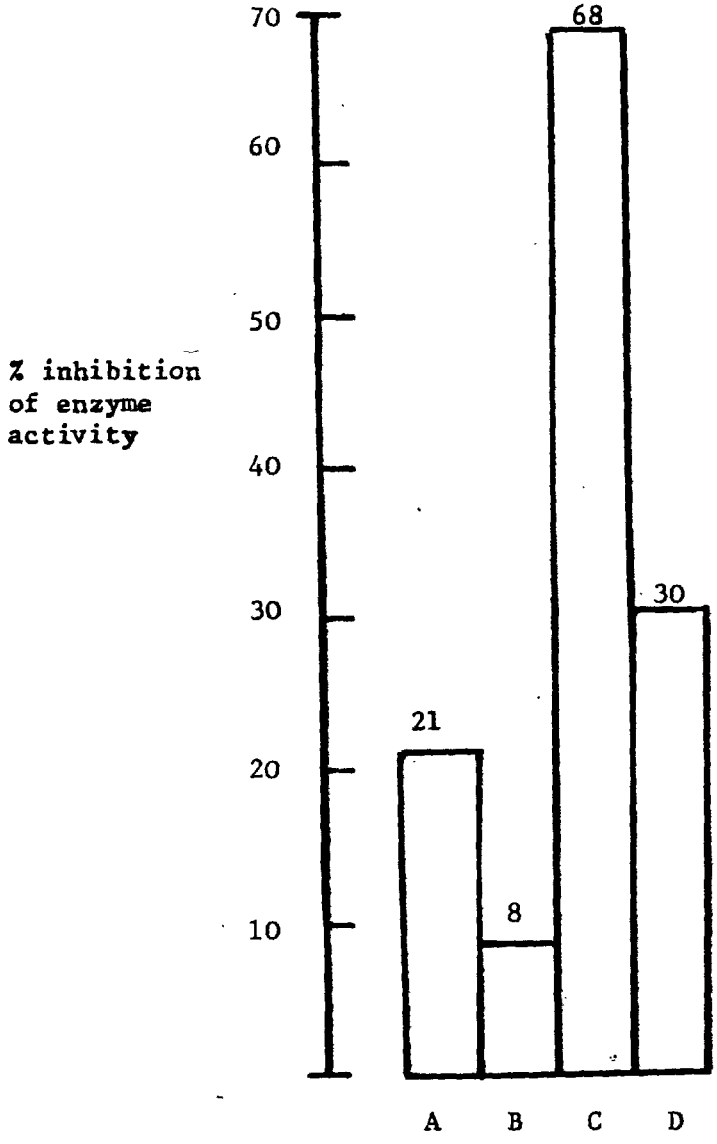


Fig. 19. Enzyme inhibition by aqueous extracts of sweet potato. Enzyme activity determined by viscometry.

A = Healthy tissue extracted with 0.85% NaCl.

B = " " " " antioxidant

C = Rotted " " " 0.85% NaCl.

D = " " " " antioxidant/ml of

Inhibition caused by 5g fresh weight tissue on PG (RVu 833)

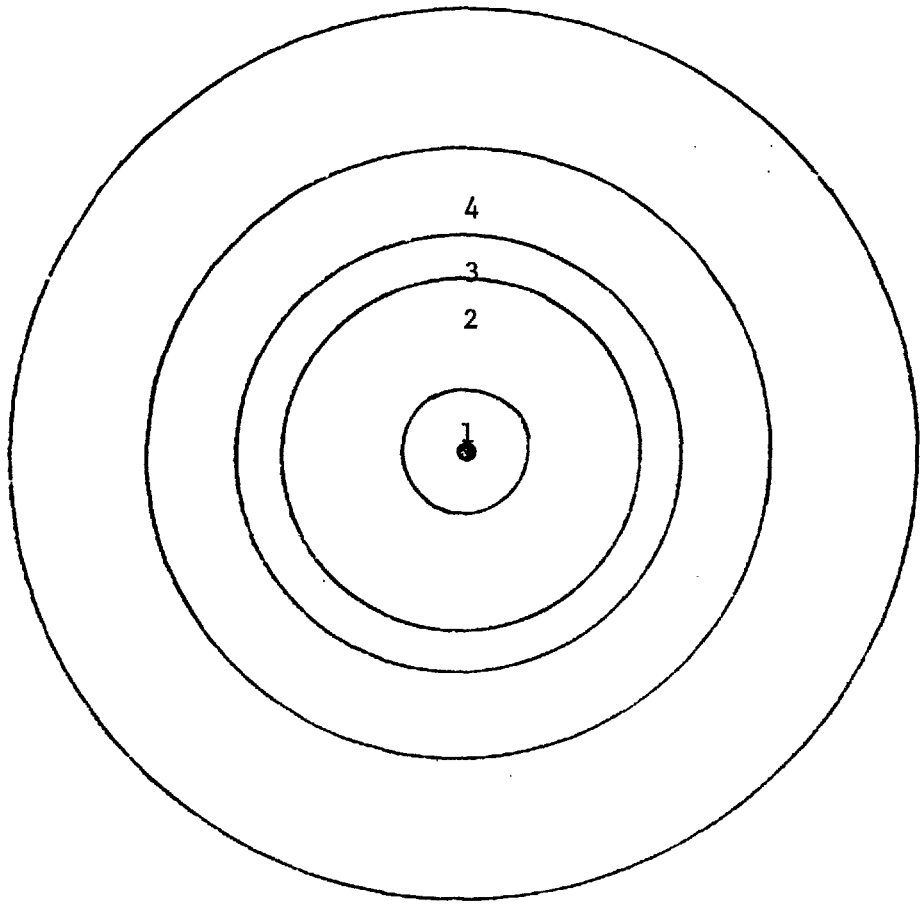


Fig. 20. Diagrammatic representation of zones of rot of sweet potato by B. theobromae, seven days after inoculation.

Zone 1 = 0.5 cm radius, zone 2 = 2cm, zone 3 = 0.5 cm and zone 4 = 2 cm.

(A) Inhibitory activity of rot zones

Each of the four rot zones was separately extracted with 0.85% NaCl and tested for their inhibitory action on PG. 20g of each of the zones was extracted both in the presence and absence of an antioxidant. The extracts were concentrated to 0.5 ml and made up to 4 ml with water. The effect of the extracts on the viscosity reducing ability of rot extract PG was tested. The reaction mixture was as described earlier.

Table 10 summarises the results.

The most inhibitory activity was found in extracts from zone three, the zone causing more than twice as much inhibition as zone 2. The oldest zone, zone 1 appears to have lost most of its inhibitory activity.

The results showed again that tissue extracted with antioxidant had less inhibitory activity on PG than that extracted without antioxidant.

(B) Distribution of PG activity in the rot zones

PG activity was assayed in the different zones and in inoculated control tissue, in terms of the release of reducing groups after 1h incubation of reaction mixture (see Materials and Methods). Table 11 summarises the results obtained.

Table 10. Inhibition of purified rot extract PG by aqueous extracts of rot zones of sweet potato.

Rot zones	% inhibition of PG	
	Extract with antioxidant	Extract without antioxidant
1	6	15.8
2	8.5	20.7
3	21.0	54.6
4	5.8	12.6
Uninoculated control	5.5	12.2

Enzyme activity was determined by viscometry. Results show enzyme inhibition caused by 5g fresh weight tissue.

Table 11. Distribution of PG, phenol oxidase and peroxidase activities and of total phenols in different rot zones caused by B.t.

Rot Zones	PG	Phenol oxidase	Peroxidase	Total phenol
1	16.4[a]	0.0043[b]	71.7[c]	215[d]
2	92.0	0.011	93.5	350
3	21.6	0.65	282.0	402
4	0	0.011	141.0	163
Uninoculated	0	0.002	36.0	126

a. PG activity as µg reducing sugar released per h per g fresh weight of tissue.

b. Phenol oxidase activity as abosrbance change (ΔA) per min per g fresh weight of tissue at 495nm.

c. Peroxidase activity as absorbance change (ΔA) per min per g fresh weight of tissue at 485 nm.

d. Total phenols as µg catechol equivalent per g fresh weight of tissue.

There was five times as much PG activity in zone 2 as in zone 1 and more in zone 3 than in zone 1.

This loss in activity in the oldest zone could possibly be accounted for by the effect of phenolic compounds or their oxidation products.

Experiments were designed therefore to measure the activity of two oxidative enzymes, phenol oxidase and peroxidase in the various rot zones.

(C) Phenol oxidase and peroxidase activities

Changes in the content of these two enzymes in tissues following infection have been reported and have been implicated in disease resistance (Stahmann et al., 1966). Their distribution in the rot zones were assayed by colorimetric methods.

Results (Table 11) showed that both phenol oxidase and peroxidase activities were highest at the periphery of the rot (zone 3). In all cases peroxidase appeared to be the more important. However, it is significant that there was almost 60-fold increase in phenol oxidase from zone 2 to zone 3, but only 4-fold for peroxidase activity. The activity of both enzymes was much increased in zone 4 by comparison with uninoculated tissue. There was relatively little activity in zone 1.

(D) Total phenol content

The total phenol content in the different rot zones was determined in terms of µg catechol equivalent per g fresh weight of rotted tissue.

Results (Table 11) showed a similar but less pronounced pattern of distribution to that for phenol oxidase activity: most of the phenols were concentrated in the outermost zone 3, with somewhat less in zones 1 and 2. There was a slight accumulation of phenols in the apparently healthy part of the tuber (zone 4) by comparison with uninoculated tissue.

Two points emerge from results of the experiments described above: (1) zone 3 appears to be an area of high physiological activity, having a much higher build up of phenoloxidase and peroxidase and phenols than the other zones. (2) Zone 4, which on visual examination was assumed healthy has a higher concentration of phenol oxidase, ~~and~~ peroxidase and total phenol than the uninoculated control. The zone must therefore be regarded as apparently healthy but not in reality, from the physiological point of view.

The study so far indicates that sweet potato in response to infection by B. theobromae makes a remarkable metabolic response especially at the periphery of the lesion. This response has been associated with defence mechanisms of

the host. The storage conditions of sweet potato are bound to influence the ability of the tubers to build up these defence mechanisms and thus resist attack by potential pathogens. Studies on the storage conditions of sweet potato were thus embarked upon.

2. STORAGE PROBLEMS OF SWEET POTATO

I Effect of environmental conditions on rot development

Observation showed that under certain storage conditions, rot either failed to develop in tissues inoculated with *B. theobromae* or was limited to a few mm from point of inoculation. It was found necessary to define more clearly the environmental conditions which induced this resistance to rot. Earlier reports (Arinze, 1974) had shown that at low relative humidities (15-60%), rot by *B.t.* was inhibited. However, at these humidities, the eating quality of the tubers deteriorated owing to high moisture loss and development of large intercellular spaces. To maintain the freshness of the tubers, therefore, high r.h^{s}. (80-90%) were kept constant and temperature/aeration were varied.

20 tubers were inoculated and stored in desiccators (r.h. 80-90%) at each of 13, 15, 20 and 25^{o} for 14 days. Air was mechanically passed continuously from a pump through a second batch of tubers stored under similar conditions.

Results (Table 12) showed that all tubers stored at 20 - 25^{o} without aeration were completely rotted. At this temperature and with aeration provided, 45% of the tubers were rotted. Tubers stored at 13-15^{o}C showed no rotting beyond 1 mm from point of inoculation both with or without movement of air (Plates 1-7).

Table 12. Effect of temperature and aeration on rot development in sweet potato tubers inoculated with B. theobromae.

Temp °C	% tubers rotted	
	Aeration	No Aeration
13	0	0
15	0	0
20	40	100
25	45	100

Tubers were stored at 85-90% r. humidity.
A tuber is taken to be rotted when lesion extends more than 2cm from point of inoculation.

Plate 1. Uninfected sweet potato tuberous roots (X 1)

Plate 2. Sweet potato tuber infected with B. theobromae. Note the eruption of the fungal pycnidia all over the surface of the tuber. (X 1)

Plate 3. X-section through s.p. tuber inoculated with B.t. and stored for 14 days at 25°C, r.h. 85%. Tuber is turned to a black mass of crumbled tissue.

Plate 4. Light microscopy of s.p. tissue infected with B.t. showing fungal hyphae penetrating inter and intracellularly. Starch grains are reduced (X 10,000)

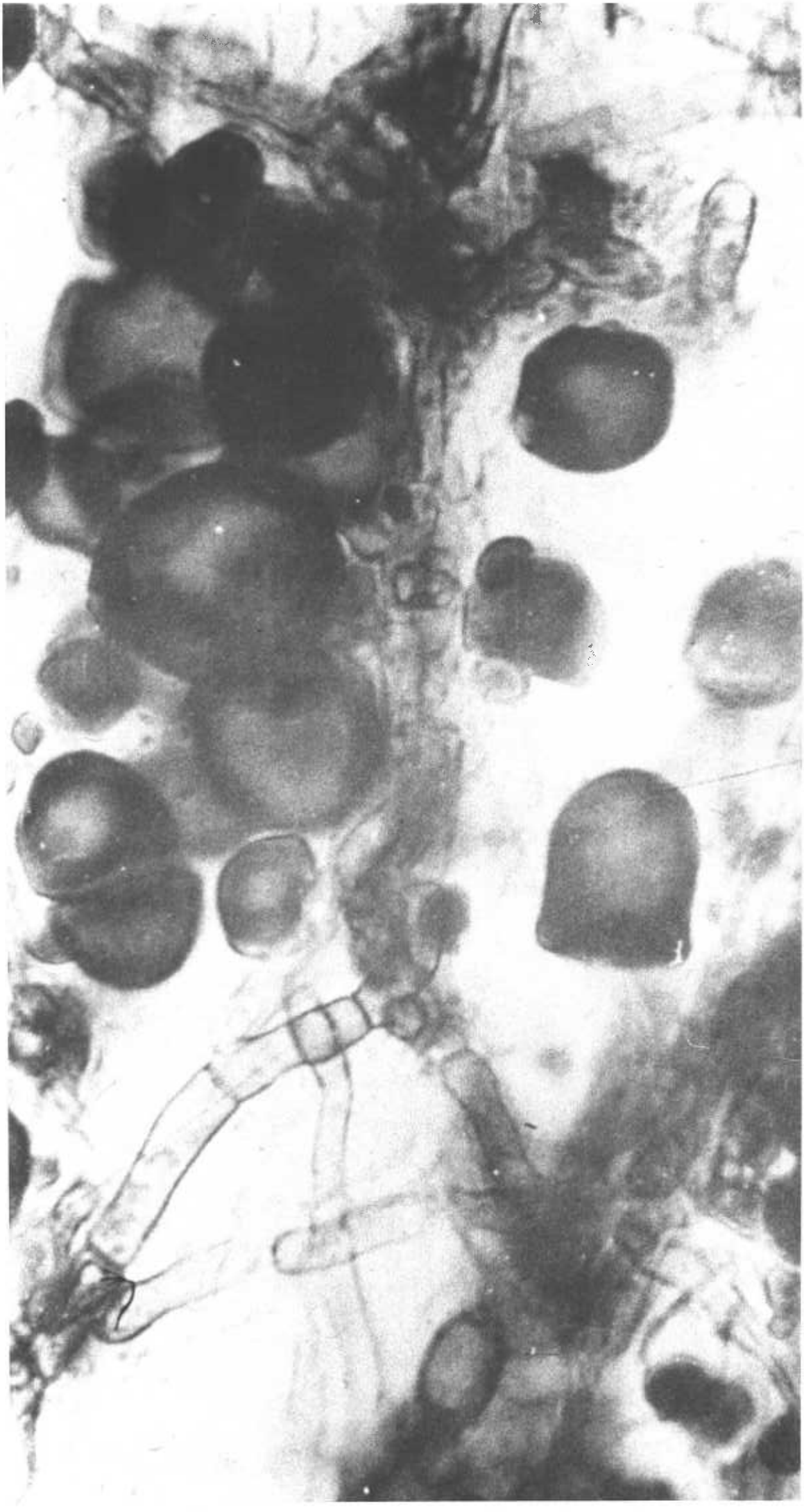


Plate 5. Light microscopy of uninfected tissue showing intact cell walls and compact starch grains (X 1,800)

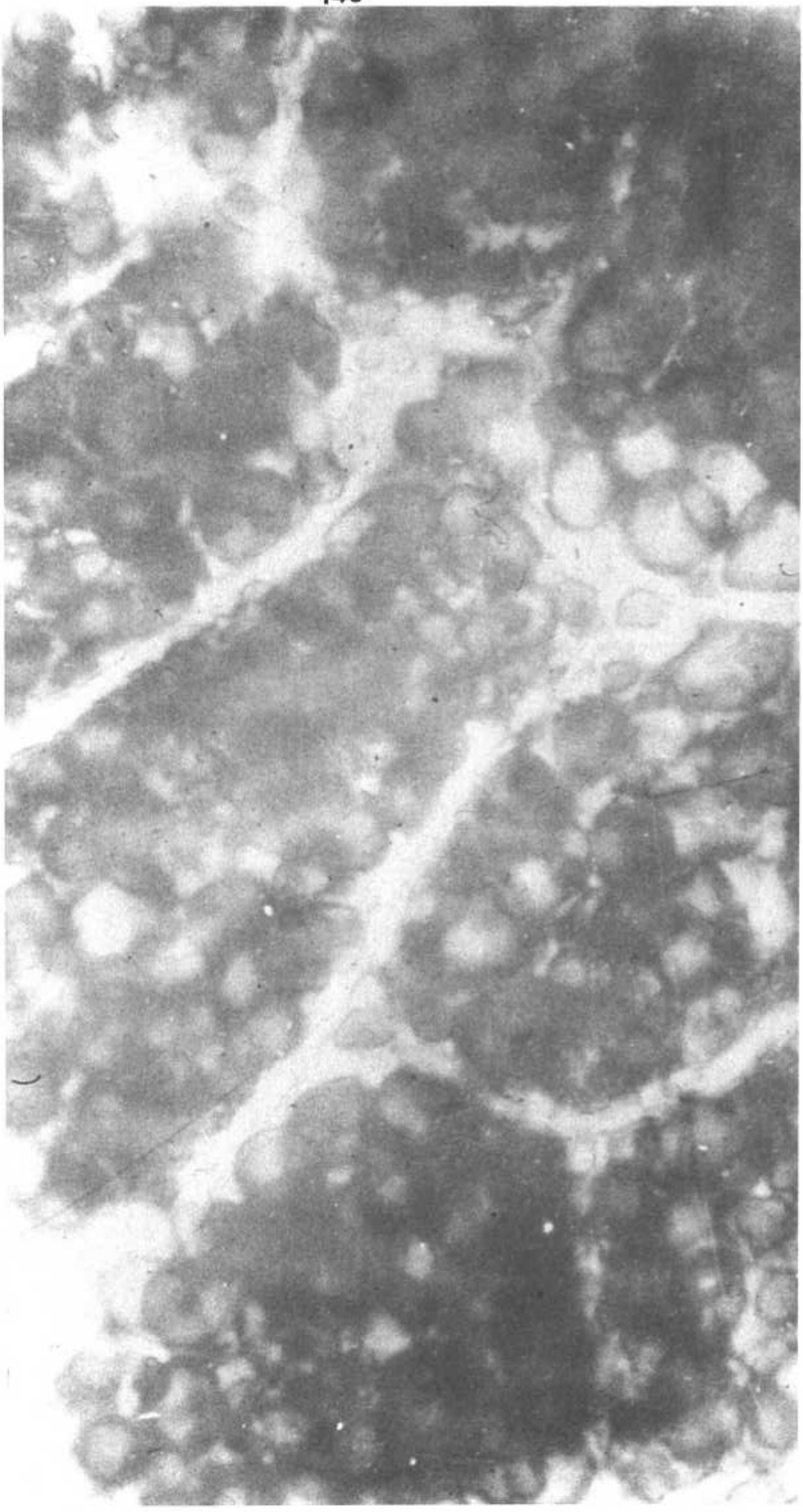


Plate 6. Cross-section of sweet potato tuber inoculated with B.t. and stored in aerated chambers at 13-15°C, r.h. 85% for 14 days.

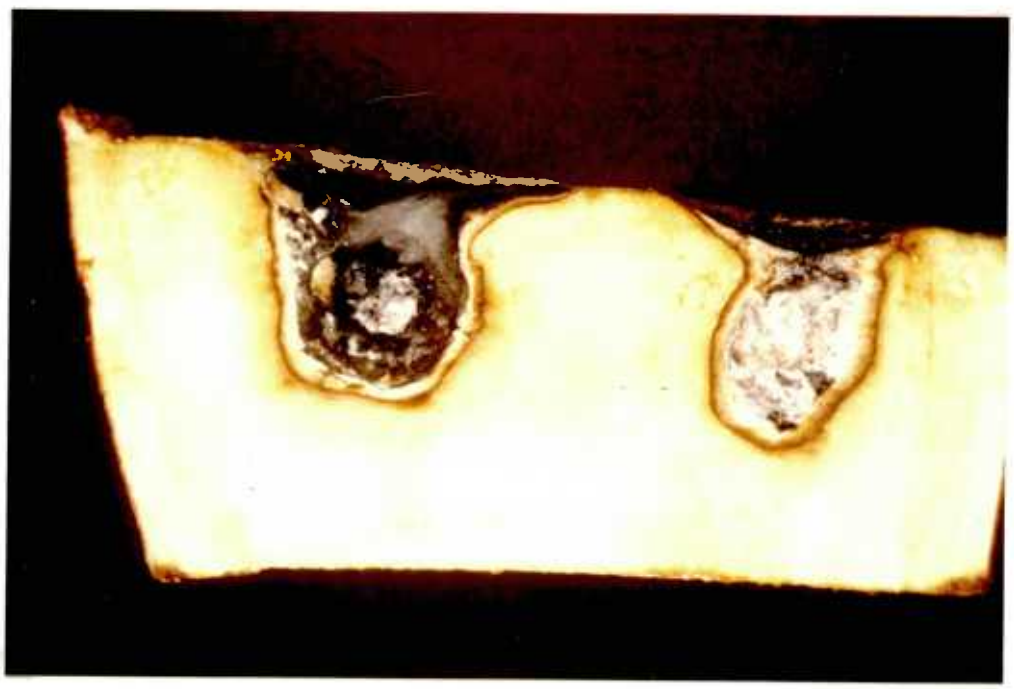


Plate 7. L.S. through same tuber as above showing localisation of fungal hyphae within the infection court.

It is obvious from the results that temperature and aeration are important factors influencing susceptibility of sweet potato to rot by B.t. The fungus had earlier been shown (Arinze, 1974) to grow poorly at temperatures below 15°C in cultures. Coupled with the accumulation of defence mechanisms by the tubers it is not surprising that rot was not even initiated by the pathogen at this temperature.

II Inoculation with other fungi

For comparative purpose, sweet potato tubers were similarly inoculated with spore/mycelial suspensions of B. cinerea (B.c.) and C. cucumerinum (C.c.) and stored at similar conditions as used for B.t. with and without aeration.

Results (Plates 8 and 9 and Fig. 21) showed that the tuber was resistant to further spread by B.c. beyond 0.5 cm from point of inoculation and did not permit C.c. to grow at all. It is interesting that even under conditions which favoured rapid rotting of the tubers by B.t. rot failed to occur with B.c. and C.c. Therefore a situation of pathogen (B.t.), weak pathogen (B.c.) and non-pathogen (C.c.) exists with reference to the sweet potato tubers.

Perhaps a reexamination of the concentration and distribution of PG, total phenols, phenol oxidases and peroxidase in tissues inoculated with these fungi would explain why the tissue is resistant to B.c. and C.c. and not to B.t.

Plate 8. Section through s.p. tissue inoculated with B.c. and stored for 14 days. Rot was initiated but remained localised.

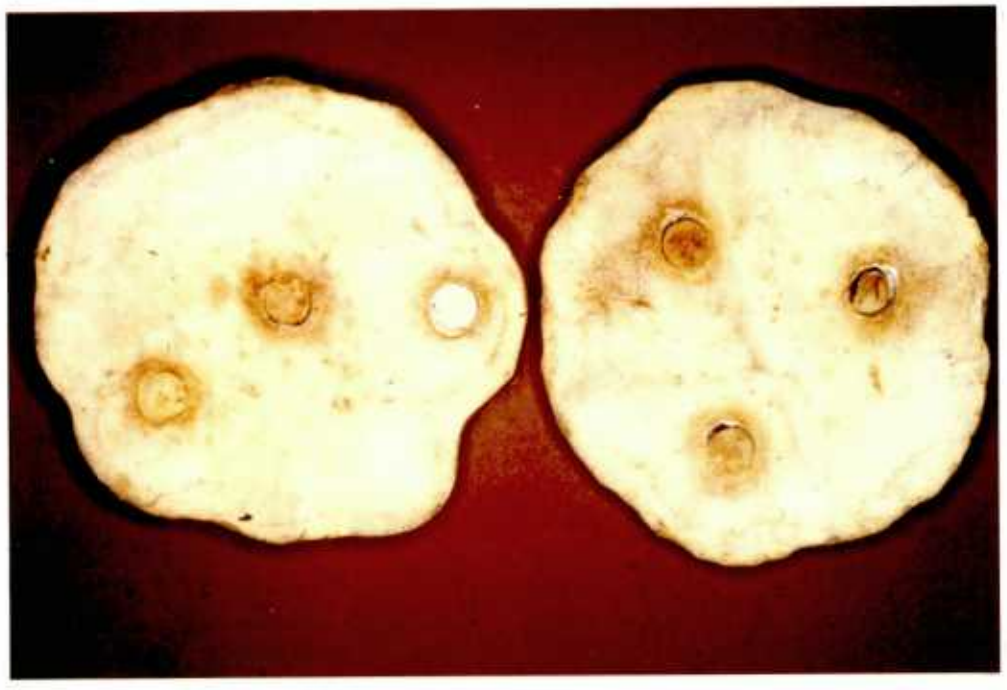
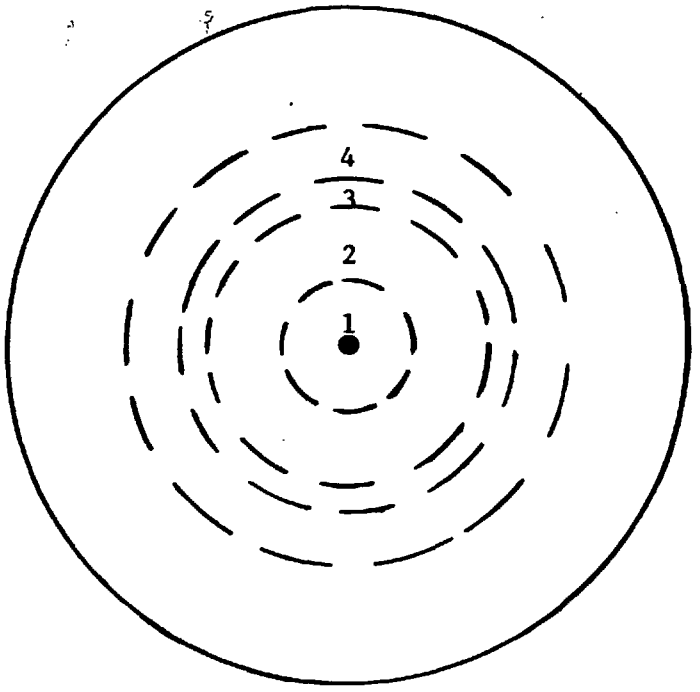
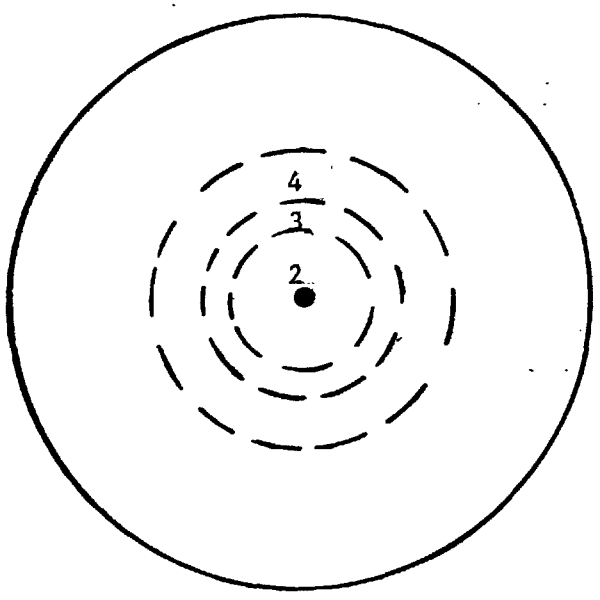


Plate 9. Section through s.p. tissue inoculated with C.c. and stored for 14 days. Tissue shows browning around the point of inoculation.



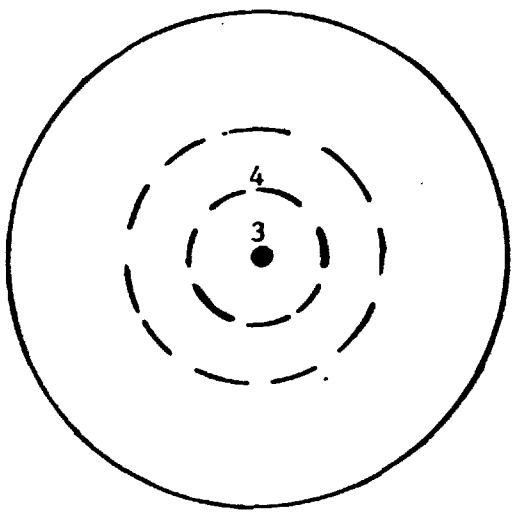
(A)

Zone 1 = 0.5 cm
2 = 2.0 cm
3 = 0.5 cm
4 = 2.0 cm



(B)

Zone 2 = 0.5 cm
3 = 0.5 cm
4 = 2.0 cm



(C)

Zone 3 = 0.5 cm
4 = 2.0 cm

Fig. 21. Diagrammatic representation of zones of rot of s.p. by (A) B. theobromae, (B) B. cinerea and (C) C. cucumerinum

(A) B. theobromae has been included here for comparative purpose.

In addition to B.c. and C.c. cultures of Fusarium solani, var caeruleum (F.s.) and Phoma exigua (P.e.) were obtained and separately used to inoculate the tubers stored at r.h. 85% at 25°C. PG content, total phenols, phenol-oxidase and peroxidases were determined in the various zones of rot which corresponded with the zones described for B.t. infected tissues.

The results (Tables 13, 14, 15 and 16) of the distribution of the variables followed similar pattern as for B.t. -infected tissue. For all the fungi, the total phenol and the oxidases were highest in zone 3 followed by zones 2 and 1 and least in zone 4. PG activity was detected in zone 2 for B.c., F.s. and P.e. infected tissues but not in zone 3. No PG activity was detected in tissues infected with C.c. There was most production of total phenols in extracts rotted least by the fungi - C.c., B.c., and F. solani.

There was not much difference in phenol oxidase and peroxidase activities of the different zones of rot caused by B.t. and those caused by the other fungi.

It appears that more phenols are produced in the most resistant situation but there is no comparative increase in the oxidases. Perhaps the maximum level of the oxidases had been reached and of more importance is the sensitivity of the fungi to their action. Of significance also is the level of PG activity recovered in tissues inoculated with these fungi. All

Table 13. Distribution of PG, phenol oxidase and peroxidase activities and of total phenols in different rot zones caused by B.c.

Rot Zones	PG	Phenol oxidase	Peroxidase	Total phenols
1*				
2	25.6	0.014	84.0	650.0
3	0	0.60	260.4	900.0
4	0	0.012	136.5	174.0
Uninoculated		0.002	36.0	126.0

*Zone 1 is non-existent for B.c. inoculated tissues. PG, phenol oxidase, peroxidase and phenols were determined as recorded in Table 11.

Table 14. Distribution of PG, phenol oxidase and peroxidase and of total phenols in different rot zones caused by C.c.

Rot Zones	PG	Phenol oxidase	Peroxidase	Total phenols
1*				
2*				
3	0	0.66	284	263
4	0	0.014	144	170
Uninoculated	0	0.002	36	126

*Zones 1 and 2 are non-existent in tissues inoculated with C.c.

PG, phenol oxidase, peroxidase and phenols were determined as recorded in Table 11.

Table 15. Distribution of PG, phenol oxidase and peroxidase activities and of total phenols in different rot zones caused by Phoma exigua.

Rot Zones	PG	Phenol oxidase	Peroxidase	Total phenols
1*				
2	15.2	0.11	85	380
3	0	0.71	291	544
4	0	0.012	138	168
Uninoculated	0	0.002	36	126

*Zone 1 is non-existent in tissues inoculated with P. exigua.

PG, phenol oxidase, peroxidase and phenols were determinsed as recorded in Table 11.

Table 16. Distribution of PG, phenol oxidase and peroxidase activities and of total phenols in different rot zones caused by Fusarium solani f. cæruleum.

Rot zones	PG	Phenol oxidase	Peroxidase	Total phenol
1*				
2	21.3	0.13	91	583
3	0	0.68	294	842
4	0	0.015	130	210
Uninoculated	0	0.002	36	126

*Zone 1 is non-existent in tissues inoculated with F. solani f. cæruleum.

PG, phenol oxidase, peroxidase and phenols were determined as recorded in Table 11.

the fungi are known to produce PG in culture. Their levels however in the tissue is very low compared with the levels obtained for B.t. infected tissue (Table 11). The low level of PG activity in the tissue might be accounted for by their inactivation by host's metabolites. Furthermore the inability of C.c. to rot sweet potato might be correlated with the poor production (or none at all) of pectic enzymes in the tissue.

III Chilling injury studies

One of the most intriguing aspects of sweet potato is the response of the tubers at low temperatures which most other tubers would tolerate. Resistance of the tubers to rot easily break down after pretreatment at temperatures below 10°C - 'chilling' injury temperatures.

Earlier reports (Yamaki and Uritani, 1972) stated that chilling of sweet potato rendered it more susceptible to rot by soft rot organisms. Experiments were designed here to investigate the effects of pretreatment temperatures on the tubers.

1. Effect of pretreatment temperatures on deterioration of tubers

Healthy tubers were surface sterilised with ethanol and kept (5 tubers for each temperature) at the following temperatures,

-22^{o} (deep freeze), 0^{o}, 5^{o}, 10^{o}, 20^{o} and 25^{o} for 48h in a desiccator 85% R.H. They were then transferred to 25^{o}C for storage for 14 days in sterile conditions at R.H. 85%. 2 cm thick slices cut out from the tubers were also incubated at same temperature in sterile petri dishes. At two day intervals both tubers and slices were assessed visually and by means of two sterile seekers for deterioration.

Results, Table 17, showed that the tubers and slices pretreated at -22^{o} were completely broken down within 24h of storage at 25^{o}. Slices stored at 0^{o} were completely broken down on the 4th day of storage. Tubers stored at 0^{o} progressively deteriorated up to the 10th day. Tubers and slices pretreated at 5^{o}, 10^{o}, 20^{o} and 25^{o} showed no sign of deterioration.

2. Effect of pretreatment temperatures on susceptibility to infection at B.t., B.c., and C.c.

The tubers were pretreated at 0, 3, 5, 7, 9, 10, 20 and 25^{o}, inoculated with the fungi and stored at 25^{o}, R .H. 80%. Rotting of the tissues was assessed at daily intervals for 2 weeks.

The result is presented in Table 18.

Tubers pretreated at 0^{o}, 3^{o} were completely rotted by the 4th day of inoculation with both B.t. and B.c. It was found that treatment of tubers at these temperatures

Table 17. Effect of pretreatment temperature on deterioration of tubers.

Pretreatment temp. (48h)	Storage period (days)						
	2	4	6	8	10	12	14
-22°	5(5)						
0	0(4)*	0(5)	1	2	4	5	5
5	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0
25	0	0	0	0	0	0	0

A degradation index 0-5 was used to measure tissue breakdown. 0 - tissue intact, no difference from control, 5 - complete breakdown of tissue shown by softness and emission of tissue juice when squeezed by hand.

* Figures in parenthesis represent index for tissue slices.

Table 18. Effect of pretreatment temperatures on susceptibility of tubers to infection by B.t., B.c. and C.c.

Pretreatment temps °C (48h)	B.t.	B.c.	C.c.
0	+ *	*	*
3	*	*	1.8[a]
5	*	*	2.5
7	*	2.4	0
9	5.3	2.0	0
10	7.1	0.5	0
20	7.4	0.5	0
25	7.0	0.5	0

a. Units represent distance (cm) of tissue rotted after 4 days inoculation.

*+ represents complete breakdown of tissue.

enabled C.c. to cause some rot although rotting was more in tubers inoculated with B.t. and B.c. By the 4th day of inoculation tubers pretreated at 3° and 5° were rotted up to 2 cm by B.c. from inoculation point while the fungus did not cause rot in tubers pretreated at 10, 20, 25°.

The result shows that low temperature treatment of sweet potato breaks down its resistance to rot, even to the usual non-pathogen, C.c.

3. Effect of low temperature treatment of tissues on maceration and cell death by purified rot extract enzyme (PG3)

Slices of sweet potato and potato were incubated at 0, 3, 5, 7, 9 and 10° for 48h and discs (0.5 x 0.05 cm) cut out from them using a cork borer. The discs were incubated for 1h (6 discs/ml) in a reaction mixture consisting of

2 ml rot extract PG3 (RVU 60)

1 ml 0.05M citrate buffer pH 5.0

1 ml H_2O

The reaction mixture containing the discs was incubated at 10, 20, 25 and 30°C in a water bath and 5 discs sampled and tested for maceration and cell death.

Results are shown in Tables 19 and 20.

Table 19. Effect of low tempterature treatment of sweet potato slices on maceration and cell death by purified rot extract enzyme (PG3).

Pretreatment temps °C (48h)	Maceration Index[a] after 1h			
	10	20	25	30°C
0	3	5		
3	3	5	5	5
5	1	3	3	3
7	1	2	2	2
9	0	2	2	1
10	0	2	2	1
25	0	2	1	1
	% cell death [b] after 1h			
0	67	98	98	98
3	60	98	97	97
5	7	77	80	82
7	13	38	45	40
9	12	35	30	35
10	4	36	35	36
25	4	30	31	37

Effect of enzyme was tested at RVu 60

(a) Maceration index from 0 (no maceration) to 5 (complete maceration)

(b) % cell death, calculated from optical density readings of neutral red extracted with methanol.

Table 20. Effect of low temperature treatment of potato tissues on maceration and cell death by purified rot extract enzyme (PG3)

Pretreatment temps °C (48h)	Maceration Index (a) after 1h			
	10	20	25	30°C
0	0	1	1	2
3	0	2	2	1
5	0	2	1	2
7	0	1	2	2
9	0	1	2	1
10	0	1	1	2
25	0	1	2	2
	% cell death (b) after 1h			
0		36	30	37
3		35	35	35
5		37	37	31
7		32	36	36
9		30	37	41
10		30	38	40
25		34	32	38

Effect of enzyme was tested at RVu 60.

(a) Maceration Index from 0 (no maceration) to 5 (complete maceration)

(b) % cell death calculated from optical density readings of neutral red extracted with methanol.

Table 21. Effect of pretreatment temperature on maceration and death of cells of sweet potato.

Pretreatment temp °C (48h)	Maceration Index [a] after storage	
	24h	48h
0	4	5
3	1	2
5	0	0
7	0	0
9	0	0
10	0	0
	% cell death [b] after storage	
0	97.4	98.6
3	0	0
5	0	0
7	0	0
9	0	0
10	0	0

(a) Maceration Index from 0 (no maceration) to 5 (complete maceration).

(b) Percent cell death, calculated from optical density readings of neutral red extracted with methanol.

There was greater maceration of sweet potato slices pretreated at 0 - 5° than those at 7 - 25°. In contrast potato slices did not show these differences. Temperatures of 0 - 5°C did not affect the maceration action of potato by the enzyme. Maceration tempearature of 10° was not conducive for maceration action of the enzyme on either potato or sweet potato.

Cell death was higher in sweet potato slices pretreated at 0 - 5° than at 7, 9, 10, 25°. Again there was no difference between potato slices stored at all the temperatures tested.

There is evidence from the results that treatment of sweet potato at 0 - 5° predispose the tissues to easy maceration and cell killing by the pectic enzyme of B. theobromae. These temperatures do not however appear to affect the response of potato tissues to the enzyme.

3. PHYTOALEXIN STUDIES

As indicated earlier, there have been reports of the accumulation of antifungal compounds (terpenes) in sweet potato infected by Ceratocystis fimbriata. These compounds limit the spread of rot in the infected tubers. Under similar conditions s.p. tubers are also resistant to infection by B. cinerea and C. cucumerinum but highly susceptible to attack by B. theobromae (Plates 3, 8, 9). The formation of antifungal compounds by s.p. in response to attack by these three pathogens and the relation between the pathogens and the compounds is the subject of this section.

I Extraction of terpenoids of infected sweet potato

1. TLC on silica gel

Lesions formed from tissues inoculated with B.t., B.c. and C.c. were taken up in chloroform-methanol and the terpenes extracted from this (See Materials and Methods). The extracts were finally taken up in 95% ethanol and run on TLC using several solvents (on preliminary) and finally using 3% chloroform in methanol. The compounds separated were firstly visualised under uv 254nm and then sprayed with Ehrlich's reagent. The bands located are shown in Fig. 22 (Plate 10).

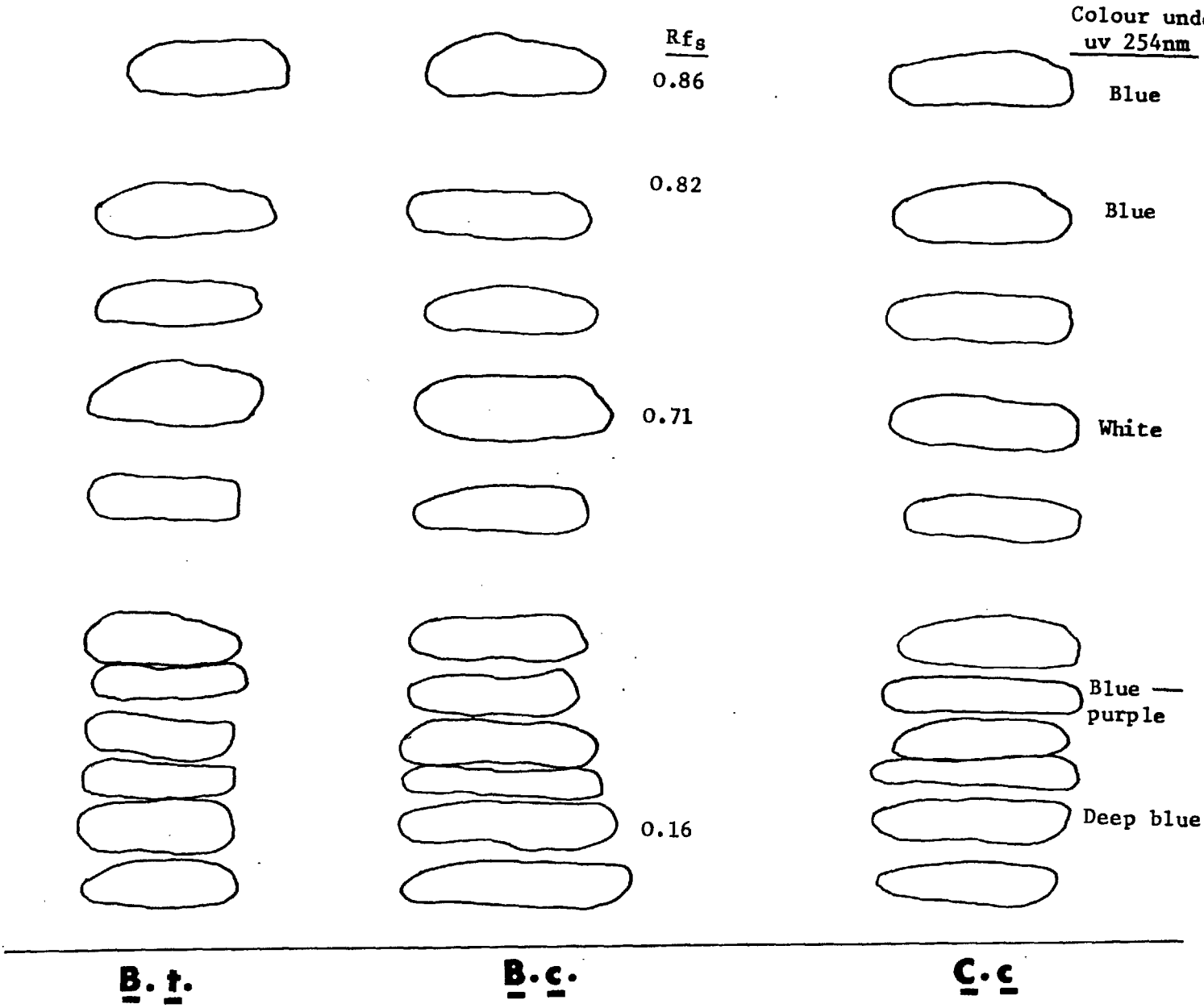


Fig. 22. TLC of terpenes extracted from s.p. infected with B.t., B.c., and C.c. Chromatogram developed in chloroform - methanol (97:3), and compounds located with Ehrlich's reagent. 1g fresh weight tissue of extract deposited on plate.

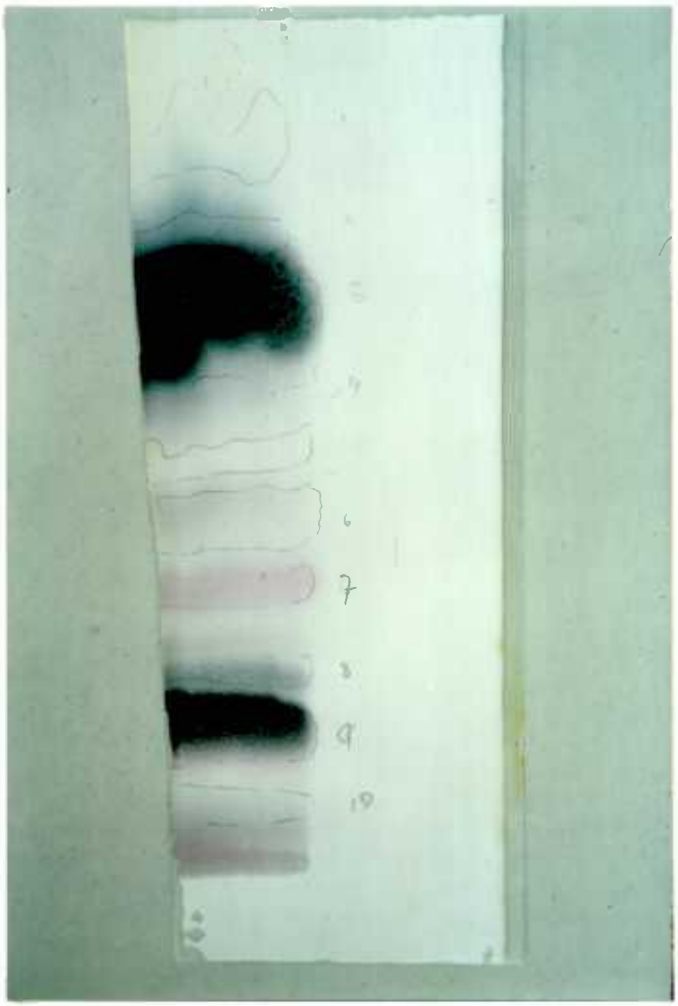


Plate 10. TLC of terpenes extracted from s.p. infected with B.t. Chromatogram developed in 3% methanol in chloroform and compounds located with Ehrlich's reagent.

The sprayed chromatogram showed that there was a total of eleven Ehrlich positive compounds present in each of the extracts. Essentially the same compounds were present in each of the three different extracts. Five of the compounds showed fluorescence under uv.

2. Isolation of s.p. phytoalexins

(A) Bioassay of phytoalexins against C.c.

The compounds separated on TLC were assayed against C.c. spores. The fungus is routinely used to assay phytoalexins on chromatograms. A dense conidial suspension of C.c. in SCA was sprayed onto the surface of a TLC plate developed in n-hexane-ethylacetate (3:2). The sprayed plate was incubated in a moist plastic box for 4 days and examined for location of antifungal compounds.

Plate 11 and Figure 23 show the results.

Of the eleven Ehrlich positive compounds separated on TLC, only four of them with r.f. values of 0.16, 0.38, 0.71 and 0.82 (using n-hexane-ethylacetate for development) were antifungal. In all cases, all the four compounds were Ehrlich positive. In these assays all the zones of rot were combined (zones 1, 2, 3, 4 for B.t., zones 2, 3, 4 for B.c. and zones 3 and 4 for C.c.). It was noted that despite the differences in the number of zones, essentially the same number of

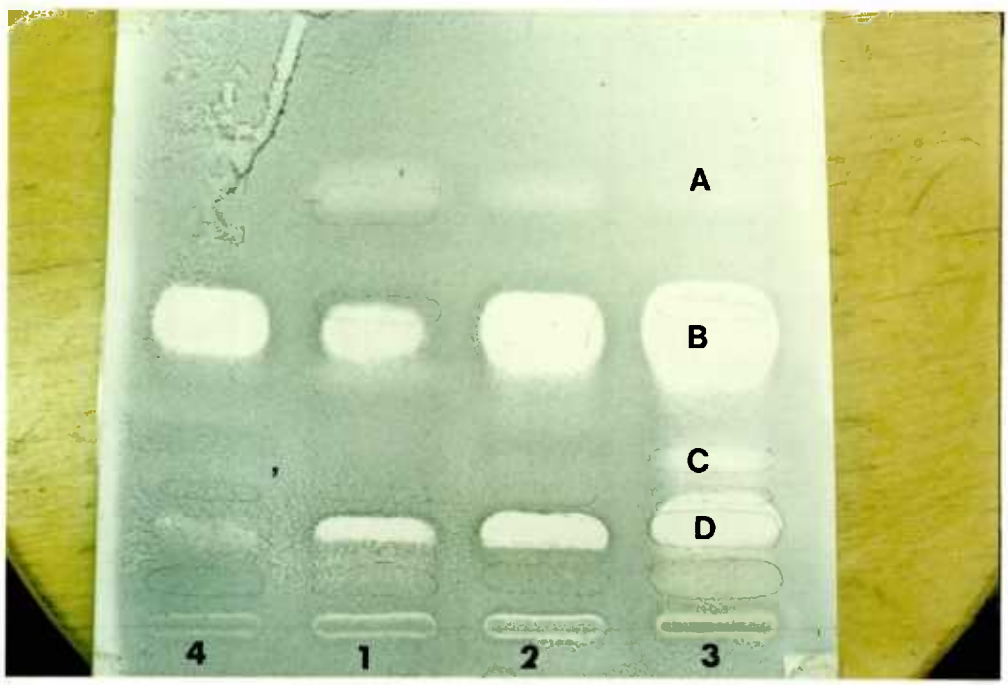


Plate 11. Cladosporium cucumerinum bioassay of chromatogram of extract of sweet potato tissue infected with B.t. The antifungal compounds A, B, C, and D were located from zone 3 of infected tissue. Compound C was not detected in zones 1 and 2. In this plate C.c. was sprayed twice.

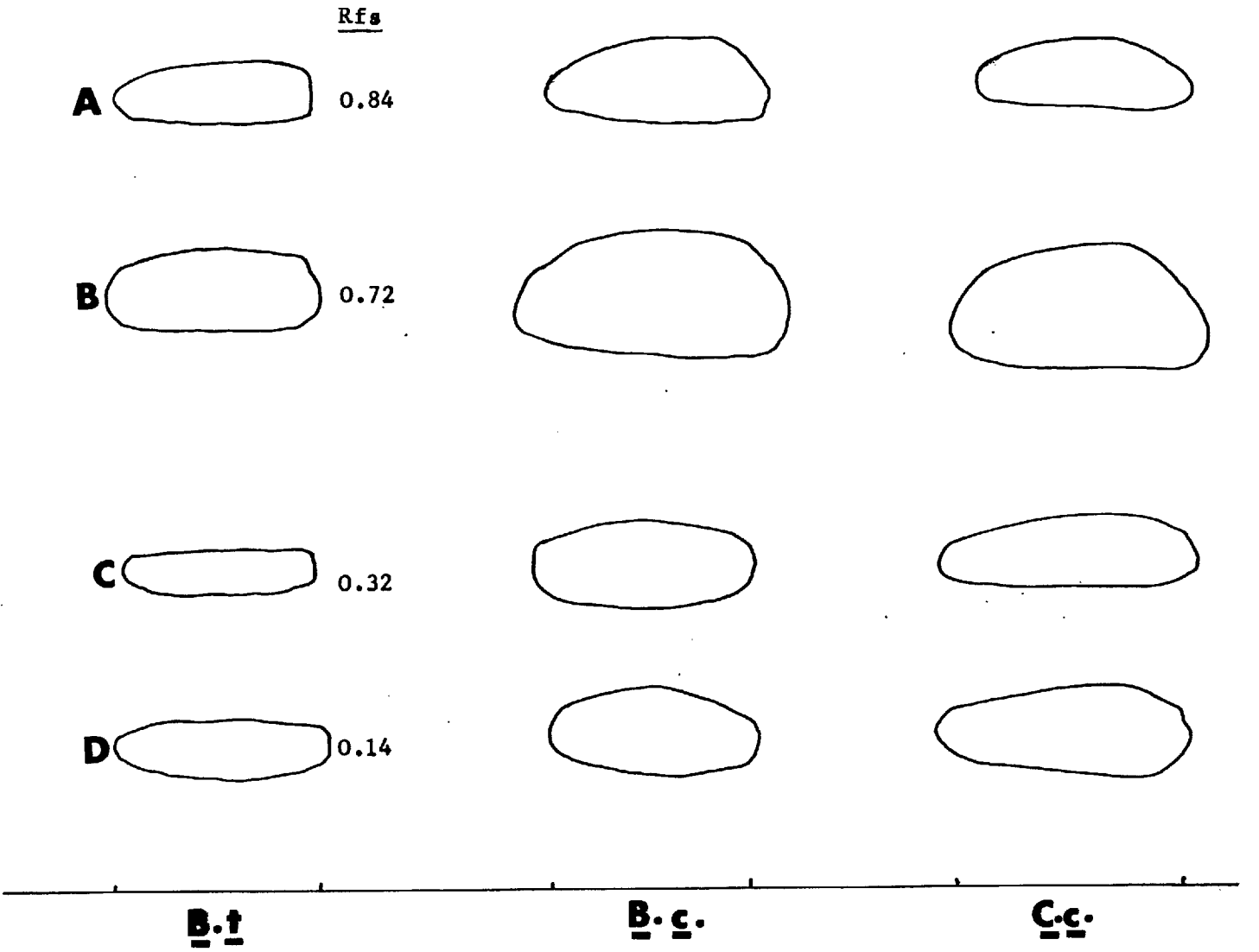


Fig. 23. Diagrammatic representation of bioassay of the terpenes (extracted from sweet potato infected with B.t., B.c. and C.c.) against C. cucumerinum spores. The compounds A, B (ipomeamarone) C, and D (ipomeamaronol) were detected as phytoalexins. 1g fresh weight of terpene extract was deposited on chromatogram.

antifungal compounds were induced by the three fungi.

(B) Identification of phytoalexins

The four bands corresponding to the inhibitory zones (as shown by the C.c. bioassay) were scraped from similarly developed T.L.C. plates and labelled compounds A, B, C and D. All the compounds were colourless and odourless. In their crude form, none of them showed a characteristic peak of absorption when scanned in a spectrophotometer. The compounds were then re-run in several solvent systems and purified further by TLC. The plate was sprayed with various visualising reagents and their colour reactions recorded. Table 22 summarises the reactions of the compounds.

Compound B was by far the largest spot on the chromatograpm with r.f. 0.72 (n-hexane-ethylacetate solvent 3:2). The uv spectrum showed an absorption maximum at 211 nm and the compound had a characteristic bitter taste. These were reported earlier by Akazawa, Uritani and Kubota (1960) and the colour shown by the Ehrlich, Lieberman and Carr-Price reactions obtained here closely matched the characteristic colour reactions of "Ipomeamarone" published by Akazawa (1960). Compound B was thus identified as Ipomeamarone. Compound D in its pure form did not show a characteristic uv absorption spectrum, was colourless and odourless. Unlike ipomeamarone, it did not

Table 22. Rf values of the antifungal compounds in varius solvents and their colour reaction to various reagents.

Substances	Solvents			Colour reaction with various reagents						uv 254nm
	I	II	III	a	b	c	d	e	f	
A	.82	.84	.66	Light brown	Yellowish brown	Light brown	Orange	Light Orange	Pink—— bluish red	Blue
B	.71	.72	.45	Dark brown	Dark brown	Orange	Light yellow	Light pink —— yellowish brown	Purplish red	White
C	.38	.32	.25	Brown	Brown	Light orange	Bright yellow	Light pink	Pink—— pinkish red	Blue
D	.16	.14	.17	Reddish brown	Reddish brown	Brownish red	Yellow—— purplish red	Bright yellow —— purplish red	Bright pinkish red	Deep blue

I = chloroform-methanol (97:3); II = n-hexane-ethylacetate (3:2); III = hexane-ether (1:1).

a = 5% ethanolic solution of 95% H_2SO_4.

b = 5% solution of 95% H_2SO_4 in acetic anhydride

c = Carr-Price reagent

d = Dinitrophenyl hydrazine reagent A

e = Dinitrophenyl hyrazine reagent B

f = Ehrlich's reagent

possess a bitter taste. The r.f. value published by Kato et al. (1973) was 0.16 using silica gel and ethylacetate-n-hexane as solvent. The same value was obtained here with similar solvent. The compound was shown to be 14-hydroxy-ipomeamarone and designated "ipomeamaronol" Kato et al., (1971).

Compounds A and C are unnamed.

(C) Qualitative determination of the compounds A, B, C, D, in different rot zones caused by B.t., B.c., and C.c.

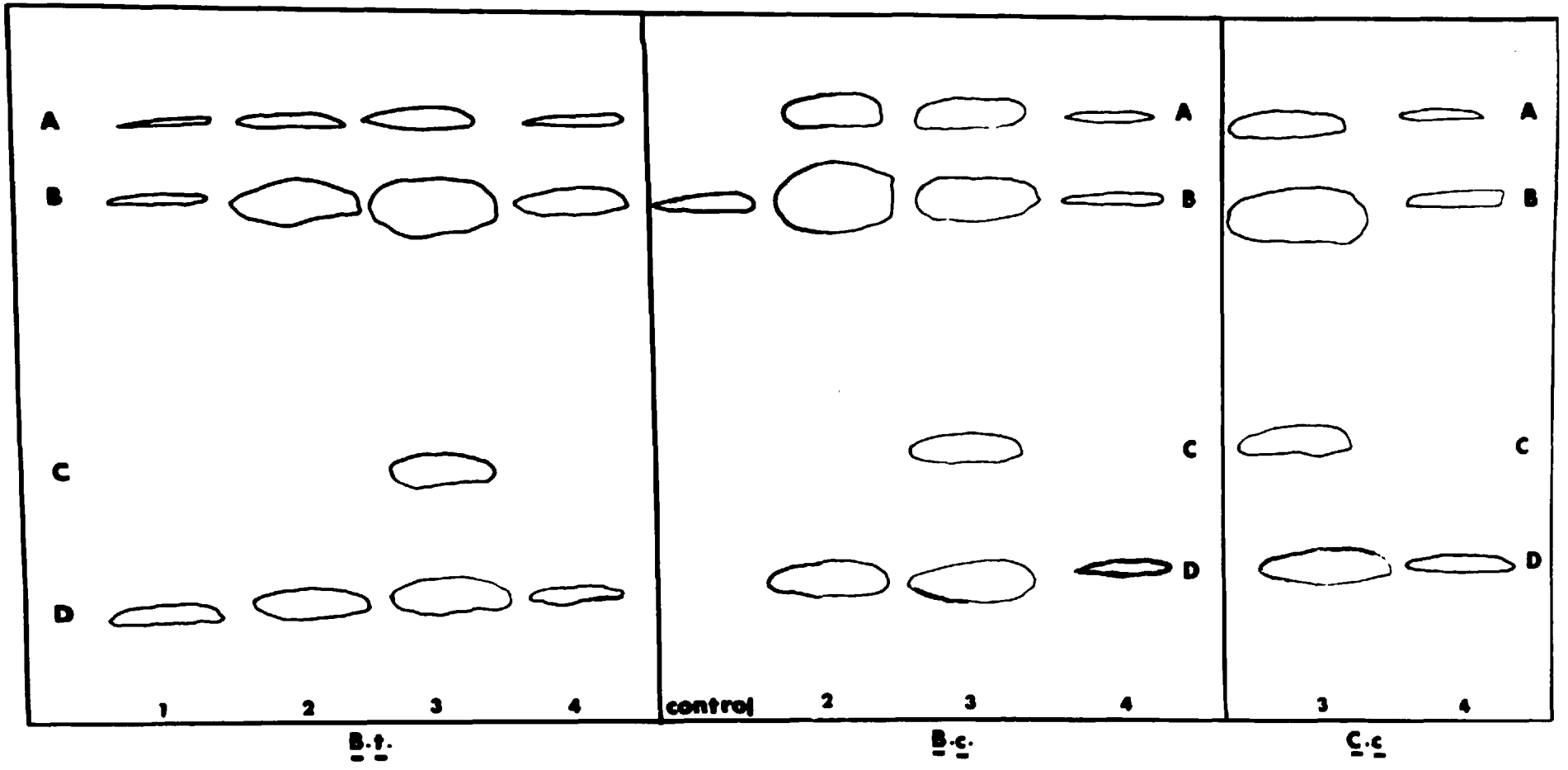
The various rot zones were extracted in chloroform: methanol (1:1) taken up in ethanol and bioassayed against C.c. spores as above. Bands corresponding to the inhibitory zones were eluted from similarly developed TLC plates into 95% ethanol and re-developed in n-hexane-ethylacetate (3:2) and sprayed with Ehrlich's reagent. Similarly developed and unsprayed plates were sprayed with C.c. spores for bioassay.

The distribution of the compounds is shown diagrammatically in Fig. 24. This separation of the different rot zones gives a picture of the distribution of the compounds. The following emerge from the chromatogram.

a. The phytoalexins A, B and D occur in all the zones of rot caused by the three fungi.
b. Compound C is located only in zone 3.
c. Zone 4 is physiologically different from uninoculated tissue and cannot therefore be regarded as healthy.

Fig. 24. Location of compounds A, B, C, D in different zones of rot (1, 2, 3, 4) caused by B.t., B.c. and C.c.

T.L.C. developed in n-hexane-ethylacetate (3:2) and compounds located with Ehrlich's reagent, and C.c. bioassay



B. theobromae B. cinerea C. cucumerinum

In view of the significance of the quantitative distribution of the compounds in the various zones, it was found desirable to quantify these compounds.

(D) Quantitative estimation of the phytoalexins formed in the different rot zones caused by B.t., B.c., and C.c.

The ethanolic eluates of the different compounds from the chromatogram were adjusted with ethanol to give a standard concentration (g fresh weight tissue). From this, aliquots were taken and made up to 4 ml with ethanol. This was added to a reaction mixture and assayed for Ipomeamarone content (see Materials and Methods). The concentration of the compounds were expressed in units of Ipomeamarone equivalents. Table 23 summarises the results.

Compound B (Ipomeamarone) occurred at highest concentration in a given zone for all the fungi. This is followed by compound D (Ipomeamaronol), C and A, where these occur together. There is a more or less similar distribution of the compounds in zones 3 and 4 irrespective of the fungus causing the lesion. There is however a fall in concentration of compounds B and D from zone 3 to 2 in lesion caused by B.t. whereas this is the reverse in B.c. infected tissues. There is a sharp drop in concentration of all the compounds from zone 2 to 1. Zone 4 contains more or less the same amount of the compounds as zone 1.

Table 23. Quantitative estimation of compounds A, B, C, D in different zones of rot caused by the three fungi

		Rot zones				Uninoculated tissue
		1	2	3	4	
B.t.	A	0.18	0.30	0.41	0.12	0
	B	0.40	1.42	2.38	0.25	0.007
	C	=*	=*	0.68	=*	0
	D	0.15	0.70	1.50	0.16	0.001
B.c.	A	=**	0.32	0.43	0.15	
	B	=**	2.80	2.26	0.22	
	C	=**	=*	0.70	=*	
	D	=**	1.64	1.50	0.17	
C.c.	A	=**	=**	0.46	0.14	
	B	=**	=**	2.10	0.24	
	C	=**	=**	0.65	=*	
	D	=**	=**	1.48	0.17	

Rot zones were extracted 7 days after inoculation. Result expressed as mg/g fresh weight tissue

=* shows compound C is absent in zones indicated

=** shows the zone is non-existent in tissues inoculated with the fungus

The concentration of the compounds ahead of lesion (i.e. zone 3) shows that the phytoalexins may be formed ahead of rot and the sharp decrease in compounds B and D from zone 3 to 2 (in tissue infected with B.t.) suggests that some mechanism might be responsible for changes in concentration of the compounds in the tissue. This is more obvious on realising that zone 3 becomes converted to zone 2 as rot progresses.

(E) Time course analysis of the phytoalexins formed by s.p. inoculated with B.t. and B.c.

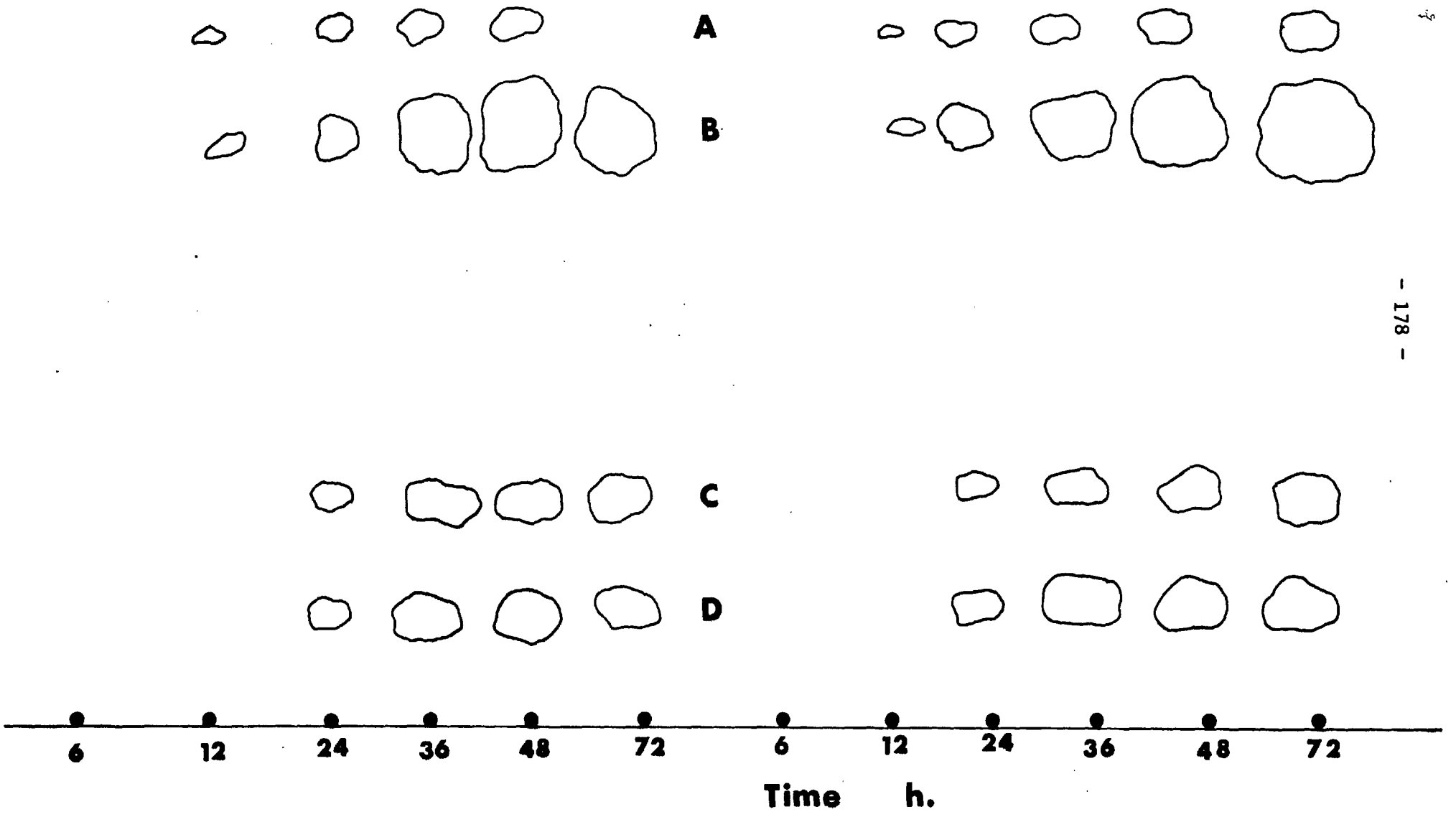
S.p. slices were inoculated with B.t. and B.c. and lesions formed were extracted with chloroform in methanol (1:1) after 6, 12, 24, 36, 48 and 72h. Aliquots of the extracts were subjected to TLC and developed in n-hexane-ethylacetate (3:2). The plate was sprayed with Ehrlichs reagent and the phytoalexins located by their rfs.

Fig. 25 shows that the formation of the compounds A and B is initiated at about 12h and C, D at about 24h after inoculation.

II Extraction of some other phenol derivatives

TLC of chloroform : methanol extracts showed that a number of compounds were stained by sulphanilic acid reagent (Grimmert & Richards, 1965) which are used to locate phenols.

Fig. 25. Time-course analysis of the phytoalexins A, B, C, D produced by sweet potato inoculated with B. theobromae and B. cinerea.



These compounds however did not show antifungal response to C.c. spores. Some phenol derivatives have however been reported to be produced by s.p. in response to infection by C. fimbriata. These derivatives were therefore investigated for their antifungal action.

1. TLC of phenol derivatives

Sweet potato was inoculated with B.t. and B.c. and extracted after 4 day incubation as described earlier (Fig. 2). The final chloroform fraction was applied to TLC on silica gel and developed successively with two solvents:

a) Ethanol-conc ammonia - H_2O 80:5:15

b) n-butanol, saturated with H_2O

The second solvent system improved the separation of the compounds. The plate was developed for 5h observed under uv (254nm) and sprayed with Benedict's reagent.

Four distinct phenol derivatives A, B, C and D, were detected on the chromatogram; two of these B_1 and C_1 fluoresced (white fluorescence) before and after spraying with Benedict's reagent. The other two, A_1 and D_1 showed blue fluorescence which was quenched after spraying the reagent (see Fig. 26).

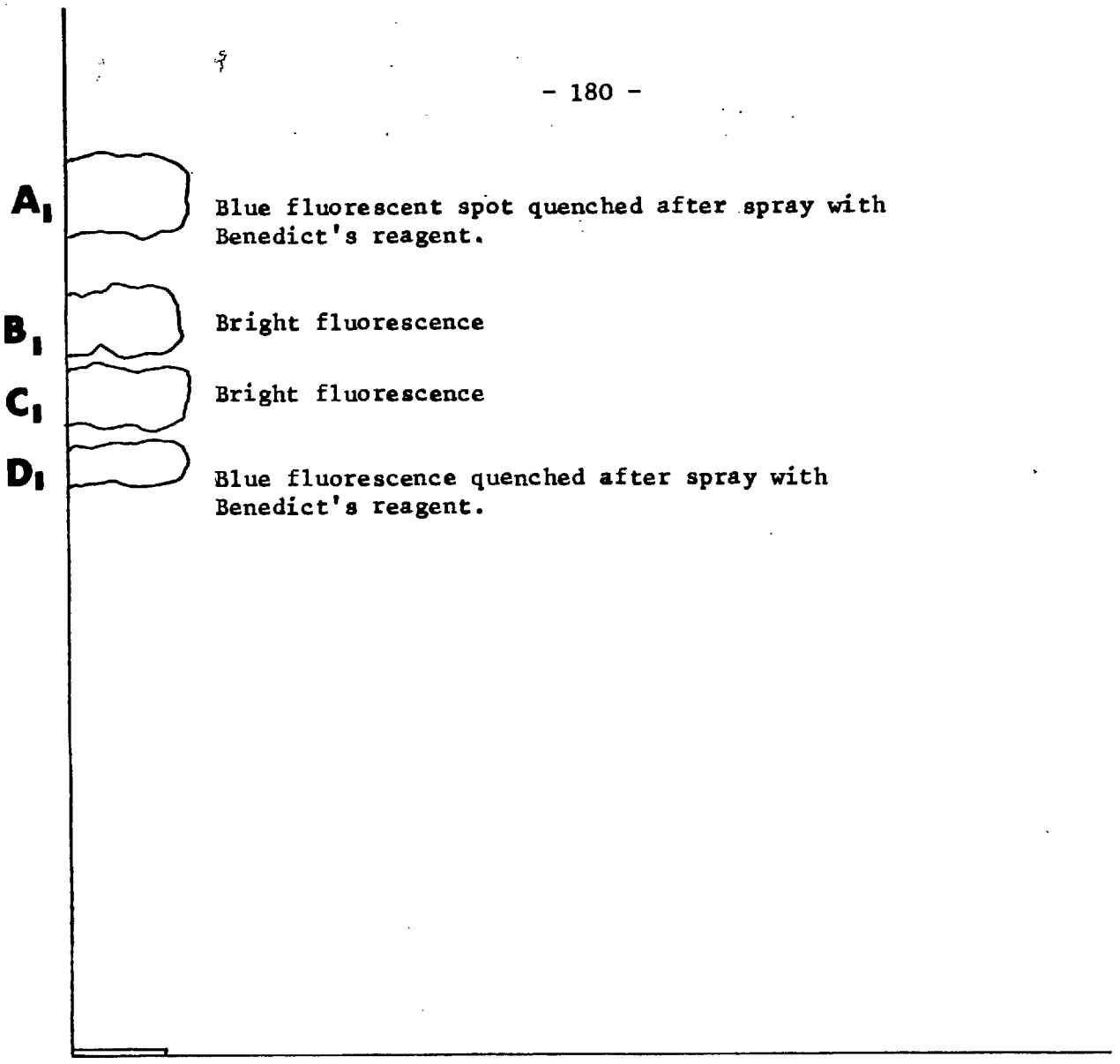


Fig. 26. Diagrammatic representation of TLC of other phenol derivatives produced by sweet potato in response to inoculation by B. theobromae. Compounds A, B, C, D, were located by uv 254 and by Benedict's reagent.

It is known that O-dihydroxy groups can be detected with Benedicts reagent and that this group is responsible for fluorescence quenching whereas susbtances which do not contain the group e.g. some compounds in the classes — coumarins, cinnamic acids, and flavonoids usually fluoresce when sprayed with the reagent (Egger, 1969). The Rf value of one of the fluorescing groups, C_1 corresponded to scopoletin, a coumarin (See Fig. 27). Uninoculated tissues did not contain these derivatives.

2. Bioassay of phenol derivatives against C.c. spores

A bioassay technique was used similar to that for the terpene components separated on TLC. Concentrations up to 4g fresh weight tissue were loaded on the chromatogram and sprayed with spore suspension of C.c.

It was observed that none of the derivatives inhibited spore germination of the fungus.

In order to raise the concentration of the compounds assays were further done on glass slides. The compounds were eluted from unsprayed chromatogram, re-run on TLC using the same solvents, re-eluted and taken up in 0.05% DMSO for bioassay. Table 24 shows that at concentrations up to 16g fresh weight tissue the separated phenol derivatives did not significantly reduce the germination of the C.c. spores.

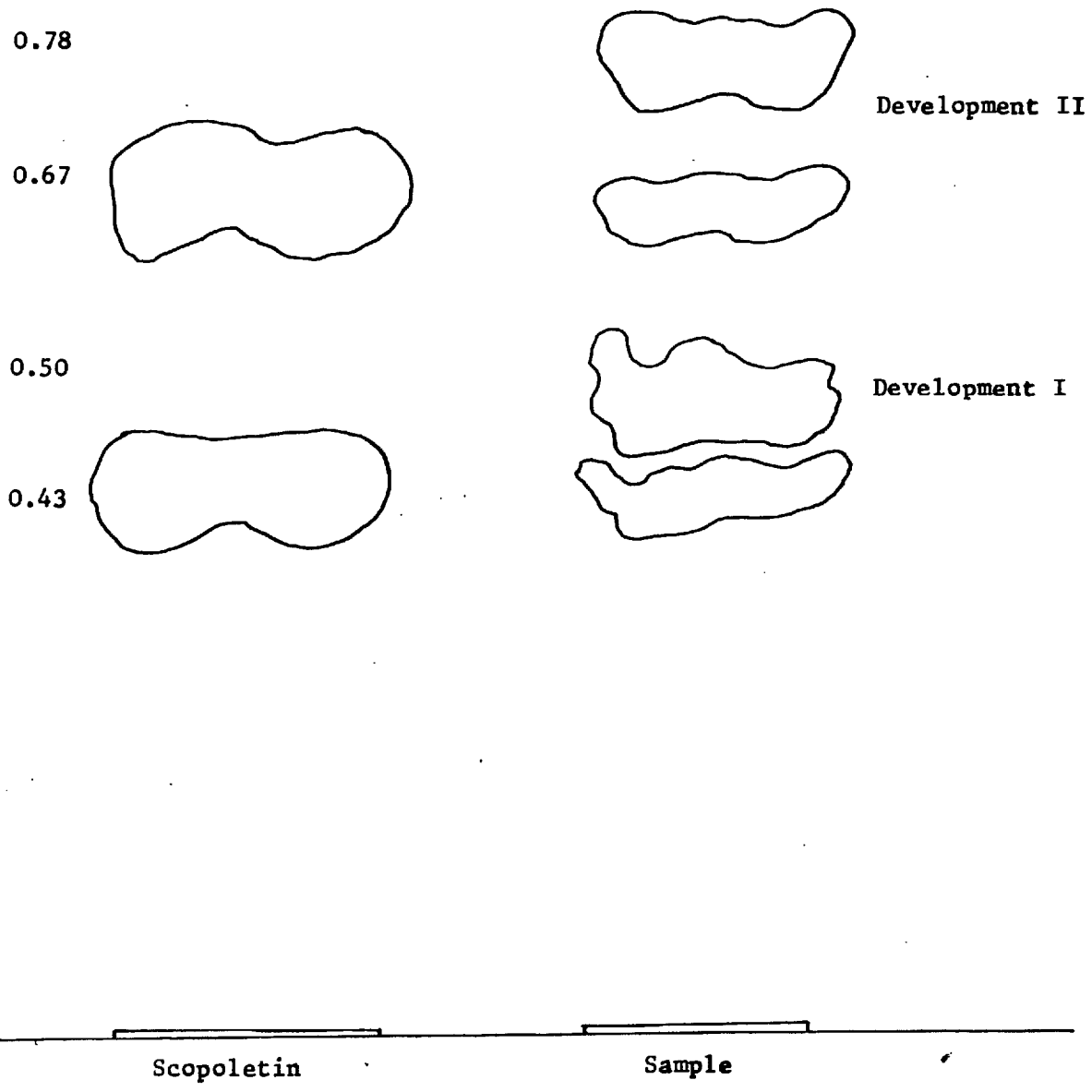


Fig. 27. Diagrammatic representation of TLC of fluorescing compounds after spraying with Benedict's reagent. Rf of one of the compounds corresponded to scopoletin.

Table 24. Effect of phenol derivatives (Compounds A, B, C, and D) on spore germination of C.c.

Compounds	% germination Concentration (g fresh weight tissue)				
	1	2	4	8	16
A_1	100	100	100	97	94
B_1	100	100	100	96	90
C_1	100	100	100	0	88
D_1	100	100	100	0	94

Compounds were eluted from unsprayed chromatogram taken up in 0.05% DMSO and spore germination assays carried out on glass slides. Spores were incubated for 24h.

3. Effect of combined phenol derivatives on germination of C.c. spores

Since the compounds were formed in the tissue only after infection, it was thought that there might be a coaction between the derivatives in causing inhibition. Aliquots of the crude chloroform extract corresponding to 0.5, 1, 2, 4, 8 and 16g fresh weight tissue were evaporated and taken up in LANS. C.c. spores were incubated in these extracts on glass slides and percentage germination of spores recorded after 24h.

The results (Table 25) shows that there was progressive inhibition of spore germination with increase in concentration of total extracts from 2 to 16g fresh weight tissue.

For comparative purpose, equivalent amounts of total terpenes taken up in 0.5% DMSO were tested for their inhibitory action on C.c. spores. Table 25 compares the results.

It is evident from the Table that C.c. is much more sensitive to the terpenes than to the phenol derivatives. Furthermore, these derivaties are more inhibitory to spore germination when combined than when separated. Perhaps there is a coaction of the compounds in causing inhibition.

From results obtained so far, sweet potato produces antifungal terpenes (Compounds A, B, C, D) in response to infection by B.t., B.c. or C.c. The tuber produces also other

Table 25. Percentage germination of spores of C.c. in combined phenol derivatives and in combined terpenes.

Extracts	Concentration (g/fresh weight tissue)					
	0.5	1	2	4	8	16
Combined phenol derivatives	100	100	85	63	30	19.5
Combined terpenes	0	0	0	0	0	0
0.5% DMSO	100					
LANS	100					

Chloroform extracts were taken up in 0.5% DMSO and LANS (Long Ashton Nutrient Solution) and spore germination assays done on glass slides. Spores were incubated for 24h.

phenols and their derivatives which may act together to have antifungal effect. However, despite these phytoalexins B.t. appears capable of rotting the tuber successfully. Experiments were therefore designed to test the sensitivity of the fungi to the phytoalexins.

III Sensitivity of B.t., B.c. and C.c. to s.p. phytoalexins

The sensitivity of the test fungi to the four phytoalexins eluted from TLC was tested by spore germination assay and by further elongation of pregerminated spores (sporelings).

1. Spore germination assays

Assays of the terpenes against spore germination of the fungi were performed using the technique of Purkayasha and Deverall (1965). The phytoalexins were taken up in 0.5% DMSO or 2% ethanol in concentrations ranging from 20ppm to 342 ppm. Percentage germination of the spores of the test fungi in the four compounds was recorded after 24h incubation and the ED_{50s} (ppm) interpolated from the graphs (Figs. 28, 29, 30, and 31).

Table 26 shows the ED_{50s} and the minimum inhibitory dose (M.I.D.) (ppm) for the compounds on the fungi. The M.I.D. is the lowest concentration which completely inhibited the germination of the spores. B.t. was less sensitive to the

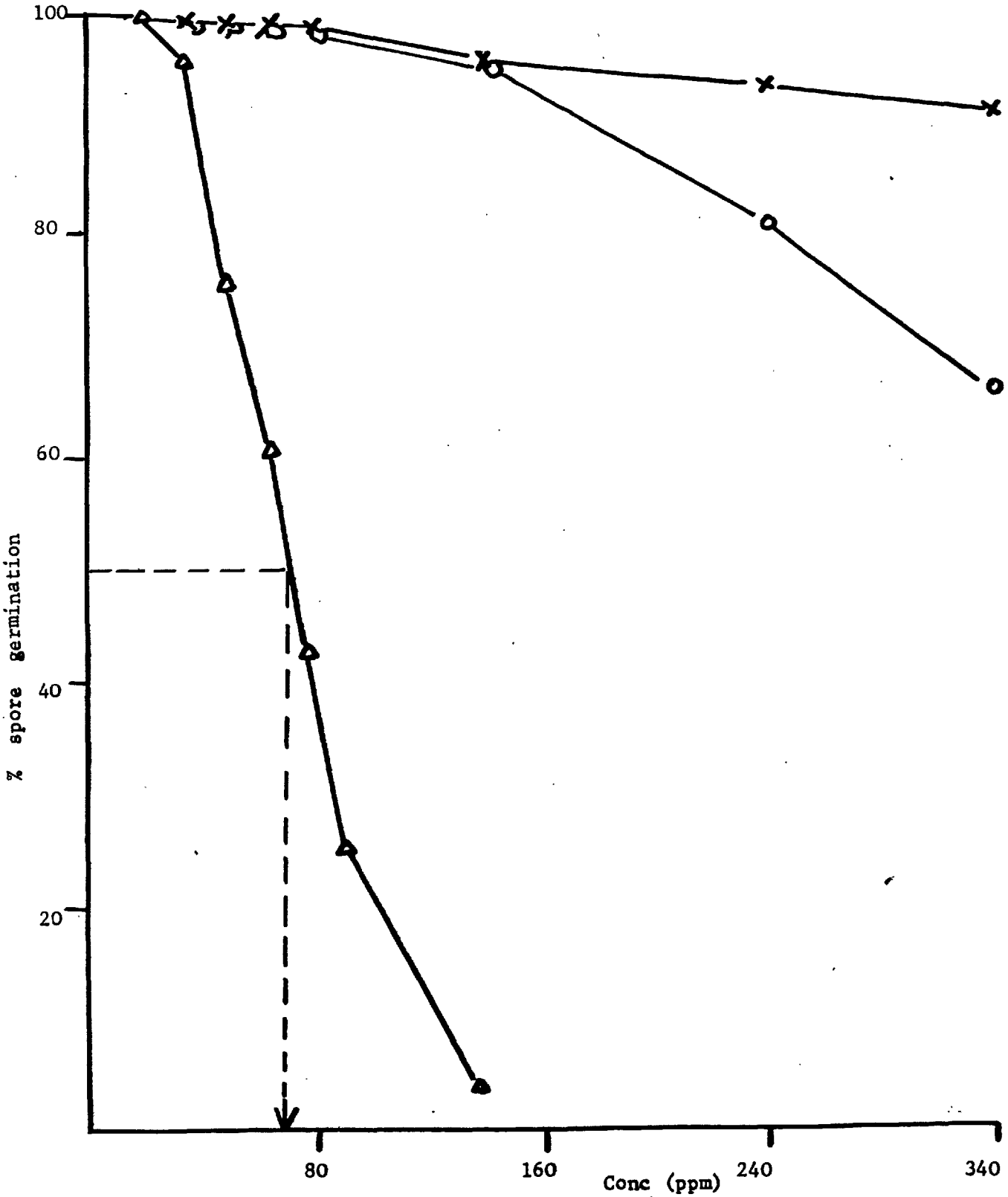


Fig. 28. Sensitivity of B.t., B.c. and C.c. to compound A determined by spore germination of the fungi.

B.t. (x——x), B.c. (o——o), C.c. (Δ——Δ).

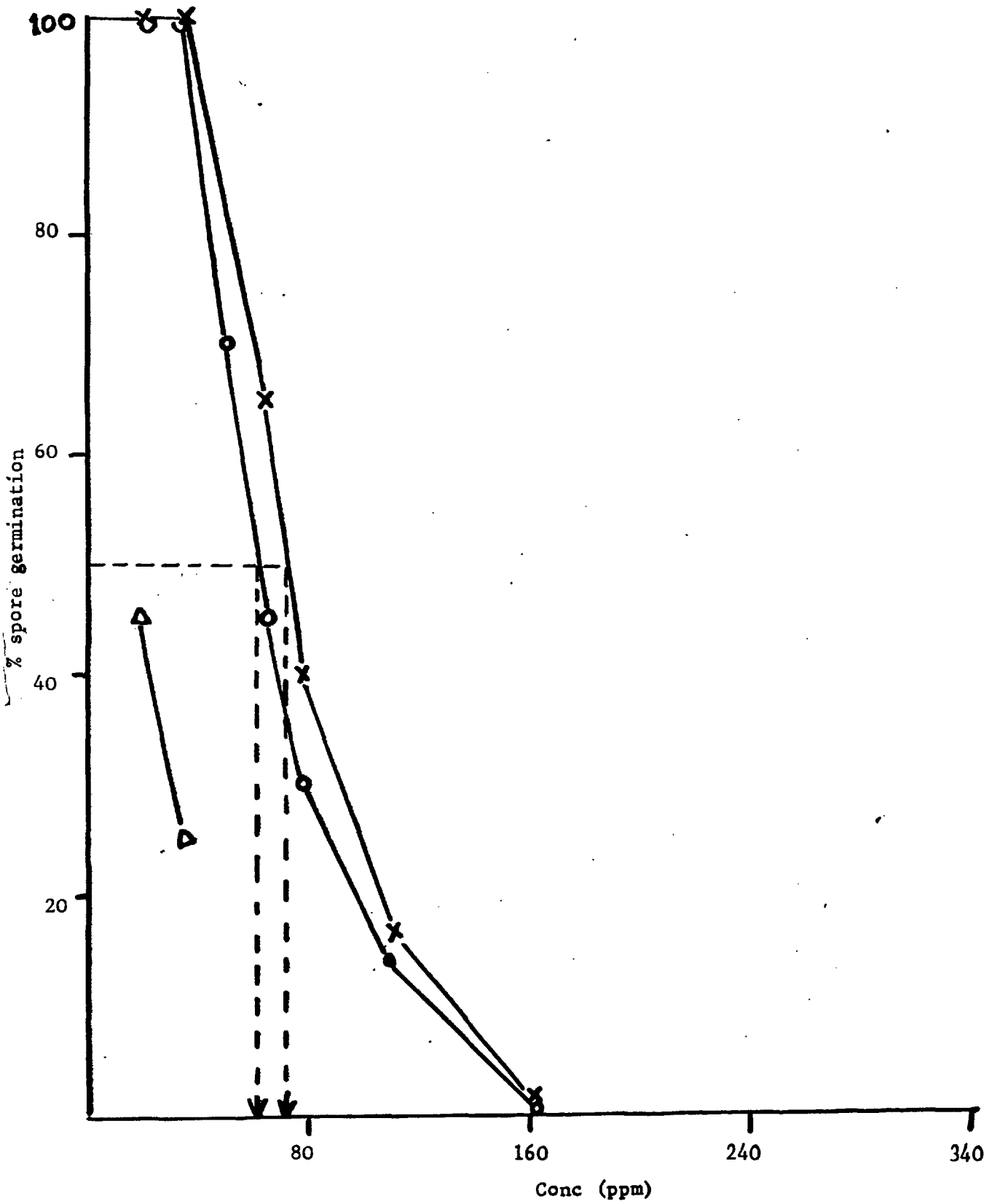


Fig. 29. Sensitivity of B.t., B.c. and C.c. to compound B (ipomeamarone) as determined by spore germination of the fungi.
B.t. (x——x), B.c. (o——o), C.c. (Δ——Δ).

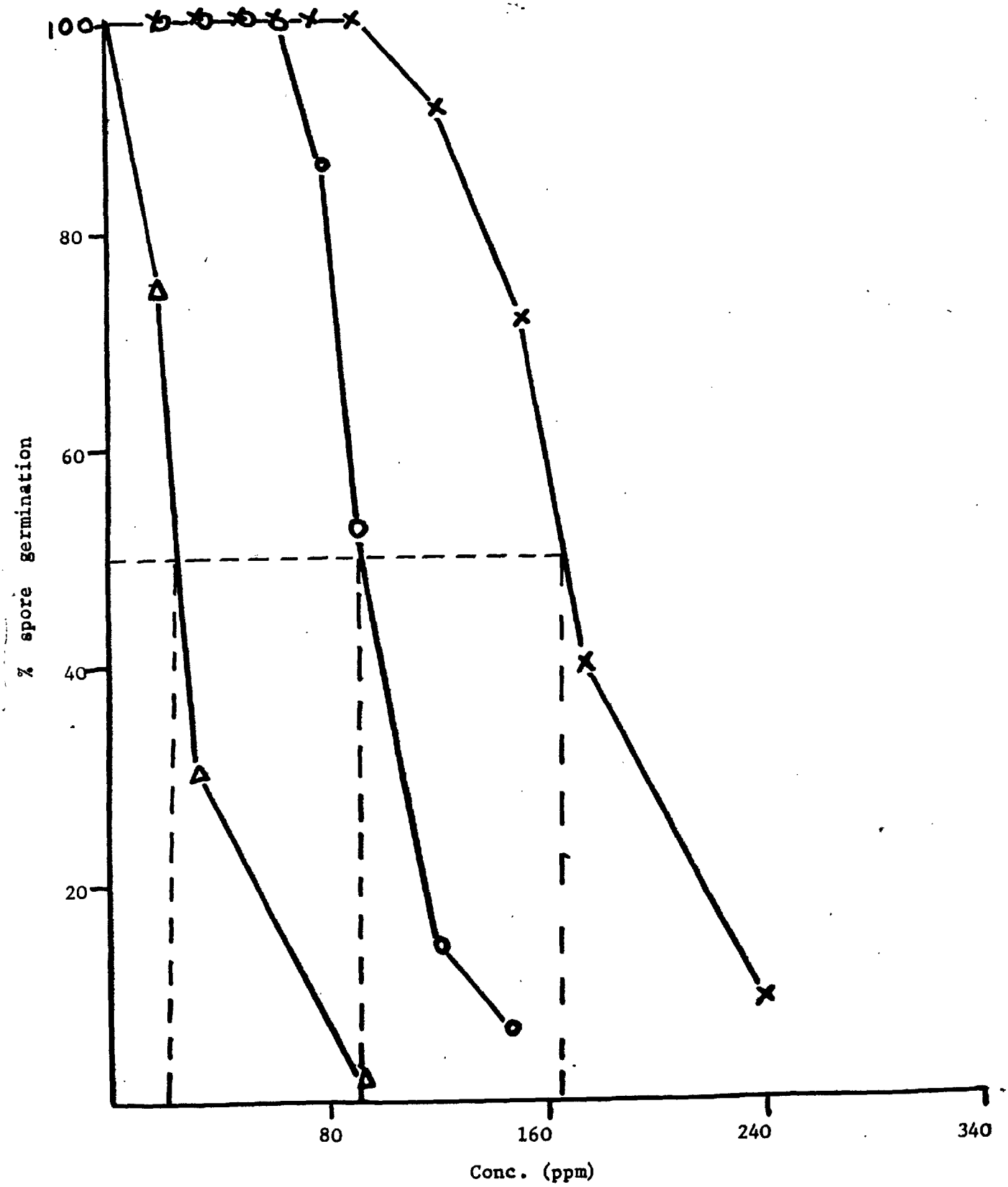


Fig. 30. Sensitivity of B.t., B.c. and C.c. to compound C as determined by spore germination of the fungi.
B.t. (x——x), B.c. (o——o), and C.c. (Δ——Δ)

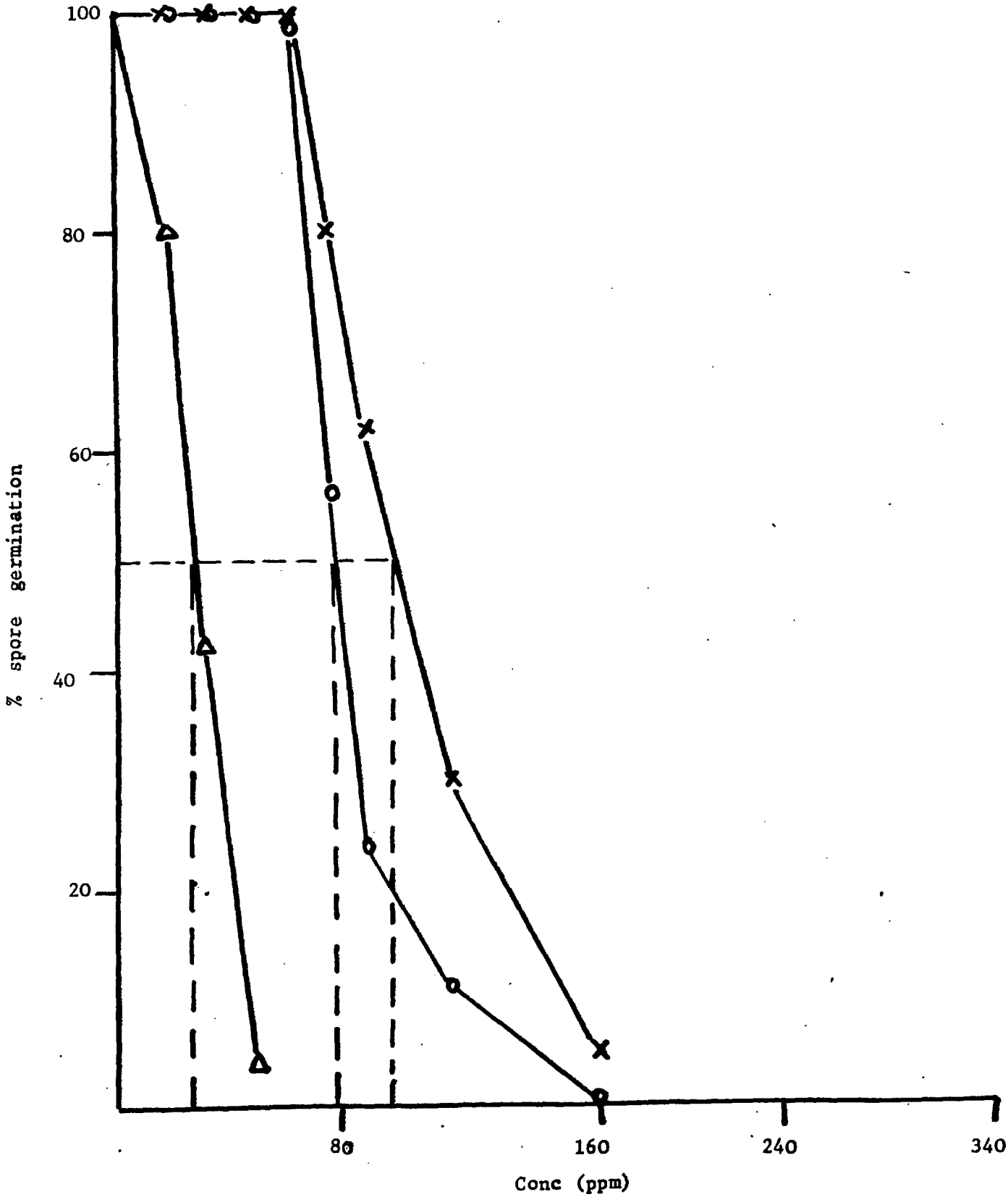


Fig. 31. Sensitivity of B.t., B.c. and C.c. to compound D (ipomeamarono1) as determined by spore germination of the fungi.

B.t. (x——x), B.c. (o——o) and C.c. (Δ——Δ)

Table 26. The ED_{50} and minimum inhibitory dose (MID) of compounds A, B, C, D for spore germination of B.t., B.c. and C.c.

Compounds	(i) ED_{50} (ppm)		
	B.t.	B.c.	C.c.
A	≥340	≥340	70
B	72	60	<20
C	164	92	<20
D	98	78	26
	(ii) MID (ppm)		
A	≥340	≥340	174
B	174	174	48
C	258	174	63
D	258	174	48

Assays were performed on glass slides and germination recorded after 24h incubation.

Minimum inhibitory dose is the lowest concentration which completely inhibited spore germination.

phytoalexins than B.c. and C.c. and C.c. much more sensitive than B.t. or B.c. B.t. and C.c. could be regarded as being on two extremes of sensitivity and B.c. regarded as intermediate.

Compound B (Ipomeamarone) had the most inhibitory effect followed closely by compound C and D. Compound A had the least inhibitory effect on spore germination of the fungi.

2. Sporeling growth assays

Assays on sporeling growth were considered more critical than spore germination since a spore has to germinate in order to cause infection by producing mycelia in the host tissue. Sporeling growth assays were similarly performed with 24h old sporelings grown on glass slides in drops of distilled water. After withdrawing the germination fluid, 20 ml of the phytoalexin was added and incubated for 5 days. The length of the sporelings was taken every 24h. The average lengths of the sporelings after 5 days growth was as follows: B.c. = 682μm, B.t. = 644μm, and C.c. = 620μm. Figs. 32, 33, 34 and 35 show the percentage growth of the sporelings taken on the 5th day of incubation in various concentrations of the compounds and Table 27 shows the ED_{50}s and M.I.D. of the compounds for sporeling growth of the fungi.

As was the case for spore germination, compound B

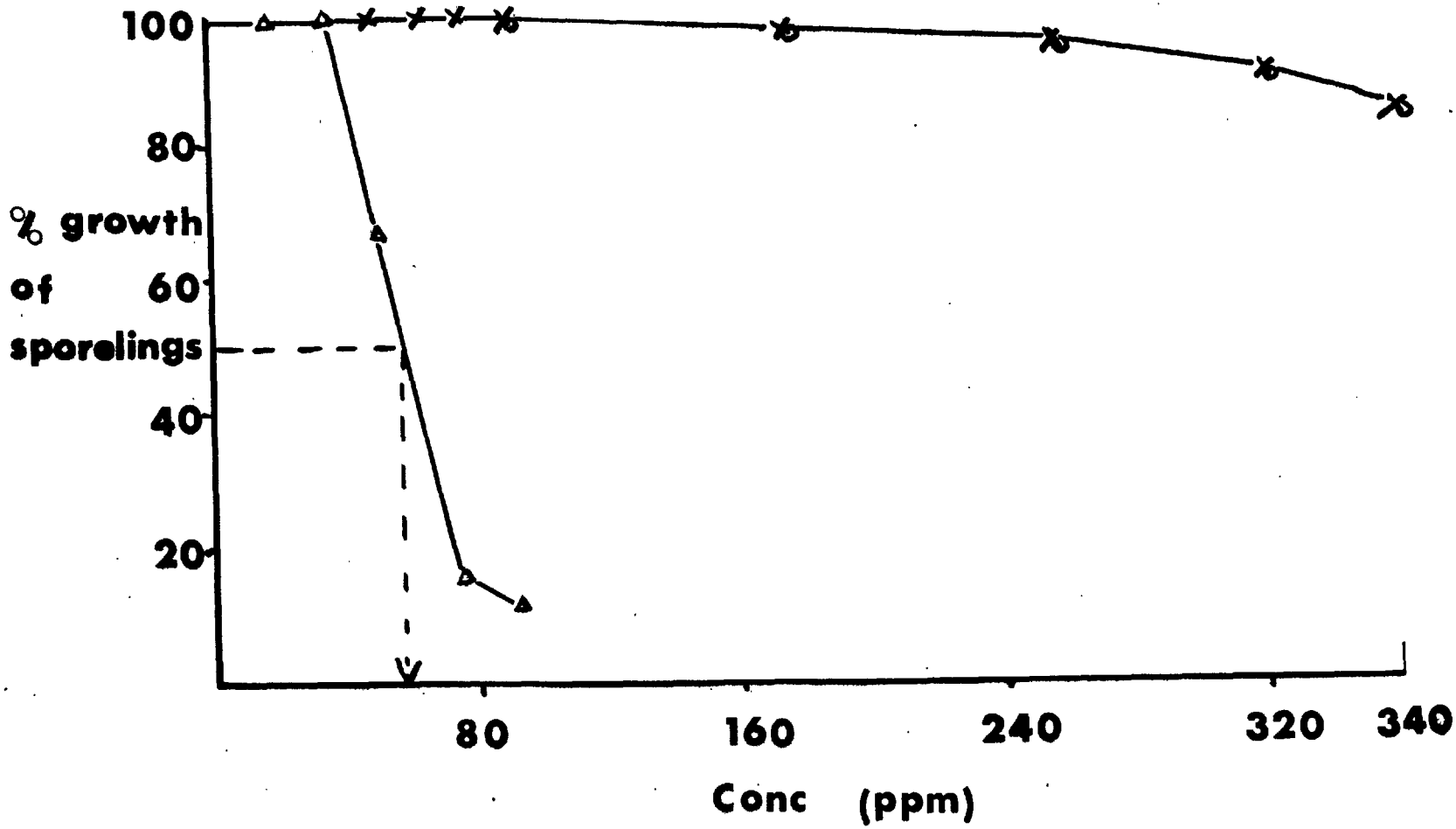


Fig.32. Sensitivity of B.t., B.c. and C.c. to compound A as determined by sporeling growth. B.t. (×——×), B.c. (o——o) and C.c. (Δ——Δ)

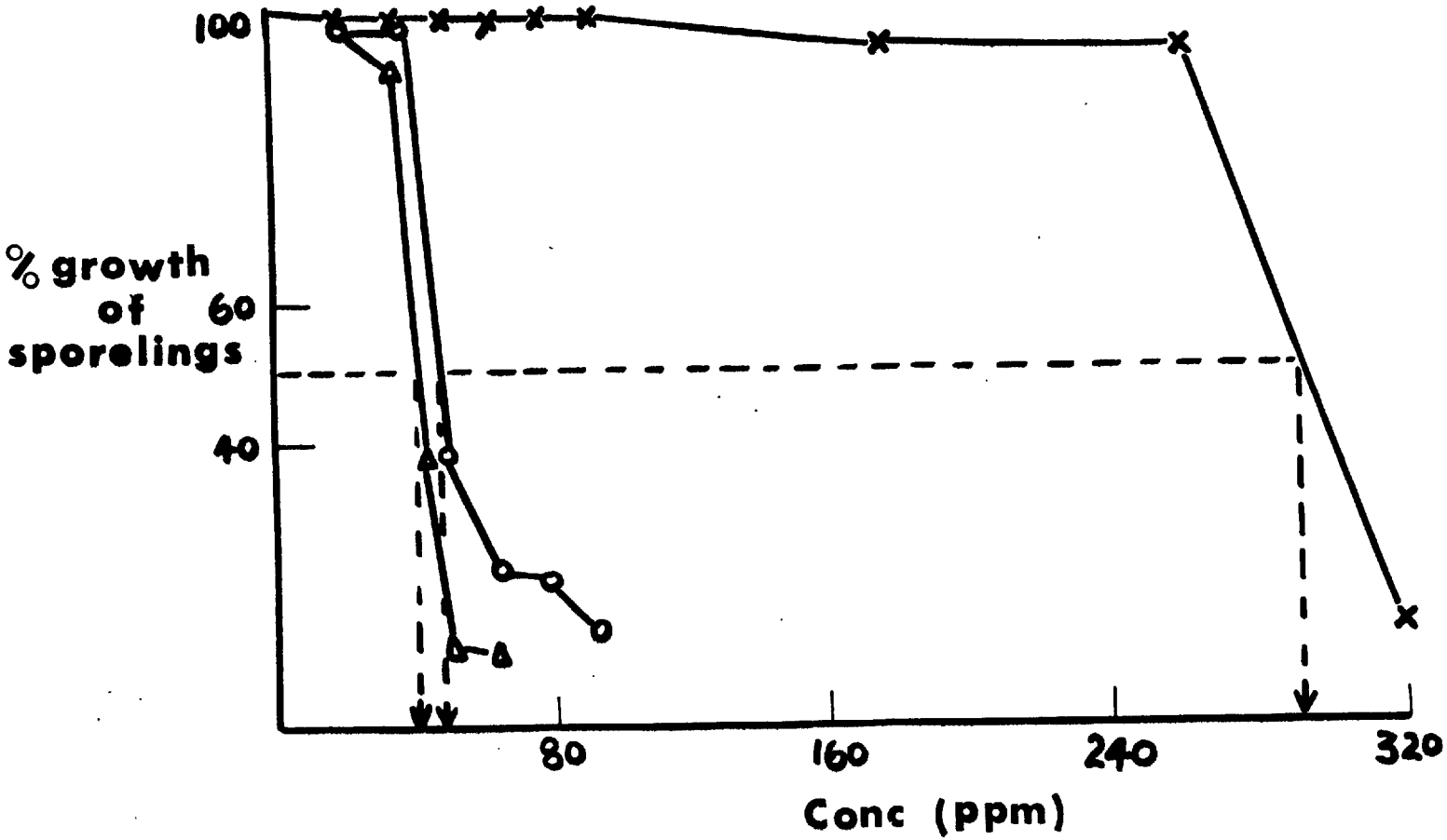


Fig. 33. Sensitivity of B.t., B.c. and C.c. to compound B (ipomeamarone) as determined by sporeling growth.

B.t. (×——×), B.c. (o——o), and C.c. (Δ——Δ)

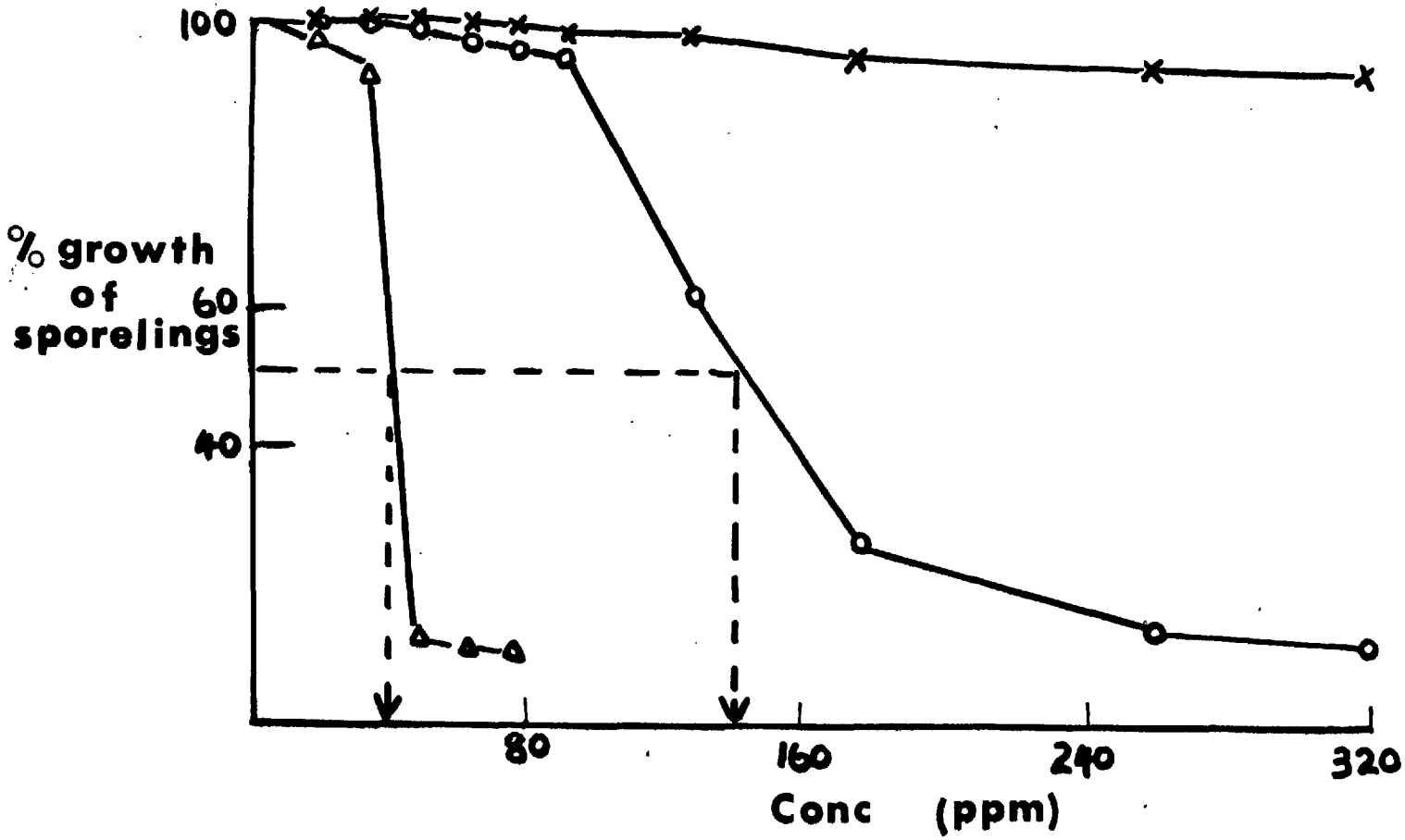


Fig. 34. Sensitivity of B.t., B.c. and C.c.to compound C as determined by sporeling growth B.t. (×——×), B.c. (o——o) and C.c. (Δ——Δ)

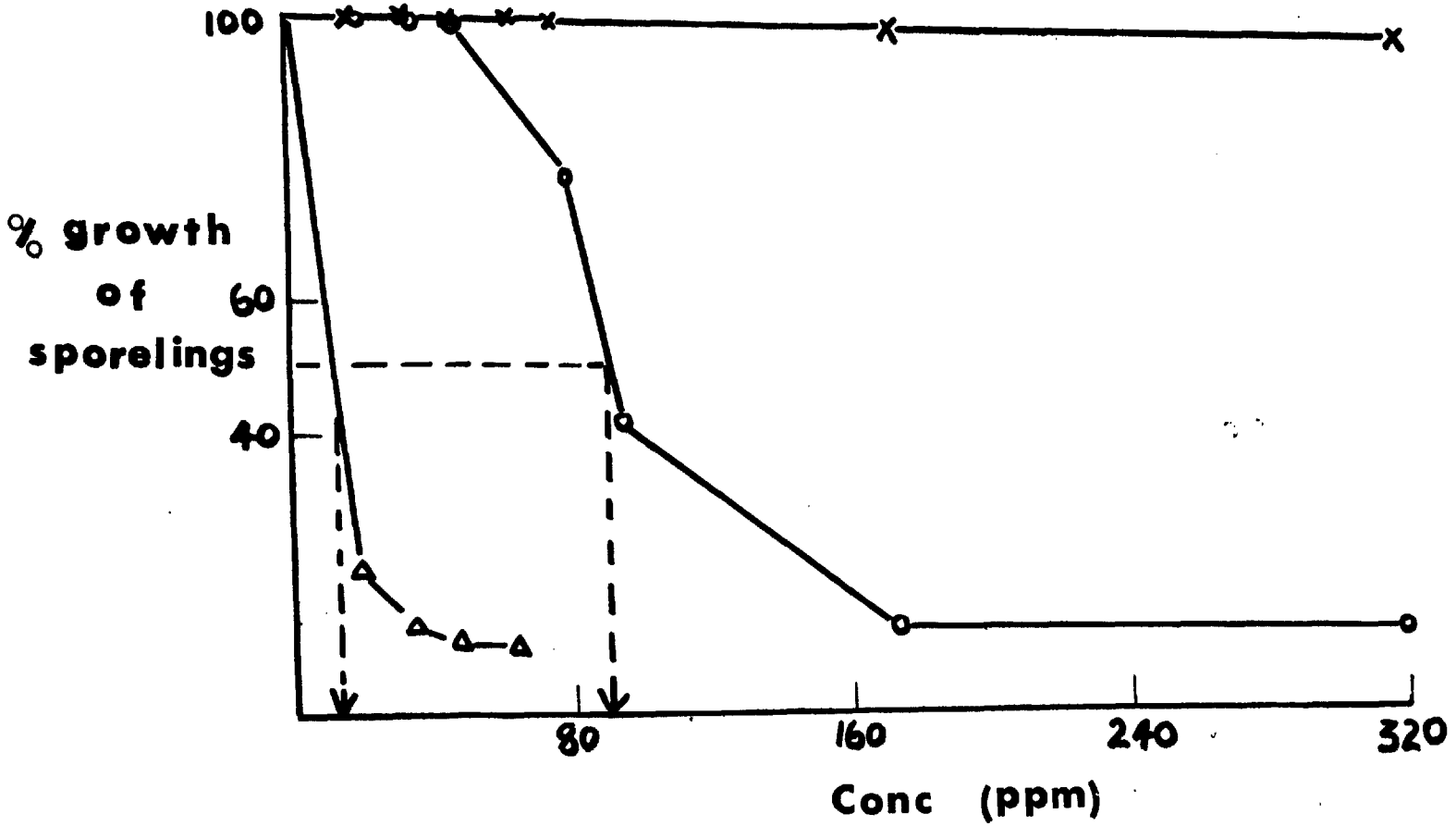


Fig. 35. Sensitivity of B.t., B.c. and C.c. to compound D (ipomeamaronol) as determined by sporeling growth.

B.t. (×——×), B.c. (o——o) and C.c. (Δ——Δ)

Table 27. The ED_{50} and MID of the compounds A, B, C, D for sporeling growth of the fungi.

(i) ED_{50} (ppm)

Compounds	B.t.	B.c.	C.c.
A	≥340	<340	68
B	292	46	40
C	≥340	138	40
D	≥340	92	14

(ii) MID (ppm)

A	≥340	≥340	76
B	≥340	90	48
C	≥340	258	63
D	≥340	174	20

Assays were performed on 24h old sporelings on glass slides in drops of distilled water. Sporeling lengths were taken on 5th day of incubation.

(Ipomeamarone) was most inhibitive to sporeling growth of B.t. and B.c. followed by Compound D (Ipomeamaronol), C and A respectively. Compound D inhibited C.c. sporeling more than B, C or A. With the exception of Ipomeamarone none of the compounds inhibited the growth of B.t. at the concentrations tested. At all these concentrations, C.c. was more sensitive than B.c. All the compounds had lower ED_{50s} for sporeling growth of C.c. than for B.c. It is noteworthy here that the observed tissue concentration of the phytoalexins is far is excess of the concentrations which inhibited growth of the fungi. (Compound A=187.5ppm Compound B=1,265ppm Compound C = 350ppm and Compound D=785 ppm. The comparatively low ED_{50s} for both spore germination and sporeling growth of C.c. and B.c. reflect the high toxicity of the compounds to these fungi.

On the whole, the assays show that B.t. is less sensitive to all the compounds followed by B.c. and C.c.

3. Activity of total terpene extract from different rot zones against sporeling growth of B.t., B.c. and C.c.

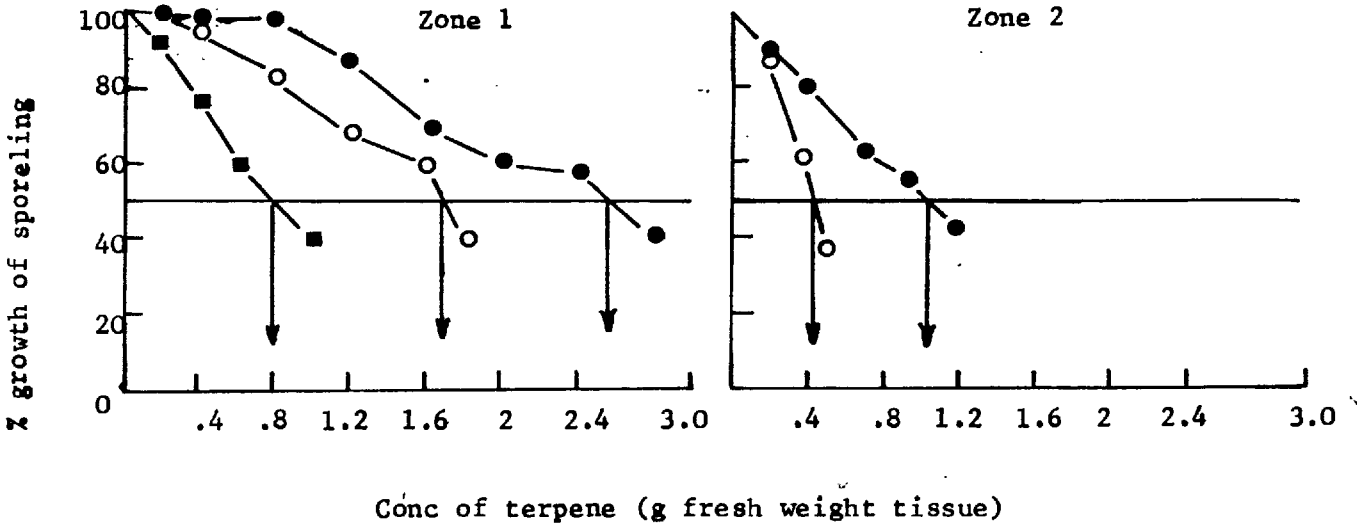
Most of the terpenes had been shown to be concentrated in zones 2 and 3 of the rotted tissue. An experiment was set up to see if there is a relation between this distribution and the effect of the zones on sporeling growth of the fungi.

The total terpene content of the zones of rot caused by B.t. were extracted after seven days of inoculation and their effect on sporeling growth of B.t., B.c. and C.c. tested. Growth assays were performed with 24h old sporelings using the standard technique described earlier. The lengths of the sporelings were measured on the 5th day of inoculation. Fig. 36 summarises the results.

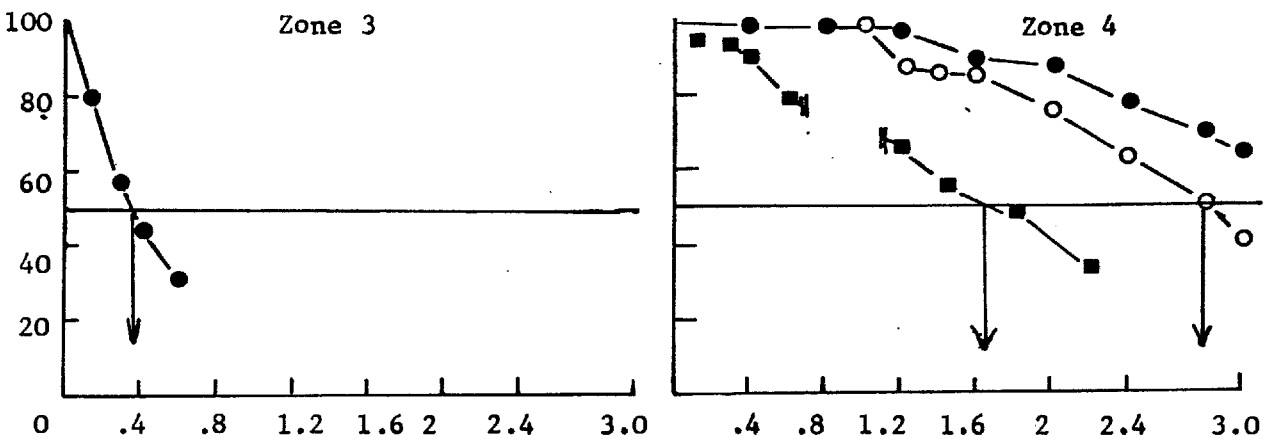
Zone three was the most inhibitory followed by zones 2, 1 and 4. Zone 3 caused more than 8 times as much inhibition to B.t. sporelings as zone 2 and more than 4 times when B.c. sporelings were tested. Zone 1 had less inhibitory action than zones 2 and 3 for all the fungi. Zone 4 showed inhibitory action against B.c. and C.c. with ED_{50}s of 2.80 and 1.64g fresh weight total terpene equivalent respectively (Table 28).

The sensitivity experiments described so far indicate that B.t. is much less sensitive to the phytoalexins and the total terpenes produced by s.p. than either B.c. or C.c. Results on the growth of the sporelings in the individual phytoalexins particularly indicate that B.t. may be able to tolerate high dosage of the compounds or else has a mechanism of dealing with the compounds. One such mechanism investigated is degradation.

Fig. 36. Effect of total terpene extract from zones of rot against sporeling growth of B.t., B.c. and C.c.
●—●B.t., o—o B.c. and ■—■ C.c.



Conc of terpene (g fresh weight tissue)



CONTROL (uninoculated)

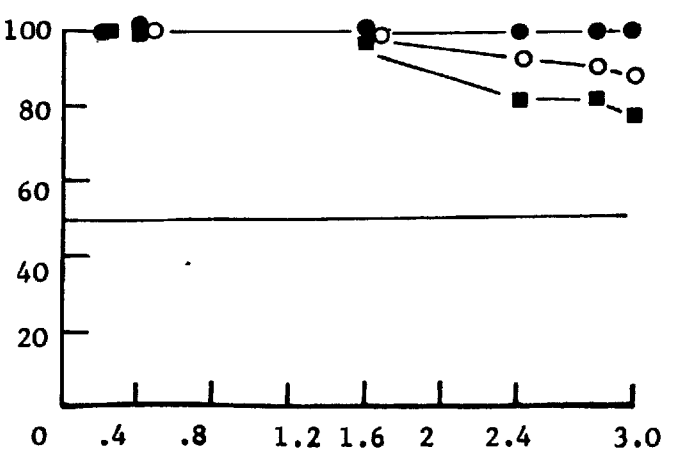


Table 28. ED_{50} of total terpene extracts from different rot zones for sporeling growth of *B.t.*, *B.c.* and *C.c.*

Rot zones	*B.t.*	*B.c.*	*C.c.*
1	2.55	1.68	0.79
2	1.06	0.46	<0.1
3	0.36	<0.1	<0.1
4	>3.0	2.80	1.64
Uninoculated tissue	>3.0	>3.0	>3.0

EDs are expressed as g fresh weight total terpene equivalent.

Extracts were taken up in 0.5% DMSO and sporeling growth assays performed on glass slides. Sporeling lengths were taken on 5th day of incubation.

IV Degradation of phytoalexins by B.t., B.c. anc C.c.

1. Qualitative determination of loss of compounds

Studies on phytoalexin degradation were undertaken to see if this might explain the relative insensitivity of B.t. to the compounds compared to B.c. and C.c.

To 5 ml homogenised mycelium of either B.t., B.c. or C.c. was added total terpene extract equivalent to 6.25g fresh weight tissue and incubated at 25° in specimen tubes. At intervals of ½, 1, 2, 4, 8, 12 and 24h three replicate tubes were harvested and the supernatant partitioned twice with ethylacetate, evaporated to dryness and taken up in 5 ml ethanol. Aliquots of the ethanolic extract (0.625g fresh weight tissue) were run on TLC and sprayed with Ehrlich's reagent. The location of the antifungal bands was mapped out on the chromatogram.

The results Fig. 37 showed that there was a decrease in the amount of all the compounds incubated with B.t., 30 min after incubation. Compound A was completely lost in the culture after 2h and B decreased with time much more rapidly than compounds C and D. All the compounds were practically lost in the culture 24h after incubation. There was comparatively little loss of phytoalexins when the extract was incubated with B.c. (Fig. 38). Compared to control, there was some loss after 24h. When C.c. was incubated with the extract

Fig. 37. Qualitative determination of loss of phytoalexins A, B, C, D, during incubation with B.t. for 24h. Chromatogram was run in n-hexane-ethylacetate (3:2) and compounds located with Ehrlich's reagent.

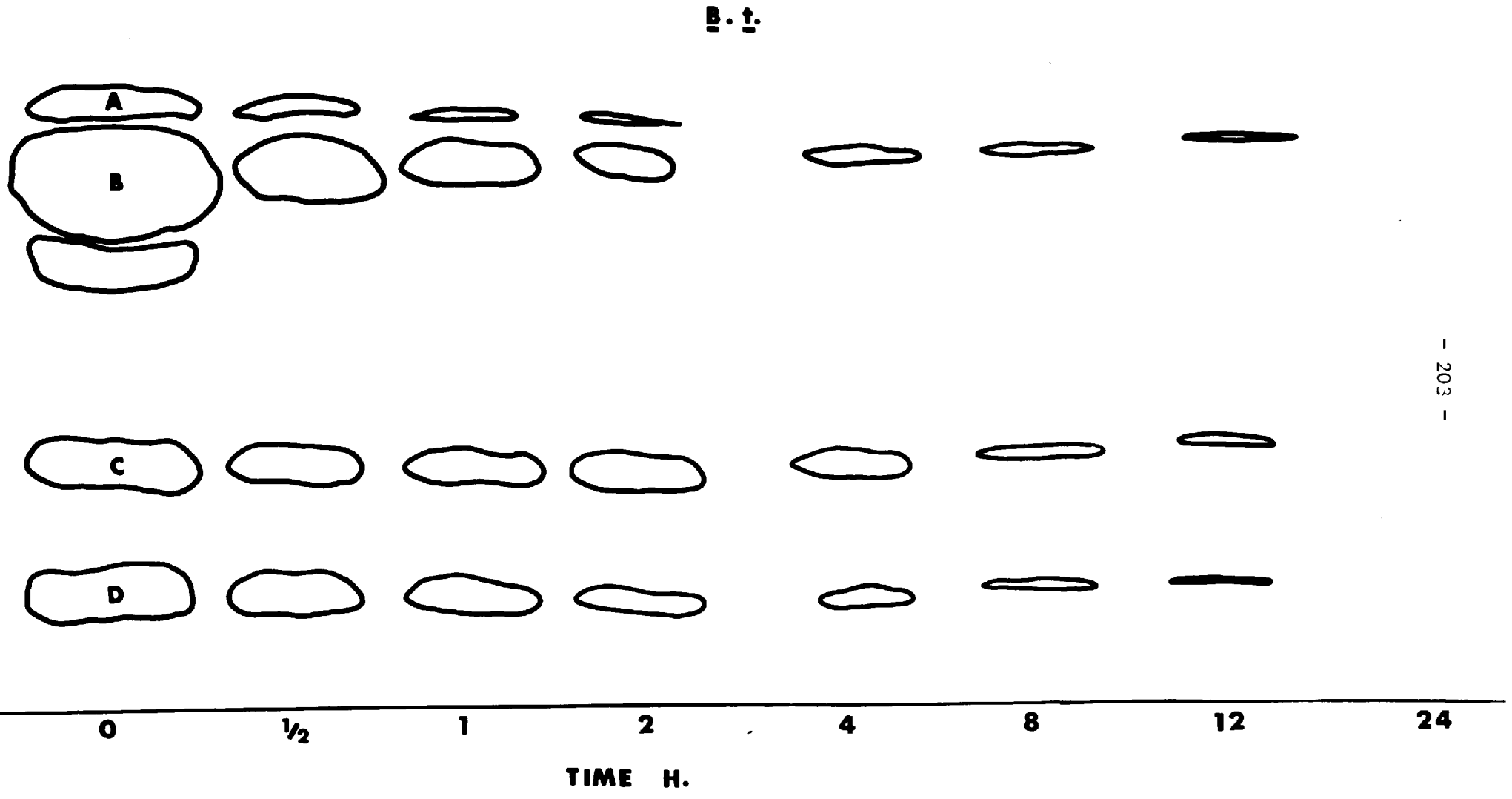
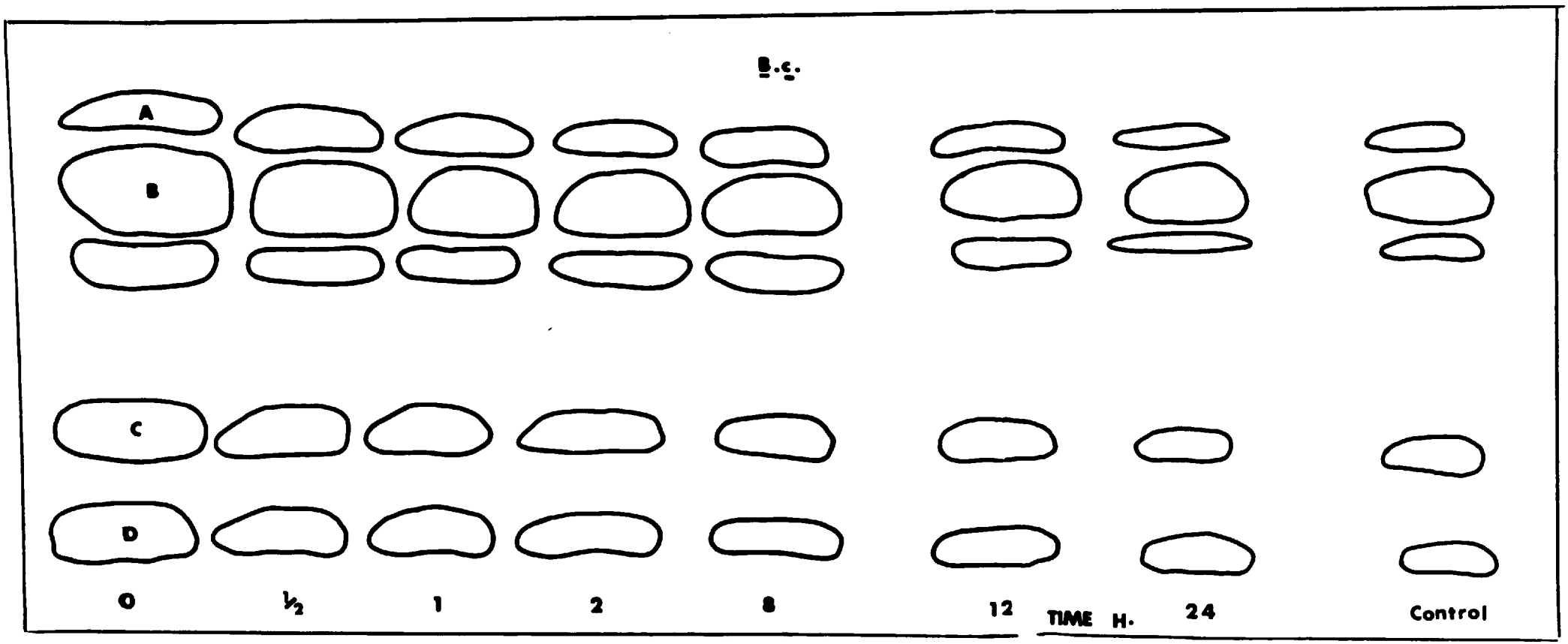


Fig. 38. Qualitative determination of loss of phytoalexins, A, B, C, D, during incubation with B.c. for 24h. Chromatogram was run in n-hexane-ethylacetate (3:2) and compounds located with Ehrlich's reagent.



there was no noticeable difference between the compounds and the control using autoclaved mycelium (Fig. 39).

2. Quantitative evaluation of loss of total terpenes

15.7 mg total terpene taken up in 0.5% DMSO was added to 5 ml mycelial suspension of each fungus and incubated at 25°. As above, at intervals of ½, 1, 2, 4, 8, 12 and 24h three replicate tubes were harvested and supernatant partitioned twice with chloroform-methanol (1:1), concentrated and taken up in ethanol for determination of total terpene content.

Fig. 40 shows the total terpene recovered at intervals up to 24h. There was a rapid loss in total terpene for the 1st 4h in extract incubated with B.t., and the loss continued up to 24h when only 5% of the total terpene was recovered.

Extracts incubated with B.c. showed gradual decrease in total terpene and after 24h incubation 58.6% was recovered.

Extracts incubated with C.c. however showed little deviation from control and 92.3% of terpene was recovered (Table 29).

Fig. 39. Qualitative determination of loss of phytoalexins, A, B, C, D, during incubation with C.c. for 24h. Chromatogram was run in n-hexane-ethylacetate (3:2) and compound located with Ehrlich's reagent.

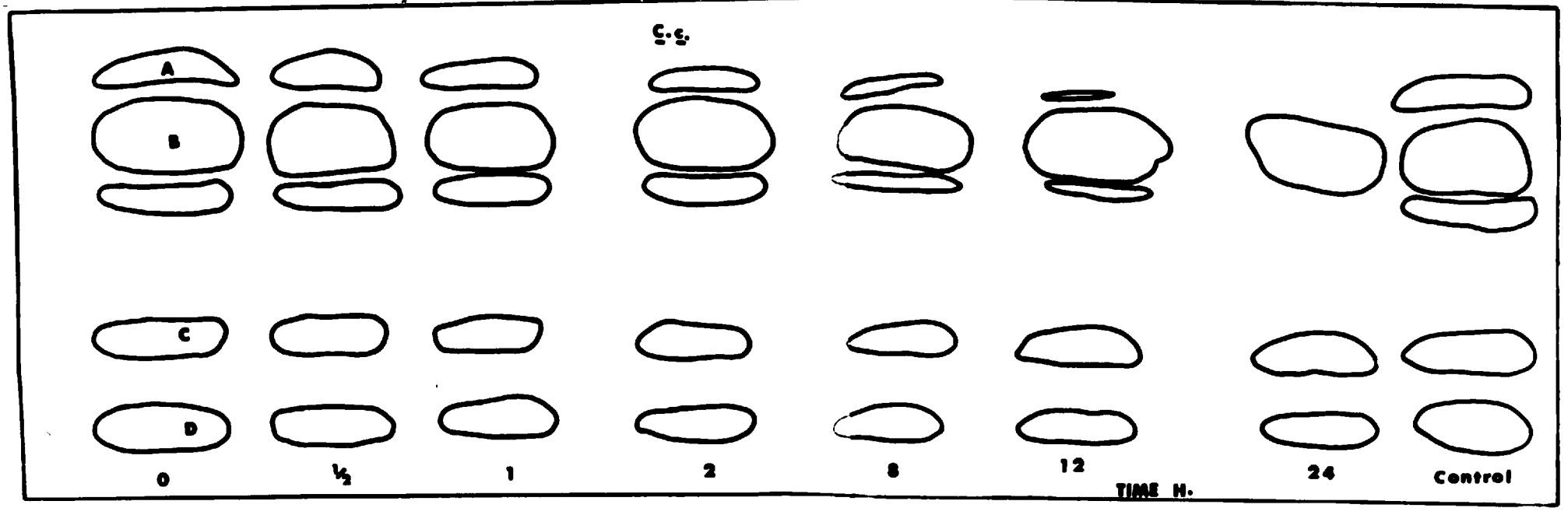


Fig. 40. Amounts of total terpenes recovered at time intervals during incubation with B.t., B.c., and C.c.

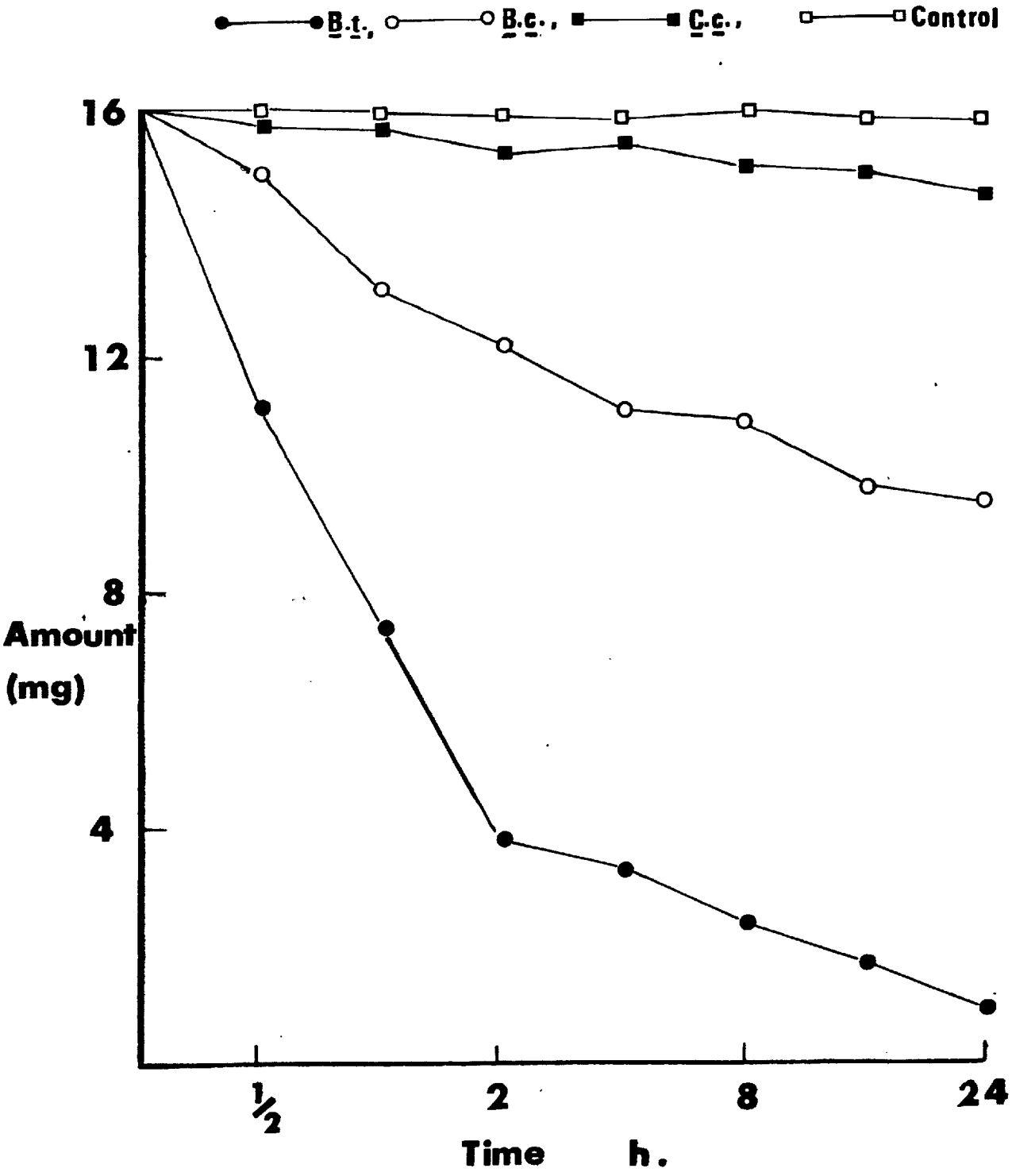


Table 29. Percent recovery of total terpene and ipomeamarone after 24h incubation with B.t.

	Total terpene	Ipomeamarone
B.t.	5.0	0.4
B.c.	58.6	73.1
C.c.	92.3	94.3

Total terpene and ipomeamarone were separately added to cultures of the fungi, incubated at 25° and the cultures re-extracted at intervals.

3. Quantitative evaluation of loss of ipomeamarone

Ipomeamarone (1.23 mg) eluted from chromatogram was taken up in 0.5% DMSO and added to 5 ml mycelial suspension of each fungus and incubated in specimen tubes at 25°. At intervals, three replicate cultures were extracted with chloroform-methanol (1:1) and taken up in 4 ml ethanol and ipomeamarone content determined quantitatively (Materials and Methods).

The amounts of ipomeamarone recovered at given intervals are shown in Fig. 41 and the percentage recovery stated in Table 29. As for the total terpenes, B.t. rapidly reduced the concentration of ipomeamarone supplied in culture. The reduction was almost linear with time for the 1st 8h of incubation and at the end of 24h, only 0.4% of the compound was recovered.

There was some loss of the compound in the B.c. culture though this was far less significant than for B.t. 73% of the compound was recovered after 24h. With C.c. culture - 94% of the total compound added was recovered after 24h.

It appears, from our present findings, that phytoalexin degradation is an important feature in the successful rotting of sweet potato by B.t. and that the failure of B.c. and C.c. to rot the tubers arises from

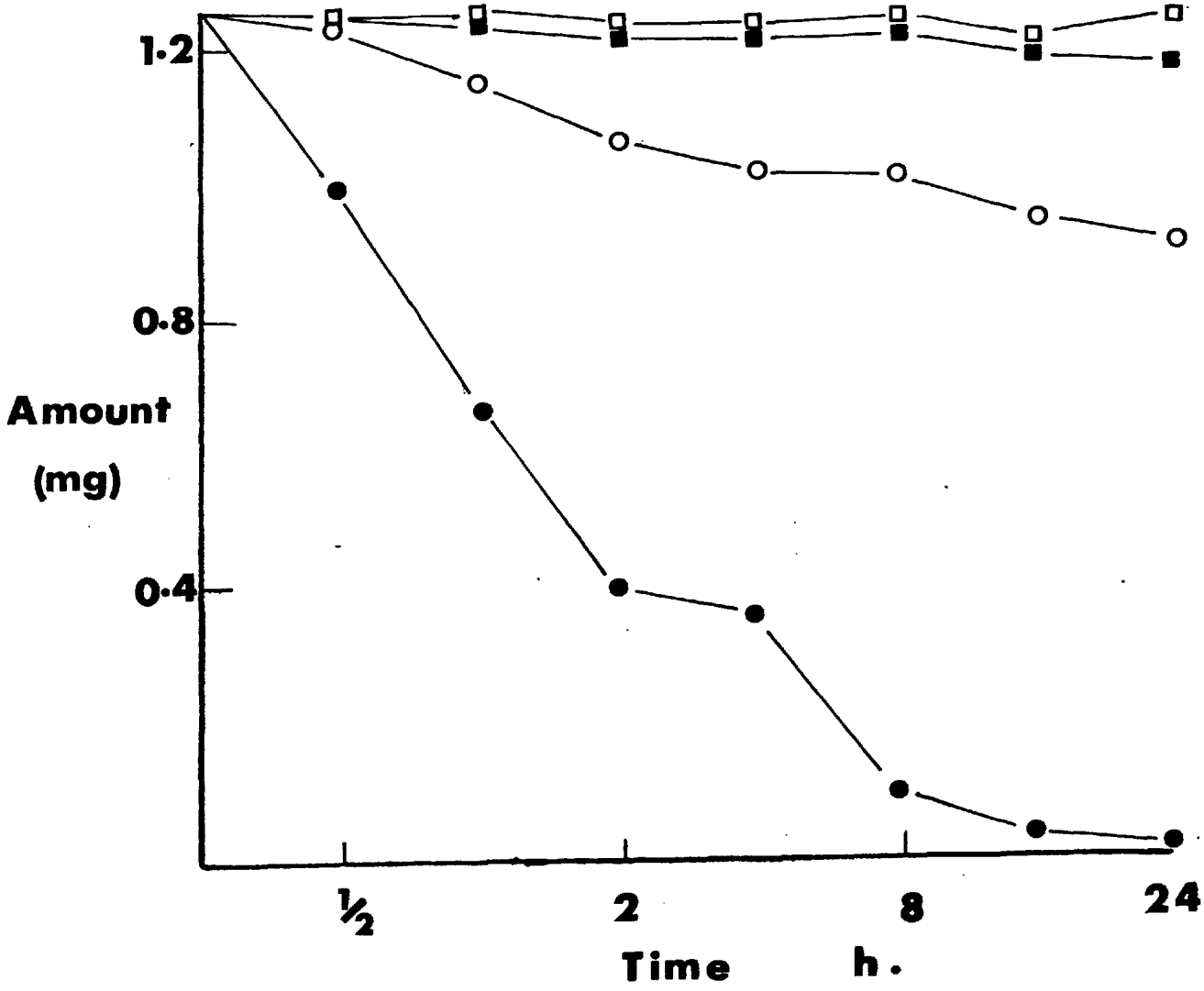


Fig. 41. Amounts of ipomeamarone recovered at time intervals during incubation with B.t., B.c. and C.c.
●——● B.t., o——o B.c., ■——■ C.c., □——□ Control

their inhibition by the phytoalexins which they are poorly able to degrade.

V Effect of chilling injury on ipomeamarone content of sweet potato

From the results obtained earlier (Tables 18-21) it is clear that storage of sweet potato at temperatures below 7°C reduced the resistance of the tubers to infection by pathogens and non pathogens alike. Ipomeamarone content of the tissue after pretreatment at low temperature (3°) was estimated to see if there was any effect of chilling on the ability of the tuber to produce the phytoalexin.

Slices were pretreated at 3°, 15°, 25° for 48h and then inoculated with B.t. and B.c. before incubating for 24h at 25°, r.h. 85% in a desiccator connected to an air pump. Rotted tissues were extracted with chloroform-methanol (1:1) and taken up in ethanol. The ethanolic extract was separated by chromatography (TLC) and ipomeamarone content determined (Materials and Methods).

Results (Table 30) showed that up to five times more ipomeamarone was induced by B.t. and B.c. after pretreating at 15° or 25° than at 3°C. The same amount of the phytoalexin was induced at 25° and 15°C.

Table 30. Effect of low temperature treatment of sweet potato on ipomeamarone accumulation.

	Pretreatment temperature		
Inoculum	3°	15°	25°
B.t.	0.29	1.8	1.70
B.c.	0.65	3.5	3.5

Ipomeamarone content expressed as mg/g fresh weight tissue

D I S C U S S I O N

In many plant disease situations, a pathogen causes infection by penetrating and at some stage dissolving the cell walls of the host tissue. Pectic enzymes have been implicated in a number of these diseases and have become an important feature of host-parasite relations. These enzymes are most important in diseases involving parenchymatous tissue where the middle lamella is known to consist essentially of pectic materials.

Botryodiplodia theobromae produced in culture four inducible isoenzymes of PG with isoelectric points at pH 4.1, 4.3, 5.1 and 5.8 These enzymes have a similar pattern of degradation of sodium polypectate (endo type) but degraded the substrate at different rates. None of the isoenzymes was exo PG. Bateman (1972) showed that Sclerotium rolfsii produced a PG complex and Cervone et al (1977) recently isolated two forms of PG from Rhizoctonia fragariae. Swinburne and Corden (1967) working on Fusarium oxysporum f sp lycopersici and Tani and Nanba (1969) on Botrytis cinerea had earlier shown that polymorphism in fact occurs in all the pectic enzymes produced by these fungi. It was further indicated that different isolates of Erwinia chrysanthemi may vary in the number of components of a givenpectic enzyme they produce (Garibaldi and Bateman, 1971).

While it is now certain that pectic enzymes have a role in pathogenesis, it is not clear what contribution the various isoenzymes make in this respect. In this work, PG 3 and PG 4 produced by B. theobromae caused tissue maceration, electrolyte loss and cell death of sweet potato. PG 1 neither macerated, killed nor caused electrolyte loss from sweet potato tissue. Within the 5h period, PG 2 caused substantial electrolyte loss, slight death of the cells but did not macerate the tissue at all. Similar results were obtained by Garibaldi and Bateman (1971) who showed that with the exception of one, all the PTE isoenzymes of Erwinia chrysanthemi caused electrolyte loss, cell death and maceration of potato tissue. The exception, although it readily degraded pectic substances in culture apparently had no effect on the tissue.

The isoenzymes of B.t. which macerated sweet potato also macerated and killed potato tissue. Not all isoenzymes behave in this way. Tani and Nanba (1969) showed that the pectic enzymes of B. cinerea differed in their ability to macerate tissues of different plant species. Again Garibaldi and Bateman (1971) demonstrated that among the PTE isoenzymes of E. chrysanthemi, there were differences in the susceptibility of different tissues to maceration; some macerated potato, carrots and cucumber readily while one had little effect on carrots but readily macerated potato and cucumber.

The type of discrimination mentioned above must be important in the host specificity of the pathogens. Cellular recognition of host tissue by enzymes of the pathogen has been suggested as a basis for pathogenesis. This recognition is tested for by the ability of the enzyme to bind unto the surface of the tissue. In this work, PG 3 (PI, 5.1) obtained from rot extract was tested for its binding capacity on several tissues and results showed that the enzyme was adsorbed on all the tissues to varying degrees. Cervone et al. (1977) showed that two isoenzymes of Rhizoctonia fragariae showed specificity in binding for strawberry tissues which the fungus is specific in attacking. Since B. theobromae could under favourable conditions infect all the tissues used for binding, it was difficult to assess selective binding and still more difficult to attribute cellular recognition with pathogenesis. Perhaps the ability of PG 3 to bind on to a wide variety of tissues could account for the wide host range of the fungus. However, until tissues resistant to infection by the fungus are tested we cannot be absolutely positive about the relation between the binding phenomenon and pathogenecity of B. theobromae.

An interesting aspect is that whereas the purified isoenzymes macerated and killed sweet potato discs, they did not bring about the same effect in intact whole tubers. The crude rot extract however caused a limited lesion (probably a hypersensitive response) around the inoculation point. This

suggests that perhaps a toxic factor was present in the crude extract or that a coaction of the enzymes was involved in nature.

Of the four isoenzymes of B. theobromae, only one, PG 3, was detected in rotted sweet potato. It is not certain whether the other isoenzymes were inhibited or inactivated during infection. Possibly they were denatured during the process of extraction. Byrde et al. (1971) recovered in vivo two of the four PG isoenzymes of Sclerotinia fructigena produced in culture - and of the two components of ∝-L-arabinofuranosidase produced in vitro only one was detected in vivo. It is now recognised that a complicating factor in detection of pectic enzymes in tissues is the susceptibility of the enzymes to inactivation by the host's constituents. Chief among these are the polyphenols and/or their oxidation products. The PG isoenzyme obtained from rotted sweet potato, however, did have the ability to macerate and kill the same tissue.

Compared with uninoculated tissue there was a marked accumulation of total phenols and the oxidative enzymes in the rotted tissue. In their study on the rot of sweet potato by Ceratocystis fimbriata, Uritani et al. (1959) showed that the infected tissue accumulated a number of polyphenols and cuomarin derivatives both in the lesion and in tissues surrounding it. They speculated that these are metabolic responses geared towards resisting infection by the parasite. In this study, the significance of phenolic oxidation was demonstrated from the inhibitory action of

of aqueous extracts of sweet potato; these extracts were obtained with and without antioxidants. Extracts of rotted tissue in the absence of antioxidant caused more than twice as much inhibition of PG than extracts obtained with antioxidants. Healthy tissue extracted without antioxidant also caused more than twice as much inhibition as that extracted with antioxidant. Byrde et al. (1960) demonstrated that catechol, chlorogenic acid, catechin and epicatechin which had little or not effect before oxidation caused substantial inhibition of Sclerotinia fructigena PG after oxidation has occurred. Increased rates of respiration have also been shown to occur in sweet potato inoculated with C. fimbriata (Akazawa and Uritani, 1955). The nature of the changes which precipitate the increase are not defined but such factors as the synthesis of phenols and the build up of antifungal compounds in and around the infection court can account for increased respiration. Furthermore, the respiratory changes may involve the action of such oxidases as phenol oxidases and peroxidases which were shown here to accumulate in and around lesions.

Despite the above, the role of the phenols which accumulated during infection is not clear, partly because their degree of oxidation in rotted tissue is uncertain. Their oxidation products are known to inhibit PG in vitro.

Nevertheless, one PG isoenzyme can be recovered from rot extracts and the fungus manages to rot the tissue successfully. There are several possibilities to account for this success. Oxidation of the accumulated phenols may not occur to any great extent in zones 2 and 3, notwithstanding the high levels of oxidative enzymes in zone 3. It is on conversion of zone 3 to zone 2 as the rot progresses that one might expect the obvious high potential for phenol oxidation of zone 3 to be expressed. However, PG activity in zone 2 is high and though phenol content is still fairly high, oxidative enzyme activity has fallen, especially phenol oxidase. In addition, zone 2 tissue did not discolour rapidly on exposure to air (unlike zone 3). Phenol oxidation could in fact be inhibited by a dehydrogenase of the pathogen in the manner demonstrated by Lovrekovich et al. (1967) for Erwinia carotovora in potato or else the oxidative enzymes themselves may be inactivated by a product of the pathogen. It is also possible that the fungus can convert some of the phenols to inactive products as suggested by a lower phenol content in zone 1. This could however be due to insolubilization of phenols by their oxidation and polymerization. It would appear, in fact that phenol oxidation (and thus discolouration) had belatedly occurred in zone 1 and caused a marked fall in PG activity found in this zone.

The spatial distribution of PG, phenols, peroxidase and phenol oxidase shows that the host tissue makes a remarkable metabolic response to infection at the periphery of the lesion. The interplay of host's metabolites and fungal PG at this zone must be crucial in determining successful infection. Together with the factors, the rate of PG production may exceed the rate of its inactivation thus allowing maceration and cell death to occur in spite of the host's defence mechanisms.

One may ask, if the non-pathogens produce PG, why do they not rot the tissue. A comparison of Tables 11, 13, 14, 15 and 16 shows that a much greater amount of PG activity was detected in the rot zones caused by B.thgobromae than in the corresponding zones of the tissues infected with the other fungi. In fact, PG activity was not detected in zone 3 of the tissues infected with any of the other tested fungi. Presumably the rate of PG induction in vivo is lower than the rate of inactivation by the host's metabolites. Although C. cucumerinum is known to produce pectic enzymes in culture (Kuć, 1962), no pectic enzyme was obtained from tissues inoculated with the fungus nor was the fungus established to any significant degree to cause lesion. Enzyme repression must be more important than its induction in this situation.

In all cases, there was most accumulation of total phenols in tissues rotted least by the fungi (compare Tables 11,

13, 14, 15 & 16. The only exception was with tissues inoculated with C. cucumerinum where the value for total phenols compared with values for corresponding part of lesions caused by B. theobromae and were in fact less. This can be accounted for by the fact that the interaction between the pathogen, C.c., and the host failed to set off the biochemical reactions which would lead to higher accumulation of phenols. Deveral and Wood (1961) suggested that phenols are released following death of cells. The pectic enzymes secreted by C.c.in vivo (if at all) did not accumulate in sufficient quantity to trigger off the sequence of reactions which would result in phenol build up. The death of cells of the inoculation point could have resulted in the phenols recovered around this zone.

In explaining the susceptibility of sweet potato to B. theobromae and its resistance to B. cinerea and C. cucumerinum, there is a possibility of interactions between pectic enzyme induction and repression, release of wall bound enzymes (e.g. the oxidases) and the phenols. The dyanamic interaction between these and the parasite would determine which direction the scale would tip - susceptibility or resistance.

One aspect of sweet potato storage is the reaction of the tubers to temperature below $10^{o}C$. Most tubers would tolerate storage temperatures less than $10^{o}C$; potato, in fact, is best stored at $4-5^{o}C$. Treatment of sweet potato at such low

temperatures predispose them to rot. A knowledge of the critical temperature below which storage becomes poor is desirable. This is specially significant for shipment of the roots across long distances during the winter months when temperatures do drop even below freezing.

Results obtained here showed that although tubers exposed to 5°C for 48h do not appear to have deteriorated they become more susceptible to infection by B. theobromae and even to the usual poor pathogens, B. cinerea and C. cucumerinum. This is in agreement with the finding of McClure (1959) that chilled sweet potato was more readily rotted by Rhizopus stolonifer than non-chilled ones.

Chilling alone at 3 - 7°C neither killed nor macerated the tissues but increased the rate at which the PG from B. theobromae macerated and killed the tissues. It appears that there must be some permanent biochemical change at 0 - 7°C which predispose the tubers to easy breakdown by the pathogen's pectic enzymes. An interesting question here is how could maceration (a phenomenon due to effects on cell walls) be affected by temperature. It looks like this is an indirect evidence of a direct action of PG on protoplasts.

Low temperature treatment also affected the ability of the tubers to secrete ipomeamarone in response to infection by B.t. and B.c. Up to five times more ipomeamarone was produced after pretreatment at 15° or 25° than at 3°.

Cruickshank and Perrin (1963) showed that resistance of pea pods to M. fructicola and their capacity to form pisatin was lost within 6 days after storage at 20°C while at 4°C, it was retained for up to 27 days. Thus, whereas pea pod requires chilling temperature for optimum formation of pisatin, sweet potato requires a higher temperature for ipomeamarone production. It appears that in providing "proper" environmental conditions for sweet potato storage (temp, 13-16°; r.h. 80-90%; aerobic condition) we are in effect creating proper conditions for optimum production of antifungal compounds i.e. optimum conditions for resistance.

Several points emerged from the reaction of sweet potato inoculated with B. theobromae, B. cinerea and C. cucumerinum with respect to phytoalexin production. Firstly, all the three fungi were capable of inducing the production of the same fungitoxic compounds in sweet potato. This confirms reports that phytoalexin induction in sweet potato is non-specific and that induction can even be generated by non-biological stimulation e.g. wounding and the use of some chemicals at appropriate concentrations (Uritani et al., 1960). However such non-biological stimulations do not lead to accumulation of compounds at levels inhibitory to the pathogens. All the fungi tested here induced levels of phytoalexin which were inhibitory to the growth of B.c. and C.c. in vitro.

The spatial distribution of the phytoalexins in the lesions caused by the fungi showed that there was accumulation

both at the centre and at periphery of lesion. Contrary to claims that phytoalexins may be bound within dead cells, (Sato and Tomiyama, 1969; Bailey and Deverall, 1971), evidence here shows that tissues up to 1cm from the dead cells contained phytoalexins at up to toxic levels. This is not surprising since earlier production of phytoalexins during the drop diffusate techmique did not involve killing of cells (Muller, 1956; Smith, 1970). It appears that the death of cells as known in hypersensitive reactions is not a pre-requisite for phytoalexin accumulation in sweet potato.

Having shown above that B.t., B.c. and C.c. all induce phytoalexin accumulation the question to be answered is how are they implicated in the resistant and susceptible situations involving the fungi. Generally, B.t.(the virulent pathogen) was much less sensitive to the phytoalexins than B.c (the weak pathogen) and C.c. (the non-pathogen). C.c. was more sensitive to the compounds than B.c. Considering that the amounts of the compounds tested were far less than the observed tissue concentration, it is not surprising that the tissue is resistant to attack by B.c. and C.c. Cruickshank and Perrin (1960) reported that M. fructicola, a non-pathogen of pea was much more sensitive to pisatin induced in pea than Aschyta pisi, a pathogen of pea. Wyerone acid has also been shown to be more active against B. cinerea than against the more aggressive B. fabae, having much more effect on growth

of germ tubes and hyphae than on spore germination (Mansfield, 1972).

B. theobromae degraded ipomeamarone and the total terpene extract of sweet potato in vitro within 24h of incubation. There was comparatively little degradation by B. cinerea. C. cucumerinum did not degrade the compounds. This agrees with the findings of Higgins and Millar (1969) that Stemphylium botryosum and Helminthosporium turcicum are capable of inducing medicarpin in alfalfa but that S. botryosum (the pathogen) and not H. turcicum (the non-pathogen) could degrade the phytoalexin. The phytoalexin induced by S. botryosum did not accumulate in the infection court because it was degraded by the fungus as rapidly as it was formed. The spatial distribution of the compounds in the different zones caused by B.t. and B.c. shows that less of the compounds were found in the older parts of lesion caused by B.t. than the corresponding parts caused by B.c. The compounds did accumulate at a greater rate than the degradation but accumulation did not reach levels toxic to B.t.

Insensitivity and phytoalexin degradation accounted for variation in the ability of B.t., B.c. and C.c. to rot sweet potato. The results compare to the findings of Nonaka (1967) Uehara (1964) and Christenton (1969) that non pathogens of pea either do not degrade pisatin or do so more slowly than the pathogens. Furthermore the difference in the virulence between

Botrytis fabae and B. cinerea on broad bean has now been accounted for by the differences in their ability to detoxify the phytoalexins in the host (Mansfield, 1972). Cruickshank and Perrin (1963) showed that when infected with Septoria pisi, the pea produced only 10 µg/ml of pisatin in the infected tissue whereas other fungi (non pathogens) induced as much as 116 µg/ml. In the light of present investigations, we might very well now question whether Septoria was not in fact degrading the phytoalexin, thus reducing the concentration in the tissue.

Undoubtedly, there must be a complex interaction of the host's metabolites in the overall resistance of sweet potato to infection. The increase in the level of phenols and their derivatives (shown to occur in sweet potato) may be important here. Perhaps there is an interaction (additive or synergistic) between these and the furanoterpenoids in the total defence mechanism. Sweet potato produces these compounds nonspecifically. Its susceptibility or resistance to a pathogen must be seen both from sensitivity of the pathogen to the toxic compounds or from its ability to degrade or detoxify them.

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