MECHANISMS OF PATHOGENESIS OF SCLEROTINIA SCLEROTIORUM ON TOMATO

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A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Faculty of Science in the University of London

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SEPTEMBER 1983

ABSTRACT

Infection of tomato (Lycopersicon esculentum) by Sclerotinia sclerotiorum resulted in tissue maceration and development of a watery soft rot. Penetration of host tissue was mainly by mechanical rupture of the cuticle by both simple and multicellular appressoria. After penetration, the fungus produced hyphae which grew both intercellularly and intracellularly throughout the infected tissues. Stem tissue was highly susceptible to the fungus during the first 3 to 4 weeks of growth but the plants became more resistant with age. The resistance was associated with the formation of a dark brown zone between healthy and infected tissues which appeared to limit the lesion expansion. Phenolic compounds accumulated within the lesion and at its darkened boundary.

Extracts from diseased stems contained the following enzymes: polygalacturonase, pectinmethylesterase, arabanase, xylanase, carboxymethlycellulase and α-L arabinofuranosidase. Except for pectinmethylesterase, none of these enzymes could be detected in healthy stem tissues. The same range of enzymes was also produced by the fungus *in vitro* when grown on sodium polypectate or pectin as the sole carbon source. Synthesis of those enzymes tested was sensitive to catabolite repression by glucose. The pH optima for the activity of the enzymes were in the range 3 - 5 and their production *in vitro* was optimal at pH 6. The enzymes produced *in vitro* and those produced *in vivo* were similar based on evidence obtained by isoelectric focusing, gel filtration and chromatography of reaction products. Polygalacturonase, arabanase and/or α-L arabinofuranosidase were able to degrade isolated stem cell walls. Their abilities to do this decreased with increasing age of the host plant from which the walls were extracted.

Compared with the other enzymes, polygalacturonase showed the greatest activities both *in vivo* and *in vitro*. This enzyme was capable of causing tissue

maceration, cell death and cell leakage. Its macerating ability synergised by oxalic acid and its activity was not affected by the protein inhibitors extracted from tomato stems or by the crude plant extract. Different fractions of electrofocused extracts of infected tissues exhibited both inhibitory and stimulatory effects on the enzyme activity. Phenolic extracts from healthy and diseased stems of 4- and 8-week plants partially inhibited polygalacturonase Those extracts obtained from the infected stems of the older plants were the most inhibitory.

ABBREVIATIONS

c.	circa
cm	centimetre
diam.	diameter
g	gram(s)
h	hour(s)
l	litre(s)
µg	microgram(s)
µl	microlitre(s)
M	molar
m	meter
mA	milliampere(s)
mg	milligram(s)
min.	minute(s)
ml	millilitre
mm	millimetre(s)
mM	millimolar
nm	nanometre
pI	isoelectric point
ppm	parts per million
p.s.i.	pounds per square inch
Rg	Rglucose
t.l.c.	thin-layer chromatography
uv	ultra-violet
v or vol.	volume
V	volt(s)
w	weight

Abbreviations for the cell wall-degrading enzymes are given in the text.

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GENERAL INTRODUCTION

1. The fungus (Sclerotinia sclerotiorum)

1.1 Taxonomy and nomenclature

The fungus was first described by Madame M. A. Libert (1837) as Peziza sclerotiorum. In 1870 Fuckel described the genus Sclerotinia and renamed Peziza sclerotiorum with a new binomial, Sclerotinia libertiana. As this binomial was inconsistent with the International Rules of Botanical Nomenclature Wakefield (1924) cited G. E. Massee as the proper authority for S. sclerotiorum (Lib.) Massee because he had used that binomial in 1895. Since de Bary used the same binomial as early as 1884 (De Bary 1884), he should be cited as the proper authority for this fungus. S. sclerotiorum (Lib.) de Bary was designated as the type species of Sclerotinia by Whetzel (1945), by Korf and Dumont (1972) and by Buchwald and Neergaard (1973, 1976). Although Eriksson in 1880 described the pathogen of clover stem rot as Sclerotinia trifoliorum (Erikss.) and Jaggar in 1920 described the small-sclerotial type which had been isolated from lettuce, celery and other crops in several locations in New York State and from lettuce in Sanford, Florida as Sclerotinia minor, these two pathogens were considered by Smith (1900) to be identical with Sclerotinia libertiana (S. sclerotiorum). Similarly, Purdy (1955); Walker (1969); Morrall et al., (1972) and Price and Colhoun (1975) included the two fungi in the one species Sclerotinia sclerotiorum.

Other less important species of Sclerotinia include: S. intermedia Ramsey; S. serica Keay; S. sativa Drayton and Groves and S. trifoliorum Eriks. var. fabae Keay (Purdy 1979).

Because of their wide distribution and great economic importance, S. sclerotiorum, S. trifoliorum and S. minor have been studied extensively (Willetts and Wong 1980). The taxonomic positions of these three species have

been assigned on the basis of traditional morphological and physiological criteria such as gross cultural charteristics, sclerotial size, ascus and ascospore dimensions, time of apothecial development in the field and host association (Jaggar 1920, Whetzel 1945, Buchwald and Neergaard 1973). According to Willetts and Wong (1980), S. minor and S. sclerotiorum had similar host ranges including vegetable and forage legumes. These authors concluded that host association was not a reliable criterion for recognition of the three species. Although the sclerotial size has been an important characteristic in the taxonomy of Sclerotinia species, Purdy (1955) reviewed the relevant earlier literature and observed that the size ranges for the sclerotia of the three species overlapped and there was a continous intergradation in sclerotial diameter from the small ones of S. minor (0.25 to 5 mm) to the larger ones of S. trifoliorum (0.3 to 10 mm) and S. sclerotiorum (1 to 30 mm). Similarly, he could not distinguish the asci or ascospores of anyone species from those of the others when he compared his measurements with those given in the original descriptions. Purdy (1955) again showed that the measurements of the sclerotia, asci and ascospores obtained by Ramsey (1924) and Drayton and Groves (1943) for S. intermedia and S. sativa respectively were within the same range as those given by other workers for S. sclerotiorum, S. intermedia and S. minor. He, therefore, concluded that the differences in the sizes of the sclerotia, asci or ascospores were unreliable criteria and could not be considered to have specific value. Wong and Willetts (1973) used polyacrylamide gel electrophoresis to study 15 isolates, four of which were identified as S. minor, four as S. trifoliorum and 7 as S. sclerotiorum. They reported that, the electrophoretic patterns for soluble proteins, aromatic esterase, acid phosphatase, glucose-6-phosphate dehydrogenase (NADP-linked), NADP dehydrogenase and malate dehydrogenase (NAD) separated the isolates into three distinct groups with only small intergroup differences. Similar results were obtained when isolates from a wider variety of crops were studied (Wong and Willetts 1975b) and when cytological studies were carried out (Wong and Willetts 1979). The grouping

of the isolates agreed with their findings when sclerotial ontogenesis (Willetts and Wong 1971) and mycelial interaction (Wong and Willetts 1975) were used as criteria. They concluded that the three groups corresponded to three distinct species, S. minor, S. trifoliorum and S. sclerotiorum. All three belong to the family Sclerotiniaceae (Whetzel 1945) in the class Discomycetes.

1.2 Geographic distribution and economic importance

S. sclerotiorum, S. trifoliorum and S. minor are worldwide in distribution but they are most common in temperate regions (Reichert 1958). S. sclerotiorum has been reported from many countries located in all continents (Purdy 1979). According to Chamberlain, (1932), sclerotium disease (also known as Sclerotinia disease or sclerotial disease) was a serious disease of tomatoes grown out of doors in New Zealand. It occurred in epiphytotic form on various hosts in the years 1919, 1923 and 1927, being particularly severe on tomato in 1923. In a more recent report in the literature (Purdy 1979) the average annual losses due to S. sclerotiorum in potatoes and tomatoes in Florida were in the ranges 25 - 50% and 4 - 6% respectively. Cook et al., (1972) reported that estimates of yield loss in beans in Western Nebraska averaged 9% in 1970 and 1971. According to Lumsden et al., (1975), 20% of the dry bean crop was lost in 1973 due to S. sclerotiorum. Wide spread losses have been reported in Alberta, California, Ontario and Japan on crops such as beans, rape, potatoes and tomatoes (Jones 1976). The measured losses from lettuce drop reported by Beach (1921) during a 3-year period in Philadelphia county, are given in Table (1) below.

1.3 Host range

S. sclerotiorum and S. minor have wide host ranges including both vegetable and forage legumes, (Wong and Willetts , 1975a, 1975b). Legumes

have been reported as the main hosts of S. trifoliorum (Purdy 1955). According to Purdy (1979) (personal communication from P. B. Adams), the host range of S. sclerotiorum comprises 64 plant families, 225 genera, 361 species and 22 other (cultivars etc) for a total of 383 species and other categories. The main hosts of S. sclerotiorum include plants of the families Solanaceae Cruciferae, Umbelliferae, Compositae, Chenopodiaceae and Leguminoseae (Willetts and Wong 1980). Reported hosts in the family Solanaceae span 19 species in 10 genera. Price and Colhoun (1975), inoculated eleven species of host plants with 19 isolates of S. sclerotiorum and found that tomato (cv. Orange Sunrise) was the most susceptible host. These authors did not find evidence for physiologic specialization of this pathogen. In general, S. sclerotiorum appears to be among the most nonspecific, omnivorous and successful of plant pathogens.

TABLE (1)

Losses from lettuce drop during a 3-year period in Philadelphia county

Year	Month	% Disease
1918	June	18
	October	75
1919	June	8
	October	17
1920	June	40
	October	17

1.4 Symptoms of some of the important diseases caused by S. sclerotiorum, S. trifoliorum and S. minor

(a) Lettuce drop

This disease was recorded in the late 1890's on glasshouse lettuce in Massachusetts and it was attributed to a small-sclerotial type of S. sclerotiorum (Smith, 1900). However, the disease apparently was not attributed to S. minor until 1920 (Jagger 1920). Several authors reported infection of lettuce by sclerotial mycelium (Smith, 1900; Adam and Tate, 1975; Purdy, 1952) , but Newton and Sequira (1972), reported infection by ascospores. As mentioned by Purdy (1979) infection of lettuce takes place often at the leaf base and on the stem at or near the soil line. The infected outer leaves wilt and lay flat on the soil surface while the heart leaves remain erect. A soft watery decay follows and subsequently white mycelium and black sclerotia are produced on, in and around infected plants.

(b) Clover stem rot

The first report of clover stem rot in the United States was in 1890 but the disease was known in Germany in 1857 (Wolf and Cromwell 1919). Although Smith considered the causal fungus to be S. libertiana (S. sclerotiorum) (Wolf and Cromwell 1919), Eriksson in 1880 described the pathogen as S. trifoliorum. According to Purdy (1979), the symptoms of the disease are as follows: the infected leaves become yellow, die and fall to the soil and from them the fungus infects the stem; the stems decay and a watery soft rot develops. White mycelium may develop on the infected tissues and on soil where the rotted plant parts lay and the sclerotia form in the mycelial mass.

(c) Bean white mold

White mold is a disease usually initiated by wind-borne ascospores (Natti , 1971). Perennial mycelium and mycelium from sclerotia in the soil are less important sources. (Blodgett, 1946; Moore et al., 1949; Vaughn and Dana, 1949). Cook et al., (1972) reported that infection of beans in Western Nebraska caused by sclerotia as a primary inoculum. According to Natti (1971), the first symptoms consist of brown water-soaked spots in the axillary tissues of the main stem and lower branches of plants at a postbloom stage of development. Primary infections are caused by invasion of plant tissues by mycelium of the causal fungus that emerged from cast blossoms lodged in the branch axils. The infection usually progresses slowly in the main stem but advances rapidly into the branches. Leaves of infected branches turn yellow, wilt and are eventually shed, leaving dry, light-brown spurs of naked branches and petioles. Frequently, sclerotia and small white tufts of mycelium are scattered along the length of the dead branches.

Pods are invaded by mycelium that advances from infected branches , by contact with the soil in which mycelium from infected abscised leaves resides, or by mycelium originating from ascospores that germinate and advance through nonliving plant parts, i.e. dead blossoms, into the pods (Purdy 1979).

(d) Sunflower stalk rot

This disease is highly destructive in the largest sunflower seed producing countries, namely, the Soviet Union, Argentina, and Romania as well as in the United States (Orellana, 1975). Jones (1923) stated that stalk rot of sunflower was caused by S. libertiana

(S. sclerotiorum). According to Morris and Swingle (1921), infection probably occurs from ascospores and sclerotia in soil. Lesions develop on the stem at the soil line. The bark around the crown and root is killed. The leaves become yellow and die, infection spreads up the stalk and eventually the whole plant dies, only vascular tissue and disorganized parenchyma remain. Flowers or the entire flower head are susceptible to infection by ascospores. A white mycelium develops and sclerotia are formed on the plant surface and also in the pith cavity of the stalk (Purdy 1979).

1.5 Life cycle of S. sclerotiorum

A generalized life cycle for the Sclerotinia spp is shown in Fig. (1). The fungus survives from one favourable crop period to another as sclerotia in the soil (Walker 1957). Primary inoculum consists in part of mycelium arising from sclerotia (Purdy, 1952), or from infected tissues (Abawi et al., 1975) but primarily of ascospores discharged from apothecia into the air (Cook et al., 1975).

1.5.1 Production of the sclerotia

Production of the sclerotia is influenced by several nutritional and non-nutritional factors. According to Purdy and Grogan, (1954), growth and sclerotial formation of S. sclerotiorum occurred only when P, K, Mg and S were added to the medium and it was enhanced by the addition of inorganic micronutrients. Vega and Le Tourneau, (1974) reported the importance of Zn in the growth medium for production of the sclerotia. The C/N ratio and the form of nitrogen in relation to the C/N ratio may also affect sclerotium production (Markukaw et al., 1975). Bedi (1963) found that the sclerotia grown on solid media of high nutrient content were almost or completely sterile with

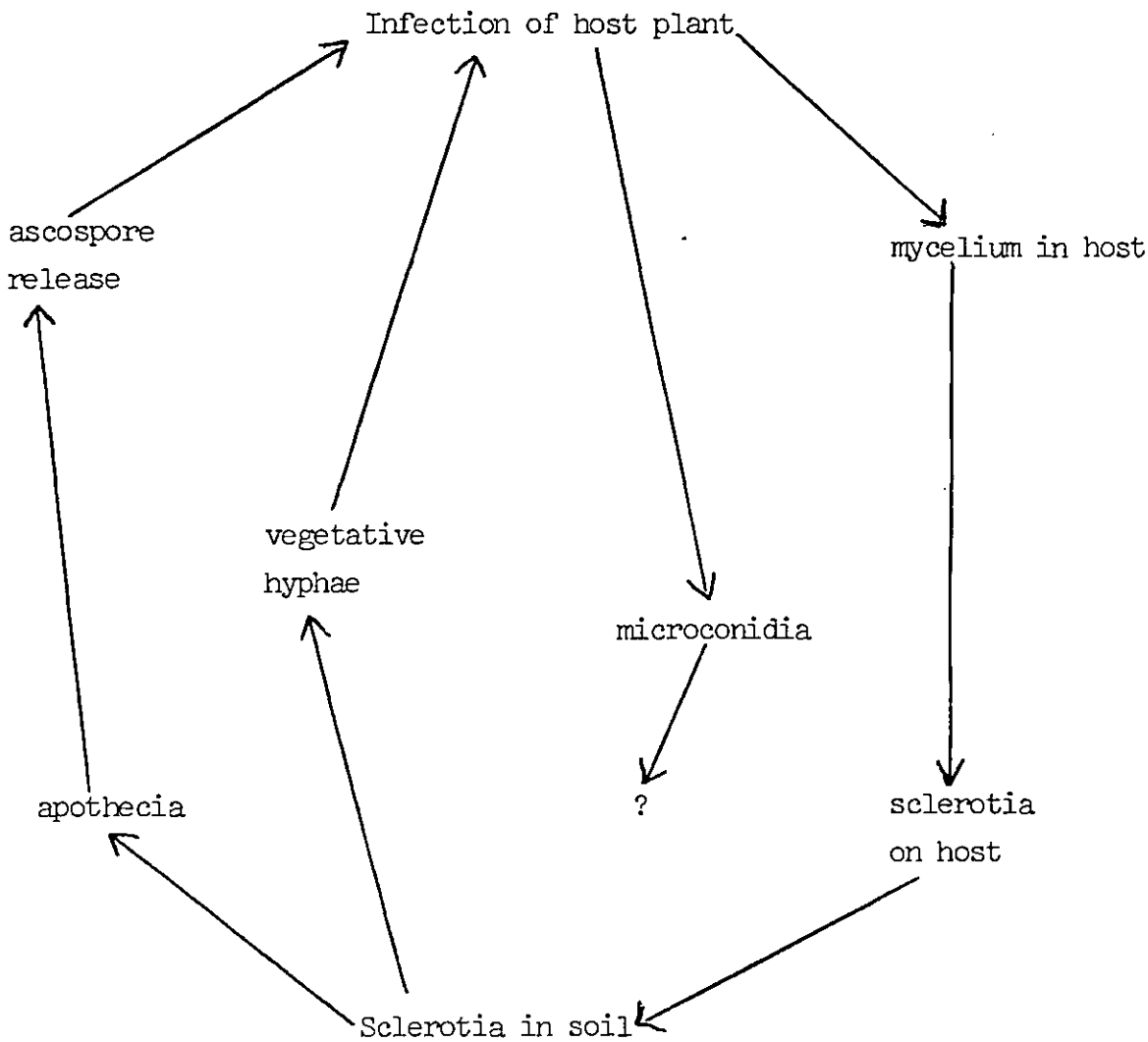


FIG. (1) Life cycle of Sclerotinia spp. (Willetts and Wong, 1980)

respect to the potential for apothecial production, while those cultured on media of optimal nutritive value for fungal growth were very fertile. Other factors that have been suggested as contributing to the induction of the sclerotia include: light, temperature, H-ion concentration of the substrate, and moisture. According to Le Tourneau (1979), production of the sclerotia can occur over a temperature range from near 0°C to 30°C. In studies on the effect of moisture levels on sclerotial production, Grogan and Abawi, (1975), found that sclerotia developed at -65 bars but not at -73 bars. More sclerotia were produced in the light than in the dark, (Humpherson-Jones and Cooke,1977). Marukawa et al., (1975) reported the production of sclerotia over a wide initial pH range (2.5 - 9).

1.5.2 Germination of the sclerotia

The sclerotia may germinate by producing apothecia (carpogenic germination) or mycelium (myceliogenic germination).

(i) Carpogenic germination

Apothecia are most readily produced in moderately cool climates when soil moisture is adequate (Walker, 1957). The optimum temperature range for incubation of the sclerotia for apothecial production is 10°C - 20°C, (Hawthorne, 1976; Saito, 1977; Lane and Sproston, 1955). According to Morrall, (1977) apothecia were produced at several water potentials in the range 0 to -7.5 bars but not below -7.5 bars. Drying of sclerotia greatly delays or possibly completely inhibits apothecial production (Abawi and Grogan, 1975). Henson, (1940), reported that differentiation of the stipes into disk and the full expansion of the apothecia took place only in light. According to Honda and Yunoki, (1977), light below 390 nm is effective in the induction of apothecium

formation. Details of apothecial genesis, ascospore production and cellular structures of the apothecia, asci and ascospores have been reported by several investigators (Saito, 1973; Kosasih and Willetts, 1975 ; Codron, 1974; Tu and Colotelo, 1973 and Jones, 1974).

(ii) Myceliogenic germination

According to Adams and Tate, (1976), there are two types of myceliogenic germination: (a) hyphal germination and (b) eruptive mycelial germination. Hyphal germination was observed as individual hyphae which emerged through the rind of the sclerotia and was common on moist sponge or sand (Saito, 1977) or when sclerotia were incubated in distilled water (Purdy 1958). Eruptive mycelial germination is distinguished by dense mycelium erupting from the sclerotium and it can occur in or on natural soil, on filter paper and occasionally on agar media. This type of germination was observed with the small sclerotial isolates (that is S. minor), (Adams and Tate, 1976).

1.5.3 Infection of susceptible host plant

If sufficient moisture is available, germination of ascospores can take place 4 to 6 hours after their release from the apothecium (Newton and Sequeira 1972). Optimum temperature for germination of the ascospores and growth of the fungus are 25°C (Abawi and Grogan, 1975) and 15 to 22°C (Willetts and Wong, 1980) respectively. Sclerotinia spp can infect susceptible hosts over a range of temperatures from 0 to 25°C with an optimum at 15 to 20°C (Chupp and Sherf, 1960). Moisture is essential for initiation and development of infection (Abawi and Sherf, 1975). The requirements of exogenous nutrients for infection of healthy tissues by S. sclerotiorum has been reported by several authors (Purdy and Bardin, 1953; Purdy, 1958; Natti, 1971). According to Purdy (1958), freshly

ejected ascospores of clover isolates penetrated directly and infected leaves of lettuce, broccoli, Brussels sprouts, ladino clover, red clover, broad bean and bean in 3-4 days, except for tomato which were infected only after 10-11 days. Ascospores of lettuce and tomato isolates infected only partially senescent bean leaves. Disorganization of cellular contents of guard cells prior to penetration was observed when ejected ascospores of clover isolates germinated 1-2 cells away from the stomatal opening. Although Jones, (1976) observed penetration of potato leaves via open stomata by mycelium of S. sclerotiorum, this mode of penetration seems to be rarely utilized. More usual is direct penetration of the host tissue from appressoria which maybe either simple or multicellular (Purdy, 1958; Abawi et al., 1975; Prior and Owen, 1964; Lumsden and Dow, 1973). The more complex multicellular appressoria grade in complexity into infection cushions. Upon contact with the host, the hyphal strands branch dichotomously to form a hand-like structure and the terminal cells become enlarged at the tips (Purdy 1958). The infection cushion initials develop into dome-shaped infection cushions, composed of three distinct types of hyphae: (i) dark-safranin-stainable hyphae near the top of the cushions; (ii) inflated, granular, light-safranin-stainable hyphae in the centre of the cushion and (iii) dichotomously branched penetration hyphae that stain approximately the same colour as the hyphae in the interior of the cushion (Lumsden and Dow, 1973). Multicellular appressoria are variously referred to as large appressoria (Boyle, 1921), appressorial masses (Purdy, 1958);cushion-shaped appressoria (Abawi et al., 1975) or infection cushions (Prior and Owen, 1964; Lumsden and Dow, 1973). The formation of the appressoria requires mechanical contact (De Bary, 1896; Abawi, et al., 1975; Purdy, 1958). The infection cushions adhere tightly to the host surface by means of a mucilage (Boyle, 1921).

Prior and Owen (1964) found that among the methods of entry of S. trifoliorum into forage legumes, the degradation of the cuticle and epidermis by enzymes produced from the infection cushion was most important. In contrast,

Lumsden and Dow (1973) studied the histopathology of infection of beans by S. sclerotiorum and S. minor and concluded that penetration was by means of multiple penetration pegs which forced their way through the cuticle and not by enzymic dissolution. According to Lumsden and Dow (1973), after penetration of the cuticle, inflated, granular vesicles were formed between the cuticle and epidermal cells and from them greatly inflated infection hyphae developed radially and invaded host tissue in an intercellular manner. The subcuticular hyphae oriented themselves parallel to one another, branched and formed an organized infection front beneath the cuticle. The radial hyphal front eventually broke into clusters of 18-20 hyphae, became oriented to the hypocotyl axis and developed more rapidly upward than downward or transversely. After colonization of the host tissue by the infection hyphae, small-diameter hyphae (ramifying hyphae) developed on the infection hyphae about 55 µm behind the hyphal tip. They branched and extensively invaded host tissue both inter- and intracellularly and they were thought to have the role of obtaining nutrients. After the fungus had become established within the host, the ramifying hyphae emerged, primarily through stomates or breaks in the cuticle. The emerging hyphae (Lumsden and Dow, 1973) eventually formed a cottony growth on the surface of mature lesion with the formation of the sclerotial initials which gave rise to mature sclerotia in 3-7 days.

1.6 Enzymes associated with disease development

1.6.1 Pectolytic enzymes

The involvement of cell wall-degrading enzymes in pathogenesis by S. sclerotiorum was originally suggested by de Bary (1886); others have reported a change in the composition of the cell walls of infected tissues, (Boyle, 1921; Hancock, 1966).

The characteristic symptom of infection of plants by S. sclerotiorum and related species is the maceration of tissues and development of a watery soft rot (Willetts and Wong 1980). The pectolytic enzymes are known to be responsible for tissue maceration and can damage cell membranes and kill cells, (Bateman and Basham, 1976). The association of these enzymes with diseases caused by Sclerotinia spp has been reported by Barkai-Golan (1974); Echandi and Walker, (1957); Hancock, (1966); Lumsden, (1976) and Marciano et al., (1982). Most studies have dealt with polygalacturonase produced in culture (Held,1955; Colotelo et al., 1971; Maxwell et al., 1972) and in diseased tissue (Barkai-Golan, 1974; Hancock, 1966; Morrall et al., 1972). Echandi and Walker (1957) reported the production of polygalacturonase and pectinmethylesterase on wheat bran. According to Lumsden (1976), S. sclerotiorum and S. minor produced two types of polygalacturonase: endo-polygalacturonase and exo-polygalacturonase. Both were secreted in culture on autoclaved bean stems, on sodium polypectate medium and in diseased tissue. The endo-polygalacturonase was produced as early as 12 hours after inoculation of bean plants and it was closely associated with the advancing margins of very young but not with the margins of older maturing lesions. This result suggested that the endo-polygalacturonase might be essential for successful ingress of the pathogen during the very early stages of pathogenesis and it probably contributed to hydrolysis of the middle lamellae one to two cells in advance of invading hyphae. The enzyme readily macerated susceptible bean and cucumber tissues but did not macerate potato tissue and its production was repressed by addition of glucose to the medium. The exo-polygalacturonase was detected 2 days after inoculation of diseased tissue and after 3-4 days in culture. Although possibly a mixture of exo-polygalacturonase and endo-polygalacturonase, the predominant activity was probably exo-polygalacturonase. This enzyme was capable of rapid maceration of bean and cucumber as well as potato tuber tissue and its production was not suppressed by glucose. The increases in activity of the exo-polygalacturonase which was correlated with time of incubation and with rapid mycelial growth

in vivo and *in vitro* suggested that this enzyme might contribute to the nutrition of the rapidly advancing infection hyphae. A comparable enzyme activity was reported by Hancock, (1966) in 2 to 4 day old infected sunflower tissue. He found that the enzyme could break down pectic substances in sunflower and tomato tissues. A reduction of 74-93% in the methoxyl content and 64 - 82% in pectic acid content of the infected tissues of the hosts was found within 2 to 4 days of symptoms developing. Morrall *et al.*, (1972), using 38 isolates of *Sclerotinia* from different hosts, found that most isolates produced both exo- and endo-polygalacturonase with the latter being more prevalent. Although several workers reported a lack of correlation between polygalacturonase and virulence (Held, 1955; Morrall *et al.*, 1972; and Newton, 1972), Lumsden (1976) found that the ability to produce large quantities of the endo-polygalacturonase was an attribute of isolates of *S. sclerotiorum* that were most virulent on bean.

Echandi and Walker, (1957), found that the optimum pH for the pectolytic activity of the culture filtrate was pH 4 when determined by rate of maceration of carrot root tissue and by reduction in viscosity of sodium polypectate solution According to Bateman and Beer (1965), the activities of both exo- and endo-polygalacturonase were near optimal at pH 4.5. Hancock, (1966), found that pectic acid was the preferred substrate for both exo- and endo-polygalacturonase. Marciano *et al.*, (1982), studied the pattern of polygalacturonase isoenzymes produced in culture (Czapek's liquid medium with pectin or polygalacturonic acid as inducers) and in infected tissues (sunflower stems or apple fruits). Only one polygalacturonase isoenzyme was produced in each culture with a molecular weight of either 30,000 or 33,000. The carbon source in the medium affected the molecular form of the isoenzymes since with polygalacturonic acid it had an isoelectric point (pI) of 4.8 and mainly an exo-type of activity, whereas with pectin it had a pI of 5.1 and the enzyme was a typical "endo" enzyme. Another isolate of *S. sclerotiorum* when grown in Czapek-pectin liquid culture

produced a single PG isoenzyme but with pI 8.8 it was thought that S. sclerotiorum was similar to Botrytis cinerea (Magro et al., 1980), with different isolates producing different polygalacturonase isoenzymes. When extracts from infected tissues were purified by electrofocusing, two peaks of polygalacturonase activity were obtained, a major peak at pI 8.3 and a minor one at pI 4.8. Extracts from sunflower stems 40 hours after inoculation gave a third peak with a pI of 6.9. The polygalacturonase isoenzyme (pI 4.8) from apple had a molecular weight of 40,000. Two of the polygalacturonase isoenzymes obtained from inoculated tissues (pI 4.8 and 8.3) were typical endo-enzymes but the third isoenzyme (pI 6.9) from inoculated sunflower showed a high percentage hydrolysis of the sodium polypectate substrate at 50% loss of viscosity and the production of free galacturonic acid after short incubation periods. All the polygalacturonase isoenzymes when assayed on sunflower stems and apple parenchyma caused maceration, cell death and release of K^+.

Maxwell and Lumsden (1970), found that oxalic acid was produced by S. sclerotiorum in culture and diseased bean tissue and it appeared to act synergistically with pectolytic and cellulolytic enzymes in the degradation of host tissue. A positive correlation was found between disease severity index and oxalic acid accumulation in infected bean hypocotyls. It was suggested that oxalic acid enhanced the activity of polygalacturonase and the other cell wall-degrading enzymes by lowering the pH of the infected tissue to the optimum for these enzymes. Synergistic action of oxalic acid with pectolytic enzymes has independently been demonstrated for Sclerotium rolfsii (Bateman and Beer, 1965), a fungus with pathogenic behaviour similar to that of S. sclerotiorum (Lumsden , 1976). Noyes and Hancock, (1981), found that oxalic acid produced by S. sclerotiorum in infected tissue of greenhouse-grown sunflower plants moved systemically to the leaves where it apparently accumulated to a 'critical' level and elicited the wilt syndrome. When healthy plants were fed oxalic acid through the hypocotyl, they showed foliar symptoms identical with those

associated with fungal infections, and the concentrations of oxalic acid in leaves of treated plants were equivalent to those in infected plants.

Lumsden (1976) detected pectinmethylesterase in extracts from diseased tissue and thought it was likely to be the same enzyme as that produced in culture by S. sclerotiorum rather than bean plant pectinmethylesterase. Fungal pectinmethylesterase was not activated by salts as was pectinmethylesterase of healthy tissue (Van Buren et al., 1962). In addition, the optimum pH of the fungal enzyme was pH 5 while that of the healthy tissue enzyme was pH 8 (Van Buren et al., 1962). Elevated levels of pectinmethylesterase were associated with the advancing margins of lesions up to two cells in advance of the invading hyphae. It was thought that both pectinmethylesterase and endo-polygalacturonase were produced by the infection hyphae of S. sclerotiorum.

Echandi and Walker (1957) found that the activity of Sclerotinia pectolytic enzymes (polygalacturonase and pectinmethylesterase) was destroyed on exposure for 10 min. to a temperature of 55°C or when mixed with onion extract. Van den Berg and Yang (1969) studied the effect of relative humidity on production of the pectolytic enzymes by S. sclerotiorum and Botrytis cinerea in a nutrient medium on the surface of carrots. Both workers found that low relative humidity increased pectolytic enzyme production. At 94 - 96% relative humidity enzymic activity was 2-7 times greater than at 98 - 100%.

Production of pectolytic enzymes was inhibited almost completely when dextrose was added to the culture medium (Van den Berg and Yang 1969). Neither pectate lyase (Hancock, 1966; Marciano et al., 1982) nor pectin lyase (Hancock, 1966; Morrall et al., 1972; Barkai-Golan, 1974; and Lumsden, 1976) is produced by S. sclerotiorum.

1.6.2 Cellulolytic and Hemicellulolytic enzymes

These enzymes often have been associated with Sclerotinia spp and pathogenesis (Barkai-Golan, 1974; Hancock, 1967; Lumsden, 1969; and Newton, 1972)

Their involvement in pathogenesis is less well understood (Baker et al., 1979). Lumsden (1969) demonstrated that S. sclerotiorum produced cellulolytic enzymes capable of degrading insoluble or "native" forms of cellulose as shown by its ability to utilize cotton fibres and filter paper as sole sources of carbon. A soluble form of cellulose, carboxymethylcellulose (CMC), also induced production of cellulase and served as a carbon source for growth. These facts suggested that S. sclerotiorum produced the series of enzymes suggested by Reese et al., (1950) to explain the degradation of native celluloses. These include the C_1 enzyme capable of degrading native cellulose to linear anhydroglucose chains; C_x that hydrolyzes the chains to soluble low molecular weight products, predominantly cellobiose and possibly a β-glucosidase or cellobiase that degrades the product to glucose. According to Lumsden, (1969), S. sclerotiorum produced abundant cellulase(s) in diseased bean tissue and the content of α-cellulose in diseased tissue decreased substantially during the invasion of bean hypocotyls and the decrease correlated positively with the severity of symptom expression. The activity of the enzyme extracted from diseased tissue or obtained from culture filtrate was maximal at pH 3.

Fuchs et al., (1965), reported that S. sclerotiorum produced arabanase when grown on groundnut-meal - a known source of araban (Hirst and Jones 1947). The enzyme released L-arabinose from commercial araban (Light and Co.) and had an optimum pH in the range 3.6 - 5.8. When groundnut-meal was replaced by glucose the fungus showed noticeable arabanase activity indicating that the enzyme was constitutive.

Hemicellulolytic enzymes were studied by Hancock (1967). He found that the araban and galactan fractions were degraded extensively in Sclerotinia-infected sunflower hypocotyls. Although xylanase was detected in infected tissues at concentrations that should have been capable of degrading the native xylan, breakdown of xylan occurred to a lesser extent compared with both araban and galactan. It was thought that absorption of xylanase to undegraded cellulosic material might be partially responsible for these results as it was shown by

Jansen et al., (1960) that cellulosic cell walls have the capability to bind protein nonspecifically. Therefore, it was possible that the encrusting substances in cell walls such as xylan could be protected from enzymic breakdown, whereas substances present in the middle lamella such as araban and galactan should be readily accessible to degradative enzymes secreted by intercellular hyphae.

Bauer et al., (1977) reported the production of an endo-β-1, 4 galactanase by S. sclerotiorum when grown in a liquid mineral salts medium supplemented with 2.0 g of Difco yeast extract and 2.0 g or 6.0 g D-galactose per litre. The enzyme hydrolysed the substrate in an essentially random fashion. The purified enzyme had a pI of 8.3, a pH optimum of approximately 4.5 and a molecular weight of 22,000 to 24,000. When incubated with isolated sycamore and potato cell walls, the enzyme readily solubilized substantial amounts of carbohydrates including a major portion of the galactan component without the prior action of a pectolytic enzyme. However, the enzyme failed at a concentration of 0.3 units/ml to cause maceration of potato tuber tissue.

S. sclerotiorum also produces α-L-arabinofuranosidase when cultured on a medium containing arabinose as the sole carbon source (Baker et al., 1979). The enzyme had properties similar to those of α-L-arabinofuranosidases produced by other plant pathogenic fungi (Fuchs et al., 1965; Sturdy and Cole, 1975) except that its pI was 7.5 while those of the other fungal α-L arabinofuranosidases are in the pH range 3.0 to 6.5 (Laborda et al., 1974; Dekker and Richards, 1976). According to Baker et al., (1979), S. sclerotiorum α-L arabinofuranosidase had an optimum pH in the range 4 - 4.5 and its molecular weight was about 63,000. The enzyme released arabinose from arabinan and from isolated cell walls of bean and rice but failed to macerate potato tuber or cucumber endocarp tissue. Its activity was inhibited in a competitive manner by L-arabono-1, 4-lactone and D-galactono-1, 4-lactone.

1.6.3 Miscellaneous enzymes

Phosphatidase B and protease are produced by S. sclerotiorum (Newton, 1972; Khare and Bompeix, 1976). These enzymes can degrade membrane constituents (Lumsden, 1979) and therefore may disrupt cell membranes during pathogenesis (Willetts and Wong, 1980; Hancock, 1972; Newton et al., 1973) resulting in leakage of nutrients from cells. pH optima for the activies of the two enzymes are 4, and 3 respectively (Lumsden, 1979).

2. The host (Lycopersicon esculentum (Mill.)

2.1 Origin and optimal environment

Tomato is a member of the large family Solanaceae, predominantly tropical in distribution but well represented in temperate regions. Of 75 or more genera and more than 2000 species, a considerable number are cultivated as ornamentals as well as for food and drugs, (Bailey 1949). Although the Solanaceae is a family of worldwide importance, its greatest concentration of diversity is to be found in South America. For this and other reasons most authorities believe that the family originated in this area, spreading to other continents at a very early period, (Hunziker, 1979).

The genus Lycopersicon is a small genus of six species of soft herbs, usually perenial. With the exception of L. esculentum, which is the cultivated tomato, and L. cheesmanii, which is endemic in the Galapagos Islands, they grow wild in a narrow coastal strip extending from Ecuador to Northern Chile (Purseglove, 1977). According to Knott and Deanon, (1967), the use of the tomato is very ancient. It is a native of Western South America where it was known and highly prized even before the discovery of the continent by Columbus.

The optimum mean temperature range for tomatoes is about 21°C to 24°C day temperature and 15°C to 20°C night temperature. High temperatures with

high relative humidity often cause serious losses in yield due to the tendency for more foliage diseases to develop. On the other hand, high temperature accompanied by low relative humidity may cause crop failure due to poor fruit set. High solar radiation intensity hastens flowering, increases the number of flower/plant and fruit set and stimulates fruit development while low solar radiation intensity causes excessive elongation of the style and abnormal development of various flower parts. It also reduces the amount and viability of pollen, diminishes fertilization, causes flower drop and reduces fruit set (Sogi et al., 1979). Tomato withstands drought conditions to some degree although frequently growth cracks and blossom-end rot are observed on fruits as a result of changes in plant water status (Knott and Deanon, 1967).

2.2 Cultivars and Uses

A great many varietes have been developed particularly in temperate countries. Varieties may be determinate or indeterminate in growth, they vary in the size, shape, colour, flavour and vitamin content of the fruits, the degree of earliness, method of growth which may be erect to sprawling and in resistance to diseases and root-knot nematode. Although the varieties are distinct in themselves, there are numerous strains, selections and re-selections of each.

According to Allerton (1954), the Stonor varieties: Moneymaker, Exhibition, X-ray, Vanguard, M.P., Prolific and All clear, which are much grown in the U.K. now, are chiefly characterized by the conspicuous pale-green fruit with very even colouring to a light red and the virtual absence of greenback. On steamed soil, growth is often rank and leafy and fruit then has a tendency to hollowness. Moneymaker is particularly good under cold-house cultivation.

The great variety of uses of tomato leads to its popularity. Tomato fruits are eaten raw or cooked, and are extensively used in the canning industry.

Green tomatoes are used for pickles and preserves. Tomatoes grown in the tropics tend to be rather coarse and lacking in flavour compared with those grown in temperate countries. Tomato seeds contain approximately 24% oil. This semi-drying oil is used as a salad oil and in the manufacture of margarine and soap. The residues of the canning industry are used for stock feed and fertilizer, (Knott and Deanon, 1967; Purseglove, 1977).

2.3 Structure

Tomato is a herbaceous plant, 0.7-2m tall, erect with thick solid stems, or spreading and later becoming prostrate, coarsely hairy, glandular with a characteristic strong odour. The plant has a strong tap-root often damaged at transplanting and a dense system of fibrous and adventitious roots is formed. Branching is usually sympodial although the base of the stem may be monopodial. In the former the terminal bud aborts or produces an inflorescence and the axis is continued by the development of the axillary bud.

The tomato stem is roughly triangular in section. Externally, the stem is bounded by the epidermis which contains, apart from the typical epidermal cells, guard cells, small capitate glandular hairs and long pointed trichomes. The cortex in the young stem consists of thin-walled parenchyma and collenchyma. Internal to the cortex is the vascular system of the stem which consists of two types of tissues : the phloem and the xylem. The phloem is external and internal to the xylem. The internal or intraxylary phloem is present as separate strands on the border of the pith, (Bewley, 1950; Purseglove,1977; Fahn, 1974).

The arrangement and structure of the stem tissues of young and mature tomato stems are shown in Fig. (2).

2.4 Cell Wall Composition

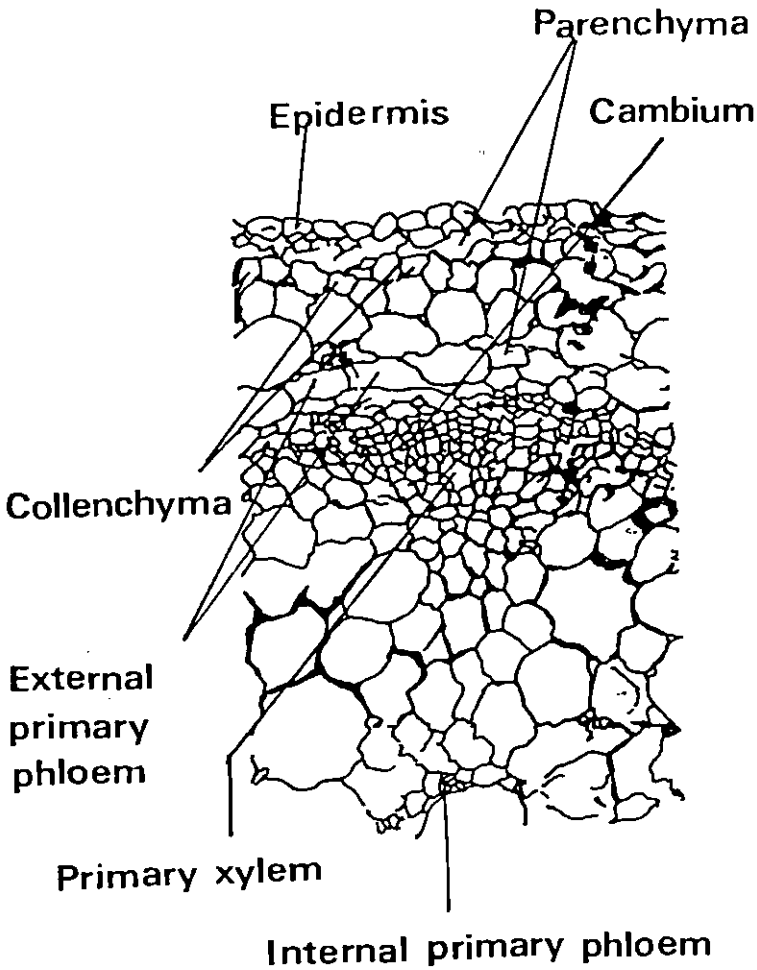
Some of the sugar constituents of cell walls of stems from 30-day old

FIG. (2)

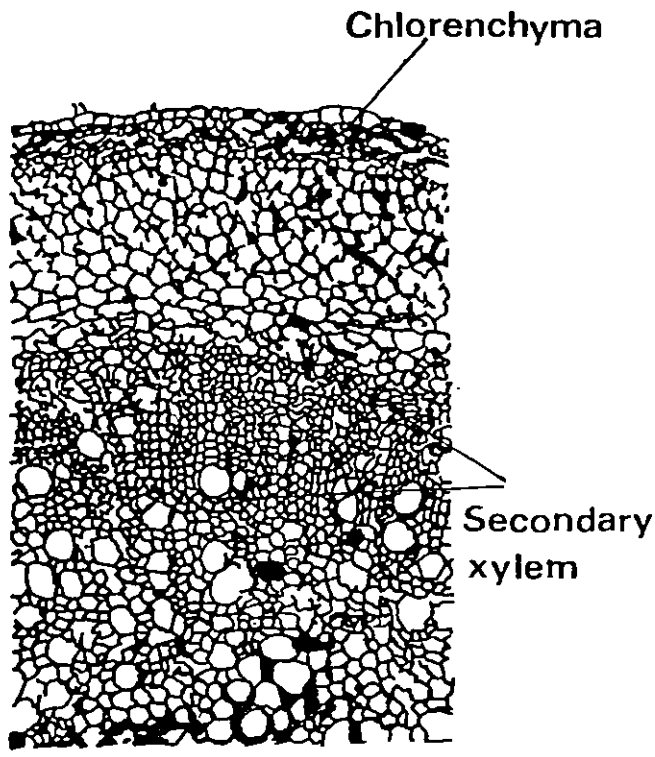
Portions of cross-section of a stem of Lycopersicon esculentum.

(A) A young stem in which internal phloem can be distinguished.

(B) A mature stem in which it is possible to discern that the cortical parenchyma below the epidermis has developed into chlorenchyma and that a fair amount of secondary xylem has been produced. (After Fahn, 1974)


Parenchyma
Epidermis
Cambium
Collenchyma
External primary phloem
Primary xylem
Internal primary phloem

A


Chlorenchyma
Secondary xylem

B

plants of two tomato varieties are shown in Table (2), taken from Jones et al (1972). Figures are given in mg/100 mg of cell wall material.

TABLE (2)

Polysaccharide composition of tomato stem cell walls

Sugar	Tomato variety	
	Clark's Special	Jefferson
Rhamnose	1.0	1.0
Arabinose	3.6	3.1
Xylose	5.9	4.7
Mannose	0.9	1.0
Galactose	5.6	5.4
Glucose	0.7	1.0
Galacturonic acid	18.2	18.0
Glucuronic acid	0.2	0.3

A group of proteins, isolated from the cell walls of tomato stems has been shown to inhibit the action of fungal polygalacturonases (Albersheim and Anderson, 1971). According to Jones et al., (1972) inhibitor preparations from the stems of Clark's Special and Jefferson possessed approximately equal abilities to inhibit the polygalacturonase of Fusarium oxysporum. Inhibitor corresponding to 100 µg protein was sufficient to cause 50% inhibition of the polygalacturonase activity. The two varieties contained approximately equal amounts of the inhibitor. Wood (1961) also demonstrated the presence in tomato stems and leaves of a heat-labile inhibitor of the polygalacturonase produced by verticillium albo-atrum.

2.5 Occurrence of cell wall-degrading enzymes in healthy tissue

The occurrence of polygalacturonase, pectinmethylesterase and cellulase in healthy tomato fruit tissue has been reported and is thought to contribute to the loss of tissue firmness which occurs during the ripening of fruit. The physical changes associated with the ripening of fruits have been attributed mainly to a breakdown in pectic substances, (Branfoot 1929). According to Hobson (1967), pectic materials are found in the cell wall and middle lamella, closely associated with cellulose, hemicellulose and lignins. They form the bulk of the intercellular binding material and until maceration they occur largely in an insoluble form known as protopectin. The classical mechanism thought to be involved in tomato softening is the progressive solubilization of pectic substances in the cell wall, with subsequent de-esterification by pectin-esterase and degradation by polygalacturonase (Hobson 1969). Cellulose breakdown by the action of cellulase probably also plays a part (Sobtka and Watada, 1971). Hall (1963), stated that the presence of a cellulolytic enzyme in tomatoes was extremely important in softening.

Goodenough and Kempton, (1976) reported the presence of α-galactosidase, β-glucosidase and β-xylosidase in the healthy root tissue but neither polygalacturonase nor cellulase could be detected. Although polygalacturonase is not considered a normal constituent of stems and leaves (Blackhurst and Wood, 1963), Deese and Stahmann (1962), reported traces of the enzymes in extracts of uninfected plants. Pectinmethylesterase, however, is a commonly occurring enzyme in tomato and other higher plants (McColloch and Kertesz 1947).

2.6 Involvement of phenolic compounds in the plant's resistance mechanism

Accumulation of phenolic compounds in tomato tissues in response to infection is considered to be a disease resistance mechanism (Matta et al., 1967, 1969). Retig and Chet (1974) noted that after treatment of susceptible

tomato plants with catechol which led to a marked accumulation of total phenols in stem tissue, symptom expression by Fusarium oxysporum f. sp. lycopersici was prevented. This enhancement of resistance was accompanied by an increase in the peroxidase and polyphenol oxidase activity of the treated plants. According to Carrasco et al., (1978), pretreatment of tomato plants with precursors of phenolic compounds such as quinic acid or phenylalanine increased both their phenolic content and their resistance to Fusarium oxysporum. Other compounds unrelated to phenolics, were ineffective. The resistance mechanism involved in quinate-treated plants was not clear, but preliminary results suggested that oxidized phenols might have a fungitoxic effect and the increased lignification might facilitate water transport and strengthen defence barriers in the xylem.

Many phenolic compounds have been identified in tomato. The presence of rutin, chlorogenic and quinic acids was reported by Tso et al., (1974) in tomato shoots. No polyphenols could be detected by these authors in the root system and both scoplin and scopletin were also absent in both shoots and roots. According to Wardale (1973), the green tomato fruit contains caffeic, coumaric, sinapic and ferulic acids and naringenin. Hunt and Baker (1980) reported the presence of chalconaringenin, naringenin, naringenin -7-glucoside and m- and p- coumaric acids in the fruit cuticles of the tomato cultivars Ailsa Graig, Alicante and Grower's Pride. Neochlorogenic and 3-o-feruloylquinic acids were identified for the first time in the leaves, stems and roots of tomato plants by Bragt et al., (1965). Kumar and Mcintyre (1973) reported that pyrogallic, cinnamic and caffeic acids were the major phenols detected in scattered cells external to the conducting elements of the phloem of verticillium infected Bonny Best and Loran Blood tomato cultivars.

MATERIALS AND METHODS

1. Plant material

1.1 Host cultivar

Tomato plants, (Lycopersicon esculentum cv. Moneymaker), grown from seeds obtained from Hurst Gunson Cooper Taber Ltd., were used in all experiments.

1.2 Growth of plants

Seeds were sown in John Innes No. 3 compost in plastic trays, 35 x 22 x 5 cm), each containing approximately 40 seeds. The trays were placed on a glasshouse bench until germination had occurred. This took 7 - 10 days. The plants were pricked out when they were about 2-week old into plastic pots containing compost (John Innes No. 3). In the summer months (April to September) the plants were grown under natural light in an unheated greenhouse. In winter, supplementary lighting provided by 6 lamps (WOTAN 240, Watt High Pressure Metal Halide) for 18 hours a day was used, the greenhouse temperature being maintained at 25 $\pm$ 2^{o}C. The plants were fed from first flower onwards with "Liquid tomato plus^{ICI}" at intervals at the recommended dilution.

2.0 Source and maintenance of Sclerotinia sclerotiorum cultures

2.1 Origin of cultures

The isolate of S. sclerotiorum used in all experiments was RC/802 from cabbage. It was obtained with two other isolates as sclerotia provided by Mr R. Coulter (ADAS, Reading). The other two isolates were RC/801 from winter oil seed rape and W4074 from chrysanthemum. For production of

apothecia, four other isolates of *S. sclerotiorum* were used in addition to the first two. All isolates are listed in Table (3) below.

TABLE (3)

Isolates of *S. sclerotiorum* used for production of apothecia

Isolate	Isolated from	Date of isolation	Supplied by
Ss_1	Runner bean	14/Sept./76	K. Price
Ss_2	Sunflower	3/Sept./76	K. Price
Ss_3	Chrysanthemum	Nov./76	K. Price
Ss_4	Stored carrots	9/Jun./78	K. Price
RC/802	Cabbage	17/Nov./80	R. Coulter
RC/801	Oil seed rape	17/Nov./80	R. Coulter

2.2 Culture media

(A) Malt Agar (Blakeslee formula, Tuite 1969) (MA)

Malt extract	20 g
Agar	20 g
Dextrose	20 g
Mycological peptone	1 g
Distilled water	1 l

(B) Sucrose-casamino acids (SCA) (Debnam 1975)

Casamino acids (vitamin free)	4.0 g

KH_2PO_4	1.0 g
$MgSO_4 . 7H_2O$	0.5 g
Sucrose	15.0 g
Glucose	0.5 g
Trace element solution	10 ml
Distilled water	1 l

The solution for trace elements was prepared as follows:

Stock solution	Final concentration of the salt in the medium (ppm)
0.02 g $FeSO_4.7H_2O$	0.2
0.10 g $ZnSO_4.7H_2O$	1.0
0.002 g $NaMoO_4.2H_2O$	0.02
0.002 g $CuSO_4.5H_2O$	0.02
0.002 g $MnCl_2.4H_2O$	0.02

Distilled water to one litre

(C) Sodium polypectate-tartarate (Byrde and Fielding 1968)

Sodium polypectate	10.0 g
Ammonium tartarate	15.0 g
KH_2PO_4	1.0 g
$MgSO_4.7H_2O$	0.5 g
Distilled water	1 l

(D) Potato Dextrose Agar (PDA)

Potato	200 g
Dextrose	20 g
Agar	20 g
Distilled water	1 l

This medium was prepared following the instructions in Plant Pathologist's Pocketbook CMI.

(E) Water Agar (Lingappa and Lockwood 1960) (WA)

Agar	15 g
Distilled water	1 l

(F) Modified Richard's agar medium (Davis 1964)

KNO_3	10.0 g
KH_2PO_4	0.5 g
$MgSO_4.7H_2O$	2.5 g
Sucrose	50 g
Agar	15 g
Distilled water	1 l

Microelements and iron were added at the concentrations of Hoagland and Knap solution (modified) (Chen et al., 1961).

(G) Bread crumbs-prune juice medium

For production of large sclerotia by S. sclerotiorum, a medium composed of commercial bread crumbs and prune juice was used. Bread crumbs were put into 250 ml flasks to a depth of ½ inch and moistened with distilled water and prune juice (1:1 v/v).

Routine sterilization was by autoclaving for 15 minutes at 15 p.s.i. (121°C).

2.3 Maintenance of cultures

Cultures were maintained on MA or PDA in Petri dishes or MaCartney bottle slopes. For long term storage they were kept under sterile liquid paraffin at room temperature.

3. Harvesting of cultures and determination of mycelial dry weight

Mycelial mats were collected on muslin, washed in running water and transferred to small aluminium foil cups of known weight. Mycelia thus collected were dried in an oven at 80°C for 24 hours, cooled over $CaCl_2$ in a desiccator and weighed.

4. Techniques used to induce production of apothecia

Isolates used for apothecial production were grown on bread crumbs-prune juice medium in 250 ml flasks. Each flask was inoculated with an agar plug cut with a no. 7 cork borer from the leading edge of the mycelium of 4-day old cultures on MA medium. All cultures were incubated in the dark at 21°C. After 30 days, sclerotia were removed, surfaced sterilized with sodium hypochlorite solution (10% chlorox) and incubated in sterile distilled water in Petri dishes and on moist washed silver sand in transparent plastic boxes at 15°C in an incubator which had a light period of 14 hours per day (Abawi and

Grogan 1975).

5. Inoculation techniques

5.1 Preparation of inoculum

The following inocula were used: (a) mycelial disks, (b) ascospore suspension and (c) germinating sclerotia.

Mycelial disks were cut with a no. 7 cork borer from the active margin of 3-4 day old cultures growing on MA in the dark at 20 $\pm$ 1°C. They were used for the inoculation of intact plants and stem cuttings as described below, (sections 5.2 and 5.3).

Ascospore suspensions were used for inoculating intact plants under glasshouse conditions and cuttings and detached leaves in the laboratory. Ascospores were collected by allowing them to eject into dry Petri dishes. After their release, the ascospores were suspended in SCA liquid medium or sterile distilled water with or without 0.1% (w/v) glucose, 0.01% (w/v) sodium citrate and 0.01% (v/v) tween 80. The concentration of ascospores in the final suspension was measured with a haemocytometer slide and then adjusted to the desired concentrations. Stem cuttings and detached leaves were inoculated with 10 µl droplets of the spore suspension using an Agla Micrometer Syringe. Intact plants were inoculated as described below (section 5.2).

Sclerotia were used for inoculating stem cuttings and detached leaves under laboratory conditions. They were allowed to germinate in SCA liquid medium in sterile Petri dishes at 26°C. This took about 4 days after which the germinating sclerotia were used as inoculum (see sections 5.3 and 5.4).

5.2 Inoculation of intact plants

The technique used was that of Price and Colhoun (1975). The soil

around the plant in each pot was covered by a polythene sheet allowing only the stem to penetrate. Each plant was inoculated at the base of the stem with a mycelial disk using sterile forceps. The inoculation site was covered with moistened sterile cotton wool and small piece of polythene to maintain a high humidity througout the infection period. When ascospore suspensions were used for inoculation of intact plants, they were atomized on the leaves and the stems Inoculated plants or the inoculation sites were covered with polythene bags for 2 - 3 days to maintain 100% relative humidity.

5.3 Inoculation of stem cuttings

Stem cuttings were surface-sterilized in sodium hypochlorite solution (10% chlorox) for 3 minutes and washed successively in three changes of sterile distilled water. The cut ends of each stem cutting were covered with soft paraffin so as to minimize water loss from stem tissues, and then inoculated with mycelial disks or germinating sclerotia which were placed on the surface of the stem segment towards the top and near the base. After inoculation, the stem cuttings were placed on sterile glass rods in small sterile clear plastic boxes containing small amount of water. The boxes were sealed and placed in a lighted room at 26°C.

5.4 Inoculation of detached leaves

Detached leaves were surface-sterilized in sodium hypochlorite solution for 1 - 2 minutes and then washed in sterile distilled water as mentioned above. Each of two small leaves were placed in a sterile Petri dish containing benzimidazole solution (75 ppm) to slow the rate of senescence. Large or old leaves were placed in sterile plastic boxes on perforated plates with their petioles connected to absorbent tissues immersed in the benziminazole solution. To create humid conditions, the boxes were lined with moistened blotting papers.

Each leaflet was inoculated in the centre with a germinating sclerotium or a mycelial disk placed on the surface of the leaf tissue.

6. Disease assessment

Disease index values were calculated using an arbitary 0-5 scale. Since the transverse movement of the fungus in the stem tissues resulted in collapse of the whole plant, the scale used for disease assessment was based on the thickness of the infected stem (Table 4).

7. Histological techniques

7.1 Preparation of whole mounts

Two techniques were used. The first was according to Shipton and Brown (1962), in which the inoculated tissues were cut into small pieces and then immersed in 12 ml of 1 part lactophenol cotton blue to 2 parts 95% (v/v) alchohol. Lactophenol cotton blue contained:

Phenol	10.0 g
Glycerine	10 ml
Lactic acid	10 ml
Aniline blue	0.02 g
Distilled water	10 ml

The mixture was brought to boiling and simmered for 1½ minutes. After boiling the tissues were kept in stain for 48 hours at room temperature, then rinsed in water and placed in chloral hydrate (5 g chloral hydrate, 2 ml water) for 30 - 50 minutes and then mounted in 50% (v/v) glycerine.

TABLE (4)

The scale used for disease assessment

Disease index	Symptoms
0	no attack
1	infected part of the stem with water-soaked lesions but without change in its thickness
2	thickness of infected part of the stem is more than 75% of that of the upper healthy portion
3	thickness of infected part of the stem is more than 50% but less than 75% of that of the upper healthy portion
4	thickness of infected stem is 50% or less than that of the upper healthy portion and the plant remains erect; or in mature plants the infected stem becomes hollow and can easily be broken
5	infected stem is collapsed; plant is prostrate

The other technique used was that of Diener (1955). Small pieces of the inoculated tissues were put in equal parts of glacial acetic acid and 95% (v/v) ethanol for two days, and then in 80% (v/v) lactic acid for 4 days. The material was removed and placed on a slide in a drop of 0.1% (w/v) cotton blue in lactophenol. After about 20 minutes the excess was removed and clear lactophenol was added.

Although the first technique was successful for clearing tomato tissues, it was found that many spores were usually removed during boiling of the inoculated tissues. No great loss was found by employing the second clearing method.

7.2 Preparation of sections (Johansen 1940)

Small pieces were cut from the inoculated sites. They were killed and fixed in Formalin-Propiono-Alchohol which was prepared as follows:-

50% (v/v) ethyl alchohol	90 ml
Propionic acid	7 ml
Formalin	3 ml

After 22 hours tissue pieces were dehydrated in a standard ethanol-butanol series. They were then infiltered with paraffin at 58-65^{o}C. After infiltration, the peices were embeded in paraffin wax and sectioned at 12 µm with a Cambridge-Frigister-Microtome. The sections were affixed to the slides with Haupt's adhesive which was prepared as follows:

Gelatine, 1 g, was dissolved in 100 ml of distilled water and then 2 g of phenol crystals and 15 ml of glycerine were added, stirred well and filtered.

The wax was removed from the sections with xylene for 5-10 minutes. The sections were then transferred to equal parts of xylene and absolute ethanol, followed by 95% (v/v) ethanol and finally they were stained with aniline blue in 90% (w/v) ethanol for 1 minute. Excess stain was washed off with clove

oil and the sections were mounted in balsam.

8. Preparation of stem and root homogenates

Stems and roots were homogenized separately in 5 vol. of distilled water in a Waring blender for 3-5 minutes. The supernatants were collected by filtration through several layers of cheesecloth and used for preparation of culture media.

9. Preparation of root exudates

Roots were obtained from 3-week old tomato plants grown in Long Ashton solution (Hewitt, 1962).

Excised roots were surface-sterilized in 0.1% (w/v) $HgCl_2$ solution for 1-2 minutes, washed in sterile distilled water and then transferred into conical flasks containing sterile distilled water. The roots were incubated in the dark at 26°C for 6 days. Incubates showing signs of bacterial contamination were discarded. The exudates were concentrated under reduced pressure at 40°C to give the equivalents of 10, 20 or 30 mg dry weight of root per millilitre and then filter sterilized (0.2 μm diam. pore size).

10. Enzyme preparation

Unless otherwise indicated, filtrates utilized for enzyme activity assays were obtained from cultures of the fungus grown on sodium polypectate-tartrate liquid medium for 13 or 14 days, and all the operations were carried out at 4°C. At the end of the growth period, cultures were harvested and the filtrates were strained through several layers of cheesecloth and centrifuged for 15 minutes at 3300 g. The clear supernatant liquids were combined and dialysed for 20-24 hours against 30 vol. of distilled water or 50 mM sodium

citrate or acetate buffers (pH 4.6) with 2-3 changes of the water or the buffer. Dialysed enzyme preparations were assayed for enzyme activity immediately after dialysis or kept under toluene or at -20°C until assayed.

10.1 Enzyme concentration

10.1.1 Freeze drying

This method was used for concentrating crude enzyme preparations. Preliminary work showed that freeze-drying did not give a satisfactory yield when used for concentration of bulked culture filtrates, so this procedure was discarded.

10.1.2 Dialysis against polyethylene glycol (PEG)

This method was used in preference to the freeze-drying procedure as it was more rapid and gave much higher enzyme yields. Culture filtrates and plant extracts were concentrated to half or third the initial vols. against 20 vols. of 20% (w/v) PEG (type 4000) with two changes of PEG solution. Visking tubings - 2.5 and 7.8 cm in diameter, obtained from the Scientific Instrument Centre Ltd., and Medical International Ltd., respectively - were used.

10.2 Enzyme purification

10.2.1 Iso-electric focusing

Electrofocusing of enzyme preparations was carried out in an LKB 110 ml apparatus for 48 to 72 hours at 5°C. The procedure detailed in the LKB instruction manual was followed using carrier ampholytes at a final concentration of 1% (w/v) to establish a pH gradient over the required range. The enzyme preparation was added in the light solution of the sucrose gradient. The lower anode solution contained 1% (v/v) phosphoric acid and the upper cathode 8% (w/v)

sodium hydroxide. The initial voltage applied was 200 V giving a current of approximately 10 mA, increasing to 600 V with a final current of about 1 mA. The column was drained by gravity flow. Three ml fractions were collected and their pH was determined when they had attained room temperature.

10.2.2 Sephadex G 100 gel filtration

For purification and molecular weight determination of the enzymes, gel filtration was performed using Sephadex G 100 superfine (Lot no. 178 - 0130), in a 45 x 25 cm column. The slurry was prepared and the column was packed and equilibrated as described by Curling (1970). The eluant used was 50 mM sodium acetate buffer (pH 4.6) containing 0.1M NaCl at a flow rate of 10 ml/hour. The rate of flow was controlled by using a peristaltic pump. The void vol. of the column (60 ml) was determined using blue dextran 2000 at a concentration of 2 mg/ml. The column was calibrated using proteins of known molecular weights eluted through the column under the same conditions as used for the unknown samples. The reference standards used, their molecular weights and their concentrations were as follows:

Bovine serum albumin	67,000	8 mg/ml
Ovalbumin	45,000	10 mg/ml
Trypsinogen	24,000	12.5 mg/ml
Ribonuclease A	13,000	15 mg/ml

The compounds were applied to the column either separately or in combination in 2 ml samples. The effluent was collected in 5 ml fractions using an automatic fraction collector. The elution vols. of the proteins were determined by spectrophotmetric readings at 280 nm. All gel filtration was done at laboratory temperature. When not in use, the column was stored in the presence of 0.02% (w/v) sodium azide in buffer to prevent microbial growth.

10.3 Enzyme assays

Unless otherwise noted, all enzyme assays were performed at 26°C. Each assay was performed at least in duplicate and the results reported are the mean of those obtained in a particular experiment. Buffers were prepared according to Cyril (1968).

10.3.1. Polygalacturonase

This enzyme was assayed by three different methods.

10.3.1.1 Reducing group method (Nelson, 1944)

Polygalacturonase activity was measured by the increase in reducing groups released from buffered solution of sodium polypectate or pectin. The method is described in section (12.1). The reaction mixture contained 1 ml of 1% (w/v) sodium polypectate or pectin solution; 0.4 ml 0.1M sodium acetate buffer (pH 4.6); 0.4 ml distilled water and 0.2 ml enzyme sample. Both, the enzyme and the buffer, were preincubated at 26°C. After a suitable period of time depending on the enzyme activity being measured, the reaction was terminated by addition of Somogyi reagent and the reducing groups were measured.

When pectin was used as a substrate, the reaction mixture was chilled at 2-3°C for 1 hour after addition of Somogyi copper reagent and before heating (for de-esterification of pectin).

Units of enzyme activity were expressed as micromoles of reducing sugars released per minute under the assay conditions. Sodium polypectate and pectin were washed several times with acidified (0.1M HCL) 80% (v/v) ethanol before use. When the washings were free of pigment and reducing sugars, the solid material was collected by filtration under reduced pressure. It was dried using absolute ethanol, ethanol; ether (1:1 v/v) and finally

by heating at 60°C for 24 hours.

10.3.1.2 Viscosity reduction method

Polygalacturonase was also estimated viscometrically using Cannon-Fenske, Technico visometers size 200 at 30°C. Reaction mixtures consisted of 5 ml 2% (w/v) sodium polypectate; 2 ml 0.1M sodium acetate buffer (pH 4.6); 2 ml distilled water and 1 ml enzyme preparation. Substrate solution (9 ml) was introduced into the viscometer and allowed to equilibrate to 30°C. The efflux time was then noted for substrate without enzyme after which 1 ml of temperature-equilibrated enzyme solution was added and mixed by bubbling air through it, concurrent with the starting of a stop clock Measurement of the efflux time was made as soon as possible after this mixing, usually within 1 or 2 min., of the start of the reaction. The reaction time was taken from the point of mixing of substrate and enzyme to the midpoint of the efflux time of the viscometer. Controls of buffer plus enzyme and buffer plus sodium polypectate were always used for each enzyme sample. Relative activity was determined by multiplying by 1000 the reciprocal of the time in min. necessary for 50% reduction in viscosity. Viscosity standards comprised sucrose solutions of the following (w/v) compositions = 0.5%; 4%; 7%, 11%; 13%; 15%; 17%; 20%; 24% and 28%. The flow time for each concentration was measured and graphs were plotted of the concentration and relative viscosities of sucrose solutions against the efflux time in seconds. Other graphs were plotted of the reaction time in min. for each enzyme preparation against the percent loss of viscosity. The relative units of enzymic activity were determined from the resultant curves.

10.3.1.3 Cup-plate method (Dingle et al., 1953)

An agar plate assay was used as the third assay method. A medium

containing sodium polypectate 1% (w/v); agar 2% (w/v); ammonium oxalate 0.5% (w/v); thiomersal 0.01% (w/v) and glacial acetic acid and sodium acetate (0.05M) to give pH 4.6 was used. The solid constituents were dissolved by heating in deionized water at 100°C. After the temperature of the medium had dropped to 60°C, glacial acetic acid was added with stirring. The medium was then steamed for 1 hour and poured into 9 cm Petri dishes, 30 ml in each. Enzyme samples and standards were introduced into wells made with a no. 3 cork borer. After 20-hour incubation, the plates were flooded with 6M HCL. The diameter of the opaque haloes appeared at the sites of polygalacturonase activity were then measured.

An aqueous solution (0.1% v/v) of a commercial pectinase was used as a standard. Enzyme activity was expressed relative to the standard which was defined arbitrarily as having 100 units/ml.

The relationships between the activity of the enzyme and the size of the hydrolysis ring was studied using varying concentrations of the former. Enzyme activity was directly related to the antilogarithm of the corrected diameter (corrected ring diameter = total diameter in mm - diameter of the well (7 mm).

10.3.2 Pectinmethylesterase

10.3.2.1 Qualitative determination

A qualitative assay method was employed to detect the presence or absence of pectinmethylesterase. Reaction mixtures consisted of pectin (Sigma Grade 1) 5.0 g; phenol 2.0 g; NaCl 5.8 g; bromocresol purple 0.1 g; water to 1 litre. To 5 ml of the substrate mixture 0.5 ml of the enzyme preparation or culture filtrate was added and the pH was adjusted to 6.2. The liberation of carboxyl groups from pectin by the enzyme was indicated by a colour change of the solution from blue-purple to orange-red within 12 hours.

10.3.2.2 Quantitative determination

The activity of the enzyme was determined quantitatively by measuring the amount of acid produced in the reaction mixture. The same mixture was employed except that the indicator was omitted and the phenol concentration was reduced to 1.0 g/litre. The enzyme (1 ml) and the substrate (10 ml) were transferred to a 25 ml beaker and the pH was adjusted to 5. The beaker was covered with parafilm and the pH of the reaction mixutre was checked periodically with a pH meter (South West Optical Instruments Ltd.) and readjusted to the initial value using a 0.01M NaOH.

Enzyme activity was expressed as microequivalents of alkali consumed per hour under the specified assay conditions.

10.3.3 Arabanase, Xylanase, galactanase and cellulase assays

Activities of arabanase, xylanase, galactanase and cellulase were assayed by measuring the increase in reducing groups according to Nelson's procedure (see section 12.1) in reaction mixtures containing respectively 1% (w/v) araban, xylan, galactan or carboxymethylcellulose as substrate. 100 mM sodium acetate buffer at pH 4.6 for arabanase, galactanase and cellulase, and pH 5 for xylanase was employed. The pH of the reaction mixtures corresponded to the optima of the four enzymes.

10.3.3.1 Standards

The increase of the reducing materials was determined by comparing the measured optical densities with those from standard curves prepared respectively with L-arabinose; D-xylose; D-galactose and D-cellobiose in the range 0.7-7 micromoles/ml. Enzyme activities were expressed as in (10.3.1.1).

10.3.3.2 Purification of substrates

Araban : Araban was found to contain considerable quantities of reducing sugars as determined by Nelson's procedure. Two methods were used for its purification.

(a) Washing procedure

Due to its solubility in water, araban was purified by washing with 95% (v/v) acidified ethanol, followed by drying in the usual way.

(b) Purification by ion-exchange chromatography

A chromatographic method (Knee, 1970) was used for further purification. Araban was dissolved in 0.005M phosphate buffer (pH 6.5) at a concentration of 50 mg/ml. The solution (10 ml) placed on a DEAE-cellulose column pre-equilibrated with the same buffer containing 0.001M EDTA. The column was eluted at a flow rate of 50 ml/hour and 5 ml fractions were collected. Each fraction was assayed for total carbohydrate content using the phenol-sulphuric acid procedure as described in section (12). The peak fractions of the total carbohydrates were pooled, subjected to dialysis overnight at $4^{\circ}C$ against distilled water and then freeze-dried. The final weight and the yield of araban were determined.

Xylan was purified using the method of Strobel (1963). Two grams of xylan were mixed with 180 ml water in a Waring Blender. The suspension was centrifuged at 10,000 g for 5 min. and the precipitate was washed twice with 100 ml of absolute ethanol and once with 100 ml diethylether and then dried at $80^{\circ}C$ for 24 hours. Carboxymethylcellulose and galactan were used without further purification.

10.3.4 α-L arabinofuranosidase and β-D Galactopyranosidase

Activities of α- arabinofuranosidase and β-D galactopyranosidase were assayed in reaction mixtures containing 0.5 ml enzyme sample, 0.5 ml of 0.1% (w/v) p-nitrophenyl-α-L arabinofuranoside or β-D galactopyranoside and 2 ml 100 mM sodium acetate buffer at pH 4.2 and 3.4 respectively. After a suitable interval, the reaction was terminated and the yellow colour of the phenate ion developed by adding 1 ml of saturated Na_3Co. The optical absorbance was read at 403 nm with a Beckman DB or DB-GT spectrophotometer. Units of enzyme activity were expressed as micromoles p-nitrophenol produced by reference to a calibration graph over the range 0.02 - 0.8 micromoles per ml.

10.3.5. Pectin and pectate lyases

Two assay methods were used to detect pectin and pectate lyases.

10.3.5.1 Spectrophotometric determination

Lyitic cleavage of pectin substrates was examined by following the increase in absorbance of reaction mixtures at 232 nm for pectate lyase (Ayers et al., 1966) and at 240 nm for pectin lyase (Archer 1973). Sodium polypectate or pectin were used as substrates respectively. The substrate solutions were clarified by centrifugation at 35000 g for 30 min. using a high speed 18 Refrigerated Centrifuge (MSE). Reaction mixtures contained 1 vol. 2% (w/v) substrate; 2 vol. distilled water and 2 vol. of buffer. The activity of each of the two enzymes was assayed at pH 5 and 8 with respectively 100 mM sodium acetate and 100 mM Tris-HCL as buffers. Enzyme preparation, 0.2 ml, was added to 4 ml of substrate mixture and the extinction read immediately and after 3-hour incubation at 30°C. One unit of enzyme activity is defined as that amount of enzyme that produces an increase in absorbance of 0.1 in 100 min. under the assay

conditions described.

10.3.5.2 Thiobarbituric acid test

A colorimetric method was also used to detect pectin and pectate lyase activities by the production of a characteristic coloured thiobarbituric acid-reactive end product having an absorption maximum at 550 nm (Ayers and Papavizas, 1965). Reaction mixtures comprised 4 ml of 1% (w/v) polygalacturonic acid or pectin adjusted to pH 5 or 8 with NaoH, 1 ml of 100 mM sodium acetate buffer (pH 5) or 100 mM Tris-HCL (pH 8) , with or without 1 ml of 0.01M $CaCl_2$ and 4 ml of dialysed enzyme preparation. A drop of toluene was added to the reaction mixtures which were incubated at 30°C for 24 hours. The reaction was stopped and enzyme protein and excess substrate were precipitated by adding successively 0.6 ml of 9% (w/v) $ZnSO_4$-9HO and 0.6 ml of 0.5M NaOH (Ayers et al., 1966). The mixture was vigorously shaken and then clarified at 16000 g for 15 min. Five ml of the clarified reaction mixtures were added to tubes containing 3 ml of 0.04M thiobarbituric acid (TBA), 1.5 ml of 1M HCL and 0.5 ml of distilled water. The tubes were placed in boiling water bath for 30 min., cooled and the absorbance of the solution was determined over the range 490-560nm.

10.3.6 Macerating activity

Tissue maceration tests were made using tomato stem disks or 0.5-1 mm thick. All disks used in an experiment were obtained from the same parts of stems of plants at the same stage of growth in an effort to reduce variation. The degree of maceration was estimated at intervals by determining the ease with which the disks could be pulled apart with dissecting needles. A macerating index with a 0-5 linear scale was used. A rating of "0" indicates no maceration and a rating of "5" indicates a complete loss of coherence of the tissue. Intermediate values indicate intermediate degrees of tissue maceration.

Treatment of tissue disks with enzyme preparations is described in Results (section 4.11).

10.3.7 Cell death

Cell death was investigated by using the Neutral Red procedure of Tribe (1955). After treatment with enzyme preparations for various periods of time triplicate disks were transferred to 0.1M phosphate buffer (pH 7) containing 1M KNO_3 and 0.1% (w/v) neutral red. This solution was freshly prepared so as to avoid the slow precipitation of the neutral red which occurs in alkaline solution. After immersion for 20 min., the tissue disks were transferred to the same solution without neutral red. Cell death was estimated using a 0-5 rating system - a rating of "0" indicates that virtually all cells in the tissue slice accumulated and retained the dye (comparable to control tissue at zero time) and a rating of "5" indicates that no cell retained the dye.

10.3.8 Cell leakage

Changes in the permeability of plant cells were measured by determining the loss of electrolytes from tissue disks during treatment. Disks were cut, washed in water and then transferred to specimen tubes. Reaction mixtures contained 12 washed disks, 10 ml of distilled water and 2 ml of dialysed fresh or autoclaved enzyme preparation. Controls with water and without enzyme were also included. Reaction mixtures were mixed and the increase in conductivity of the bathing solution was measured at intervals after the addition of the enzyme using a Chandos conductivity meter. Electrolytes leaked were expressed as a percentage of the total present in the tissues. For determination of the total electrolytes the tubes were finally heated on a boiling water bath, cooled and then the conductivity of the supernatant was

measured.

11. Estimation of total proteins

Protein content of culture filtrate or plant extract was determined by following the procedure of Lowry et al., (1951). This is a colour reaction between Folin-Ciocalteu reagent and the phenolic groups on tyrosine residues in the protein. To a sample of 0.1 ml of the protein solution; 1 ml of a reagent (made by adding 1 ml 0.5% (w/v) $CuSO_4 - 5H_2O$ in 1% (w/v) sodium citrate to 50 ml 2% (w/v) Na_2CO_3 in 0.1M NaOH) was added. The reagent was freshly prepared and discarded after 1 day. The solution was mixed well and left to stand for 10 min. at room temperature, after which 0.1 ml of the Folin-Ciocalteu reagent (commercial reagent (BDH) diluted 2-fold with distilled water to give a 1M acid solution) was added and mixed rapidly. After 1 hour, the absorbance of the sample was read at 750 nm. A standard curve relating absorbance at 750 nm to protein content was prepared with bovine serum albumin over the range 10-500 mg/ml.

12. Estimation of total carbohydrates

The method used was that of Dubois et al., (1956). To 1 ml of a carbohydrate-containing solution an equal vol. of 5% (w/v) aqueous phenol was added and mixed. Concentrated sulphuric acid, 5 ml, was added quickly and the solution was mixed thoroughly. The reaction tubes were allowed to cool and then the absorbance was read at 490 nm against a reagent blank. Standards containing from 0 to 900 µg/ml of glucose were treated similarly.

12.1 Estimation of reducing sugars

Total reducing sugars in enzyme reaction mixtures were analysed by

Nelson's (1944) modification of the Somogyi method. To 2 ml sugar solution, 2 ml of Somogyi copper reagent was added. The mixture was heated rapidly to 100°C for 10 min., cooled to 22°C and then 2 ml of arsenomolybdate reagent was added. After thorough vortex mixing extinction was read at 500 nm on a Beckman DB- spectrophotometer against a reagent blank treated identically except that the enzyme sample was added after the addition of Somogyi reagent. If necessary, the excess substrate was precipated by centrifugation at 3000 g for 3 min.

Calibration curves were prepared with galacturonic acid in the range 0.8-5 micromoles/ml and glucose in the range 0.5-5 micromoles/ml.

The copper and arsenomolybdate reagents were prepared according to Archer (1973).

13. Estimation of total phenols

Total phenols were determined quantitatively by employing the Folin-Ciocalteu reagent (Bray and Thorpe 1954) and measuring the intensity of the blue colour spectrophotometrically at 650 nm (Retig and Chet 1974). A suitably diluted portion of the phenolic extract was transferred to a boiling tube and made up to 10 ml with water. Folin-Ciocalteu, 1 ml and 20% (w/v) sodium carbonate solution, 2 ml, were added. The tube was heated in a boiling water bath for 1 min. and then cooled in water to room temperature. The blue solution was diluted to 25 ml before spectrophotometry.

13.1 Estimation of o-dihydroxyphenols

The o-dihydroxyphenols were measured according to Johnson and Schaal, (1957). To 1 ml of the phenol-containing ethanolic extract, 1 ml 0.5M-HCL; 1 ml Arnow's reagent (10 g of $NaNO_2$ and 10 g of $NaMoO_4$ in 100 ml distilled water) and 10 ml of distilled water were added. The pink colour developed after

addition of 2 ml 1M NaOH to the mixture was measured at 515 nm.

Both Folin-Ciocalteu and Arnow's reagents were replaced by water in the blank samples used for the adjustment of the spectrophotometer (Beckman DB) reading to zero absorbance. Chlorogenic acid was used in both methods for preparation of standard curves in the range 110-670 µg/ml for total phenols and in the range 12-198 µg/ml for o-dihydroxyphenols.

14. Estimation of oxalic acid

The oxalic acid content of culture filtrates and plant tissues was estimated by titration against $KMnO_4$. Filtrates from 13-day old cultures on sodium polypectate and extracts from healthy and diseased plants were used. Culture filtrates and plant extracts were concentrated by freeze-drying respectively to one third the original volume and to a volume equal to the fresh weight of the extracted tissues. To 1 vol. of the concentrated sample, 2 vol. of cold acetone were added and mixed and the mixture was allowed to stand overnight at - 20°C. The supernatant liquid was collected by centrifugation at 4000 g for 15 min. and the precipitates were washed with acetone: water (2:1 by vol.) Washings were added to the supernatant and acetone was removed by evaporation under reduced pressure at 35°C. Samples (15 ml) of the aqueous residue were put into 30 ml centrifuge tubes. Three ml of saturated $CaCl_2$ solution were added to each tube, mixed and the tubes were left to stand for 3 hours and then centrifuged at 7000 g for 20 min. The supernatant liquid was discarded and the pellet was washed with a little very dilute ammonium hydroxide and centrifuged again. The contents of the tubes were transferred to a 100 ml beaker and 10 ml 2M H_2SO_4 were added to dissolve the sediment. The solution was heated to boiling and titrated while hot to a faint pink coloured end point with 0.01M potassium permanganate and the amount of oxalate present was calculated as the free acid.

14.1 Preparation of potassium permanganate

Since distilled water usually contains traces of reducing substances which react slowly with permanganate to form hydrous manganese dioxide which promotes the autodecomposition of permanganate (Kolthoff and Sandell, 1943), the solution (0.1M potassium permanganate) was boiled gently for 30 min. (Arthur, 1961) and then allowed to cool to the laboratory temperature before filtering through a sintered-glass filter crucible. The filtrate was transferred to a clean bottle of dark brown coloured glass. 0.01M potassium permanganate was prepared by dilution of this solution. The dilute solution was prepared immediately prior to use and was not stored because of the relatively rapid rate decomposition.

15. Chromatographic techniques

15.1 Paper chromatography

One-dimensional descending paper chromatography was used to detect oligogalacturonides in enzyme digests. The chromatogram was developed on a sheet of a Whatman no. 1 paper, cut 30 cm wide and 50 cm long. A starting line was pencilled approximately 10 cm from the end of the paper and the individual starting points for the various samples being investigated were arranged at 3.0 cm intervals along this line. The samples were applied to the paper with the aid of an Agla Micrometer Syringe. Each sample (50 µl) was applied in several batches at regular intervals with sufficient time for evaporation between each application. For rapid evaporation, a hair-dryer was employed. The wet spot was not allowed to exceed 1 cm in diameter. After application of the samples, the paper was folded back above the starting line and hung from a trough in a tank which had been prepared by putting a small amount of solvent mixture at the bottom. The solvent was poured carefully into the trough and

the tank was covered with a sheet of glass. All chromatograms were run at room temperature (21 - 26°C). After 18 hours, the paper was removed from the trough and dried either in a fume cupboard with a strong draught or by using a hair-dryer.

15.1.1 Solvent system

The solvent system employed was ethylacetate-pyridine-acetic acid-water in a 5:5:1:3 proportion by volume. This system was found to give the best separation and most reproducible results (Archer 1973).

15.1.2 Locating reagents

Silver nitrate reagent (Trevelyan et al., 1950) was used for detection of reducing sugars on chromatograms. The reagent comprised (1) 0.1 ml of saturated silver nitrate solution in 50 ml of acetone, (2) 0.5% (w/v) sodium hydroxide in ethanol. The paper chromatogram was sprayed liberally or dipped through reagent (1) and allowed to dry thoroughly. Chromatograms were then sprayed or dipped with reagent (2). Reducing sugars appeared as dark brown spots. Contrast could be enhanced and the pattern preserved by brief spraying with 10% (w/v) aqueous sodium thiosulphate (Hathway and Seakins 1958). Migration distances were measured relative to the distance travelled by glucose . Silver nitrate solution was kept in a dark reagent bottle.

Reducing sugars were also revealed by spraying the dried chromatograms with a solution containing: benzidine (0.5 g); glacial acetic acid (20 ml) and absolute ethanol (8 ml). Chromatograms were then heated to 105°C for 15 min. (Cramer, 1955). The separated sugars appeared as brown spots. This reagent was found to be less sensitive than the silver reagent.

15.1.3 Standards

Sugars used as reference compounds included: glucose; mono-di-; and trigalacturonic acids; arabinose; galactose and xylose. Samples of the sugar standards (50 µg) were included on each chromatogram. All sugars were stored at 4°C in 10% (v/v) aqueous isopropanol.

15.2 Thin-layer chromatography

Separation and identification of products of enzyme reactions was also achieved using cellulose thin-layer chromatography. The size of the sample, the reference standards and the application procedure were the same as described for paper chromatography. Samples were applied in spots 1 cm from the bottom of the plate and at 1.5 cm intervals. Each spot was approximately 3 mm in diameter. Plates were developed in an ascending manner for 4 hours at room temperature (21 - 26°C) in solvent-equilibrated tanks. Chromatograms were sprayed after drying with silver nitrate reagent to locate the reducing sugars.

15.2.1 Solvent systems

The following systems were tried:

A Butan-1-ol-acetic acid-water 4:1:1 (by vol.)

B Butan-1-ol-acetic acid-water 4:1:5 (by vol.) (upper equilibrated phase)

C Butan-1-ol-acetic acid-water 20:5:11 (by vol.)

D Butan-1-ol-acetic acid-water 2:1:1 (by vol.)

E Isopropanol-water 4:1 (by vol.)

The best resolution of sugars was obtained by using system (A), see appendix (I).

15.2.2 Preparation of plates

Microcrystalline cellulose (CELLEX MX) was used for preparation of the plates. Cellulose powder (20 g) was mixed to a homogeneous suspension with 50 ml distilled water using a pestle and mortar. The cellulose suspension was poured into a Shandon TLC spreader which was adjusted to give layers 0.5 mm thick.

16. Extraction techniques

16.1 Extraction of stem cell walls

The technique used was that of Cooper et al., (1981). Stem tissues were homogenized with 3 vol. of acetone for 3 min. in a Sorval-Omni mixer at setting 6. The homogenate was filtered through several layers of cheesecloth and the process repeated until filtrates appeared free of pigment. The residue was then homogenized in 10 vol. (w/v) of 100 mM potassium phosphate buffer pH 7.0 for 5 min., filtered and the process repeated a further 4 times followed by 5 washes in distilled water. Cell walls were then extracted for 5 min. with chloroform methanol (1:1 v/v); filtered; resuspended and finally washed exhaustively with acetone. They were dried at 70^{o}C for 24 hours and then ground to a homogenous powder using a CRYPHON mill.

16.2 Extraction of enzymes

Enzymes were extracted by blending diseased tissues in 2-5 vol. of 50 mM sodium acetate or citrate buffer (pH 4.6) containing 0.1M NaCl and 0.5% (w/v) ascorbic acid for 1½ min. Tissues were chilled in an ice bath during extraction of the enzymes. The macerated tissue was filtered through several layers of cheesecloth. The extract was dialysed and assayed for enzyme activity

after dialysis or kept until used as described for culture filtrate.

16.3 Extraction of phenols

The method used was based on that described by Biehn et al., (1968). Healthy and diseased stems were cut with a razor blade into small pieces, weighed and then plunged into boiling 80% (v/v) ethanol (1:5 w/v) in test tubes in a water bath. After boiling for 15 min., the stem tissues were separated and homogenized in cold 80% (v/v) ethanol for 1½ min. in a Sorvall-Omni mixer at setting 9. The mixture was boiled for another 15 min. and then filtered through a Buchner funnel. Filtrates were combined and evaporated under reduced pressure at 50°C to a small volume (1 ml/g fresh weight).

16.4 Extraction and partial purification of polygalacturonase inhibitors

The procedure used was that of Albersheim and Anderson (1971). Twenty grams of stem tissue from the basal portions of 5-week old tomato plants were chopped with a razor blade into 4 mm segments. The segments were homogenized in an ice bath in 3 vol. of 100 mM potassium phosphate buffer (pH 7) in a Waring blender for 1 min. The resulting suspension was filtered through a coarse sintered-glass filter. The supernatant solution was discarded and the residue was homogenized with 3 vol. of the same buffer. Again, the supernatant fraction was discarded and the residue was ground with 3 vol. of 500 mM potassium phosphate buffer (pH 7). The solution was collected by filtration and the residue was re-extracted with a further 3 vol. of the 500 mM buffer. The extracts were combined and $(NH_4)_2SO_4$ was added to 70% of saturation. The precipitate was collected by centrifugation at 35000 g for 10 min. The pellet was suspended in buffer: the soluble portion is expected to contain the polygalacturonase inhibitors.

16.5 Extraction of oxalic acid

Oxalic acid was extracted from healthy and diseased stem tissues by employing the same procedure used for extraction of enzymes. The extractant used was 50mM sodium acetate buffer (pH 4.6) containing 0.1M NaCl.

17. Sources and purity of chemicals

Chemicals used were mainly of analytical grade and obtained from BDH, Sigma, Hopkin and Williams Ltd. and Koch-Light Laboratories Ltd. Pectin NF was from Bulmers; lupin galactan, di- and trigalacturonic acids were kindly provided by Dr. S. A. Archer; Araban (Koch-Light Laboratories); xylan (Sigma, Chemical Company); carboxymethylcellulose (BDH Chemical Ltd.); p-nitrophenyl-α-L arabinofuranoside (Sigma, Chemical Company); p-nitrophenyl-β-D galactopyranoside (Sigma, Chemical Company); polygalacturonic acid (BDH Chemical Company) and sodium polypectate (Sigma, Chemical Company) were obtained commercially. Except for D-glucose and D-xylose which were obtained from (Sigma, Chemical Company), the other reference standards used in chromatography and enzyme assays were purchased from (BDH, Chemical Ltd.). Diethylaminoethly cellulose and microcrystalline cellulose (CELLEX MX) were obtained from Wand R. Balston Ltd., and Bio-RAD Laboratories respectively. Sephadex G 100 and all protein standards were purchased from Sigma, Chemical Company.

18. Statistical analysis of results

Statistical methods used were as described by Parker (1979). Results from the experiment on pathogenicity of different isolates of *S. sclerotiorum* and the experiment on susceptibility of tomato plants at different stages of growth to infection by the fungus were subjected to analysis of variance.

According to Parker (1979), the most important assumptions on which this method of analysis is based are;

(i) that the effects are additive, i.e. an individual value (of X) is considered to be made up of the grand mean + treatment effect + uncontrolled error.

(ii) that the error is normally distributed and has equal variance for all treatments.

Results in the form of small, whole-numbered counts tend to have a variance proportional to their mean (or equal to it). So, in order to render the variances independent of the means, the original data can be adjusted or transformed by means of some mathematical function. An appropriate transformation when counts are low or when zeros occur is $\sqrt{x + 0.5}$. Because of the occurrence of zero values in the results obtained from the two experiments mentioned above, the original data were transformed according to this formula before analysis.

The significance of differences between means in these experiments and in the experiments on the effects of host factors on growth in vitro of S. sclerotiorum was estimated using F or t tests. Individual differences between treatment means were examined using: (i) Least significance differences L.S.D. or (ii) a multiple range test.

Numerical and graphical data are presented together with an estimate of the standard error or the standard deviation as indicated in each case. Results from isoelectric focusing, gel filtration, etc, were in general not statistically analysed.

RESULTS

1. Pathogenicity testing and observations on disease development

The type of response of a host to infection with a pathogenic micro-organism reflects the nature of the interaction between them. The development of symptoms results from changes at cellular and sub-cellular levels, due in turn to physico-chemical changes brought about by the fungus. The age of the whole plant or of its organs may influence the development of disease. Effects may be qualitative (differences in appearances of symptoms) or quantitative (differences in numbers or intensity of symptoms per unit of inoculum).

The prime objective of the work in this section was to describe symptoms of the disease incited by S. sclerotiorum and to test susceptibility of tomato plants at different stages of growth.

1.1 Infectivity of different isolates of S. sclerotiorum

A pathogenecity test was conducted using three isolates of S. sclerotiorum and 4-week old tomato plants. The isolates used were RC/802, RC/801 and W4074. The plants were arranged in a randomized block design comprising four treatments and five replicates. The treatments included the three isolates plus a control in which the plants were inoculated with disks taken from sterile MA medium incubated at the same temperature and for the same period of time as the cultures of the fungus. The plants were inoculated using the technique of Price and Colhoun (1975). The severity of the disease was assessed four days after inoculation.

As shown in Table (5), the three isolates were virulent on tomato. No significant difference was found between them in degree of pathogenicity

TABLE (5)

Susceptibility of tomato plants to infection with three isolates of S. sclerotiorum

Isolate	Mean disease index ± SE (Transformed values *)
RC/802	2.2 ± 0.1
RC/801	2.0 ± 0.1
W4074	2.1 ± 0.1

* Original data were transformed according to the formula $\sqrt{x + 0.5}$ (Parker 1979).

to tomato. Isolate RC/802, being the most consistently aggressive, was used throughout the rest of the work reported in this thesis.

1.2 Plant age and susceptibility to the fungus

As this study involved a comparison of young and old tissues and since tissues of widely different ages occur on a single plant, all comparisons were made on tissues of the basal part of the stem at different stages of growth. Every two newly produced true leaves were considered to represent a stage of growth. Twelve plants of each stage were selected at random and inoculated using mycelial disks. Disease assessment was made seven days after inoculation using the scale described earlier.

As shown in Table (6), the susceptibility of tomato plants to infection by S. sclerotiorum decreased with increasing age and stage of development. Statistical analysis of the results revealed highly significant differences ($P = 0.001$) in susceptibility at different stages of growth. The highest levels of infection occurred during the first and second stages at which the plants had average disease severity ratings (before transformation) of 4.9 and 4.1 respectively. The first visible symptoms of the disease was the formation of light-coloured, water-soaked lesions at the infection site on the stem. The basal parts of the infected stems of plants at the first two stages of growth became much thinner and softer than the stem above and this led to the collapse of some plants 2-3 days after inoculation. The lesions expanded upward more rapidly than downward. The upward progress of the disease was checked in many cases by the formation of dark brown zones around lesions and the appearance of adventitious roots, particularly at the last two stages studied in this experiment (Plate 1). No sudden collapse of the infected plants was observed at these stages. Diseased plants showed yellowing and epinasty of the lower leaves (Plate 2). Yellowing started with the lowermost leaves and proceeded upwards. At first it appeared as large interveinal yellow patches on the older leaves but later the entire leaves became yellow. White patches of mycelium appeared on the outside

TABLE (6)

Susceptibility of tomato plants at different stages of growth to infection with S. sclerotiorum

Stage of growth	Mean disease index ± SE (Transformed values *)
First	2.28 ± 0.02
Second	2.11 ± 0.07
Third	1.66 ± 0.08
Fourth	1.83 ± 0.13
Fifth	1.54 ± 0.12
Sixth	1.44 ± 0.10

* Original data were transformed according to the formula $\sqrt{x + 0.5}$ (Parker 1979)

PLATE (1)

Formation of the dark brown zone around the lesion and the appearance of the adventitious roots primordia on the infected stem of 8-week old plant.

PLATE (2)

S. sclerotiorum - infected tomato plant showing foliar symptoms (A) compared with a healthy plant (B).


A
B

of the stem with the formation of a few immature sclerotia - 1 to 3 in number - seven days after inoculation on some plants at the last three stages. The pith in some of the infected plants was replaced by the mycelium with one or two sclerotia formed in the cavity.

1.3 Susceptibility of tomato stem to infection by S. sclerotiorum

The susceptibility of different parts of tomato stem to infection by S. sclerotiorum was assessed by inoculating stem segments from different portions of mature plants with mycelial disks. The length of infected stem and the disease index value were determined at 12 hour intervals over a period of 3 days. Results obtained are given in Table (7).

The typical symptoms including water-soaked lesions surrounded in the old tissues by dark brown zones were developed in inoculated cuttings. Lesions appeared at 1 day and they were well developed at 3 days after inoculation. The highest rates of lesion expansion occurred during the first two days. On the basis of the length of lesion, susceptibility of the stem tissue increased towards the tip of the stem. However, if we consider the disease index values, almost all parts of the stem were equally susceptible to the infection by the pathogen. After incubation for 3 days, all parts reached more or less the same level of infection.

1.4 Lesion expansion on stems of 8-week old plants

Observations made during the experiments described above showed that lesion expansion on the basal parts of the stems of older plants was checked by the formation of dark brown zones and was associated with the appearance of adventitious roots. An experiment was conducted using 8-week old tomato plants to measure lesion expansion over a period of 14 days. Plants were inoculated using mycelial disks and lesion length was measured at 1-day intervals.

TABLE (7)

Susceptibility of tomato stem to infections by S. sclerotiorum

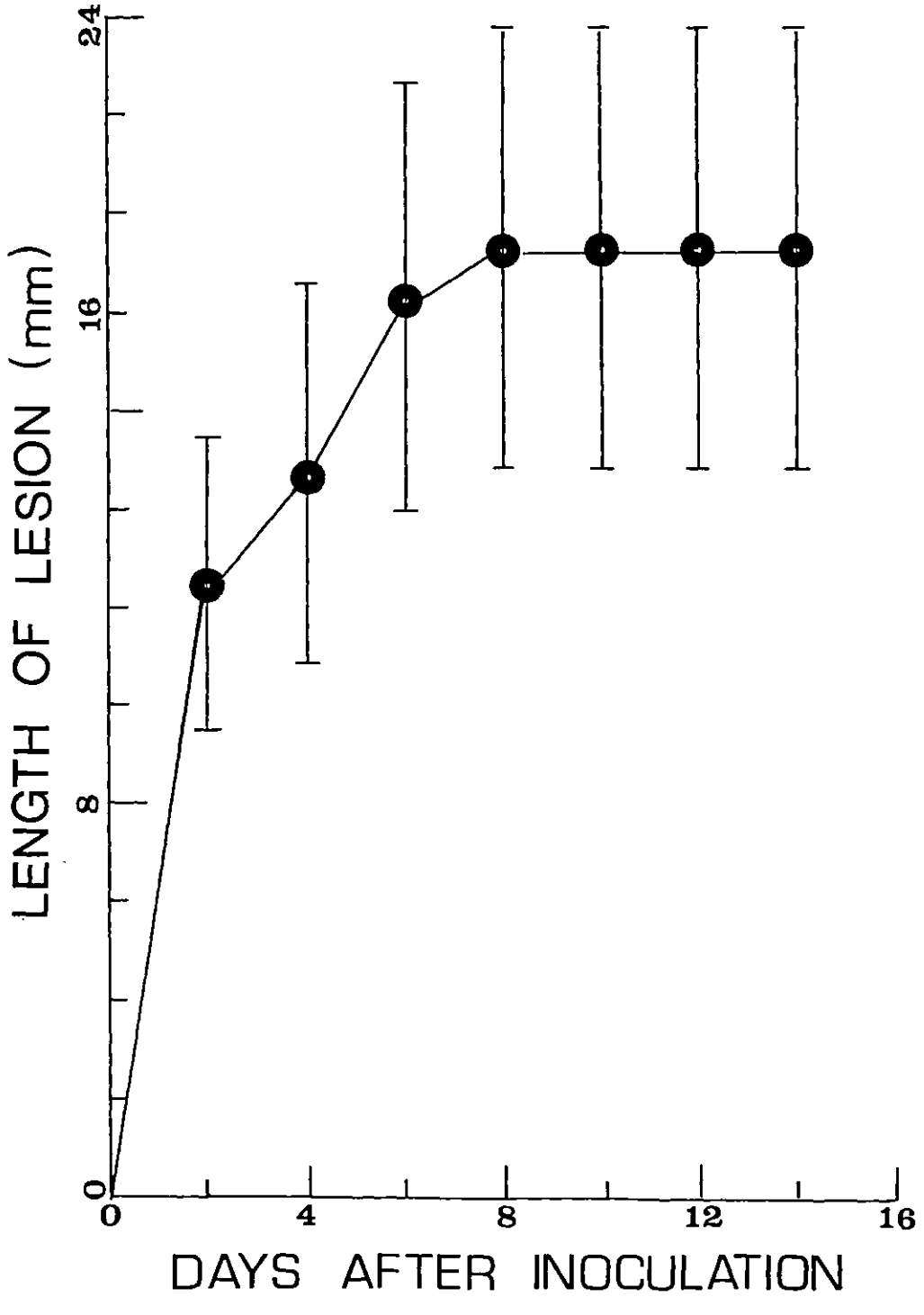
Stem part	Mean internode length (mm)	Hours after inoculation					
		12	24	36	48	60	72
Stem base	44.8	0	0.8 (.3)	1.7 (.9)	5.2 (1.7)	6.9 (1.9)	7.2 (1.9)
1st internode	56.8	0	2.6 (.8)	4.2 (1.5)	6.2 (1.8)	6.4 (1.8)	6.9 (1.9)
2nd internode	41.3	0	0.3 (.2)	3.7 (.9)	4.8 (1.3)	6.2 (1.4)	7.1 (1.7)
3rd internode	46.3	0	1.3 (.3)	3.1 (1)	7.3 (1.7)	9.2 (1.7)	9.4 (1.9)
4th internode	48.8	0	2.3 (.7)	5.8 (1.4)	8.2 (1.8)	10.5 (1.9)	11.3 (1.9)
5th internode	61.3	0	1.6 (.8)	4.3 (.8)	6.3 (1.2)	6.6 (1.3)	9.2 (1.3)
6th internode	95.8	0	1.8 (.7)	6.4 (1.3)	10.0 (1.4)	13.0 (1.9)	20.7 (1.9)
7th internode	102.5	0	1.7 (.7)	8.4 (1.5)	11.2 (1.7)	15.8 (1.9)	19.7 (1.9)
8th internode	88.8	0	2.0 (.5)	7.7 (1.1)	7.9 (1.1)	15.6 (1.2)	16.7 (1.2)
9th internode	71.3	0	0.2 (.2)	1.7 (.8)	2.7 (1.2)	4.8 (1.7)	5.7 (1.9)

Data represent mean lesion length (mm) on the stem base and each of nine internodes. Mean disease index values are indicated between brackets.

FIG(3)

Expansion and limitation of lesions on tomato stem inoculated by *S. sclerotiorum.*

Bars denote standard deviations.


LENGTH OF LESION (mm)
0
8
16
24
0
4
8
12
16
DAYS AFTER INOCULATION

As shown in Fig. (3), the development of lesions was very rapid during the early stages of pathogenesis but the process became restricted and no further expansion was observed after a week from inoculation. By this time, the formation of black or dark brown zones around lesions and the development of adventitious roots were very evident.

2. Preliminary studies on the process of penetration and infection of tomato by S. sclerotiorum

Results obtained from the experiments in the previous section suggest that partial resistance of tomato to S. sclerotiorum infection is present in the basal parts of the old stems. A series of experiments were conducted to study the mode of penetration and infection of tomato stems and leaves by the pathogen using two types of inoculum; mycelium from sclerotia and ascospores. All drawings were made with the aid of a camera lucida on a binocular research microscope (Olympus Optical Co., Ltd) fitted with a 100X oil immersion objective and a 10X eye piece.

2.1 Infection by mycelium from germinating sclerotia

For the study of the mycelial infection of tomato by S. sclerotiorum, isolate RC/802 was used. The sclerotia were germinated in sucrose - casamino acids liquid medium as mentioned in Materials and Methods.

2.1.1 Infection of detached leaves and stem cuttings

Leaves and hypocotyls were obtained from 4 or 5-week old plants and inoculated as described earlier. Four hours after inoculation and then every two hours, small pieces of the inoculated tissues were sampled, cleared in lactophenol and stained with aniline blue. The mode of movement of the fungus within the host was observed by making transverse

sections of the inoculated tissue at the same time intervals.

Microscopy of the cleared leaves revealed that some hyphae were branching dichotomously at their tips by 4 hours after inoculation, (Fig. 4 A-B). At 8 hours.a few fully-developed infection cushions and many infection cushion initials were observed (Fig. 4C) and (Plate 3). The leaf tissue beneath infection cushions became yellowish-brown in colour, indicating the collapse and the disorganization of the tissue below the points of penetration. The infection cushions were darker than their supporting hyphae. Penetration of the host tissue was also accomplished by simple appressoria. The formation of simple appressoria and the appressorial masses suggested that the mode of penetration of the host tissue was by mechanical rupture of the cuticle. On four occasions hyphal strands were observed entering through stomata (Fig. 4D). At 12 hours after inoculation large, granular, light-blue stained sub-cuticular hyphae were observed. These hyphae (modal diameter was 12.5 µm) grew radially from the points of penetration, (Plate 4). Most of the stomata around these sites were open with signs of destruction of the cellular content of their guard cells. Microscopy of the cleared tissues also revealed that the mode of emergence of the fungus from the host tissue was through breaks in the cuticle and also through stomatal openings, Plate (5) on the adaxial leaf surface.

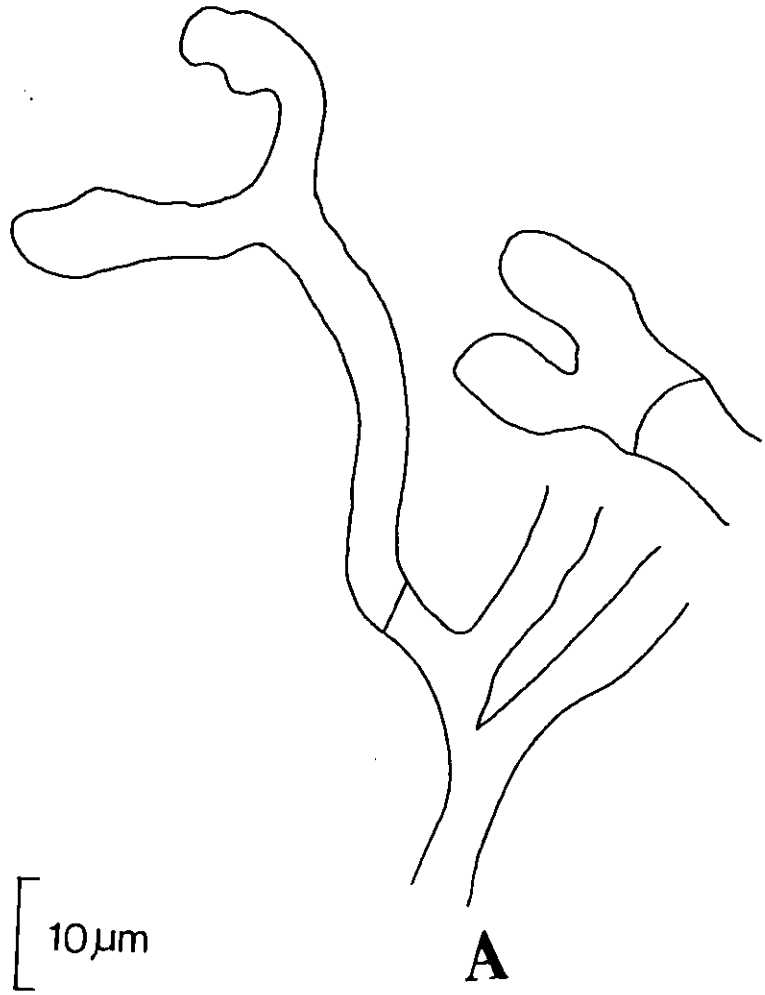
Epidermal strips from the inoculated hypocotyls were also studied microscopically after clearing. The mode of penetration was the same except that no hyphae were observed to enter through lenticels. The sub-cuticular infection hyphae were first observed at 8 hours and they had developed their characteristic parallel orientation by 22 hours after inoculation.

Paraffin-embedded sections revealed that the mycelium grew both inter-and intracellularly throughout the infected leaf and stem tissues.

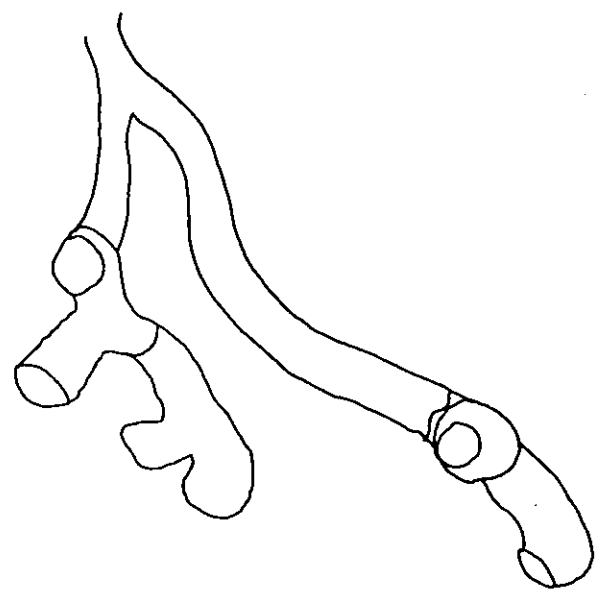
Lesions visible to the naked eye on hypocotyls were evident 8 hours after inoculation, but not until 14-16 hours on the upper surface of the leaves.

FIG (4)

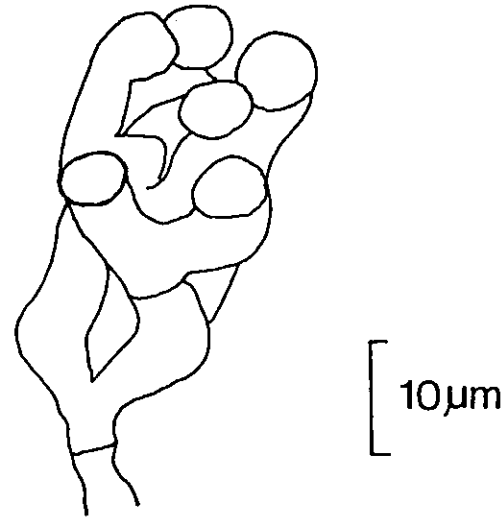
(A, B) Dichotomous branching of the hyphae of S. sclerotiorum and (C) formation of the infection cushion on tomato leaves. (D) penetration of the hyphae through the stomatal opening.



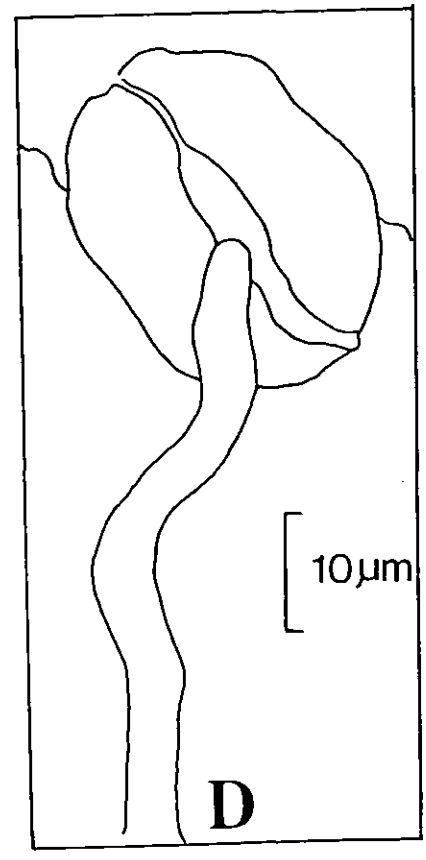
A



B



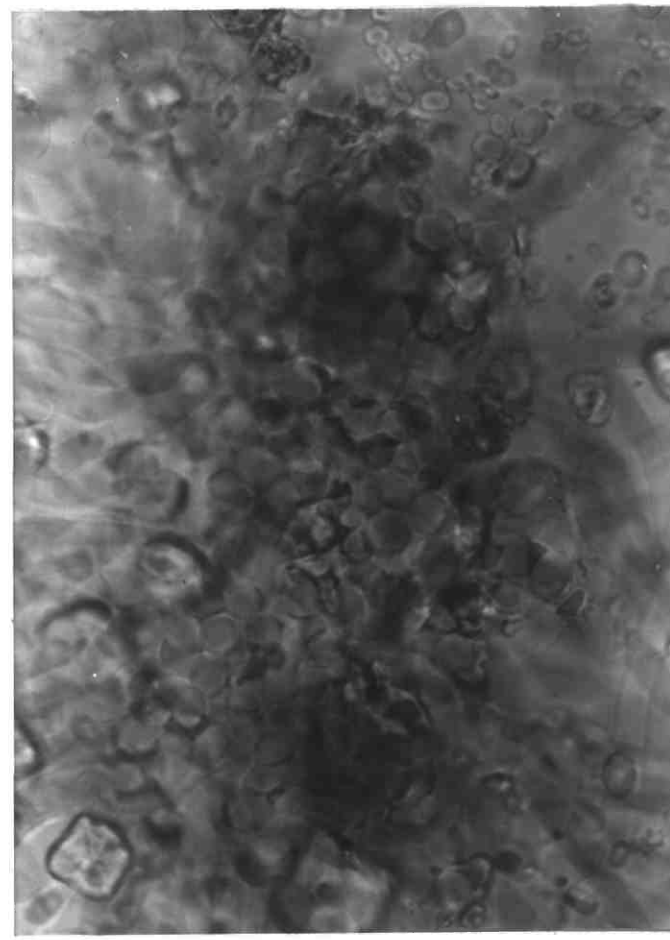
C



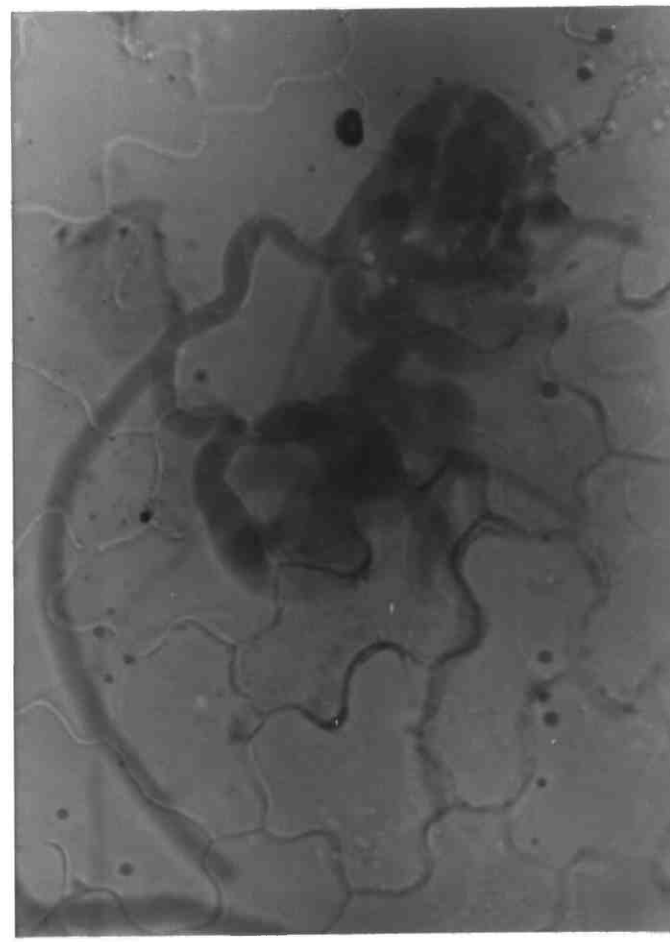
D

PLATE (3)

(1 and 2) Fully-developed infection cushions observed on cleared tomato leaves at 8 hours after inoculation. c. (x 630).



1



2

PLATE (4)

The radial growth of the subcuticular hyphae from the points of penetration. c. (x 160).

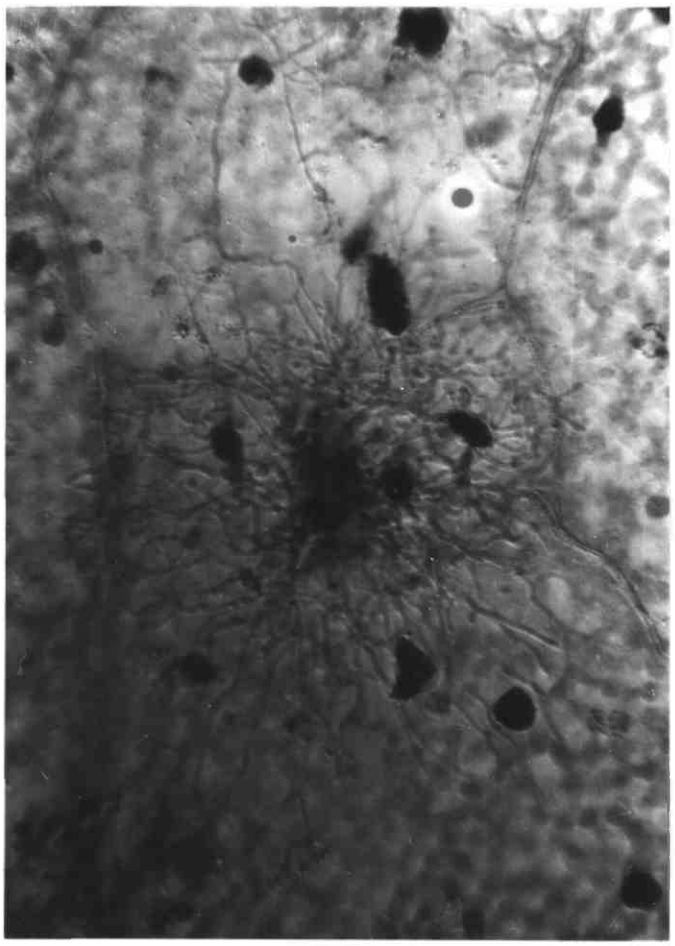
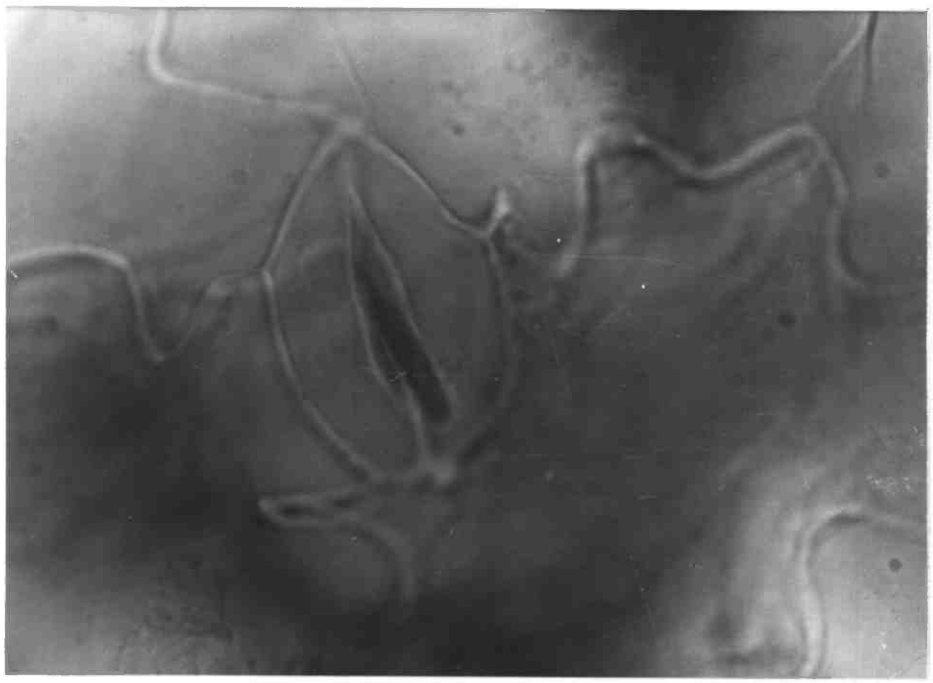


PLATE (5)

Emergence of the fungus through the stomatal opening on the adaxial leaf surface. c. (x 1600)



2.2 Infection from ascospores produced and released under laboratory conditions

2.2.1 Production of apothecia by S. sclerotiorum

Sclerotia incubated in sterile distilled water in Petri dishes did not produce apothecia. Sclerotia of some isolates (Ss_3, Ss_4) produced mycelium only, while those of the other isolates failed to germinate. Incubation on moist sand was successful in inducing apothecium formation. Sclerotia of the isolates RC/802 and Ss_2 produced many apothecial initials after a month of incubation. The number of initials produced by Ss_2 increased rapidly. The stipes elongated and after about 10 days started to differentiate into apothecial disks. Isolate Ss_1 produced numerous stipes and a few were produced by Ss_3 and Ss_4 after about 7 months. Some of the sclerotia of the last two isolates produced small secondary sclerotia.

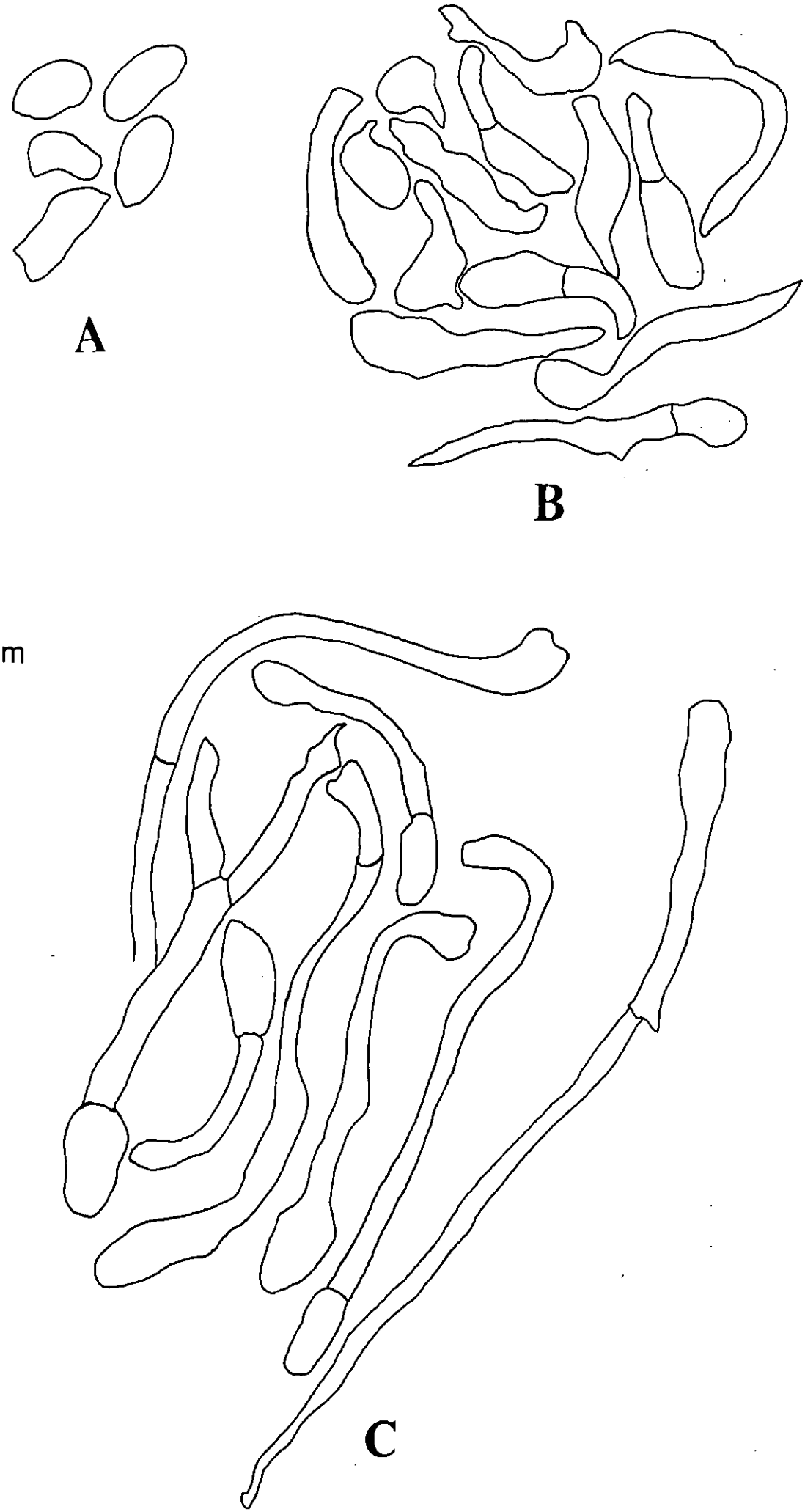
2.2.2 Germination of ascospores and penetration of leaf and stem tissues

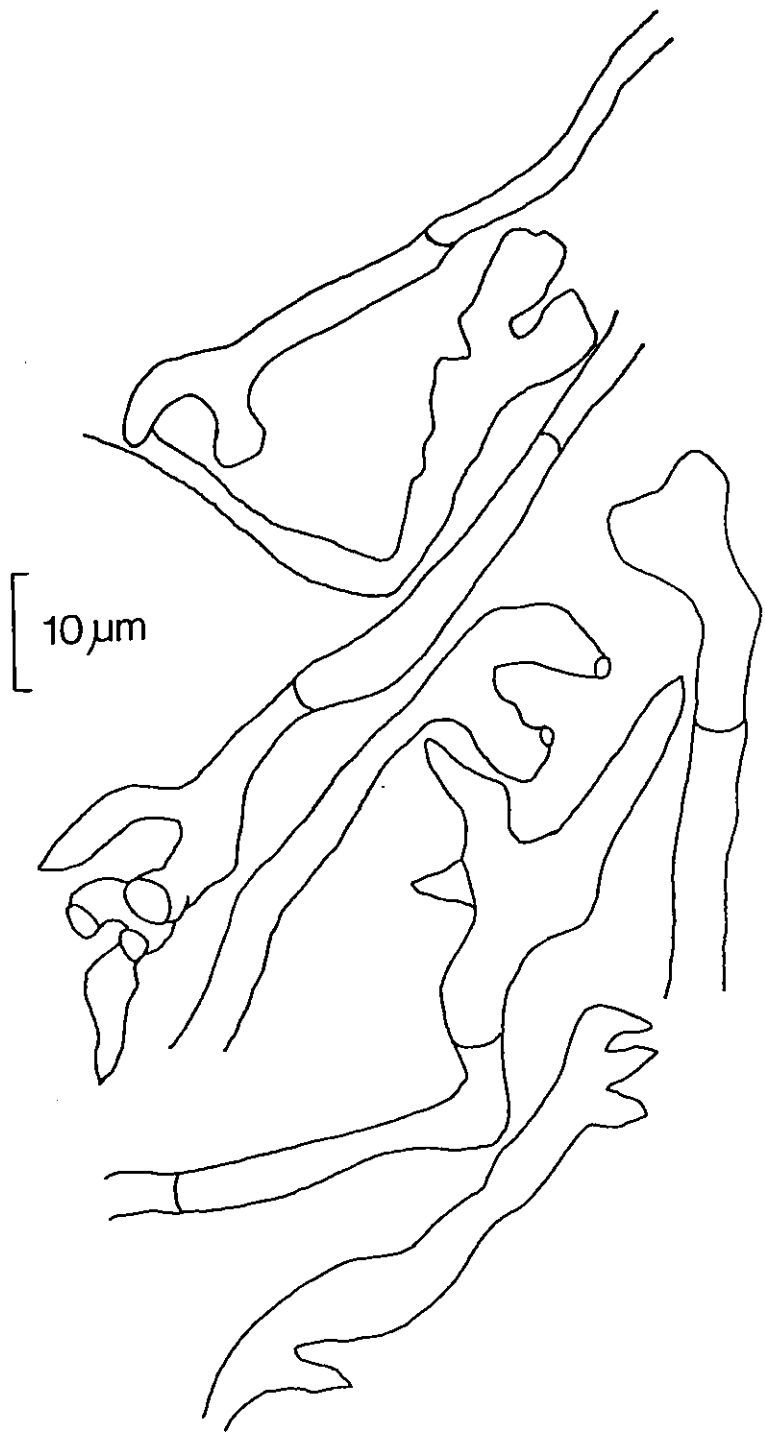
Ascospores of isolate Ss_2 were suspended in SCA liquid medium or water at two different concentrations for each i.e. 2×10^4 and 4×10^4 for SCA and 3×10^4 and 5×10^4 for water. Hypocotyls and leaves were cut from 4-week old plants and inoculated with 10 µl droplets from each spore suspension as described earlier. At 6-hour intervals portions were cut from the inoculated tissues and cleared using the technique of Diener (1955).

Germination of ascospores in water and SCA medium took place within 3-4 hours. Each ascospore germinated by producing one or two germ tubes from one or both ends (Fig. 5 B). Where more than a single germ tube developed from a spore, emergence was asynchronous. The germ tubes grew for a short distance (20-60 µm) and then they became enlarged at the tip (Fig. 5 C). At 24 hours after inoculation many of the hyphae arising from ascospores

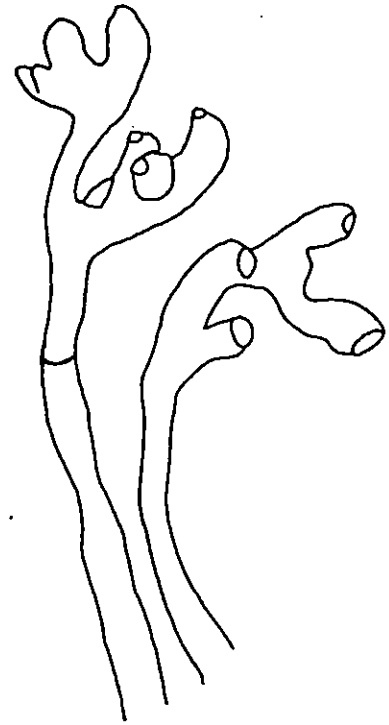
FIG. (5)

(A) Ascospores of S. sclerotiorum; (B) germination of the ascospores from one or both ends of each ascospore; (C) swelling of the hyphal tips and (D) dichotomous branching of the hyphae. (E) formation of the infection cushions on tomato leaves and (F) hypocotyls.


A
B
10 µm
C

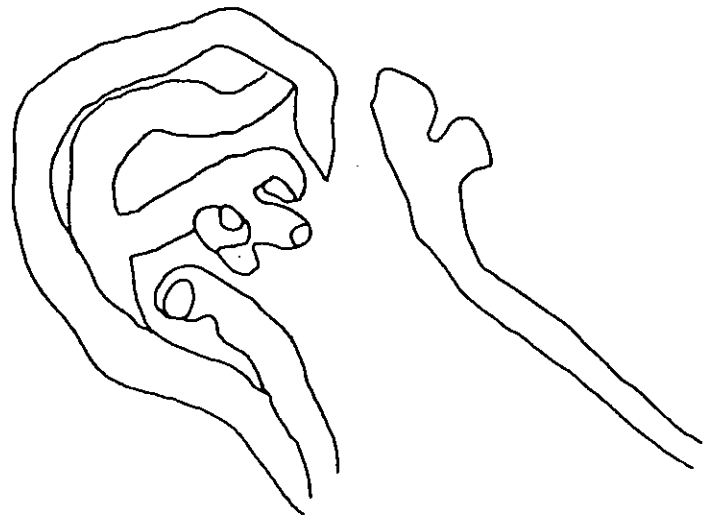


D



E

10 µm



F

germinated in SCA medium branched dichotomously at their tips forming two swollen structures (Fig. 5 D). Signs of destruction of the cuticle under these structures were observed by 24 hours after inoculation. Occasionally hyphal tips continued branching forming four or eight swollen structures, (Fig. 5 E and F) but more commonly they divided only once. Extracellular material accumulated on the surface of stomatal and other epidermal cells 12 hours after inoculation (Plate 6). This was most evident when the leaves were inoculated with ascospores germinated in distilled water. These failed to form swollen structures and no sign of infection was observed. When glucose 1% (w/v) and sodium citrate 0.01% (w/v) were added water-soaked lesions were formed on the leaves 4 days after inoculation but no sign of penetration of the host tissue was observed even when these lesions were examined after 10 days. When ascospores germinated in SCA medium, they branched successfully and formed swollen structures. Water-soaked lesions were formed on detached leaves and hypocotyls two days after inoculation and they were about 5 mm in diameter after three days.

2.2.3 Infection of intact plants

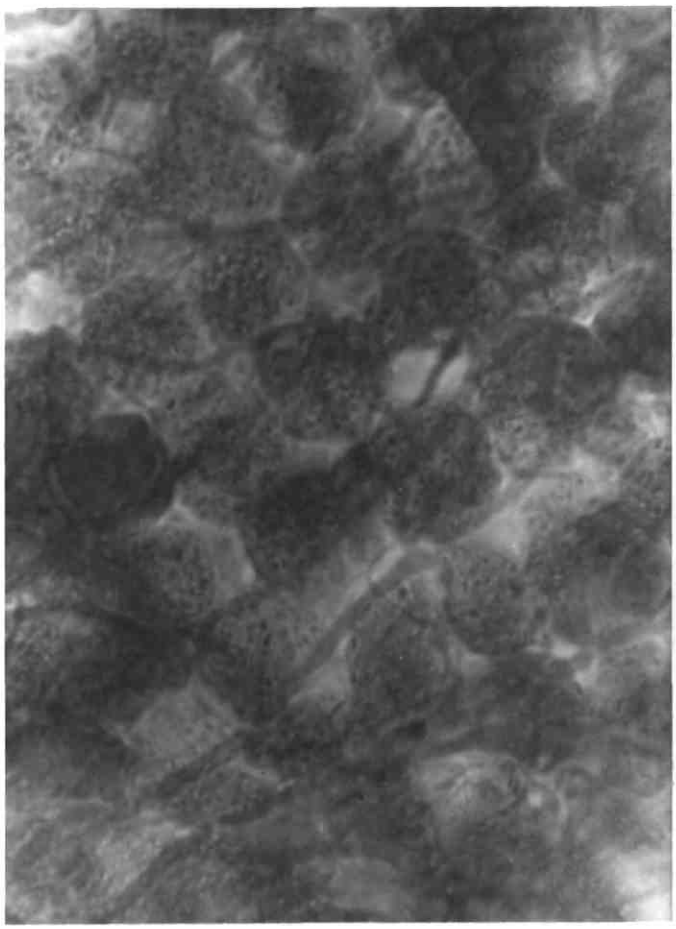
Intact 4-week old tomato plants were inoculated using ascospore suspensions at the lower concentrations, namely 2×10^4 and 3×10^4 per ml. Only a very few small lesions - 2 to 3 mm in diameter - were formed 3 days after inoculation on leaves inoculated with SCA - ascospore suspension. No symptom was observed when inoculation was made using ascospores suspended in water alone or when glucose 1% (w/v) and sodium citrate 0.01% (w/v) were added.

3. Growth and enzyme production in vitro in relation to medium composition

Infection of tomato stem with S. sclerotiorum results in maceration

PLATE (6)

Accumulation of the extracellular material on the surface of the epidermal cells of tomato leaf at 12 hours after inoculation. c (x 630).



of the infected tissues and development of a watery soft rot. Tissue maceration and cell-wall degradation during pathogenesis are the characteristic symptoms of many plant diseases incited by facultative, necrotrophic pathogens (Baker *et al.*, 1979). In plant tissues infected with such pathogens a number of different cell-wall degrading enzymes can be readily detected (Bateman and Basham, 1976). These enzymes particularly the pectolytic enzymes that degrade the α 1,4 linkages between galacturonosyl moieties in pectic substances cause extensive cell-wall breakdown and maceration (Albersheim *et al.*, 1969; Bateman and Basham, 1975).

Experiments in this section were undertaken to provide further insight into the nature of resistance to *S. sclerotiorum* infection observed on old tomato stems and to study the production *in vitro* of the extracellular cell-wall-degrading enzymes which might be associated with pathogenesis by this fungus.

3.1 Effect of host factors on growth *in vitro* of *S. sclerotiorum*

3.1.1 Effect of root segments

Segments from surface-sterilized roots of 7-day old tomato seedlings were placed in groups of two on the surface of modified Richard's agar medium (Davis, 1964) in 9-cm Petri dishes. Each dish was inoculated with a single sclerotium placed one centimeter from each of the two segments. Control plates were inoculated with sclerotia alone. All plates were incubated at 22 $\pm$ 1°C in darkness.

In 2-3 days, the advancing margin of the colony reached the segments and grew over them. In none of the replicates was there any inhibition of fungal growth. As shown in Table (8), the mycelial growth of the fungus on plates with or without root segments did not differ significantly.

TABLE (8)

Effect of root segments on growth of *S. sclerotiorum*

Time after inoculation (h)	The mean diameter of the fungal colony (mm)	
	Sclerotia with root segments	Sclerotia without root segments
24	0	0
48	13.94 ± 0.97	17.44 ± 0.76
72	43.56 ± 2.18	44.25 ± 1.21
96	78.19 ± 3.16	77.00 ± 4.56

Figures are the means and the standard errors.

3.1.2 Effect of root exudates

Root exudates were collected and concentrated as described earlier. The effect of the exudates on the growth of the fungus was examined at 26°C on water agar medium by using separately the equivalents of 10, 20 and 30 mg of the root dry weight per plate. The effect was also tested at four different temperatures i.e. 5, 15, 20 and 25°C using only the equivalent of 10 mg of the root dry weight per plate. The cup-plate technique was employed. Wells were cut out of the agar with a no. 5 cork borer. The bottom of each well was sealed with a drop of melted agar. A sample (0.2 ml) of the concentrated exudates was put in each well. In the control the same amount of sterile distilled water was used. All plates were inoculated with mycelial disks and incubated in the light at the required temperature. Linear growth of the fungus was measured at 24-hour intervals.

Results are shown in Tables (9) and (10). Root exudates had no significant effect on growth of the fungus whatever their concentration or the incubation temperature.

3.1.3 Effect of stem and root homogenates

Root and hypocotyl homogenates were prepared from healthy 4-week old plants grown in Long Ashton solution in Kilner jars. The homogenates were diluted with distilled water and then supplemented with agar (1.5g/100 ml) and autoclaved. The homogenates agar medium was poured into 9-cm Petri dishes - 10 ml in each. WA medium was used as a control in this experiment. The plants were inoculated with mycelial plugs and incubated in the light at 26°C. Homogenates were also prepared from the roots and the upper parts of stems of root-inoculated and non-inoculated tomato plants. The tap roots of the plants were inoculated with mycelial disks using the technique of Price and Colhoun (1975). At 6 days after inoculation, the diseased roots

TABLE (9)

Effect of different amounts of root exudates on radial growth of S. sclerotiorum

Root exudates (mg root dry weight equivalent/plate)	Time after inoculation (h)		
	24	48	72
0 (Control)	16.17 ± 0.44	18.17 ± 0.73	19.50 ± 0.29
10	18.00 ± 0.76	21.50 ± 0.99	23.67 ± 1.30
20	16.77 ± 1.01	20.77 ± 3.03	21.00 ± 3.18
30	18.50 ± 0.00	23.27 ± 1.01	22.93 ± 1.06

Figures are means of colony diameters (mm) and standard deviations calculated from three replicates per treatment.

TABLE (10)

Effect of root exudates at different temperatures on radial growth of S. sclerotiorum

Temperature (C°)		Time after inoculation (h)		
		24	48	72
5		0	6.2 ± 0.1	12.2 ± 0.3
	Control	0	7.3 ± 0.6	13.7 ± 0.8
15		11.5 ± 0.3	21.7 ± 0.7	33.2 ± 0.9
	Control	15.2 ± 0.8	24.3 ± 0.8	36.5 ± 0.9
20		11.8 ± 5.9	22.4 ± 7.7	31.5 ± 7.0
	Control	18.0 ± 0.5	25.4 ± 2.2	40.2 ± 0.7
25		17.8 ± 0.4	22.1 ± 1.7	38.5 ± 2.4
	Control	19.0 ± 0.5	28.7 ± 1.2	44.8 ± 0.2

Figures are means of colony diameter (mm) and standard deviations from three replicates per treatment.

were excised and homogenates were prepared from the roots and the stems. The same method described above was used except that the concentration of the homogenate was half that used above, and 15 ml of the medium was used per plate instead of 10 ml. The plates were inoculated with germinating sclerotia and incubated under the same conditions as above. The growth of the fungus was measured at 24-hour intervals.

Results are given in Tables (11) and (12). Radial growth of the fungus on the stem and root homogenates did not differ significantly at 24 hour after inoculation. At 48 hours, there was a significant increase ($P = 0.05$) in the rate of growth on the stem homogenates which became highly significant ($P = 0.01$) on the third day. When the hypocotyl was removed from the stem, (Table12), the growth of the fungus increased significantly ($P = 0.05$) on the stem homogenate from noninoculated plants 24 hours after inoculation. At 48 hours, a highly significant increase ($P = 0.01$) in growth rate was found on the stem homogenates (Table12). The fungus grew actively on stem homogenates from healthy and diseased plants whereas its growth was less on the root homogenates. After four days, growth on stem homogenates from root-inoculated plants lagged behind that on equivalent noninoculated plants.

3.2 Production of CWDE in liquid culture media

3.2.1 Enzyme activities in 13-day culture filtrates.

Crude culture filtrate of *S. sclerotiorum* grown on sodium polypectate was prepared and assayed for the following enzymes: polygalacturonase (PG), arabanase (Ar), pectinmethylesterase (PME), pectin and pectate lyases (PL, PGL), xylanase (Xy), carboxymethylcellulase (Cx), α-L arabinofuranosidase (AF) and β-D-galactopyranosidase (GP). Table (13) shows the respective enzyme activities, expressed as units/mg protein

TABLE (11)

Effect of root and hypocotyl homogenates on growth of S. sclerotiorum

Time after inoculation (h)	The mean diameter of the fungal colony (mm)		
	Root homogenate	Stem homogenate	Control (WA)
24	25.40 ± 0.98	26.10 ± 0.32	15.33 ± 1.67
48	52.00 ± 1.02	54.90 ± 0.30	17.17 ± 2.62
72	72.40 ± 0.99	77.60 ± 0.62	19.50 ± 3.62

Figures are the means with standard errors

TABLE (12)

Effect of root and stem homogenates from root-inoculated and non-inoculated tomato plants on growth of S. sclerotiorum

Time after inoculation (h)	The mean diameter of the fungal colony (mm)				Standard error
	Root-inoculated plants		Noninoculated plants		
	Root homogenate	Stem homogenate	Root homogenate	Stem homogenate	
24	8.60	12.90	10.10	18.10	± 1.73
48	11.40	25.60	13.50	31.00	± 2.34
72	15.10	39.10	16.00	46.80	± 2.48
96	18.80	55.30	17.80	65.10	± 2.71
120	20.00	67.80	18.50	78.30	± 2.79

Figures are the means and the pooled standard errors.

TABLE (13)

Activities of cell-wall degrading enzymes in 13-day old culture filtrates of S. sclerotiorum.

Enzyme	Units/mg protein in culture filtrate
Polygalacturonase	42.8
Pectinmethylesterase	54
Arabanase	34.65
β.D-galactopyranosidase	4.575
Carboxymethylcellulase	4.500
α-L arabinofuranosidase	2.525
Xylanase	1.880

Results are the means from duplicate cultures.

in the culture filtrate.

When grown on a mineral salt medium supplemented with sodium polypectate as the sole carbon source, S. sclerotiorum secreted a range of polysaccharide-degrading enzymes. PG was produced at high levels and is the dominant extracellular polysaccharidase produced by the fungus. Xy was produced at a fairly low level. Activities of Ar and Cx were respectively 18.4 and 2.4 times greater than that of Xy in terms of reducing group release. AF showed a level of activity approximately half that shown by GP. There was a relatively low level of PME and no PL or PGL activity could be detected.

3.2.2 Time course of polygalacturonase production in cultures on sodium polypectate medium

The production of PG by S. sclerotiorum was followed at regular intervals. The fungus was grown on sodium polypectate medium and cultures were harvested at 3-day intervals. The mycelial dry weight was determined and PG in the filtrate was measured using the cup-plate method. The relationship between the activity of the enzyme and the size of the hydrolysis ring in this method was studied using graded concentrations of the former. A curvilinear relationship was found between the activity of the enzyme and the diameter of the ring, the square root of its diameter or its area (Fig.6). Enzyme activity was directly related to the antilogarithm of the corrected diameter (corrected diameter = total diameter in mm - diameter of the well, (7 mm).

As shown in Fig. (7), production of PG by S. sclertiorum increased with age and with growth. Statistical analysis of the results revealed significant positive correlations between the enzyme activity and both the age and the dry weight of the fungus (Table14). The concentration of the enzyme increased rapidly between day 6 and day 10, subsequently declining on days 12 and 15. There were considerable variations in enzyme production

FIG. (6)

Relationship between ring size and enzyme activity in PG assay.

(●) Corrected diameter (CD) (mm)

(o) $\sqrt{\text{Corrected diameter}}$ ($\sqrt{CD}$)

(◉) Antilog. of corrected diameter

(▲) Area (mm^2)

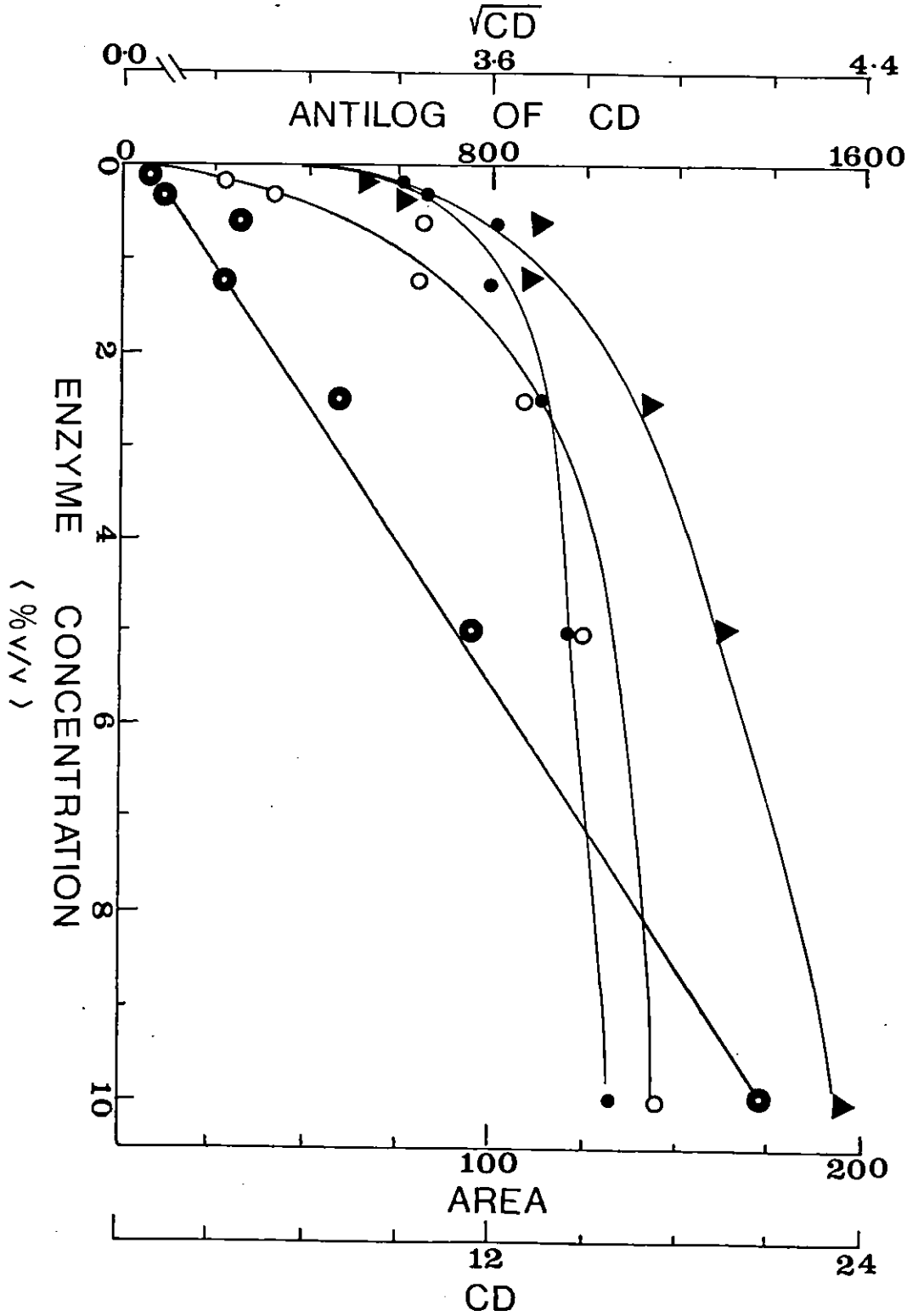

√CD
0·0
3·6
4·4
ANTILOG OF CD
0
800
1600
0
2
4
6
8
10
ENZYME CONCENTRATION
(%v/v)
100
200
AREA
12
24
CD

FIG. (7)

The increase in PG activity (measured by the cup-plate method) with the dry weight of the mycelium and the age of the culture.

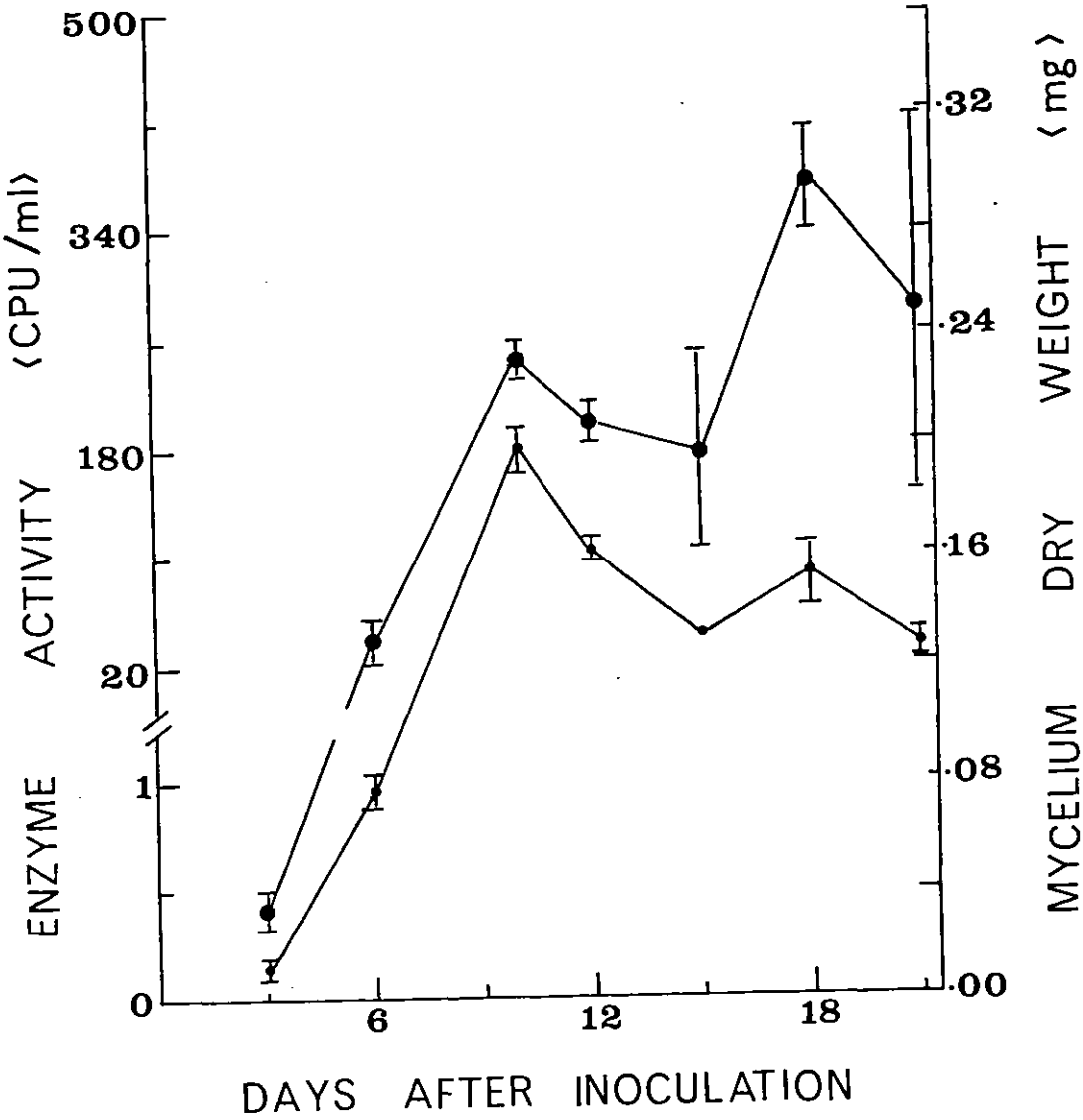
Correlation coefficients are + 0.789* and + 0.869* respectively.

(•) mycelium dry weight.

(●) PG activity.

Vertical bars show the standard deviations of the means for each value.

* Significant at 5% level of probability.


500
340
180
20
1
0
ENZYME ACTIVITY (CPU/ml)
·32
·24
·16
·08
·00
MYCELIUM DRY WEIGHT (mg)
6
12
18
DAYS AFTER INOCULATION

between replicates, particularly after 12 days. PG activity was maximal on day 18 but it decreased subsequently. Similarly, the growth of the fungus increased rapidly until the tenth day, thereafter, the fungus began to autolyse resulting in a dry weight loss. In general, the increase and the decrease in the dry weight of the fungus and in the PG activity with time occurred simultaneously and in similar fashion.

TABLE (14)

Correlation coefficients showing degree of association between PG concentration (measured by cup-plate assay method) and the dry weight of the fungus

	Correlation coefficients
Culture age	+ 0.789*
Mycelium dry weight	+ 0.869*

* Significant at 5% level of probability

3.2.3 Effect of different carbon sources on growth of the fungus and production of polygalacturonase

Cultures were grown on mineral medium supplemented with 1% sodium polypectate, pectin, polygalacturonic acid or galacturonic acid in 250 ml flasks containing 100 ml of liquid. The pH of all media was adjusted to 6.0. Production of PG was studied over a period of 14 days. At each harvest date (5, 10 and 14 days from inoculation), the mycelial mat was removed and its dry weight was determined. Culture filtrates were

assayed for PG using the cup-plate method.

Results obtained (Fig. 8) showed that pectin was the best carbon source for growth and sclerotium production. It resulted in mycelial mat weights of approximately 136 mg after 14 days. Most of the sclerotia were formed between the fifth and tenth days. Sodium polypectate and polygalacturonic acid gave similar growth response curves but the mycelium obtained weighed only approximately 97 mg after 14 day's growth, and sclerotium formation was delayed until between the tenth and fourteenth days. PG production followed a pattern similar to growth but was not in all cases related directly to mycelial weight. For example, polygalacturonic acid stimulated higher production of PG at 5 and 10 days after inoculation compared with sodium polypectate, whereas the latter supported more growth. Fungal growth was poor on the galacturonic acid containing medium; the mean dry weight of the mycelium on the 14th day was only 11.6 mg and sclerotia were never observed. PG production was low overall, although on a dry weight basis it was comparable with the sodium polypectate medium.

Most of the PG was secreted into all media between the tenth and fourteenth days i.e. at the time of greatest mycelial growth. On the pectin-based medium only, there was a rapid decline in pH. During the first five days of growth the initial pH (pH6) dropped to 4.6 followed shortly afterwards by a rapid increase in PG activity. Maximum enzyme activities were reached after minimum pH levels were attained.

Although pH shifts were less this was also true for the other carbon sources employed in this experiment. In the case of galacturonic acid the minor pH shift is in accord with the very modest growth achieved. Clearly the monomer alone (in much higher purity than in the other media) is not a very satisfactory substance for growth.

3.2.4 An attempt to induce production of pectate lyase by *S. sclerotiorum*

Results obtained from the first experiment in this section showed that

FIG. (8)

Effect of carbon source on growth and PG production by S. sclerotiorum in stationary cultures.

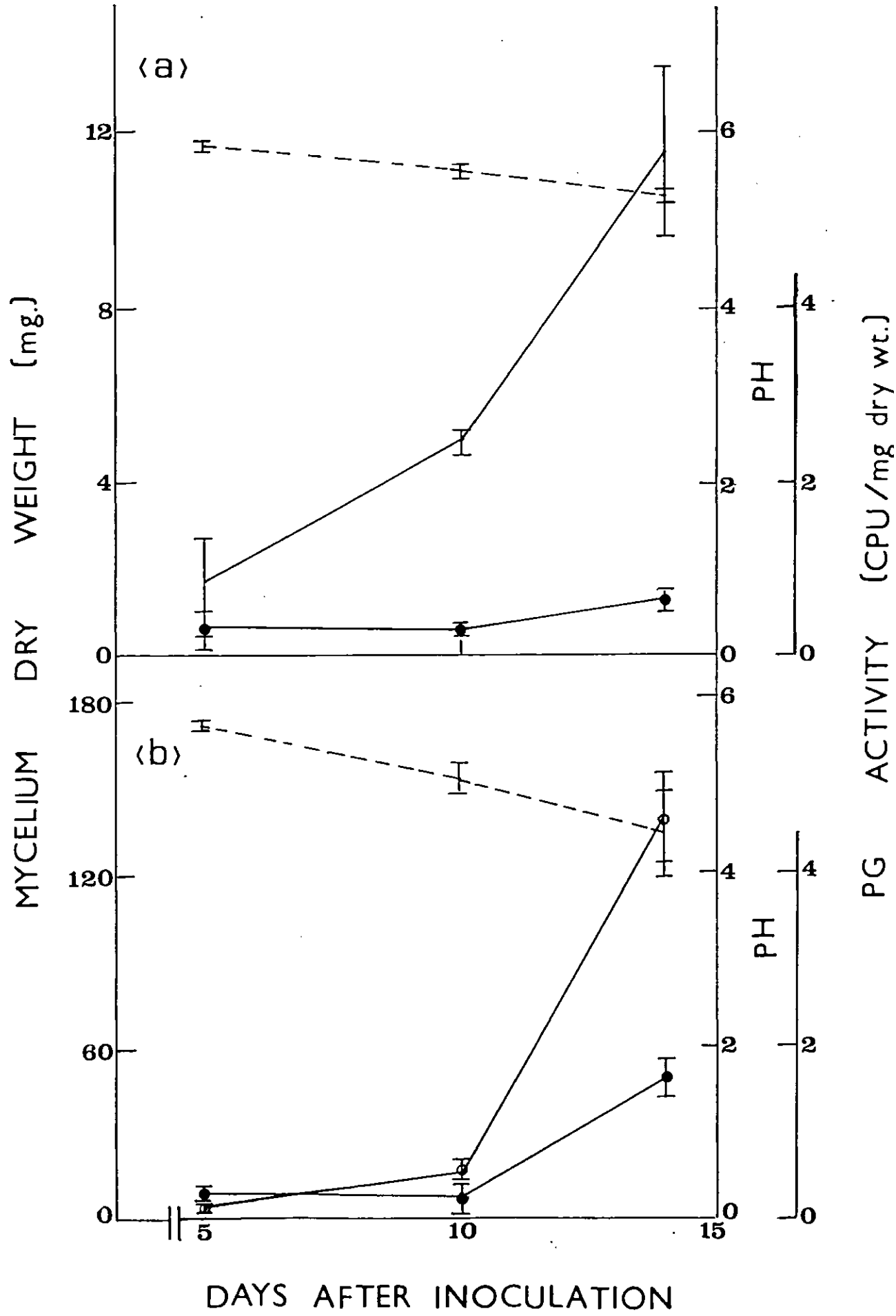
(a) Galacturonic acid; (b) Sodium polypectate;

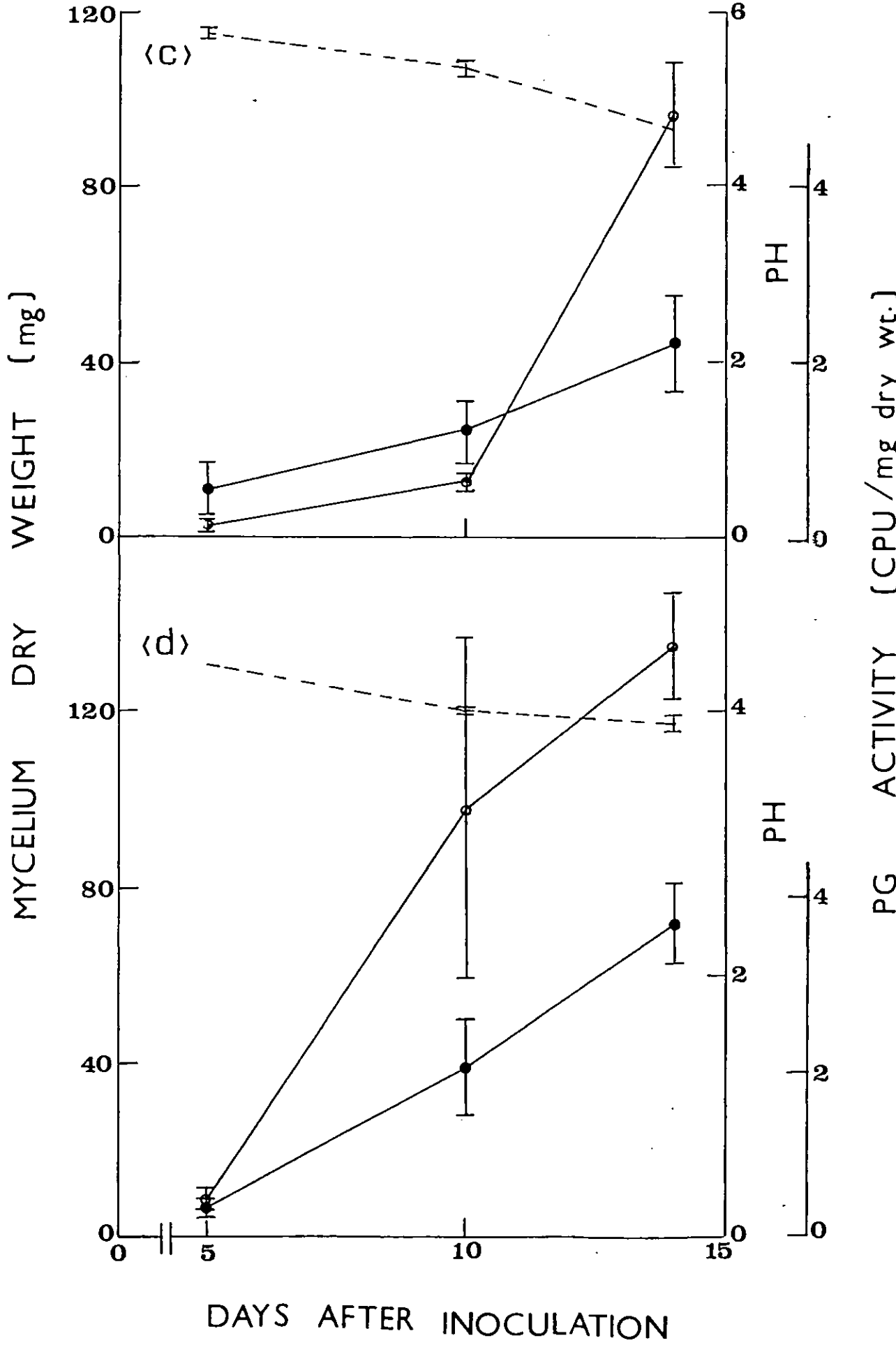
(c) Polygalacturonic acid and (d) Pectin.

(—●—) PG activity as measured by the cup-plate.

(——) Mycelium dry weight.

(- - -) pH.


(a)
(b)
MYCELIUM DRY WEIGHT (mg.)
PH
PG ACTIVITY (CPU/mg dry wt.)
DAYS AFTER INOCULATION
0
4
8
12
60
120
180
2
6
5
10
15


(C)
(d)
MYCELIUM DRY WEIGHT (mg)
PH
PG ACTIVITY (CPU/mg dry wt.)
120
80
40
0
6
4
2
0
5
10
15
DAYS AFTER INOCULATION

S. sclerotiorum did not produce PGL or PL when grown on sodium polypectate-tartarate medium. This might be related to the low pH of the medium (pH 5·7) since the induction of pectin lyases has been reported to be markedly pH dependent (Archer, 1973). As mentioned by Hancock and Millar, (1965) when Colletotrichum trifolii was cultured on a defined liquid medium containing pectic acid or on potato broth, high activities of polygalacturonate-trans-eliminase (PGL) were not encountered until the pH of each medium rose naturally to 7.5-8.3.

An attempt was, therefore, made to induce production of PGL by S. sclerotiorum by artificially raising the pH of culture. The fungus was grown first on pectate-tartarate liquid medium for six days at 21°C in the dark. Mycelial mats were then transferred aseptically to 100 ml of fresh medium in each of six 250 ml flasks. In three flasks the medium was adjusted to pH8 by using K_2HPO_4 instead of KH_2PO_4 and by addition of a drop of ammonia solution prior to autoclaving. After autoclaving, the pH was 7.6 whereas that of the medium containing KH_2PO_4 was about 5·7. All cultures were incubated for a further 10 days under the same conditions as above.

In cultures at pH 5.7 growth was more rapid than at pH 7.6, sclerotia being formed by day three compared with day eight at the higher pH. Irrespective of initial pH, all cultures had shifted to approximately pH 4.2 at harvest. Neither PGL nor PL were detected in any filtrates despite a transient period at least of incubation above pH 7.0 in one of the treatments.

3.2.5 Enzyme secretion by the fungus when grown on cell walls

Enzyme production by the fungus was compared in cultures containing nutrient salts and cell walls extracted from 4- and 8-week old plants which served both as sole carbon sources and inducers of enzyme synthesis. The medium used was that described under Materials and Methods for production

of enzymes except that sodium polypectate was substituted by cell walls. Mycelial disks were added to 250 ml Erlenmeyer flasks containing 100 ml of nutrient salts, adjusted to pH 6, supplemented with a suspension of cell walls, (0.5% w/v). The liquid cultures were incubated on a reciprocal shaker at 90 strokes per min. for 14 days. Samples (3 ml) were withdrawn daily under aseptic conditions, filtered free of fungal mycelium, dialysed overnight to remove monosaccharides and assayed for CWDE. Fig. (9) shows the respective enzyme activities, expressed/ml culture fluid.

Production of all enzymes commenced within two days of incubation, except for AF which was detectable only on the third day and cellulase which was not measured until the fourth day. For all enzymes the greatest increase in activity occurred during the first five days (Fig. 9), with the peak activities for most being obtained between days 8 and 12. The only significant exceptions were Cx, which showed little further increase after day four, and cellulase (C_1), activity of which was very low and approximately constant throughout the period of measurements. Greatest activities in terms of glycosidic bonds cleaved was shown by PG followed by Cx, Xy and Ar. Activities of Cx and Xy were greatest in cultures grown on 8-week old walls, whereas for the remaining enzymes, 4-week walls resulted in greatest enzyme yields.

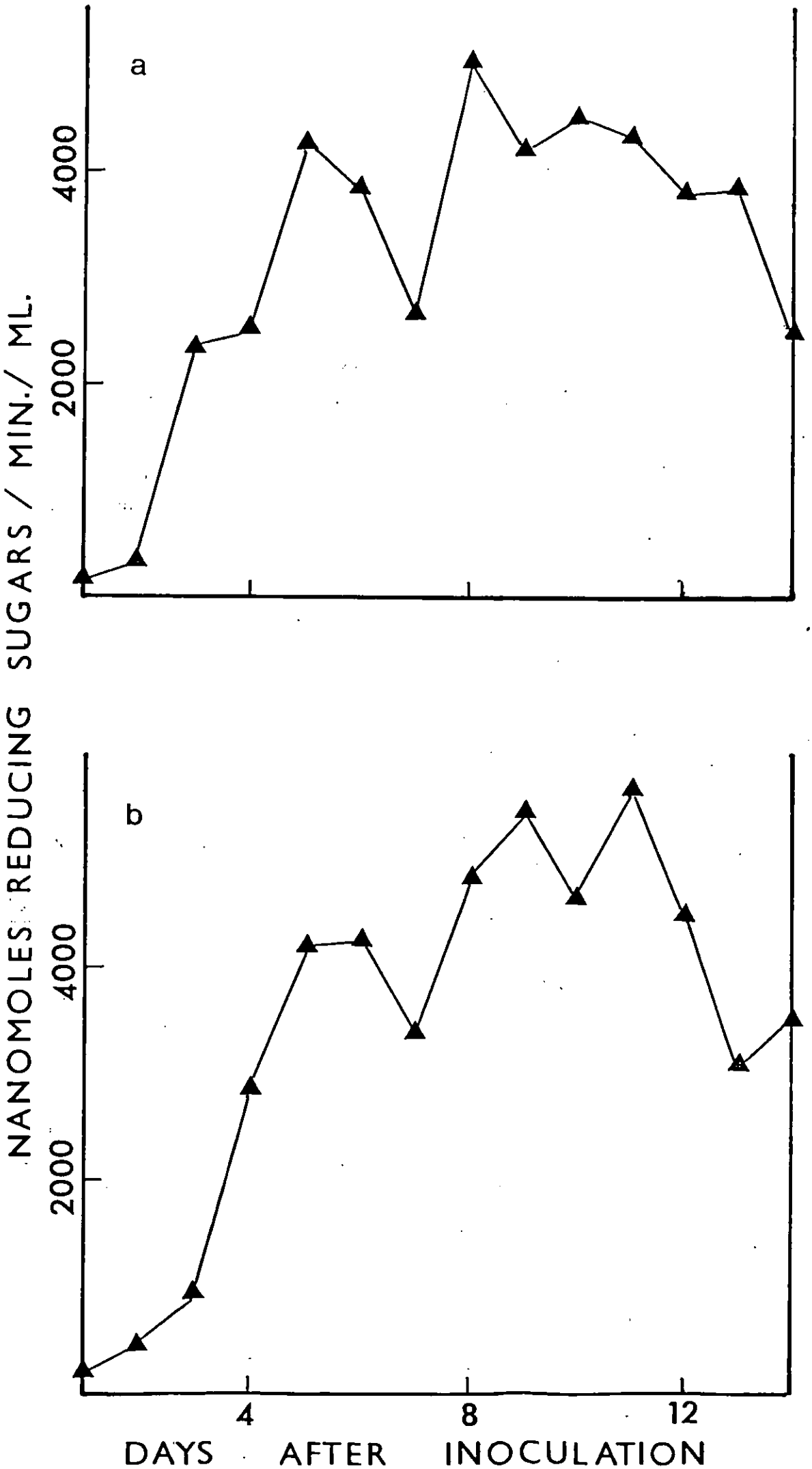
3.2.6 Influence of pH and glucose on enzyme secretion and sclerotium formation.

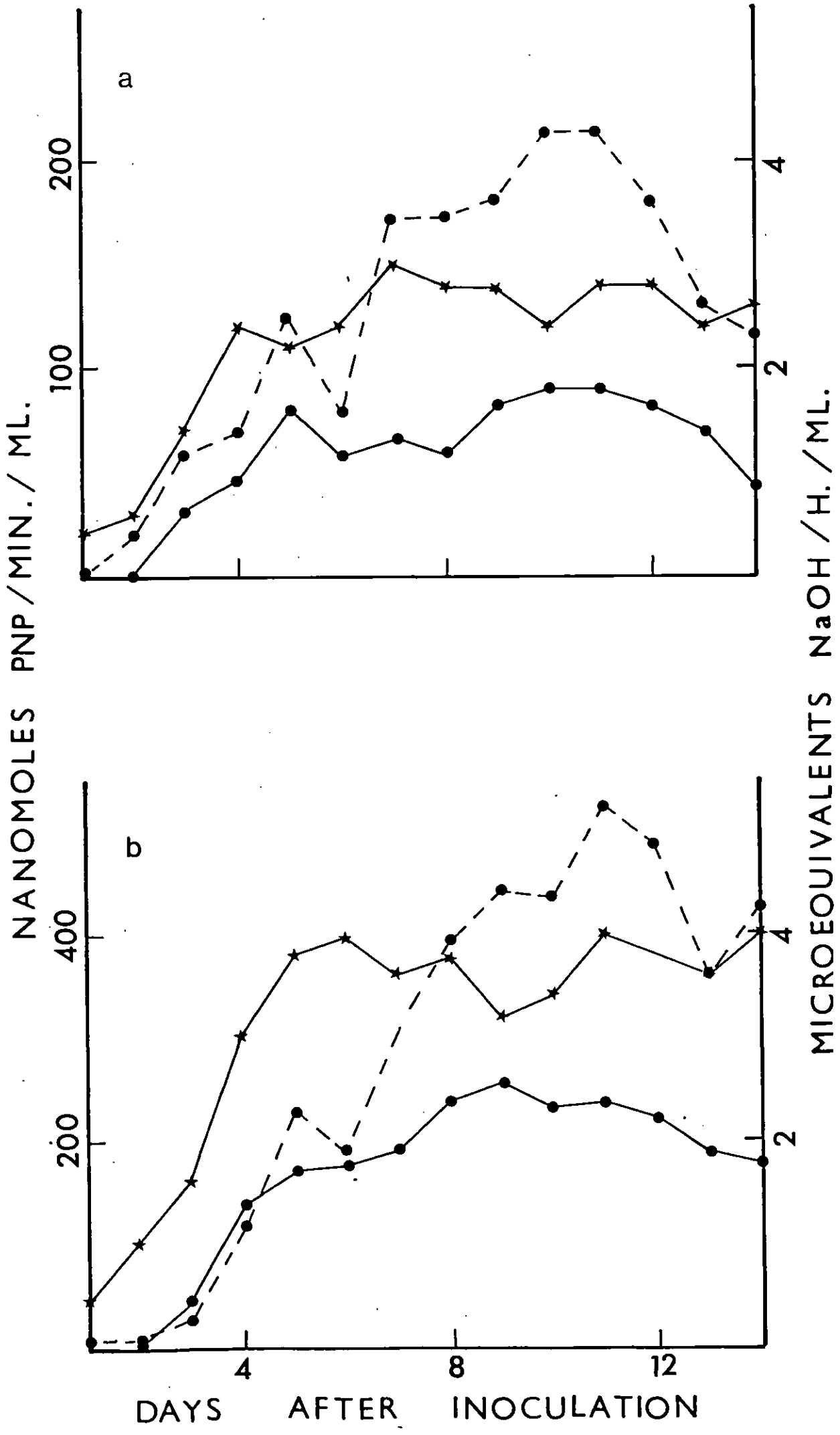
The effect of the initial pH and of glucose on the production of the different CWDE and the formation of sclerotia was investigated. The fungus was grown in stationary culture in 100 ml flasks containing 20 ml of a mineral medium fortified with glucose or sodium polypectate as the sole carbon source. The pH of the culture medium was adjusted aseptically after autoclaving to different values (pH 3, 4, 5, 6, and 7) using sterile

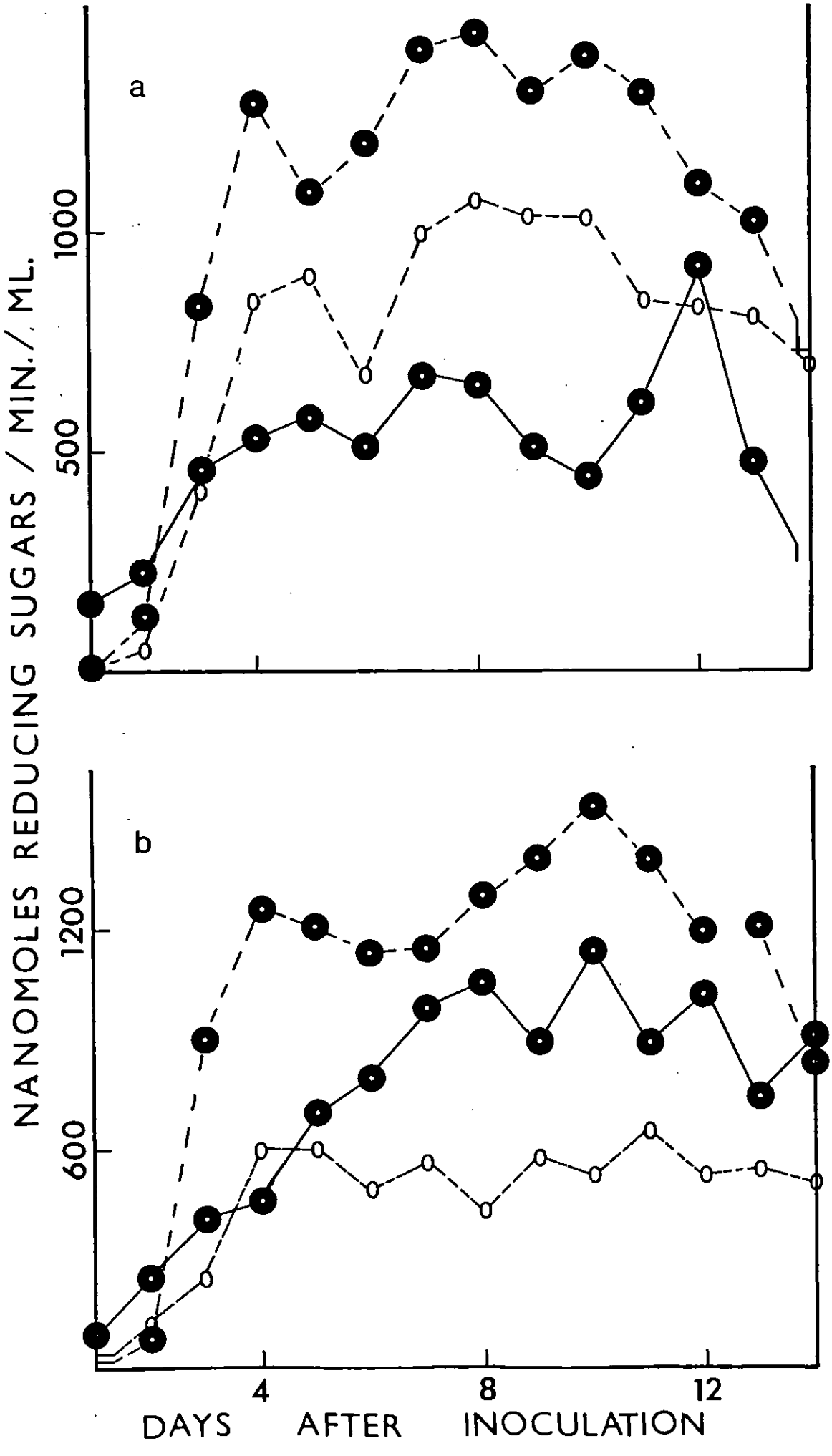
FIG. (9)

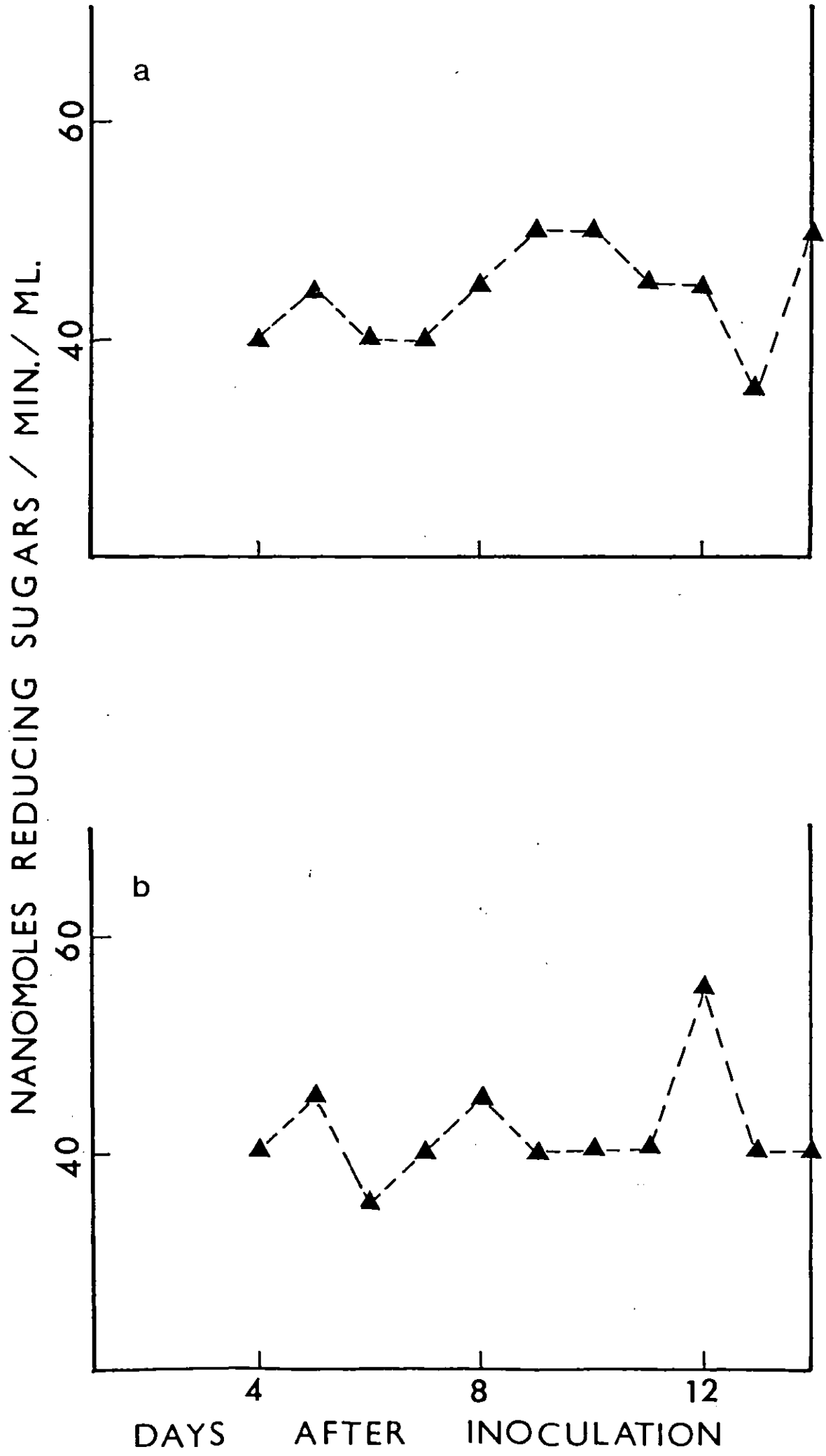
Production of cell wall-degrading enzymes by S. sclerotiorum when grown on cell walls from (a) 8- and (b) 4- week old tomato plants.

(-▲-) PG; (-✶-) PME; (-●-) AF; (-●-) GP; (-◉-) Ar; (-○-) Xy; (-◉-) Cx; (-▲-) C_1 (cellulase).


a
b
4000
2000
NANOMOLES REDUCING SUGARS / MIN./ ML.
4
8
12
DAYS AFTER INOCULATION


a
b
200
100
400
200
4
2
4
2
4
8
12
NANOMOLES PNP / MIN. / ML.
MICROEQUIVALENTS NaOH / H. / ML.
DAYS AFTER INOCULATION


a
b
NANOMOLES REDUCING SUGARS / MIN./ ML.
500
1000
600
1200
4
8
12
DAYS AFTER INOCULATION


a
b
60
40
60
40
NANOMOLES REDUCING SUGARS / MIN./ ML.
4
8
12
DAYS AFTER INOCULATION

0.1M NaOH or 0.1M HCl.

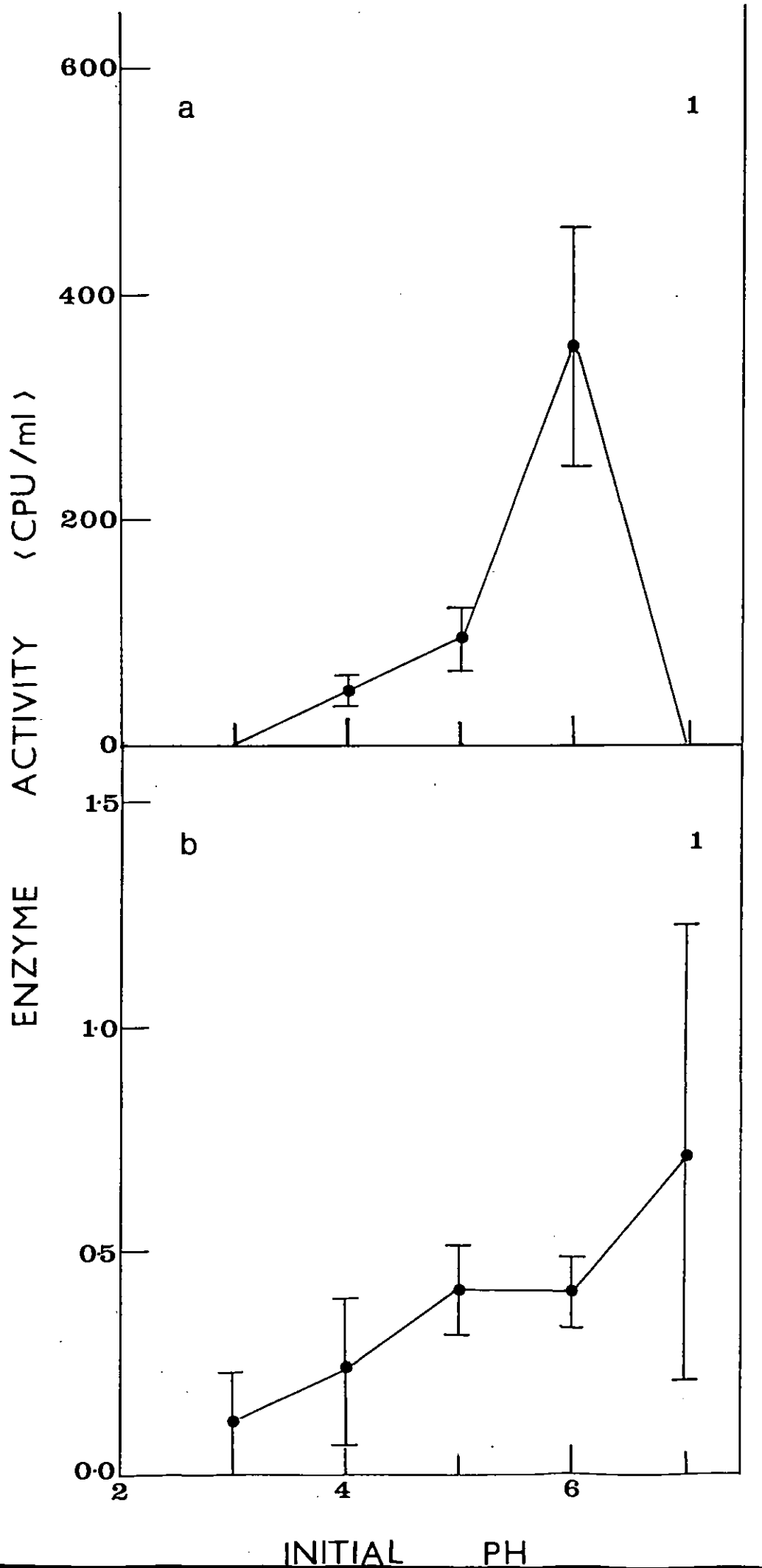
Results for the production of the CWDE on sodium polypectate and glucose at different pH values are given in Fig. (10a) and (10b) respectively. Production of all six enzymes tested for in this experiment was optimal when the fungus was grown on sodium polypectate medium at pH6. Secretion of the enzymes was markedly suppressed at initial pH values of 7 and 3. Except for Xy which was not catabolite repressed by glucose at pH3 and 5 the activity of all other enzymes was either greatly reduced or completely eliminated when glucose was used as the sole carbon source in the culture medium. The various pH optima for production of the enzymes on glucose medium (Fig. 10b) may be due to the effect of the initial pH on the strong catabolite repression exerted by glucose.

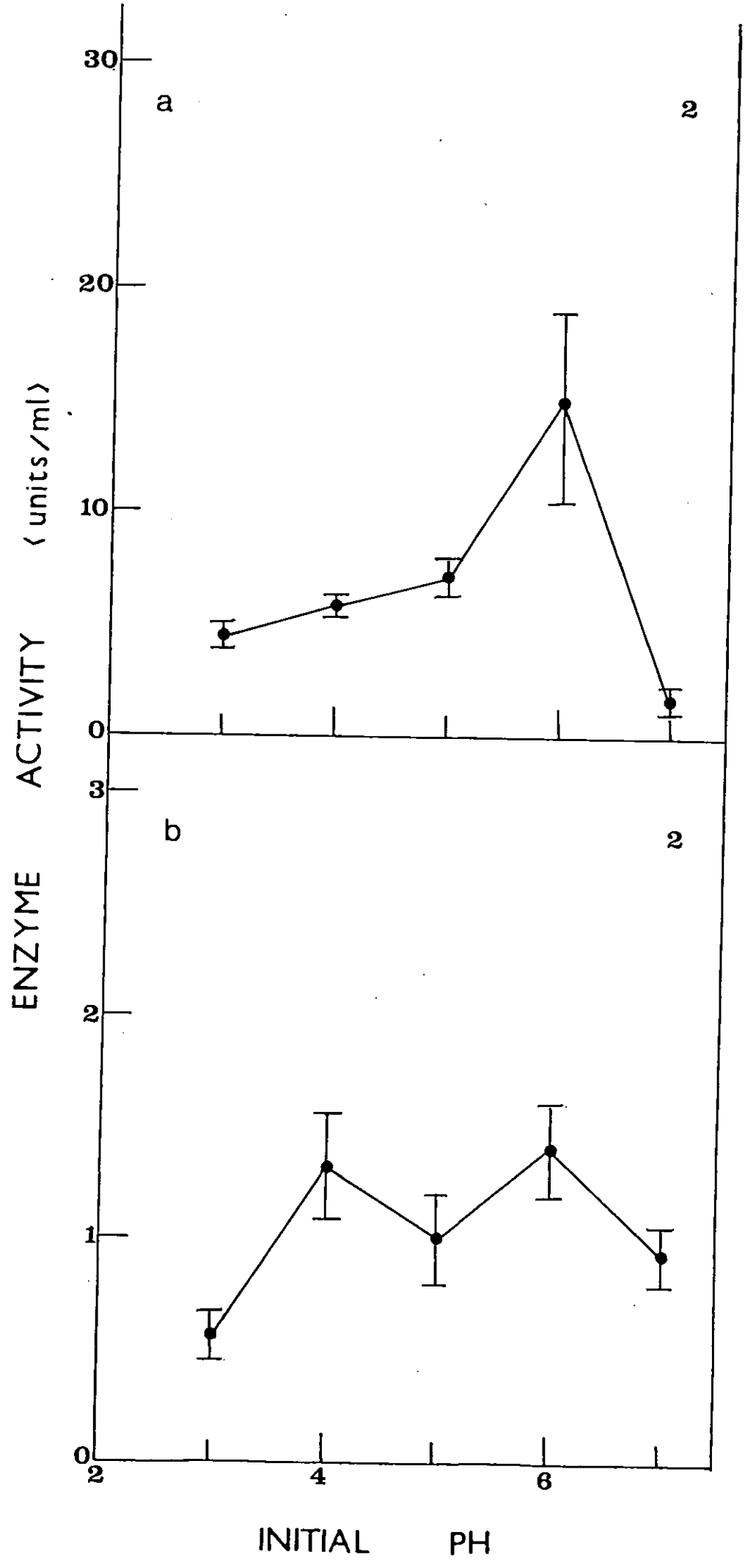
When activities of PG, PME, GP and AF were measured in dialyzed and nondialyzed filtrates from both cultures, similar results were obtained, with the exception of PG from sodium polypectate-grown cultures at pH 6 and PME from glucose-grown cultures at pH 4 which lost respectively about 60% and 50% of their activity during dialysis.

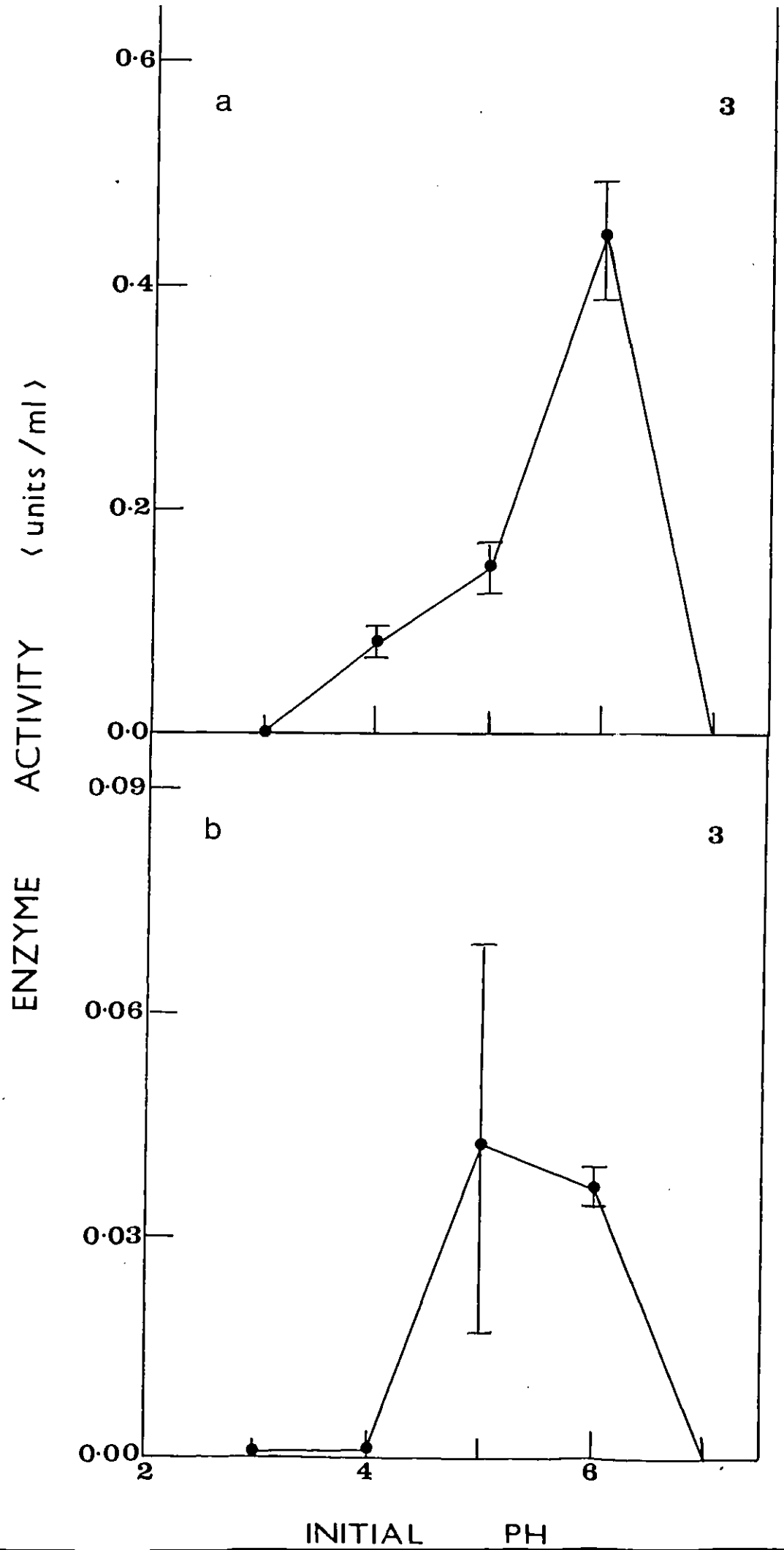
In contrast to enzyme secretion, sclerotium formation was better in the medium containing glucose (Table 15). On both media the largest number of sclerotia was formed when the pH was 6. No sclerotia were observed in cultures at an initial pH of 7. Similarly, the highest concentration of soluble protein in the culture filtrate occurred at pH 6 on glucose or sodium polypectate. Highly significant differences (P=0.01) were found in the protein concentration between the glucose and polypectate-grown filtrates at pH 6 and 7, (Table 15).

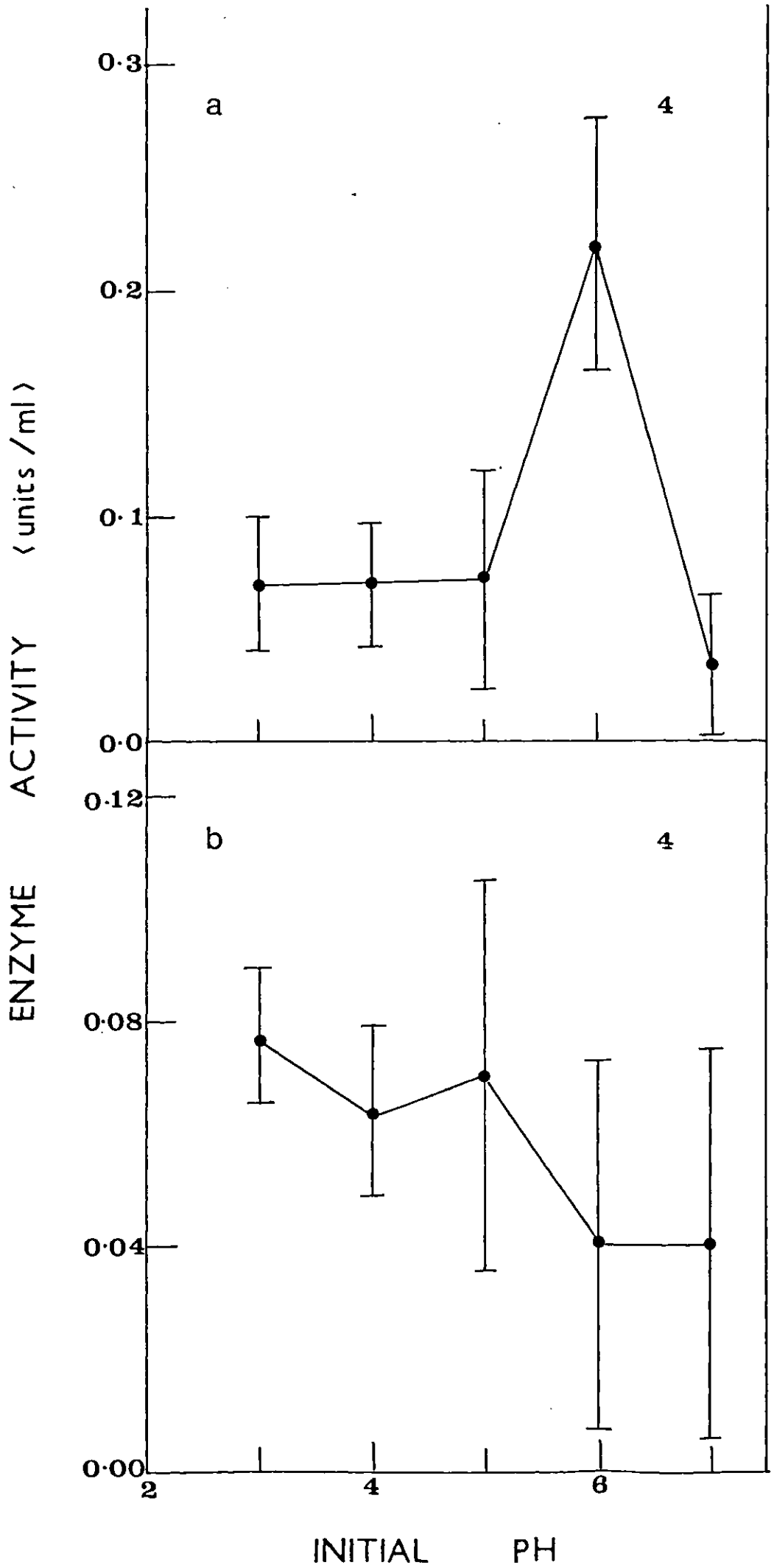
FIG. (10)

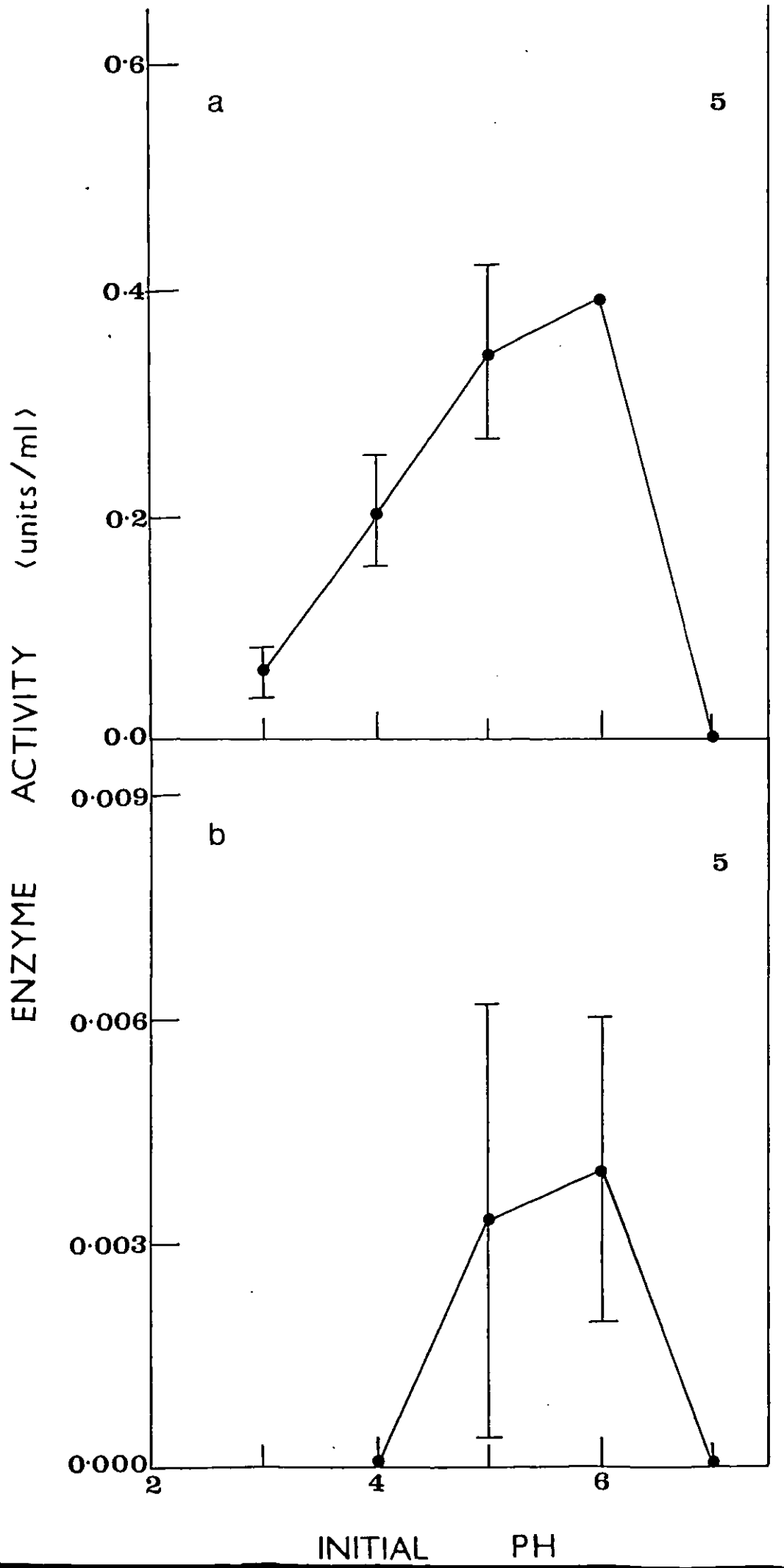
Activities of the cell wall-degrading enzymes in the dialysed filtrates from (a) sodium polypectate-grown and (b) glucose-grown cultures of *S. sclerotiorum* at different pH values - (1) PG; (2) PME; (3) Cx; (4) Xy; (5) AF; (6) GP.
Bars denote the standard deviations of the means of three determinations.


600
400
200
0
a
1
ENZYME ACTIVITY (CPU/ml)
1·5
1·0
0·5
0·0
b
1
2
4
6
INITIAL PH


30
a
2
20
10
0
ENZYME ACTIVITY (units/ml)
3
b
2
2
1
0
2
4
6
INITIAL PH


0·6
a
3
0·4
0·2
0·0
ENZYME ACTIVITY (units/ml)
0·09
b
3
0·06
0·03
0·00
2
4
6
INITIAL PH


0·3
a
4
0·2
0·1
0·0
ENZYME ACTIVITY (units/ml)
0·12
b
4
0·08
0·04
0·00
2
4
6
INITIAL PH


0·6
a
5
0·4
0·2
0·0
ENZYME ACTIVITY (units/ml)
0·009
b
5
0·006
0·003
0·000
2
4
6
INITIAL PH

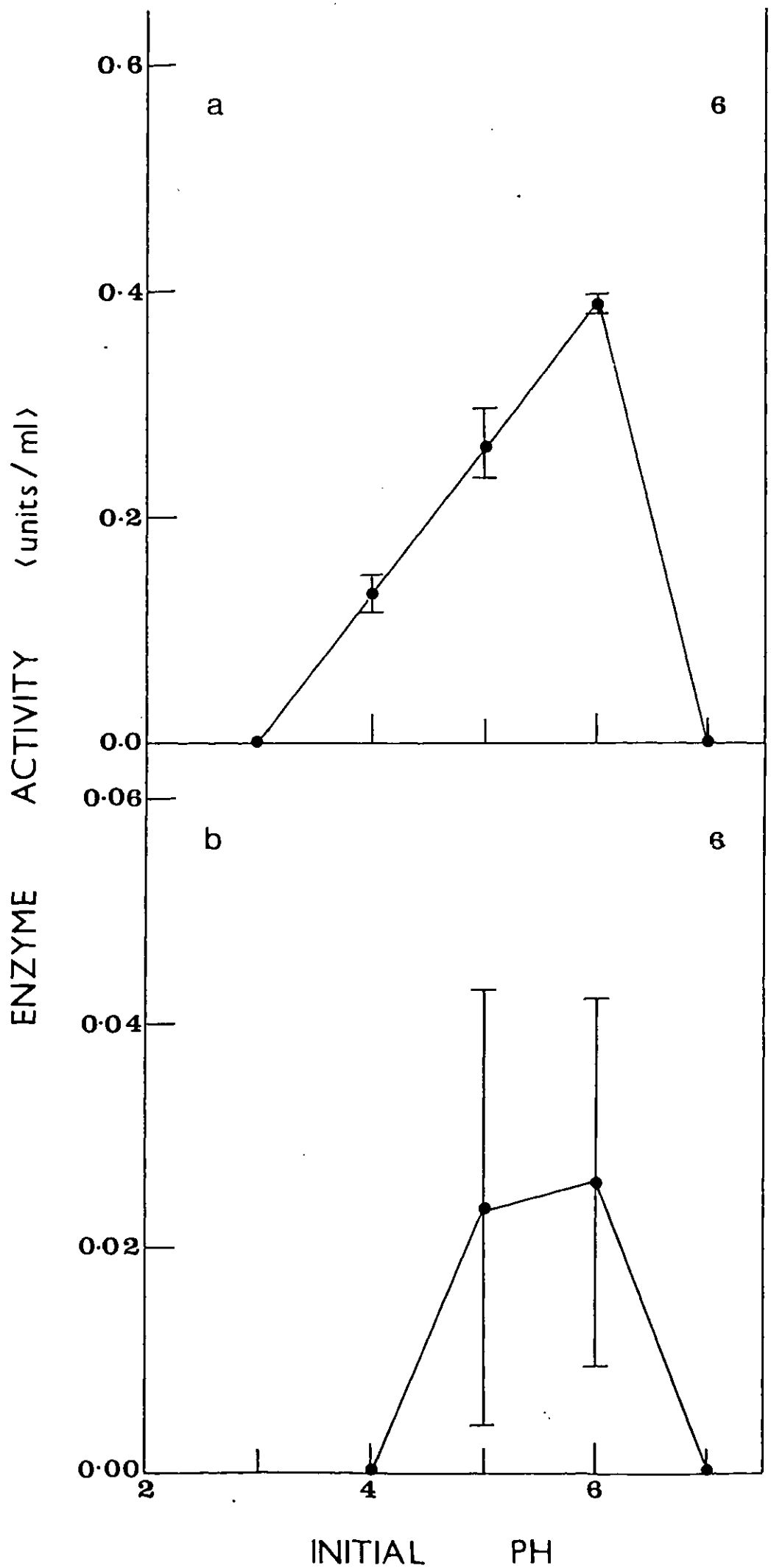

0·6
a
6
0·4
0·2
0·0
ENZYME ACTIVITY (units / ml)
0·06
b
6
0·04
0·02
0·00
2
4
6
INITIAL PH

TABLE (15)

The protein contents and the number of sclerotia in glucose and polypectate-grown cultures of S. sclerotiorum at different initial pH levels

pH	Protein content (mg/ml)*		Average number of sclerotia/flask	
	Polypectate	Glucose	Polypectate	Glucose
3	0.059 ± 0.002	0.023 ± 0.002	2	10
4	0.070 ± 0.005	0.058 ± 0.006	4	7
5	0.085 ± 0.063	0.070 ± 0.016	6	13
6	0.209 ± 0.021	0.080 ± 0.020	12	17
7	0.109 ± 0.011	0.013 ± 0.006	0	0

*Each figure is the mean (± standard deviation) of three replicates.

DISCUSSION

Growth of S. sclerotiorum was not affected by root segments or exudates. When the fungus was grown on homogenates from the plant roots and stems, significantly less growth occurred on root homogenates compared with stem homogenates as indicated by the difference in the linear growth of the fungus. Since growth of S. sclerotiorum was poor on WA medium which was used as a control, no relevant comparison could be made with the growth of the fungus on this medium. The slow growth observed on root homogenate medium is not necessarily an indication of growth inhibition by root homogenates. Equally, it could have been caused by a shortage of nutrients in the root as compared with stem-homogenates. The method of linear measurement used for estimation of the fungal growth is less well suited to a comparison of the effects of different media as it does not provide a measure of the actual amount of mycelium produced, (Hawker, 1950). The inhibitory effect of tomato root extract has been reported previously by Davies (1964) for Fusarium oxysporum. This inhibition might have been due to the presence of tomatine, an antifungal substance in stems and roots of healthy plants (McCane and Drysdale 1975). According to Arneson and Durbin (1968), S. sclerotiorum was among the most sensitive fungi to α tomatine.

When S. sclerotiorum was grown on sodium polypectate or pectin as the sole carbon source, it produced a range of CWDE, the most dominant being PG. The production of PG as well as the other enzymes was maximal at pH6. There is some evidence that enzyme production by micro-organisms can be affected by the hydrogen ion concentration (Jensen, 1969). In an earlier investigation into the effect of pH on the synthesis of pectic-degrading enzymes by fungi, Hancock (1966) mentioned that pH changes could be important in pathogenesis. Goodenough and Maw (1974) found that production of PG by Pyrenochaeta lycopersici

grown in the presence of pectic substances varied markedly with the pH of the culture medium, being maximal at 5.0 and decreasing rapidly at values above or below this. Other effects of pH on fungal enzyme synthesis have also been reported (Akinrefon 1969, Perley and Page, 1970). The rate of fungal dry matter production was also affected by pH and may be dependent on the action of the PG and other CWDE in breaking down the polypectate substrate. Growth and sclerotium formation were maximal at pH 6. This contrasts with a report that the optimum pH for growth in vitro of S. sclerotiorum is in the range of 3.4 - 4.0, (Dounine et al., 1935). When the fungus was grown on different carbon sources, PG was produced in each culture. Both growth and enzyme production were better on pectin, sodium polypectate and polygalacturonic acid than on galacturonic acid. However, when the results were expressed on a dry weight basis, PG production on galacturonic acid was comparable with that found on sodium polypectate medium. A similar effect of galacturonic acid on production of PG was also reported by Goodenough and Maw (1974) for the enzyme of Pyrenochaeta lycopersici when the fungus was grown at pH 6.0.

When glucose was used as the sole carbon source in the culture medium, the production of all the CWDE was reduced. Glucose is known to repress synthesis of pectolytic and cellulolytic enzymes in a number of fungi (Horton and Keen, 1966, Nisizawa et al., 1972). However, Mullen and Bateman (1975) found that glucose released from starch used as a carbon source in the culture medium was rapidly utilized by Fusarium roseum and did not prevent synthesis of CWDE. Cellulase production by S. sclerotiorum has been reported to be suppressed by 1% (w/v) glucose (Lumsden, 1969). The low level of enzyme activity found in this study may be attributed to constitutive enzyme secretion. Synthesis of Af and PG was also influenced by glucose in the medium, both being severely repressed. In contrast, Fuchs et al., (1965) and Lumsden, (1976) reported the production of constitutive Ar and exo-PG respectively by S. sclerotiorum.

When cultures of S. sclerotiorum were supplied with stem cell-wall material as the sole carbon source, the same range of CWDE detected in sodium polypectate or pectin cultures was produced. The enzyme secreted in greatest amounts was PG which was detected in relatively high concentrations 1 day after inoculation. The earlier and higher accumulation of PG on cell wall material was also reported by Goodenough et al., (1976) for Pyrenochaeta lycopersici when grown on walls from tomato root (cv. Moneymaker) and by Jones et al., (1972) for Fusarium oxysporum lycopersici when cultured on cell walls from tomato stem (cv. Clark's Special). Recent studies in which isolated plant cell walls have been used as carbon sources for plant pathogens suggest that CWDE are produced sequentially, with polygalacturonase the first, and cellulase the last in the sequence, (Jones et al., 1972). It has been suggested that pectic enzymes, particularly endo-enzymes which perhaps function as "wall modifying enzymes", (Karr and Albersheim, 1970), may be a necessary pre-requisite to wall degradation by other enzymes, since by degrading the amorphous matrix (especially pectic polysaccharides) they render other wall polymers more accessible to enzymic hydrolysis (Bateman and Basham, 1976). However, degradation of cell polymers by single polysaccharidases without the prior treatment of the walls with wall modifying enzymes has been reported (Archer 1973, Bauer et al., 1977, Cooper et al., 1978).

In the present study, the highest accumulation of PG occurred after 7 days of incubation. A similar result was also obtained for PG production by Botrytis cinerea, Fusarium oxysporum, f.sp lycopersici, Sclerotinia trifoliorum and S. fructigena (Monilia fructigena) on cell walls from tomato stems and petioles (Cooper et al., 1981). Lumsden (1976) suggested that S. sclerotiorum produces another unstable form of PG during the early stages of pathogenesis. There was, however, no evidence for an early peak of PG activity in this study when the fungus was grown on cell wall material. In general, the various CWDE tested for followed a similar production pattern as that shown by PG. Their

highest concentrations were found at or after 7 days of incubation with the enzymes produced on walls from 4-week old plants being approximately two-fold greater in concentration with the exception of Cx and Xy which were greatest in cultures grown on 8-week old walls. The observed fluctuations with time in enzyme accumulation may be the result of the production by S. sclerotiorum of an extracellular protease, (Khare and Bompeix, 1976). Partial inactivation or binding of some of the enzymes by the cell walls used as the carbon source in the culture medium is another possible explanation (Cooper et al., 1981; Bateman, 1963; Blackhurst and Wood, 1963).

PG and PGL were not detected in filtrates from cultures grown on any of the carbon sources used. Attempts to induce synthesis of these enzymes by S. sclerotiorum were unsuccessful. This confirms the conclusions of other workers (Hancock, 1966; Morrall et al., 1972; Lumsden, 1976; Marciano et al., 1982).

4. Characterization of CWDE produced in vitro by S. sclerotiorum

Filtrates from 13-day old cultures grown on sodium polypectate (Section 3.2.1) were used as the source material for enzyme characterization.

4.1 Loss of PG activity caused by dialysis

Since a loss of enzyme activity upon dialysis was greatest for PG, as indicated in the previous section, the effect of dialysis on the activity of this enzyme was studied in greater detail.

It is known that the dialysis tubing, as obtained from the manufacturers, contains glycerol as a plasticizer, traces of sulphur compounds and heavy metal ions (Plummer, 1978). Some of these contaminants may have adverse effects on protein solutions and must be removed before use. Impurities were removed by boiling for 30 minutes in alkaline EDTA (Na_2CO_3, 10 g/l : EDTA, 1mmol/l), followed by washing with distilled water, (Plummer, 1978). Tubing so treated was used for dialysing the culture filtrate against 0.05M sodium acetate buffer pH 4.6 or against distilled water. As a control, untreated tubing was used. After dialysis, PG activity was measured using the cup-plate assay. Pieces were cut from the treated and untreated membranes before and after dialysis and tested for their ability to hydrolyse sodium polypectate by placing them on the surface of the agar medium.

No loss in PG activity was found when dialysis was performed against acetate buffer in treated or untreated membranes, (Table 16). The enzyme lost about 55% of its activity when dialysed against distilled water. No activity was detected adhering to the membrane.

TABLE (16)

The effect of dialysis on PG activity

Dialysis membrane	Activity ratio (%)[a]	
	Dialysis against buffer	Dialysis against water
Treated	100	45
Untreated	100	45

[a] PG activity is expressed as a percentage of its activity in the nondialysed filtrate.

4.2 Loss of PG activity caused by lyophilization

PG activity did not increase as expected in culture filtrates concentrated by freeze-drying. This observation suggested that there might be some loss of the enzyme caused by freeze-drying. This possibility was tested using a 13-day old pectin-grown culture filtrate dispensed in scintillation vials and deep frozen (-20°C). Three 10 ml batches treated as follows: (1) Control (kept frozen), (2) lyophilized, rehydrated, frozen and stored for 20 days, and (3) lyophilized, stored dry at -20°C for 20 days and then rehydrated. After thawing, all were assayed for PG simultaneously using the cup-plate method. All three treatments had identical PG activity C 600 CPU, i.e. there was no loss of PG activity associated with freeze-drying. Possible reasons for the observed loss of the enzyme activity during the concentration of bulk filtrates by lyophilization are discussed later, (see Discussion section).

4.3 PH activity relationships of CWDE

The effect of pH on the activity of the extracellular CWDE was studied by determining enzyme activity on their respective substrates at different H^+ ion concentrations. The different pH levels were maintained with 0.1M citric acid-sodium phosphate buffer for pH 2.6 to 7 and 0.1M hydrochloric acid-potassium chloride buffer for pH levels below 2.6. Results are shown in Fig. (11).

High activities of all the CWDE were exhibited throughout the pH range 2.6 to 5.8. Maximum activity of AF occurred at pH 3.4 while the optimum pH for Cx was 4.6. PG and Ar activities were maximal at pH 4.6 and GP and Xy peaked at pH 4.2 and 5 respectively, (Fig.11). PME was active within the pH values of 4.2-5.8 with an optimum occurring around pH 5, (Fig.11).

Outside the optimum pH ranges, the activities of all the enzymes assayed in this experiment were markedly reduced. At neutral pH, or below pH 2.0, very little activity was exhibited by any of the enzymes.

4.4 Isoelectric points of pectin and sodium polypectate-induced CWDE

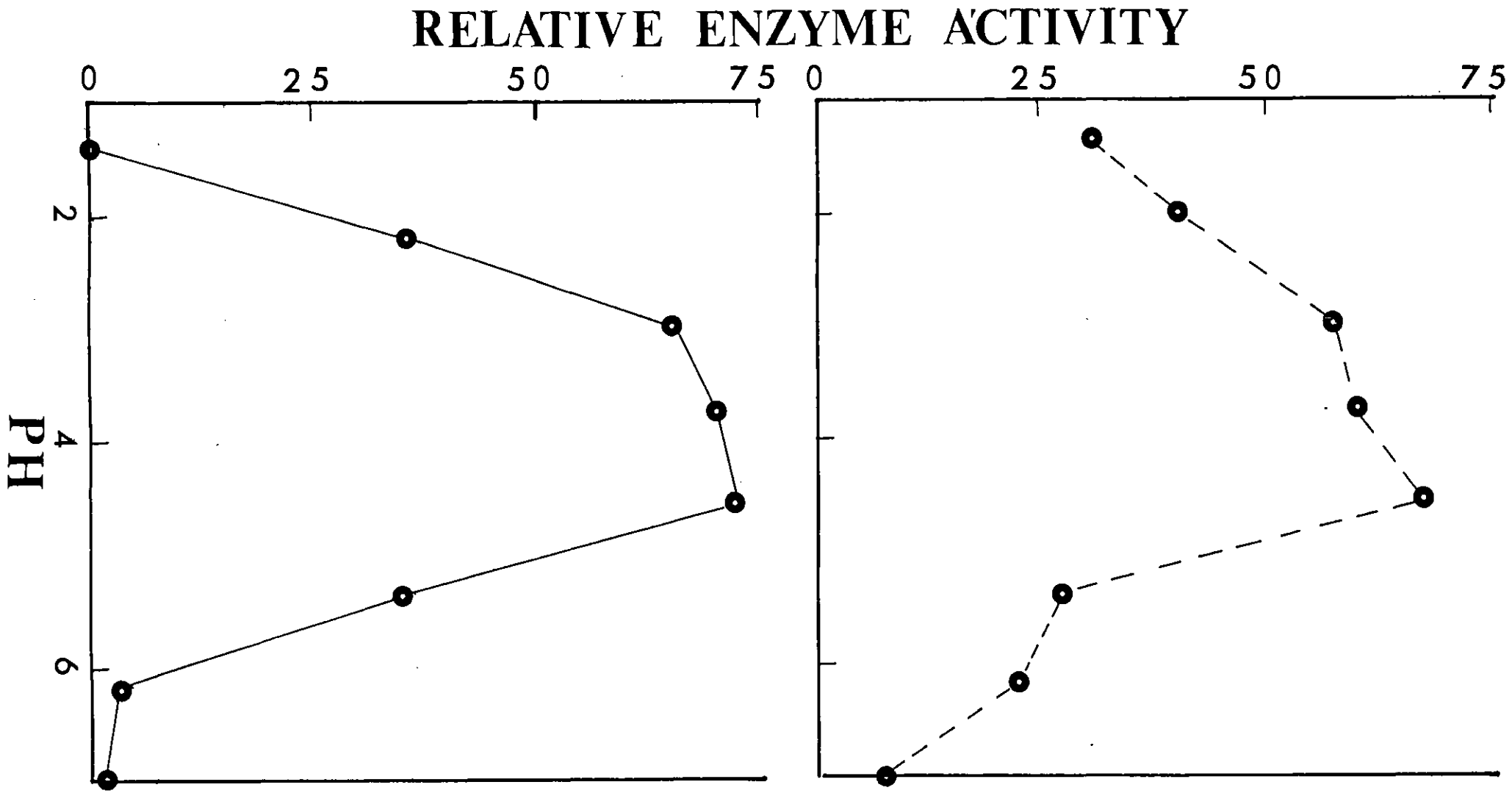
These were determined as the pH values of fractions of maximum enzyme activity. Dialysed crude filtrates from cultures on polypectate or pectin were focused on a pH 3-10 gradient for 68 hours. Fractions were dialysed first for 8 hours against 0.2 M potassium phosphate-sodium phosphate buffer (pH 6) in order to counteract the extremes of pH in some of the fractions and then dialysed against distilled water for another 8 hours. After dialysis, all fractions were adjusted to the same volume with distilled water and assayed for protein content and enzyme activity. (Fig.12) shows the electrofocusing of polypectate-grown culture filtrate in a pH 3-10 gradient.

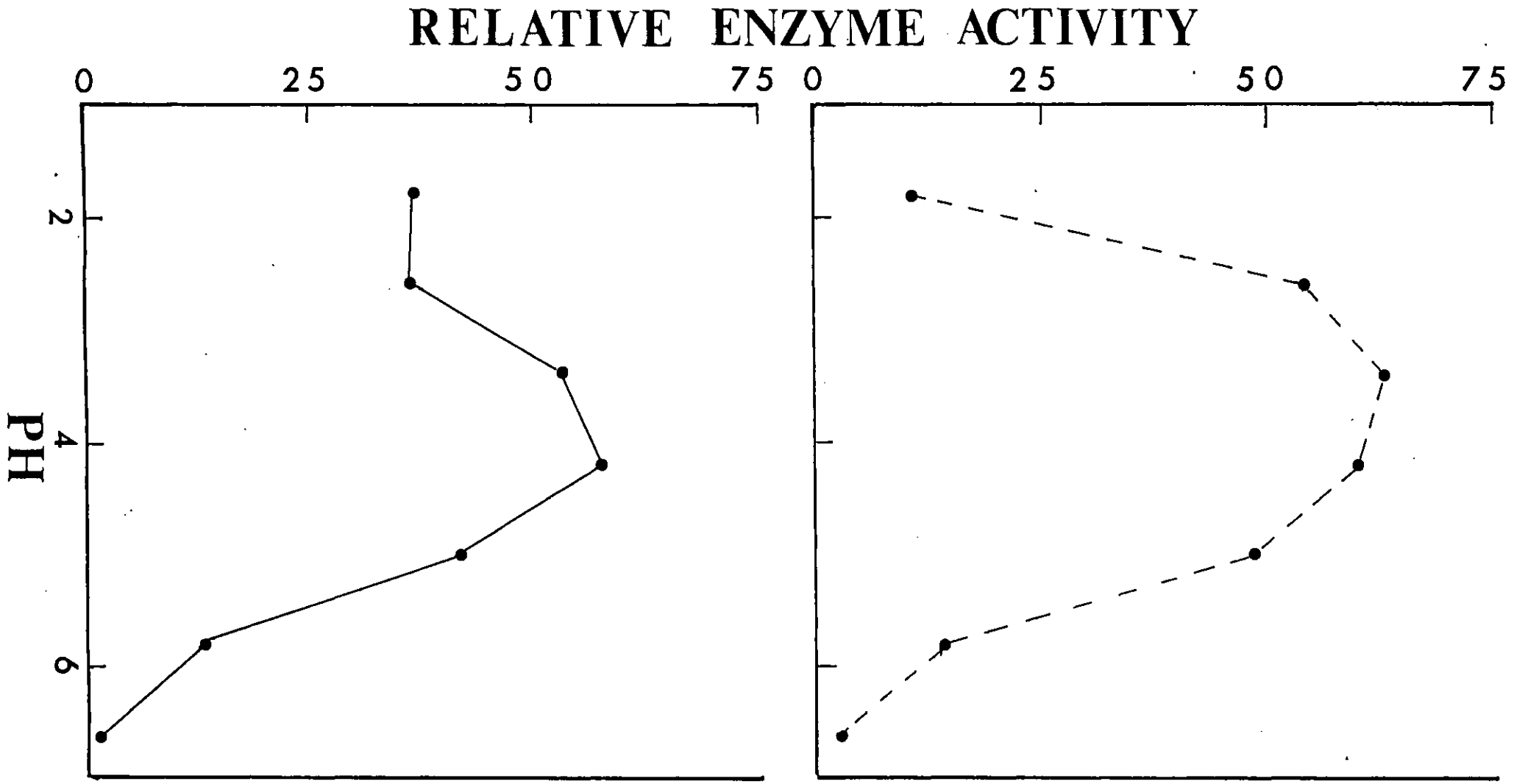
FIG. (11)

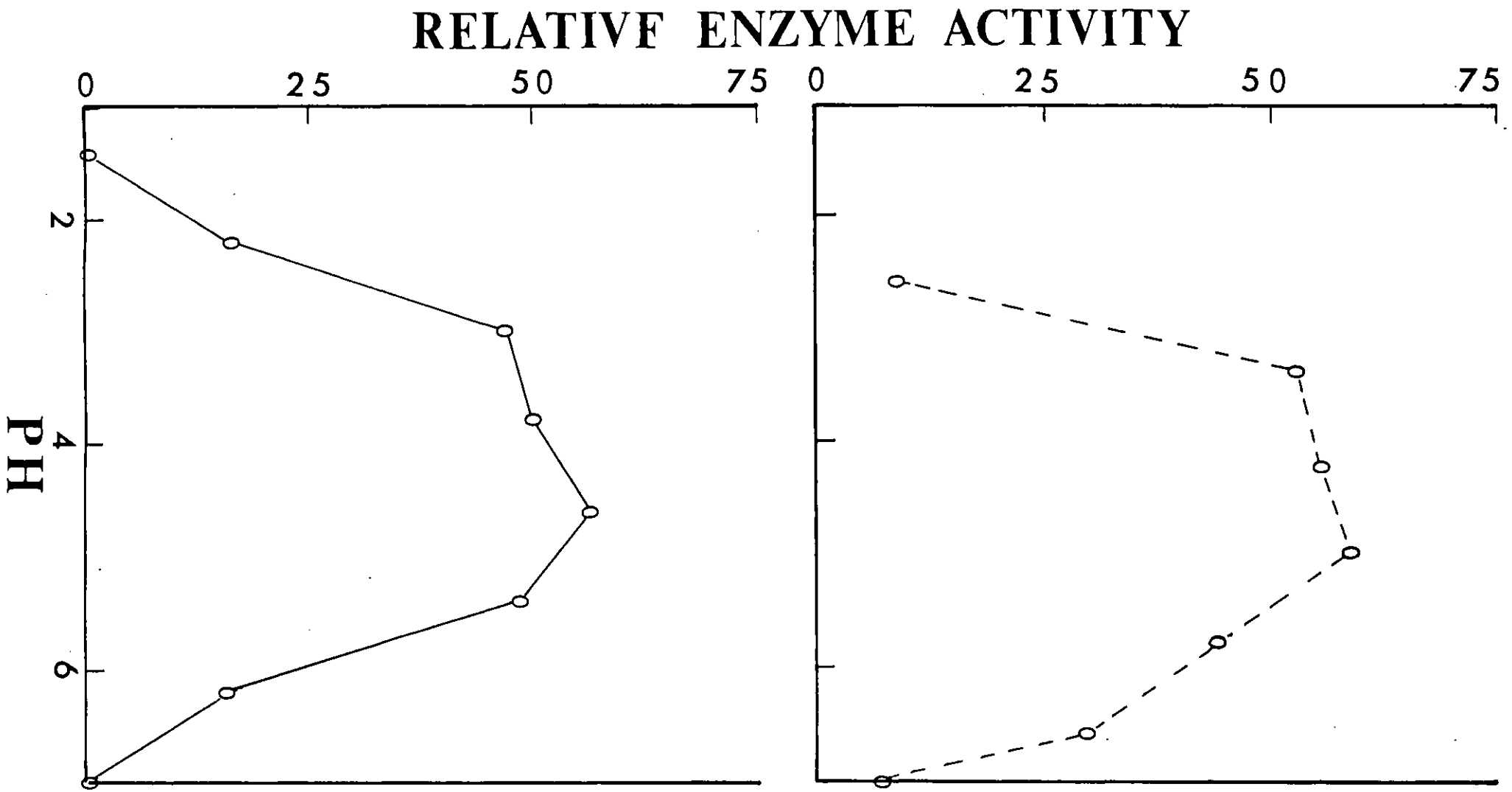
Activity of the cell wall-degrading enzymes produced by S. sclerotiorum in relation to pH.

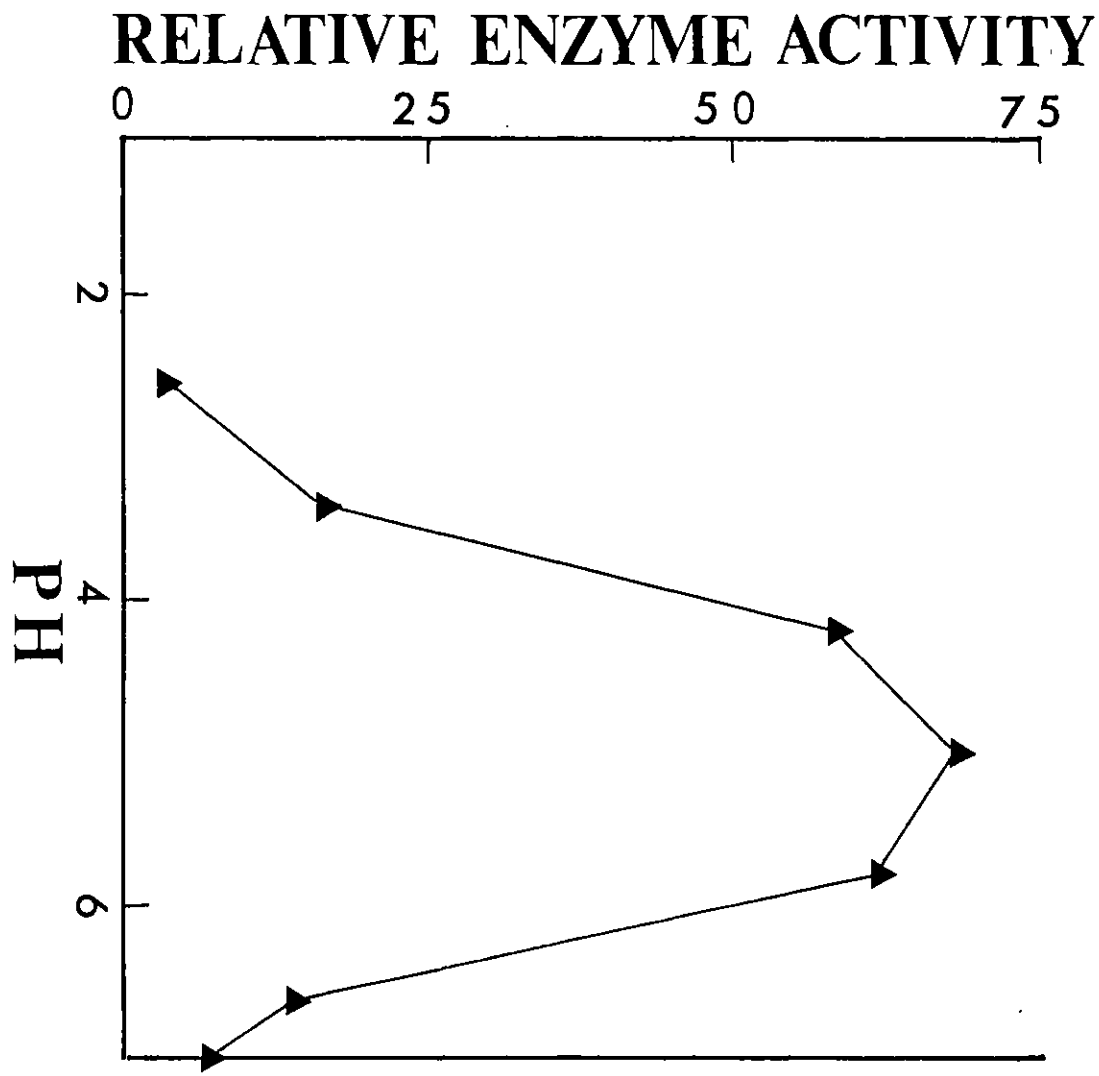
(-●-) PG; (-◉-) Ar; (-•-) AF; (—•—) GP; (--O--) Xy; (—O—) Cx; (—▲—) PME

x axis is an arbitrary scale


RELATIVE ENZYME ACTIVITY
0
25
50
75
0
25
50
75
PH
2
4
6


RELATIVE ENZYME ACTIVITY
0
25
50
75
0
25
50
75
PH
2
4
6


RELATIVE ENZYME ACTIVITY
0
25
50
75
0
25
50
75
PH
2
4
6


RELATIVE ENZYME ACTIVITY
0
25
50
75
PH
2
4
6

When the fractions were tested for PG activity a major (PG I) and minor (PG II) peaks were obtained (Fig. 12), corresponding to pH 4.8 and 5.7 respectively. PME occurred in a broad zone on the acid side of PG with an isoelectric point of 4.5. A sharp peak of AF activity was found to focus at pH 7.6. The Ar complex was composed of four peaks at 4.6, 4.8, 6.2 and 7.6. Cx showed two forms with isoelectric points at pH 3.5 and 5.9. The isoenzyme pattern of GP contained six forms at pH 2.8, 3.5, 4.9, 5.6, 6.7 and 7.6. Xy appeared in four peaks corresponding to pH 2.8, 4.4, 6.5 and 7.7.

The isoelectric points of the pectin-induced CWDE are presented in Table (17). A single peak of PG was present with an isoelectric point of 4.6. Galactanase (Gl) focused as a sharp peak at pH 8.4 and it showed three minor peaks at pH 3.5, 4.6 and 7.3. Xy, Ar and PME showed some differences but broadly the results are similar to those obtained for polypectate-grown cultures, allowing for the error introduced by collecting fractions of finite size. All fractions gave negative results when tested for PL or PGL activity.

TABLE (17)

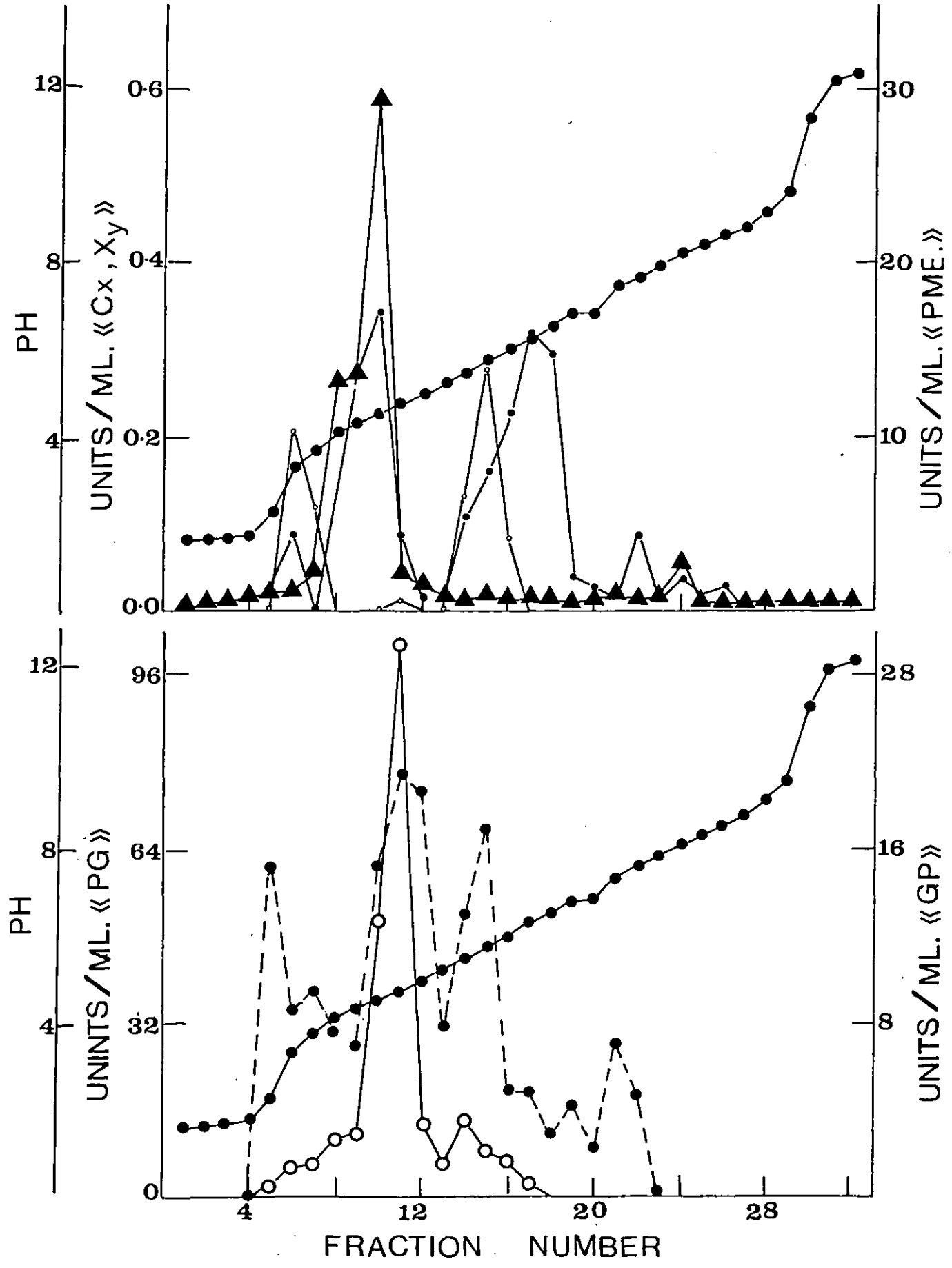
Isoelectric points of the pectin-induced CWDE produced by *S. sclerotiorum*

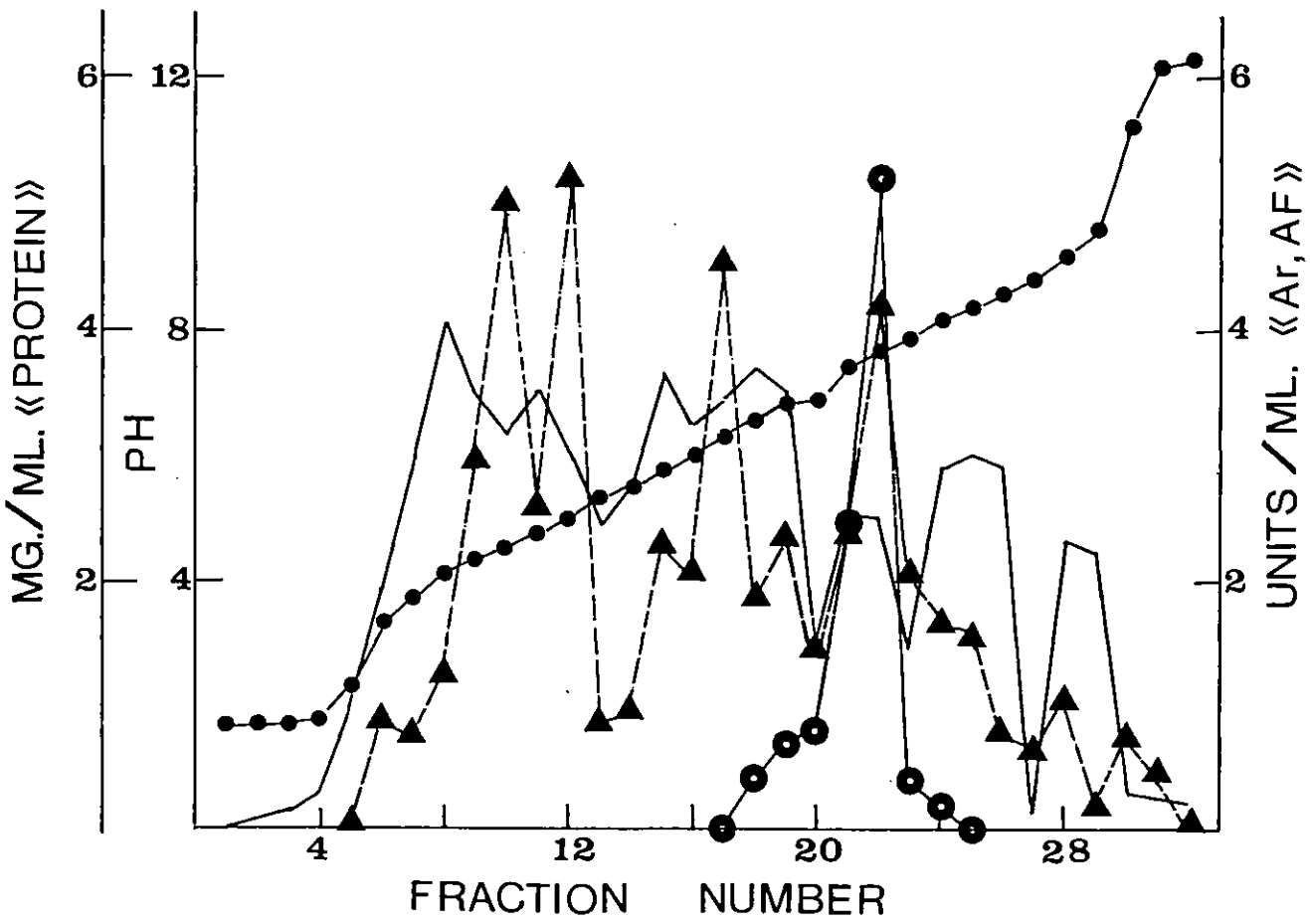
Enzyme	Isoelectric point *
Polygalacturonase	4.6
Pectinmethylesterase	3.6, 4.2
Arabanase	3.5, 4.6, 6.4, 7.5
Xylanase	2.9, 4.2, 6.4
Carboxymethylcellulase	3.5, 6.0
α-L arabinofuranosidase	7.7
Galactanase	8.4, 3.5, 4.6, 7.3

* Values are the means of two determinations

FIG. (12)

Wide range (pH 3 - 10) electrofocusing of sodium polypectate-grown culture filtrate of S. sclerotiorum - (∘) Cx; (•) Xy; (-▲-) PME; (O) PG; (-●-) GP; (◉) AF; (-▲-) Ar; (——) Protein content (-●-) pH gradient


12
8
4
PH
0·6
0·4
0·2
0·0
UNITS / ML. «Cx , Xy»
30
20
10
UNITS / ML. «PME.»
12
8
4
PH
96
64
32
0
UNINTS / ML. «PG»
28
16
8
UNITS / ML. «GP»
4
12
20
28
FRACTION NUMBER


MG./ML. «PROTEIN»
PH
UNITS / ML. «Ar, AF»
6
4
2
12
8
4
6
4
2
4
12
20
28
FRACTION NUMBER

4.1.1 Multiple forms of PG and PME detected by isoelectric focusing in a narrow pH range

The fractions which contained the bulk of PG activity from electrofocused pectin and sodium polypectate-grown culture filtrates were pooled and subjected to a further purification by electrofocusing with pH 4 – 5 ampholytes. Fractions collected were dialysed as mentioned above and then assayed for PG, PME and Ar. Results are shown in Figs. (13; 14).

Only two peaks of PG activity were obtained; one at pH 4.8 (PGI) and the other at pH 5.7 (PGII) which was a minor peak, (Fig.13). PME was resolved into three isoenzymes with isoelectric points at pH 4, 4.3 and 4.5 (Fig.13).

4.1.1.1 Relationship between viscosity reduction and substrate degradation by polygalacturonase (PG I, PG II)

Endo- and exo-forms of enzymes can be distinguished by determination of the percentage of α-1, 4 glycosidic bonds broken at 50% loss in the viscosity of the substrate. Endo-enzymes are considered to cleave 1 to 2% of the glycosidic bonds at 50% viscosity reduction, exo-enzymes greater than 10% (Archer, 1973; Cooper et al., 1978).

To determine whether the cleavage of the pectic substrate by the two forms of PG is random (endo) or terminal (exo), the relationship between reduction of substrate viscosity and substrate breakdown was examined. Times required for 50% reduction in the relative viscosity of the substrate (t_{50}), were obtained from curves of viscosity reduction against time, (Fig.15). From other curves of reducing groups released against time in similar reaction mixtures, (Fig.15) levels of substrate degradation at times corresponding to t_{50} values were calculated. For both isoenzymes

FIG. (13)

PG and PME patterns following isoelectric focusing of the peak fractions of PG in a narrow pH range (pH 4 - 6).

(—●—) PG; (--●--) PME; (●) pH gradient.

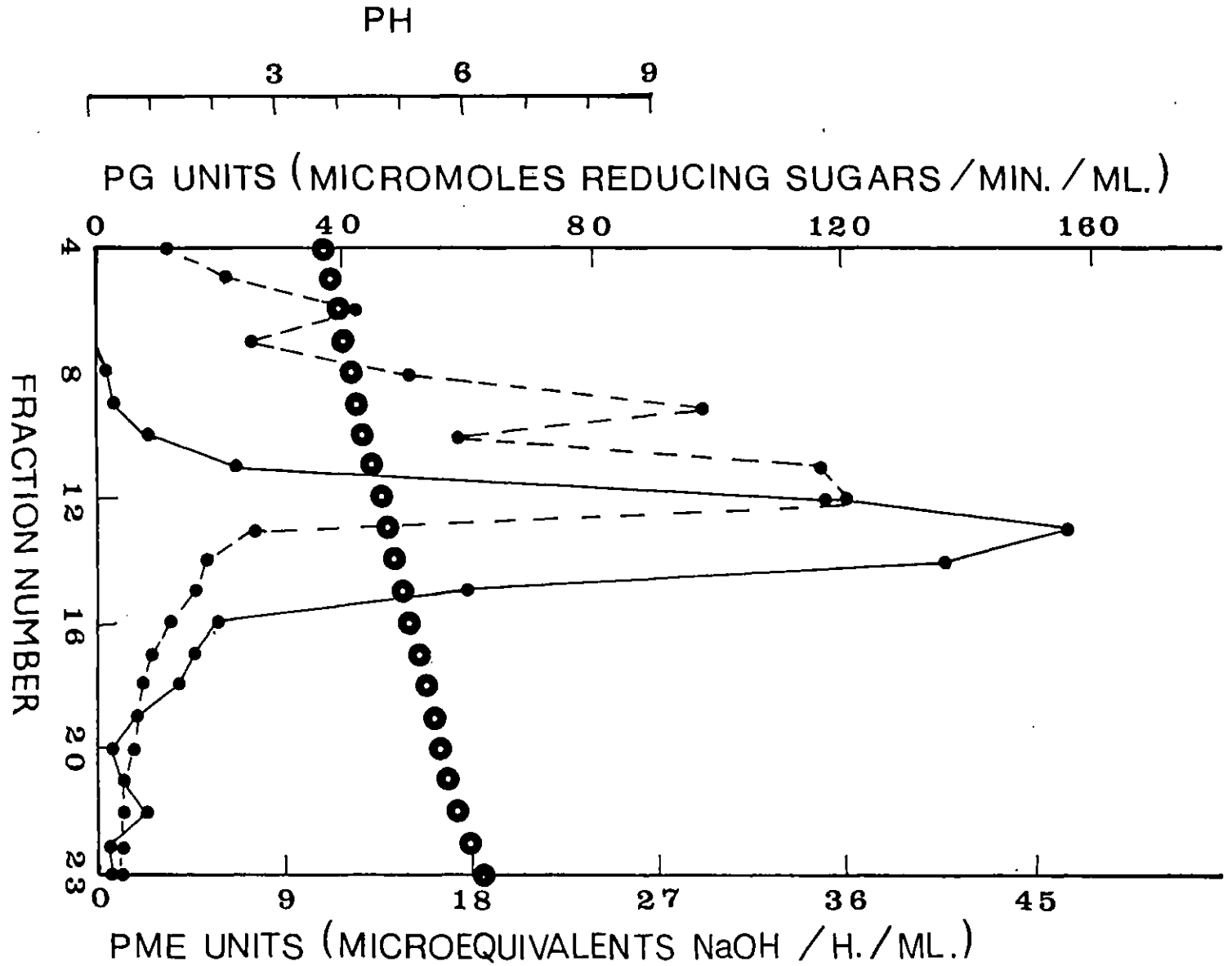

PH
3
6
9
PG UNITS (MICROMOLES REDUCING SUGARS / MIN. / ML.)
0
40
80
120
160
FRACTION NUMBER
4
8
12
16
20
23
0
9
18
27
36
45
PME UNITS (MICROEQUIVALENTS NaOH / H. / ML.)

FIG. (14)

Narrow range (pH 4 - 6) isoelectric focusing of arabanase. Dotted line = pH gradient.

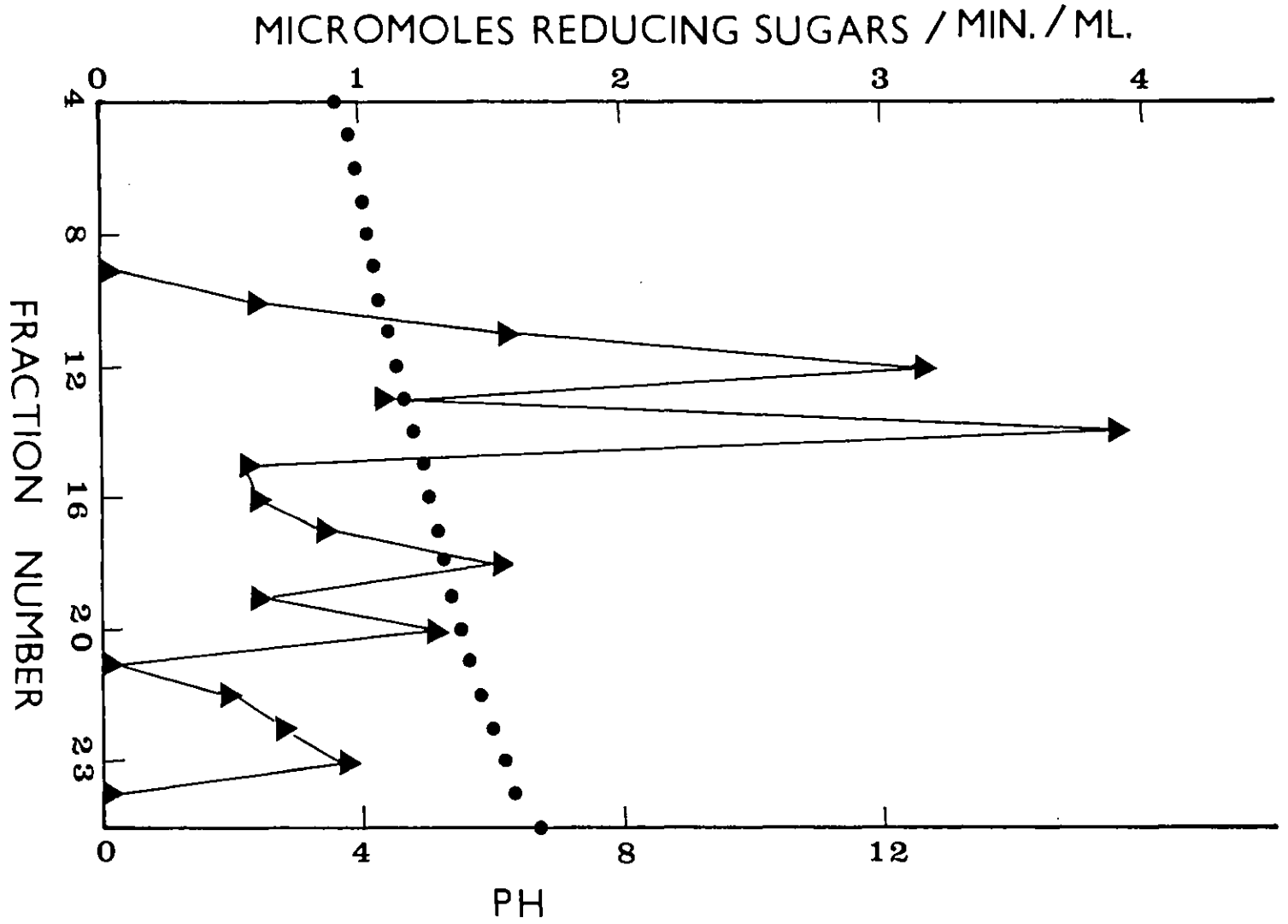
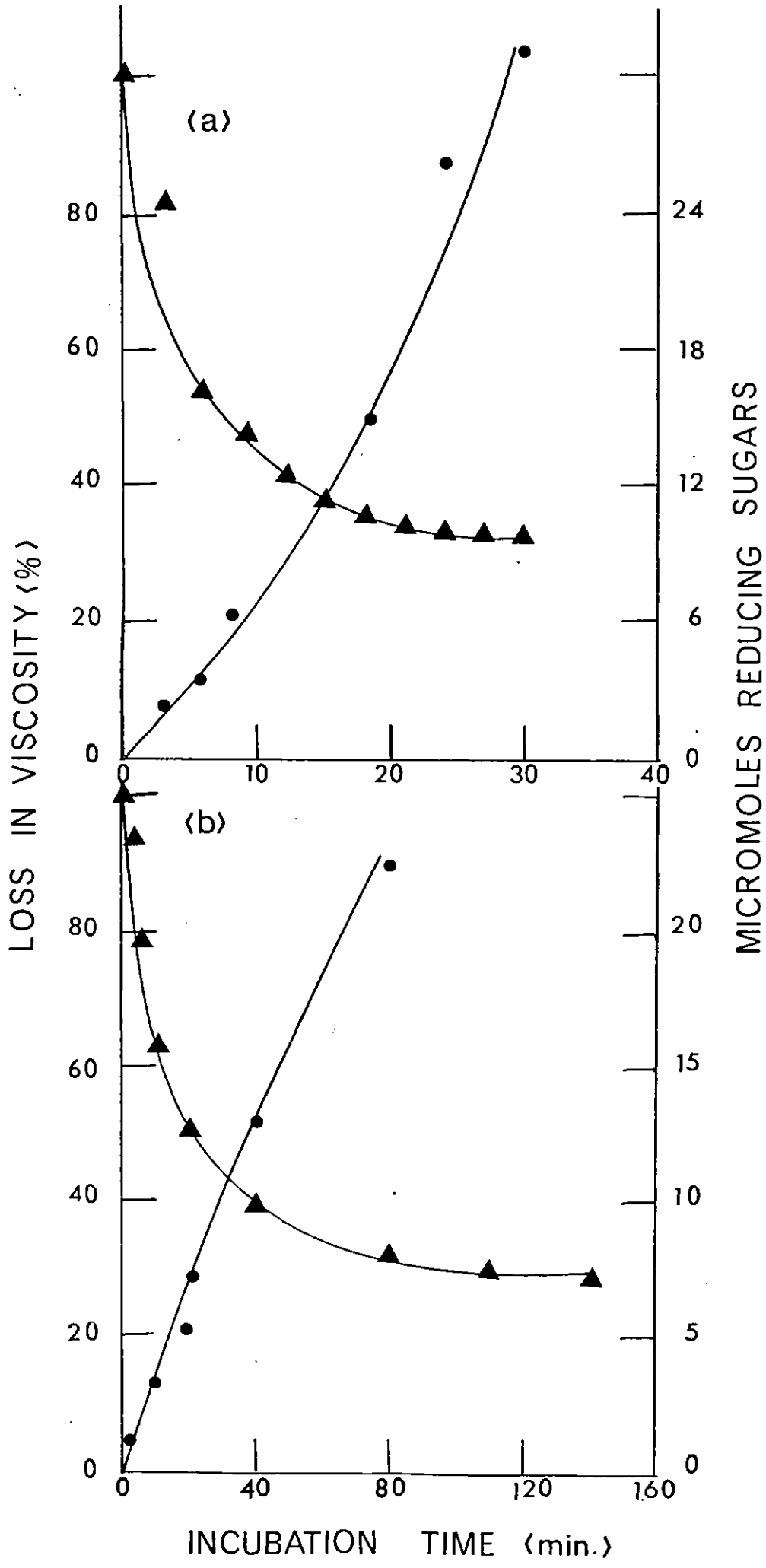

MICROMOLES REDUCING SUGARS / MIN. / ML.
0
1
2
3
4
FRACTION NUMBER
4
8
12
16
20
23
0
4
8
12
PH

FIG. (15)

Viscosity changes and hydrolysis of sodium polypectate by (a) PG I and (b) PG II.

(▲) Loss in viscosity of sodium polypectate solution.

(●) Release of reducing sugars from sodium polypectate.


(a)
(b)
LOSS IN VISCOSITY (%)
MICROMOLES REDUCING SUGARS
INCUBATION TIME (min.)
0
20
40
60
80
0
6
12
18
24
0
10
20
30
40
0
5
10
15
20
0
40
80
120
160

a result of 8% was obtained which, although higher than that for classical endo-enzymes, is below the figure expected for exo-type activity.

4.4.1.2 Correlation between different assay methods for PG

PG in the electrofocused sodium polypectate-grown culture filtrates was assayed using the three procedures described earlier. As shown in Fig . (16), both isoenzymes of PG were detected by each of the assays. The conversion ratios for the enzyme activities as determined by the assay methods are shown in Table (18) below.

TABLE (18)

Correlation of assay procedures for PG

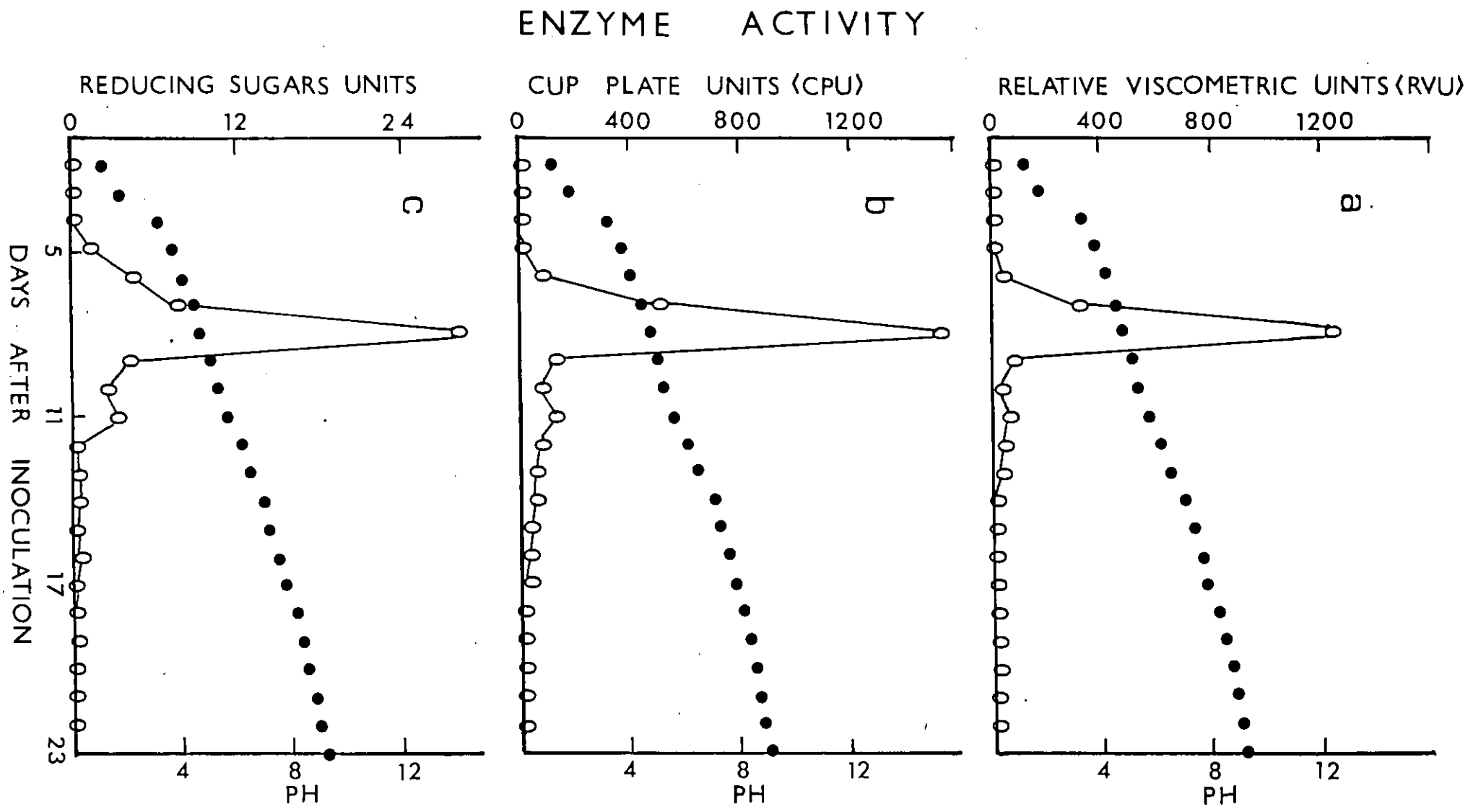
Polygalacturonase isoenzyme	Units of enzyme activity *		
	Viscometric	Agar Plate	Reducing sugar
I	1	1.3	0.023
	0.79	1	0.018
	42.82	54	1
II	1	2	0.071
	0.5	1	0.036
	14.06	28.1	1

Units

Viscometric	$1/t \times 10^3$
Agar plate	arbitrary units
Reducing sugar	µmol/min.

FIG. (16)

Activity of polygalacturonase (PG I and PG II) in the electrofocused sodium polypectate-grown culture filtrates as determined by (a) viscometry; (b) cup-plate and (c) reducing sugars.


ENZYME ACTIVITY
REDUCING SUGARS UNITS
0
12
24
c
CUP PLATE UNITS (CPU)
0
400
800
1200
b
RELATIVE VISCOMETRIC UINTS (RVU)
0
400
800
1200
a
DAYS AFTER INOCULATION
5
11
17
23
4
8
12
PH
4
8
12
PH
4
8
12
PH

4.5 Molecular weights of pectin and sodium polypectate-induced CWDE

Sephadex gel filtration was used for the determination of the molecular weights of the CWDE produced by S. sclerotiorum. Filtrates from pectin-grown cultures were dialysed extensively before being concentrated against PEG. To minimize loss of activity 5% (w/v) sucrose was added to the culture filtrate and dialysis was performed against 5% (w/v) sucrose solution under the same conditions described earlier. After partial purification of the concentrated filtrates by wide-range isoelectric focusing (pH 3 to 10), the peak fractions of PG activity were pooled, concentrated and applied to a column of Sephadex G-100. Total protein and enzyme activity were measured at different steps of the purification procedure. Samples (2 ml), from the most active peak fractions of the other CWDE were also applied separately to the Sephadex column. Their elution volumes and the increases in their specific activities were determined. Results obtained are presented in Tables (19; 20) and Fig. (17). The molecular weights of the CWDE were also determined by applying crude filtrates from pectin or sodium polypectate-grown cultures. The filtrates were concentrated using the same procedure as above.

Gel filtration chromatography of the different enzyme preparations revealed that most of the CWDE of S. sclerotiorum had similar molecular weights. The peaks of activity were detected in approximately the same fractions when the enzyme preparations from crude or electrofocused filtrates were applied to the column. The approximate molecular weights of the electrofocused pectin-induced CWDE, calculated with respect to the standards are recorded in Table (21). The elution profiles for PG, Ar, and AF demonstrate single homogeneous peaks (Fig.17) with minor shoulders and little tailing. Similar results were also obtained with Xy. The elution volumes of these four enzymes corresponded to a globular protein with a

TABLE (19)

Partial purification of PG I from pectin-grown cultures of S. sclerotiorum

Purification step	Enzyme specific activity (nmol/min./mg of protein)	Purification ratio
Culture filtrate	3.5	1
After concentration	3.3	0.94
After electrofocusing	23.9	6.83
After Sephadex G-100 gel filtration	172.6	49.31

TABLE (20)

Increase in the specific activity during Sephadex G-100 gel filtration of the most active fractions of the isoelectric-focused CWDE

Enzyme	Specific activity (units/mg protein)		Ratios of specific activity (GF/EF)
	After electrofocusing (EF)	After gel filtration (GF)	
Pectinmethylesterase (pI 4.2)	26.0[a]	147.7	5.7
Arabanase (pI 4.6)	2.08[b]	10.48	5.0
α-L arabinofuranosidase (pI 7.7)	0.408[c]	2.74	6.7
Carboxymethylcellulase;			
C x I (pI 3.5)	1.42[b]	3.62	2.5
C x II (pI 6.0)	0.38[b]	4.59	12.1

a - μ equivalent NaoH/hr

b - μ mole reducing sugar/min

c - μ mole p-nitrophenol/min.

molecular weight in the range 40,000 - 41,000. Gel filtration of the partially purified PME showed a single peak of activity with a molecular weight in the range 38,000 - 40,000. The two isoenzymes of Cx appeared to have the lowest molecular weights, Table (21).

TABLE (21)

Molecular weight ranges of the CWDE as estimated by Sephadex G-100 gel filtration

Enzyme	pI	Molecular weight range
Polygalacturonase I	4.8	40,000 - 41,000
Polygalacturonase II	5.7	40,000 - 41,000
Pectinmethylesterase	4.2	37,000 - 38,000
Arabanase	4.6	40,000 - 41,000
α-L arabinofuranosidase	7.7	40,000 - 41,000
Xylanase	4.2	40,000 - 41,000
Carboxmethylcellulase I	3.5	22,000 - 23,000
Carboxymethylcellulase II	6.0	22,000 - 23,000

When the crude filtrates from pectin- or sodium polypectate-grown cultures of S. sclerotiorum were chromatographed by gel filtration, similar enzyme activity profiles were obtained indicating that the enzyme molecules induced on the two carbon sources were of similar molecular mass.

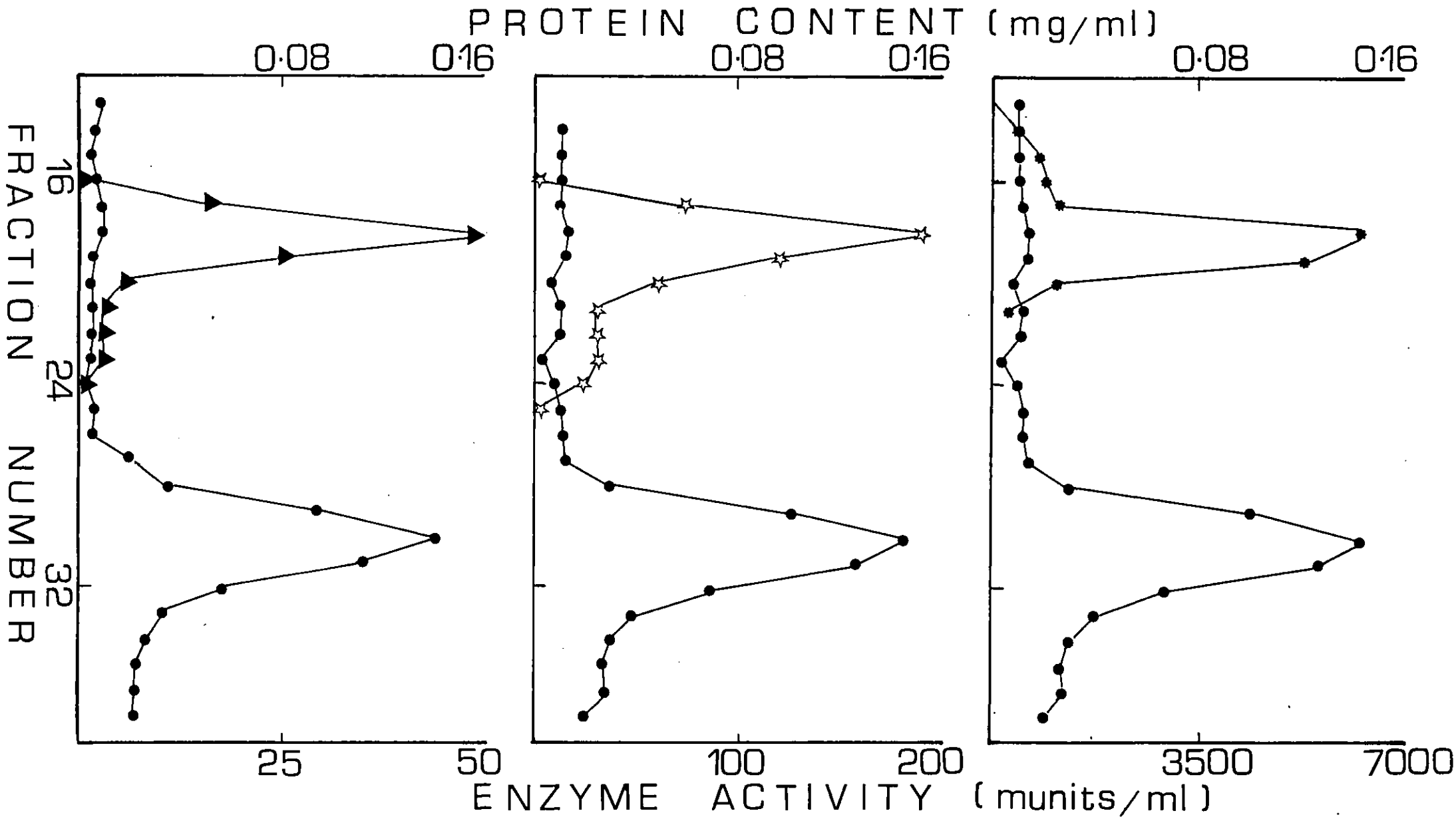
Gel filtration of the two PG isoenzymes resolved by isoelectric focusing of sodium polypectate-grown cultures revealed a single peak of activity eluted in fractions 17 to 20 with most of the activity in fraction 18. These findings indicate that the two forms of PG are structurally similar molecular species of the enzyme. The estimated molecular weight of the two isoenzymes was in the range 40,000 - 41,000.

FIG. (17)

Sephadex G100 elution profiles of PG (✱); Ar (☆); and AF (▲) activities

(●) Protein content

PG and Ar were measured using the reducing sugars assay. AF was measured as described in Materials and Methods.


PROTEIN CONTENT (mg/ml)
0·08
0·16
0·08
0·16
0·08
0·16
FRACTION NUMBER
16
24
32
25
50
100
200
3500
7000
ENZYME ACTIVITY (munits/ml)

As shown in Fig. (17), the bulk of the material which gave a strong Lowry reaction was contained in fractions 27 to 34 and was clearly of considerably lower molecular weight than the CWDE. The purification procedure used in this study resulted in an approximately 50-fold increase in the specific activity of PG over that of the culture filtrate (Table 19). Increases in the specific activity of selected CWDE achieved during gel filtration are presented in Table (20).

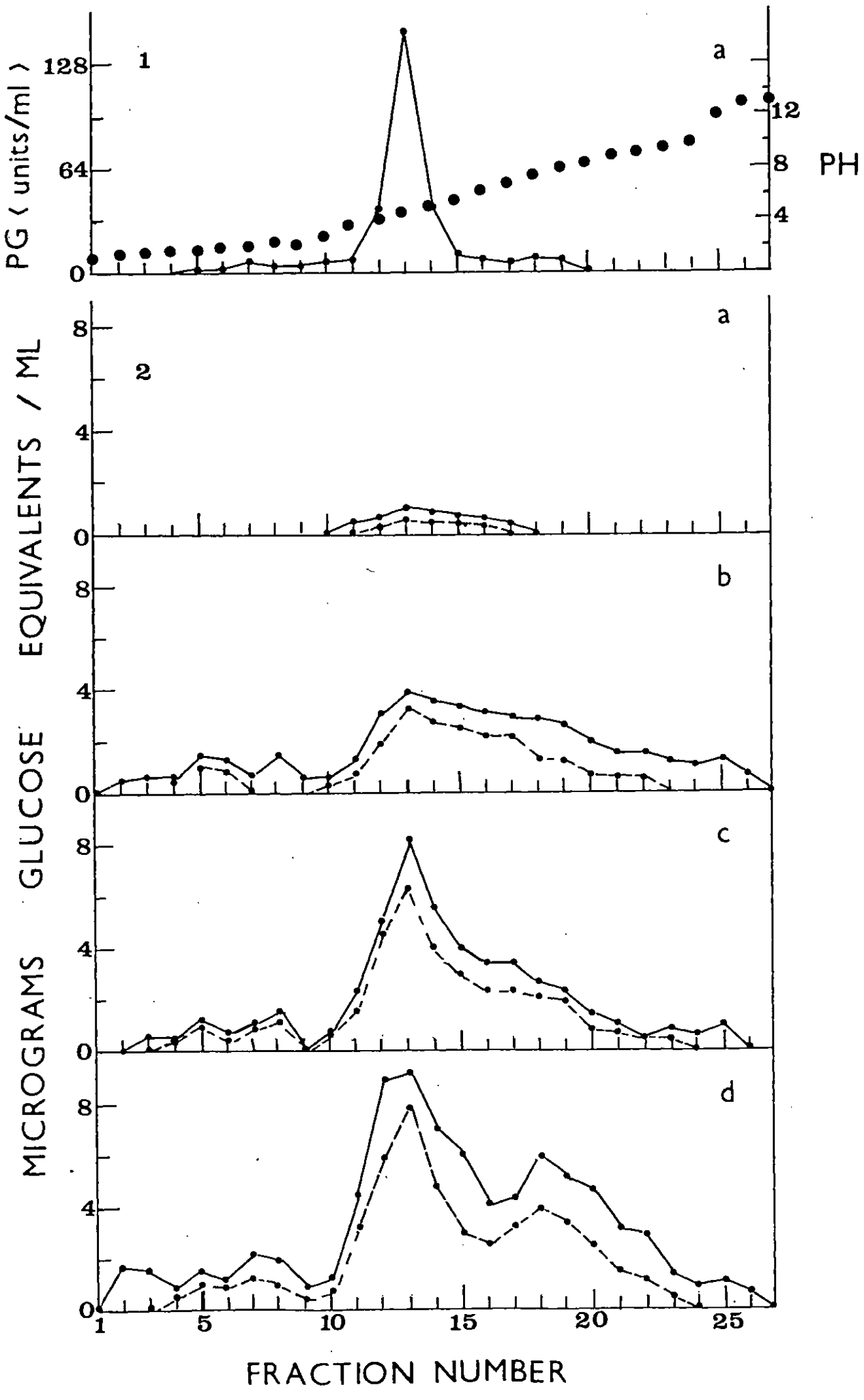
4.6 Enzymic degradation of isolated cell walls by fungal enzymes

Fractionsof the electrofocused filtrates from sodium polypectate-grown cultures were tested for their abilities to solubilize cell walls isolated from healthy stems of 4-, 8- and 12-week old plants, and from infected stems of 9-week old plants. Cell walls were incubated with samples from each fraction. The reaction mixture was the same as that described for reducing sugar PG assay but with cell walls (at 1% w/v) used as substrate. After a suitable period, the reaction was stopped by heating at 100°C for 5 minutes and the incubates were assayed for reducing sugars and for total carbohydrate. All fractions were assayed also for PG activity using the reducing sugars method.

Each batch of cell walls showed a similar digestion pattern in that fraction 13 caused the greatest release of sugars, but there were marked differences in the extent of digestion between batches (Fig. 18). Young cell walls were more easily digested than older ones. Fraction 13 corresponded with

FIG. (18)

Effect of electrofocused culture filtrate on (1) sodium polypectate and (2) tomato stem cell walls. (1a) Polygalacturonase activity determined by reducing sugars method. Dotted line = pH gradient. (2a, b, c and d) Total carbohydrates (—•—) and reducing sugars (--•--) released from cell walls obtained from infected stems of 9-week (a) and healthy stems of 12-(b); 8-(c) and 4-(d) week old plants.


1
a
128
64
0
PG (units/ml)
12
8
4
PH
2
a
8
4
0
b
c
d
MICROGRAMS GLUCOSE EQUIVALENTS / ML
1
5
10
15
20
25
FRACTION NUMBER

the peak of PG activity and it is likely that this enzyme was responsible for a substantial proportion of the wall solubilization. On 4-week old walls there was a second peak at fraction 18 coinciding with the activity peak of an isoenzyme of Ar and AF, but there was no clear coincidence between any of the minor peaks of wall digestion (fractions 5, 7, 8) and any measured fungal CWDE.

4.6.1 Hydrolytic products of cell walls treated with partially purified or crude enzyme preparations.

Products released from cell walls by the peak fractions or a mixture of enzymes from crude culture filtrates were analysed chromatographically. Enzyme preparations were incubated at 26°C with isolated cell walls from stems of 4-week plants on a reciprocal shaker (90 strokes/min.). Incubates contained: cell walls (25 mg); distilled water (5 ml); enzyme preparation, active or autoclaved for 15 minutes (5 ml) and a drop of toluene to prevent microbial growth. Samples were withdrawn from the reaction mixtures at 0, 1, 3, 6, 12 and 24 hours, boiled for 5 minutes and then examined by both descending paper and ascending thin layer chromatography.

With both culture filtrate and PG-enriched fraction 13, chromatograms of reaction products revealed that the monomer galacturonic acid and some medium molecular weight oligogalacturonides (probably pentamer or hexamer), together with compounds corresponding to arabinose, glucose and galactose were the first detectable products. Monomeric galacturonic acid which was the main initial and final reaction product, increased steadily in concentration as the oligogalacturonides decreased.

Chromatographic analysis of fraction 18 reaction mixtures revealed the liberation of 3 products corresponding to arabinose, glucose and galacturonic acid with arabinose being the predominant product.

When fractions 16 to 25 of the electrofocused filtrates were assayed for Ar and AF activities, both showed highest activities in fractions 18 and 19 (Fig.19). It was concluded that the peak of reducing substances observed at fraction 18 was mainly the result of arabinose release by Ar and/or AF.

4.7 Effect of cell walls on PG activity

Cell walls (100 mg) from 4-, 8- and 12-week plants were added separately to 3 ml of distilled water in test tubes and shaken in a reciprocal shaker (90 strokes per min.) to hydrate for 1 hour at 26°C. Excess water was then decanted from cell walls after centrifugation (1500 g, 15 min.). Samples (3 ml) from the diluted PG peak of an electrofocused culture filtrate which contained $\underline{c}$ 300 CPU/ml were added to each test tube and all the tubes were shaken for 1 hour at 26°C. Controls comprised enzyme samples maintained under identical conditions but without added cell walls.

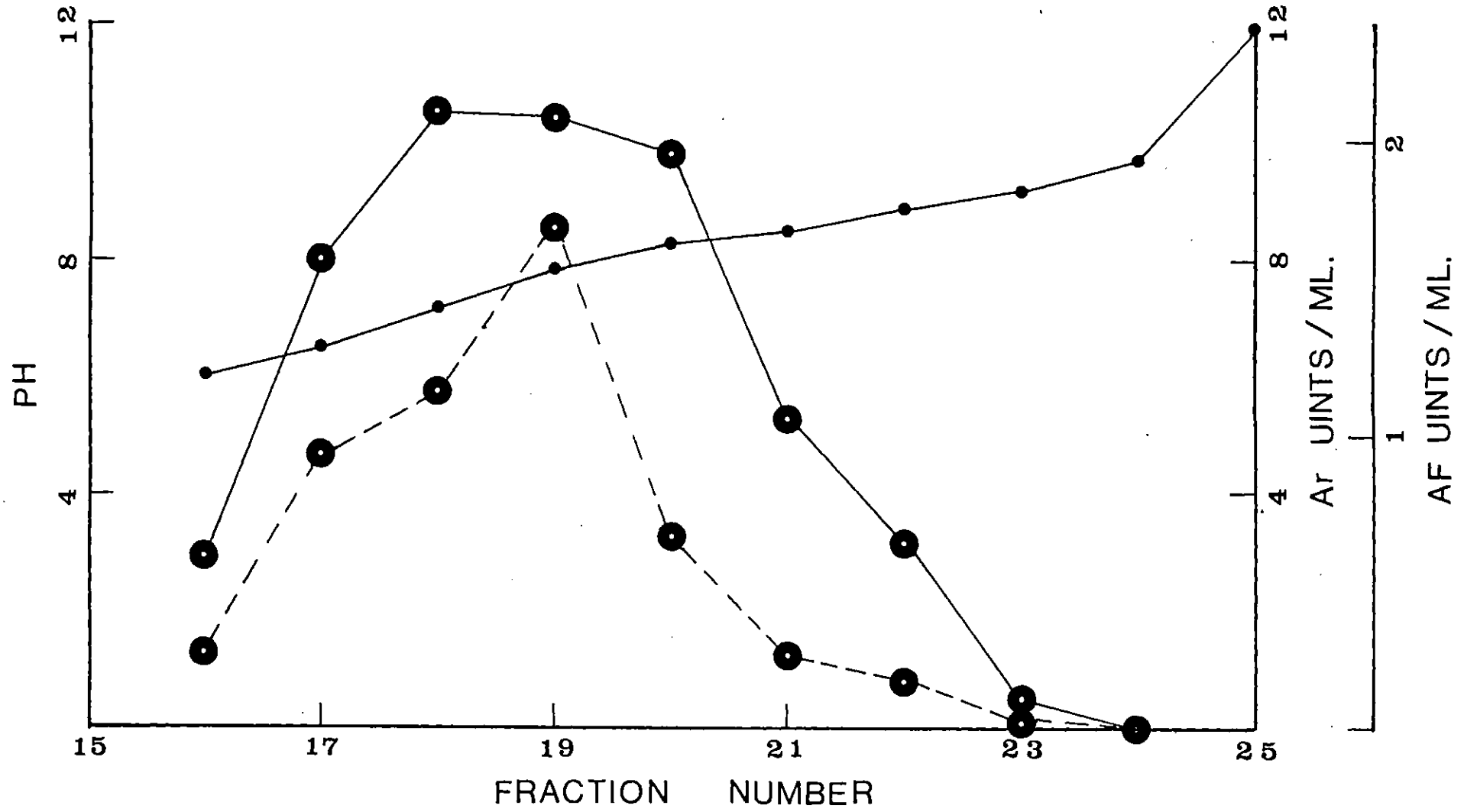
Results obtained revealed no effect on PG activity by cell walls from any of the three sources. Activity of the enzyme was nearly the same in all the treatments.

4.8 Effect of electrofocused plant extract from diseased tomato on PG activity

The isolation of proteins from the cell walls of tomato stems which inhibit fungal PG has been reported (Albersheim and Anderson, 1971; Jones et al., 1972). Two approaches were used to examine the ability of these proteins to inhibit the PG secreted by S. sclerotiorum. In the first of these, inhibitors were sought in the electrofocused extracts from diseased tomato stems. Infected 5-week old stems were harvested 3 days after inoculation and

FIG. (19)

Activities of Ar (-●-); and AF (-●-) in fractions 16 to 25 of the electrofocused culture filtrate (Fig. 18). (●) pH gradient.


12
8
4
PH
15
17
19
21
23
25
FRACTION NUMBER
12
8
4
Ar UINTS / ML.
2
1
AF UINTS / ML.

extracted using the enzyme extraction procedure. The crude extract was concentrated against PEG and then partially purified by wide range electrofocusing (pH 3-10).

Equal volumes of the fungal enzyme preparation (sodium polypectate-induced culture filtrate) and each fraction of the electrofocused plant extract were incubated for 1 hour at 26°C. PG activities of the fungal enzyme preparation, the plant extract and mixtures of both were determined using the reducing sugar procedure.

As shown in Fig. (20), the partially purified plant extract had both inhibitory and stimulatory effects on PG in the fungal culture filtrate. About 50% of the original activity was observed when PG was incubated with the most inhibitory fractions. On the other hand, the activity of the enzyme was increased by about 35% when treated with the fractions which showed the highest stimulatory effects.

4.9 Effect of partially purified PG inhibitors from healthy tomato stem on the activity of the enzyme produced by *S. sclerotiorum*

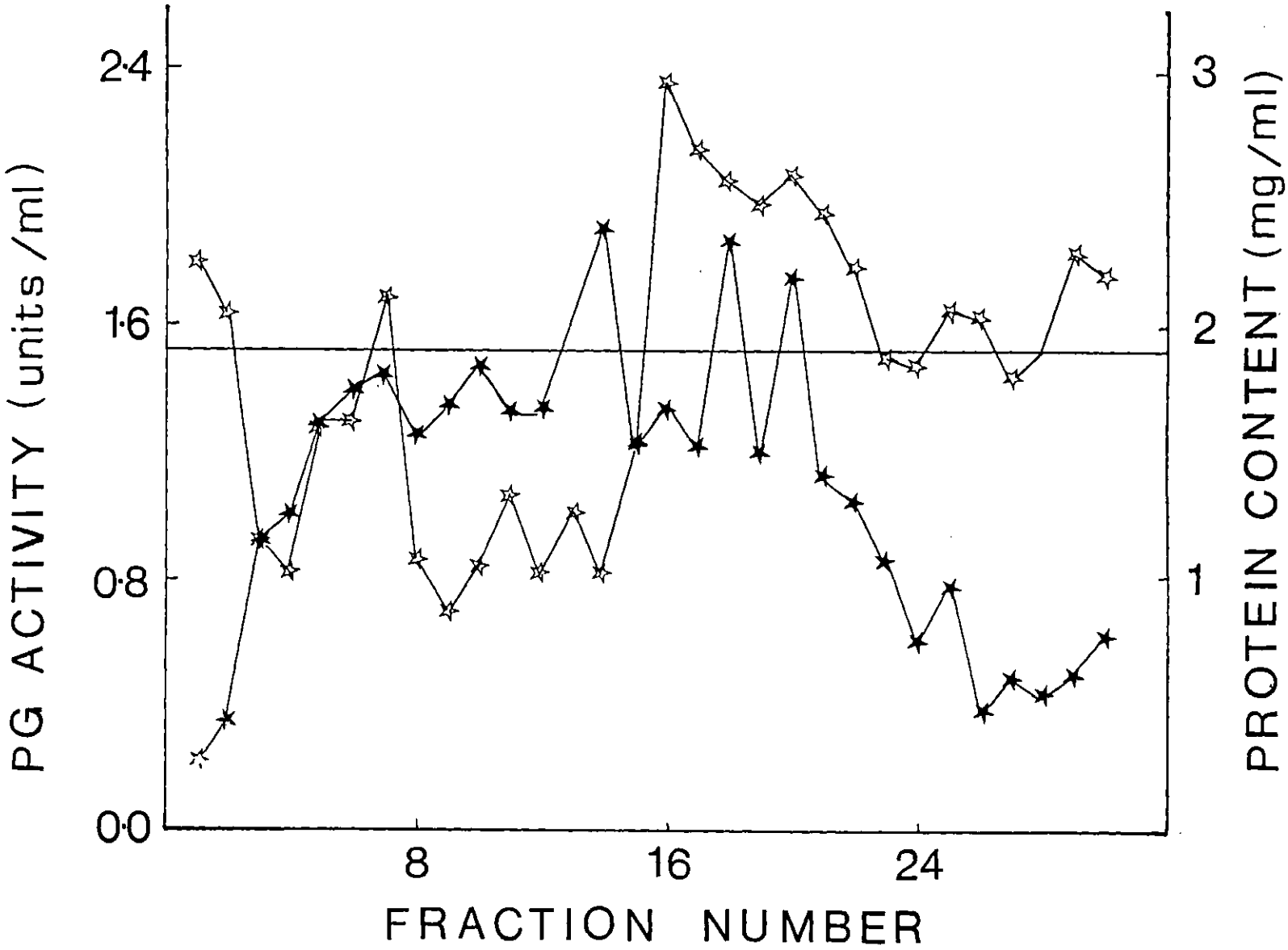
An attempt was also made to isolate the protein inhibitors of PG using the extraction procedure of Albersheim and Anderson (1971). The effect of different amounts of the inhibitor on PG activity was tested in reaction mixtures at different substrate concentrations.

Samples containing 15.5, 31 and 62 µg protein were incubated for 1 hour with PG in crude culture filtrate. Enzyme assay was started by addition of sodium polypectate to the reaction mixtures at different amounts to give the following concentrations (w/v): 0.125%, 0.25% and 0.5%. The inhibitor was replaced by buffer in the control. After half an hour the reaction was stopped and liberated reducing groups were measured.

The effect of crude extracts from healthy tomato stems on PG activity was also tested.

FIG. (20)

Effect of the partially purified infected stem extract on PG activity. (☆) PG activity as measured by the reducing sugars assay. (★) - Protein content.
Horizontal line shows the activity of PG in the culture filtrate (control).


2·4
1·6
0·8
0·0
PG ACTIVITY (units/ml)
3
2
1
PROTEIN CONTENT (mg/ml)
8
16
24
FRACTION NUMBER

Results obtained (Table22), revealed no effect of the inhibitory protein on PG activity. The enzyme exhibited more or less similar activities at the different amounts of the protein used. The observed reduction in PG activity at 0.5% (w/v) substrate concentration is clearly due to the substrate inhibition of the enzyme activity. Similarly, no loss in PG activity was found when the enzyme was incubated with crude extract.

TABLE (22)

Effect of different amounts of the proteins extracted from tomato stem cell walls on PG activity at different concentrations of sodium polypectate substrate.

Amount of protein used (ug)	PG activity (units/ml) Substrate concentration (w/v)		
	0.125%	0.25%	0.5%
0 (Control)	1.8	2.0	1.4
15.5	1.8	2.0	1.7
31.0	1.8	1.9	1.7
62.0	1.7	1.8	1.7

4.10 Oxalic acid content of culture filtrate and healthy and diseased plant tissues.

Oxalic acid contents of culture filtrate and plant tissues were determined using 13-day old sodium polypectate-grown culture filtrates and stems from 5-week old plants. Excised stems were inoculated with mycelial disks and after 3-day incubation, the diseased portions and comparable portions

from healthy stems were extracted and their oxalate contents determined.

Culture filtrate contained 2.6 mg/ml of oxalic acid and infected stem tissue the equivalent of 0.11 mg per gram fresh weight. As no oxalic acid could be detected in healthy stems, it was concluded that the oxalic acid in the diseased tissue was of fungal origin.

4.10.1 Simultaneous action of oxalic acid and PG on tomato stem tissue

Oxalic acid used in this experiment was prepared from culture filtrate. Crude culture filtrate (100 ml) was dialysed against 250 ml of distilled water for 30 hours with 6 changes of the water. In order to increase the rate of dialysis, the water was agitated with a magnetic stirer. The diffusate was concentrated under reduced pressure at 40°C to 100 ml and its oxalic acid content was determined.

The role of oxalic acid in tissue maceration and the potential for the different CWDE in crude dialysed and undialysed culture filtrates to macerate plant tissue were examined in reaction mixtures containing 10 ml of culture filtrate or oxalic acid solution or culture filtrate plus oxalic acid solution in the ratio 1:1, 1 ml 0.1 M sodium acetate buffer (PH 4.6) and 18 cross sections of hypocotyls approximately 0.5 mm in thickness obtained from 3-week old plants. Controls in this test consisted of reaction mixtures in which culture filtrates and oxalic acid solution were autoclaved for 15 minutes or replaced with water. Reaction mixtures were incubated for 3 hours. After 0.5, 1, 2, and 3 hour incubation, 3 disks of tissue were removed from each treatment and assessed for maceration.

Oxalic acid contents of the diffusate, dialysed and undialysed filtrates were 2.48, 0.09 and 2.57 mg/ml respectively. As shown in Table (23), maceration of stem tissue was first detected after 1-hour incubation. It was complete in the treatment receiving undialysed culture filtrate after

TABLE (23)

Effect of culture filtrate and oxalic acid on tomato stem tissue

Treatment	Incubation time (h) and degree of maceration			
	0.5	1	2	3
(1) Water control	0	0	0	0
(2) Undialysed culture filtrate	0	1	3	5
(3) Autoclaved undialysed culture filtrate	0	0	0	0.5
(4) Dialysed culture filtrate	0	0.5	2	4.3
(5) Autoclaved dialysed culture filtrate	0	0	0	0
(6) Oxalic acid solution	0	0.5	1	1
(7) Autoclaved oxalic acid solution	0	0	0	0
(8) Dialysed culture filtrate plus oxalic acid solution	0	1	3	5
(9) Autoclaved dialysed culture filtrate plus oxalic acid solution	0	0	0	0

3 hours and nearly complete in the treatment containing dialysed culture filtrate (rating of 4.3). Slight maceration occurred when the stem tissue disks were treated with oxalic acid alone, a maceration index reading of 1 being obtained after 3 hours. When oxalic acid was added to the dialysed filtrate, the rate of tissue maceration was the same as that caused by undialysed culture filtrate. No maceration occurred in the water control, and except for the autoclaved undialysed culture which caused slight tissue maceration after 3 hours (rating of 0.5), all treatments containing autoclaved oxalic acid or culture filtrate failed to macerate stem tissue.

4.11 Maceration of plant tissue and killing of cells by the CWDE

The ability of the CWDE produced by *S. sclerotiorum* to macerate and kill plant tissue was investigated using electrofocused filtrates and stem tissue from 4-week old tomato plants. Reaction mixtures contained 9 tissue disks suspended in 6 ml of distilled water to which 3 ml of the enzyme preparation was added. Controls, consisting of autoclaved enzyme and/or water substituted for enzyme were used for all treatments. Three disks of tissue were removed from each treatment at 1-hour intervals and assessed for maceration and cell death.

Tissue maceration and cell death occurred predominantly in fractions which contained PG activity. The association of these two processes with the enzyme activity is illustrated in Fig(21)2c'2bMaceration and cell death paralleled each other in all tests and occurred at approximately the same rate for each fraction. Autoclaved enzyme preparations or water failed either to macerate or kill the stem tissue.

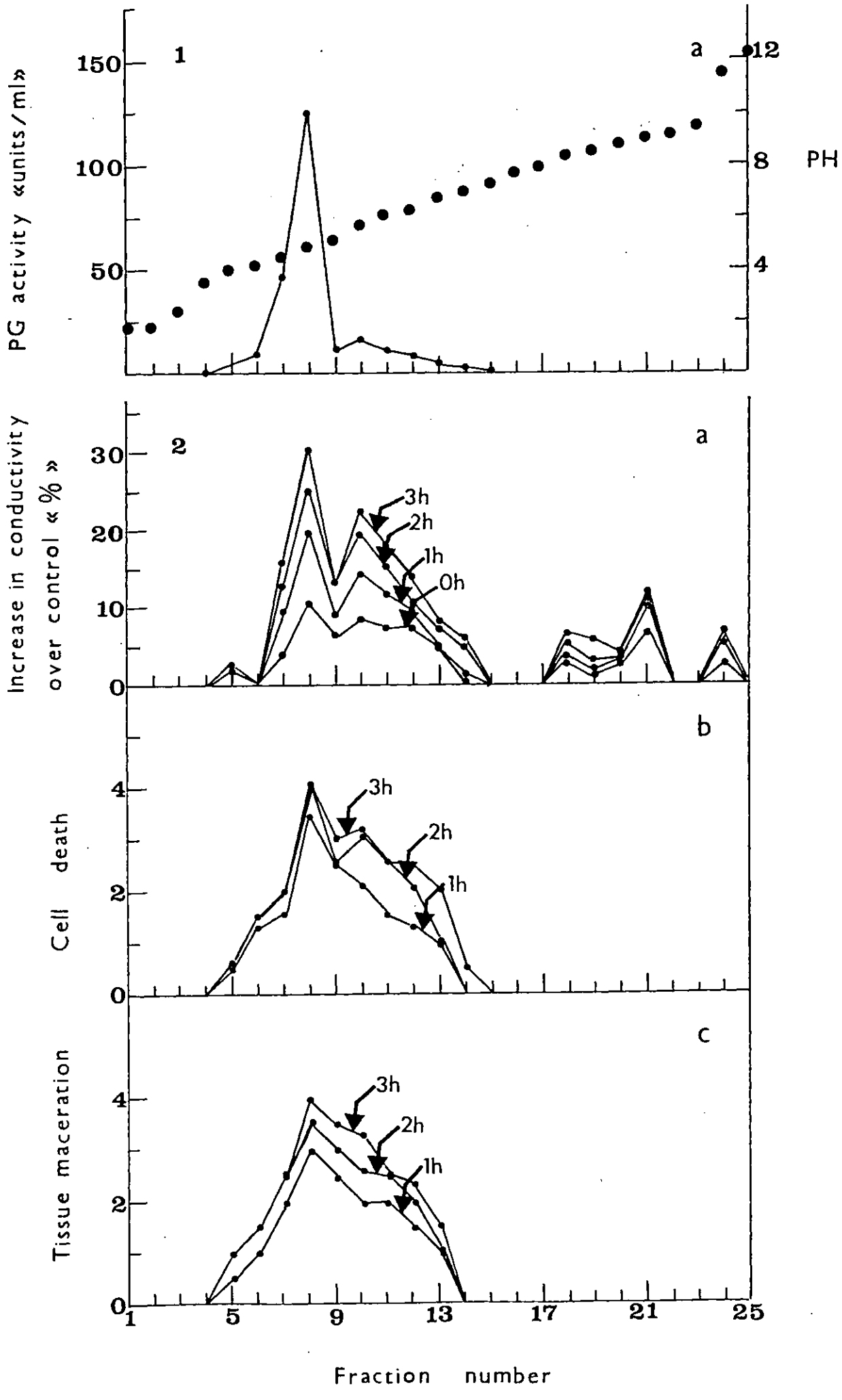
4.12 Changes in cell membrane permeability induced by CWDE

Tissue disks cut from stems of 4-week old plants were also used

FIG. (21)

Effect of electrofocused culture filtrate on (1) sodium polypectate and (2) tomato stem disks.

(1a) Polygalacturonase activity determined by reducing sugars method. Dotted line = pH gradient.
(2a) Increase in conductivity given by disks treated with active enzyme over that given by disks treated with autoclaved enzyme at 0, 1, 2 and 3 h. Results are expressed as % of total electrolytes leaked from disks after boiling.
(2b) Cell death on linear scale 0 (all alive) to 5 (all dead) after 1, 2 and 3 h. incubation.
(2c) Cell maceration on linear scale 0 (no maceration) to 5 (complete maceration) after 1, 2 and 3 h. incubation.


1
a
150
100
50
12
8
4
PH
PG activity «units/ml»
2
a
30
20
10
0
3h
2h
1h
0h
Increase in conductivity over control « % »
b
4
2
0
3h
2h
1h
Cell death
c
4
2
0
3h
2h
1h
Tissue maceration
1
5
9
13
17
21
25
Fraction number

as substrate for determining the effect of the CWDE on the permeability of plant cell membranes at 1-hour intervals for a period of 3 hours, (see section 10.3.8 in Materials and Methods).

The results of one experiment with enzymes from culture filtrate after isoelectric focusing are shown in Fig (21)2a The leakage of the electrolytes from the plant tissue occurred in all cases which may mean that loss of electrolytes is not necessarily an indication of changes in membrane permeability. However, the greatest loss was found when the tissue disks were treated with the fractions which contained greatest PG activity, (fraction 8, pI 4.8 and fraction 10, pI 5.7). The pattern of electrolytes loss with time in the water control was similar to that observed when the disks were treated with enzyme preparations. The loss of the electrolytes from the tissue which had not been exposed to enzyme might be due to physical damage to the cells caused by cutting of the disks.

DISCUSSION

The pH optima of the CWDE produced in vitro by S. sclerotiorum were comparable with those of corresponding enzymes of other plant pathogens, (Wood, 1967; Baker et al., 1977; Quantick, 1978) and agree with earlier investigations using this fungus (Hancock, 1967; Lumsden, 1976; Baker et al., 1979; Bateman and Beer, 1965), with the exception of Cx which had a pH optimum higher than that reported by Lumsden (1969). Except for Af, all other CWDE exhibited more than one peak of activity when electrofocused in a wide pH range (3-10). The resolution of the enzymes into different components indicates the presence either of isoenzymes or artifacts which can arise from a single enzyme (Cervone et al., 1978; Banks et al., 1971; Archer, 1973). Only one form of PG was detected in cultures grown on pectin whereas two were recovered from cultures of the fungus on sodium polypectate. The two PG forms designated as PG I and PG II, exhibited similar properties to one another in terms of molecular weight and type of activity but they had different isoelectric points. True isoenzymes of PG which possess similar properties have been reported by others (Cooper et al., 1978). Cervone et al., (1977) demonstrated the production by Rhizoctonia fragariae of two forms of PG indistinguishable on the basis of enzymological properties and molecular weight. The major peak of activity of pectin or sodium polypectate-induced PG (PG I), produced by S. sclerotiorum has a pI of c 4.8. Marciano et al., (1982) reported a similar result for the enzyme induced by polygalacturonic acid and for one of the isoenzymic forms of PG detected in extracts from infected sunflower stem and apple fruit tissues. In the present study the carbon source in the medium did not influence the molecular form of PG I since it had more or less the same isoelectric point when pectin or sodium polypectate was used as the sole carbon source. In contrast, Marciano et al., (1982), found that

the polygalacturonic acid-induced PG had a different isoelectric point from that induced by pectin. The results obtained in this study are similar to those reported for Rhizoctonia solani (Brookhouser et al., 1980), since in the case of this fungus the same PG was produced in culture regardless of the inducing substance.

The sodium polypectate-induced PG (PG I and PG II) reduced substrate viscosity by 50% after about 8% hydrolysis. When PG I was incubated with isolated stem cell walls it released galacturonic acid and some medium molecular weight oligogalacturonides but galacturonic acid was the main initial and final reaction product. A high percentage hydrolysis and the production of free galacturonic acid have also been reported by Marciano et al., (1982) for the polygalacturonic acid-induced PG (pI 4.8). Although the percentage hydrolysis shown by this form of PG is high it is lower than that typical of exo-activity (Rombout, 1972). This implies that the enzyme produced by the isolate of S. sclerotiorum used in this study has both endo- and exo-properties. The pattern of release of reaction products from cell walls by crude enzyme perparations is consistent with the presence of both endo- PG and exo-PG activities. Hancock, (1966), Morrall et al., (1972) and Lumsden, (1976), using preparations from S. sclerotiorum, showed the existence of two components of PG (endo-PG and exo-PG). That such components may not represent polymeric forms was indicated by the results of the molecular weight determination. Sephadex G 100 gel filtration of the crude or partially purified enzyme preparations gave a single peak of activity corresponding to a molecular weight in the range 40,000 - 41,000. Wide and narrow range electrofocusing of the enzyme preparations did not separate an exo component. The PG produced on pectin and the two forms detected in the sodium-polypectate grown culture filtrates have the same molecular weight. The molecular weight recorded for this enzyme is higher than those obtained by Marciano et al., (1982) for the PG forms induced by polygalacturonic acid or pectin but it is the

same as that found for the PG isoenzyme (pI 4.8) extracted from infected apple fruit tissue. Marciano et al,(1982) When PG I was incubated with cell walls from healthy plants at different stages of growth or walls from diseased plants, no loss could be detected in its activity indicating that this enzyme is not absorbed by tomato stem walls. Crude extracts from healthy stems also did not affect PG activity. However, when plant extract was subjected to fractionation by electrofocusing it exhibited both stimulatory and inhibitory effects. The inhibitory effect was slightly greater than the stimulatory effect. Inhibition of fungal polygalacturonases by extracts from tomato stem has been reported and attributed to proteins in the cell walls, (Albersheim and Anderson, 1971; Jones et al., 1972). Albersheim and Anderson, (1971) isolated 5 glycoproteins from cell walls of tomato stems, bean hypocotyls and suspension-cultured sycamore cells. Purification of the protein from bean plants which inhibits the PG from Colletotrichum lindemuthianum has shown that these proteins are specific for a PG from a particular fungal species. The properties of the inhibitor protein from bean plants closely resemble those of the phytohaemagglutinins and other plant glycoproteins which agglutinate transformed animal cells. Phytohaemagglutinins, or lectins as they are sometimes known, are becoming increasingly implicated as cell wall constituents (Talmadge, et al., 1975; Lamport, 1973; Keegstra et al., 1973 and Monro, 1974). Proteins isolated from tomato stem cell walls according to the technique described by Albersheim and Anderson, (1971) showed no effect on the activity of PG from S. sclerotiorum. The observed synergism between the fungal enzyme preparation and some fractions of the electrofocused plant extract was unexpected. A similar synergism was also observed between PME of Fusarium oxysporum f. sp. lycopersici and the undialysed tomato stem juice (Langcake and Drysdale, 1975) but in this case the stimulation was thought to be due to a heat stable activator of the host PME produced by the fungus in the culture. The association of the increased PG activity with increases

in the total protein suggests that the increase in the enzyme activity may have resulted from the stimulatory effect of some proteins in the plant extract. Activation of some enzymes by proteins is well-known, for example, activation of the cellulase of Myrothecium verrucaria by a wide variety of proteins (Whitaker, 1952).

In agreement with a report by Maxwell and Lumsden, (1970), S. sclerotiorum produced oxalic acid in liquid cultures and in infected tomato stem tissue. A synergistic relationship was found between PG and oxalic acid in the maceration of tomato stem tissue. One aspect of this synergism in the host tissue might be the lowering of the pH of the infected tissue to a pH value of $\underline{c}$ 4.5 which is optimal for PG activity. Hancock, (1966), found high endo-PG activity from S. sclerotiorum-infected sunflower and tomato stem was associated with a decrease in pH from 6.2 to about 4.5 and a 3-fold increase in the total acidity of Sclerotinia-infected stems. A significant increase in PG activity was also found in tissue extracts obtained from S. sclerotiorum-infected bean hypocotyls 2 days after inoculation (Maxwell and Lumsden, 1970). The pH value of the tissue extracts decreased from 6.1 on day 0 to 4.1 on day 2. It is also possible that oxalate combines with the calcium ions in the calcium pectate of the host cell wall, permitting the PG to hydrolyse more readily the pectates in the middle lamella, (Bateman and Beer, 1965). A similar synergism was also reported between endo-PG and oxalic acid secreted by Sclerotium rolfsii in bean hypocotyls, (Bateman and Beer, 1965). When the stem tissue disks were treated with oxalic acid alone, slight maceration was observed. The toxic effect of oxalic acid on plant tissue has been reported (Bateman and Beer, 1965) and it was thought that it results from the change of pH within the tissue. Autoclaved oxalic acid failed to cause maceration of the stem tissue. This result was unexpected and it might be due to loss of some of the oxalic acid at high temperatures during autoclaving.

In this study, tissue maceration , cell death and cell leakage were always associated with PG activity, confirming the results of other workers (Lumsden, 1976; Marciano et al., 1982). The leakage of the electrolytes observed in fractions 18-24 (Fig(21)2a)did not correspond to the activity of any of the CWDE tested. It was thought possible that this increase was due to the presence in the culture filtrate of toxins produced by S. sclerotiorum (c.f. Hanchey, Wheeler and Luke, 1968; Strobel, 1974). This fungus is also known to produce phosphatidase and protease (Newton, 1972 ; Khare and Bompeix 1976). The toxicity of these two enzymes on plasma membrane has been demonstrated by Stephens and Wood, (1974).

Activity of PG in culture filtrate was reduced greatly when dialysed against distilled water for 16-20 hours. A possible reason for this reduction in enzyme activity is the drop of the pH of the culture filtrate to a low value during prolonged dialysis against distilled water (Plummer, 1978). The observed loss of PG activity during the concentration of bulked culture filtrates may be due to the following reasons: (1) bulk freeze drying is a much more prolonged process giving greater possibilities for inactivation; (2) if undialyzed material is freeze-dried there are considerable possibilities for high local salt concentrations, frothing and a high ionic strength medium once it is thawed or rehydrated.

Synthesis of PME by S. sclerotiorum was induced by both pectin and sodium polypectate. The in vitro production of this enzyme by S. sclerotiorum has been reported previously (Echandi and Walker, 1957). The results obtained from the isoelectric focusing studies indicate that this enzyme exists in multiple forms the isoelectric point of which differ only slightly. From the experimentally determined molecular weights of the pectin and sodium polypectate-induced PME, it is clear that all the enzyme components are of the same molecular weight which is in the range 37,000 - 38,000. This molecular weight is comparable with that reported by Archer (1973) for one of the

isoenzymic forms of PME produced by S. fructigena.

Ar activity measured as reducing groups from araban was demonstrated in culture filtrates of S. sclerotiorum. Although electrofocusing revealed the presence of four isoenzymic forms, only one peak of activity was obtained by gel filtration, indicating that all forms may have the same molecular weight which was found to be the same as that of PG (40,000 - 41,000). The high Ar activity found in the peak fraction for PG in the electrofocused culture filtrate was first thought to be due to the PG activity. It is known that the araban substrate contains substantial proportions of galacturonic acid (Archer, 1973); and in the commercial sample the greater part of the araban still exists in the form of a pectin-araban complex (Fuchs et al., 1965). So, when cultures containing PG activity are assayed on araban, apparent Ar activity may reflect activity of PG on parts of the polymer based on galacturonic acid. However, the same result was also obtained after purification of the commercial araban using DEAE cellulose chromatography, (Knee, 1970). Ar activity was also found in the peak fraction for AF. Purification of the latter enzyme was facilitated by its high isoelectric point (pI 7.4), although it has the same molecular weight as Ar. The properties of the partially purified AF were similar to those reported by Baker et al., 1979, except for its molecular weight which is in the range 40,000 - 41,000 while that obtained by Baker et al., (1979) was about 63,000. The increase in reducing sugars observed when samples from the peak fraction for AF were incubated with araban might be the result of AF activity on this substrate. Liberation of arabinose from araban by AF action has been reported by Archer, (1973) and Laborda et al., (1974) for S. fructigena and by Baker et al., (1979) for S. sclerotiorum. As monomeric arabinose was found to be the main product released when cell walls were incubated with an enzyme preparation containing high Ar and AF activities, it was concluded that Ar and/or AF might have an exo-type of activity. This result is in agreement with those of Fuchs et al.,

(1965) and Baker et al. (1979). In contrast, Kaji et al., (1965) reported that Ar from S. libertiana (S. sclerotiorum) liberated small quantities of oligomers in addition to monomer. The isoelectric point for AF determined in this study is the same as that obtained by Baker et al., (1979).

The GP produced in vitro by S. sclerotiorum had a pH optimum of c 4.2 for the hydrolysis of p-nitrophenyl-β-D-Galactopyranoside. The enzyme was resolved into 6 forms when partially purified by electrofocusing in a wide pH range (3 - 10). Galactanase (GL) showed a major peak of activity with a pI 8.4. This result confirms that of Bauer et al., (1977). However, three other forms with isoelectric points of 4.6, 7.3 and 3.5 were also obtained. These peaks may have resulted from the action of Ar and AF on lupin galactan substrate. The release of arabinose from lupin galactan by S. sclerotiorum AF has been reported (Baker et al., (1979).

The isoenzyme pattern of Xy contained four forms when produced on sodium polypectate but it showed only three forms when the fungus was grown on pectin. This enzyme failed to degrade its substrates in tomato stem cell walls as evidenced by the absence of xylose or the oligomers of this monosaccharide when cell walls were incubated with culture filtrate having Xy activity. This result is in agreement with that reported by Hancock, (1967). However, cell walls from 4- and 8-week old plants allowed the production of this enzyme by the fungus when used as the carbon source in the culture medium.

The results obtained with S. sclerotiorum cellulase indicate that this enzyme is an inducible enzyme with a pH optimum of 4.6 which is in the range reported for other fungal cellulases (Wood, 1967). When a cell-free enzyme solution was tested for its ability to solubilize insoluble cellulose, it exhibited a considerably lower activity in comparison with its activity on carboxymethylcellulose. This result suggests that S. sclerotiorum (isolate RC/802) has a low ability to utilize the "native" cellulose in the invaded tissue. However, it is possible that the presence of the fungal mycelium could enhance the rate of reaction by constantly providing fresh enzymes (Gupta and Heale, 1970).

S. sclerotiorum has been shown to secrete a cellulase complex with a pH optimum near pH 3, which enables it to utilize cotton fibers and filter paper pulp as sole sources of carbon (Lumsden, 1969). Isoelectric focusing and gel filtration revealed the presence of two forms of this enzyme with the same molecular weight in the range 22,000 - 23,000 which approximates the molecular weight reported by Bauer *et al.*, (1977) for G1 produced by this fungus.

5. Characterization of CWDE produced in vivo

5.1 Extraction of diseased tissue for enzymes

Attempts were made to enhance enzyme recovery from infected tissue by (1) adjusting the electrolyte concentration, (2) the use of a reducing agent and (3) the use of a cationic surfactant.

In no case did the recoveries differ significantly (Table 24). The extractant used in the rest of the work in this section was 0.1M sodium acetate buffer (pH 4.6) + 0.1M sodium chloride + 0.5% (w/v) ascorbic acid.

5.1.1 Activity of CWDE detected in vivo

Extracts from healthy and diseased tomato stem tissues were dialysed against 0.05M sodium acetate buffer (pH 4.6) and assayed for CWDE activity.

In extracts of diseased tissue PG was the most active enzyme present, followed in descending order by Cx, Ar, AF and Xy (Table 25). PME was present at a much lower level and was the only enzyme of the six tested which was detected in healthy tissue.

5.1.2 Time course of PG production in diseased stem tissue

The enzyme was extracted from *S. sclerotiorum*- infected stem tissues of 4- and 8-week old plants at 3-day intervals for a period of 15 days. The extracts were dialysed against 0.05M sodium citrate buffer (PH 4.6) and assayed for PG using the cup-plate method. Results are shown in Fig. (22).

TABLE (24)

Activities of PG and AF extracted from healthy and diseased tomato stem tissues by different extractants

Extractant	Enzyme activity (mUnits/ml of plant extract)			
	Diseased tissue		Healthy tissue	
	PG	AF	PG	AF
A	967	73	0	0
B	1008	67	0	0
C	875	76	0	0
D	1017	79	0	0

Figures are the means of three determinations

(A) 0.1M sodium acetate buffer (pH 4.6) + 0.1M sodium chloride

(B) 0.1M sodium acetate buffer (pH 4.6) + 0.1M sodium chloride + 0.5% (w/v) ascorbic acid

(C) 0.1M sodium acetate buffer (pH 4.6) + 0.1M sodium chloride + 0.1% (w/v) cetylpyridinium chloride

(D) 0.1M sodium acetate buffer (pH 4.6) + 0.1M sodium chloride + 0.5% (w/v) ascorbic acid + 0.1% (w/v) cetylpyridinium chloride

TABLE (25)

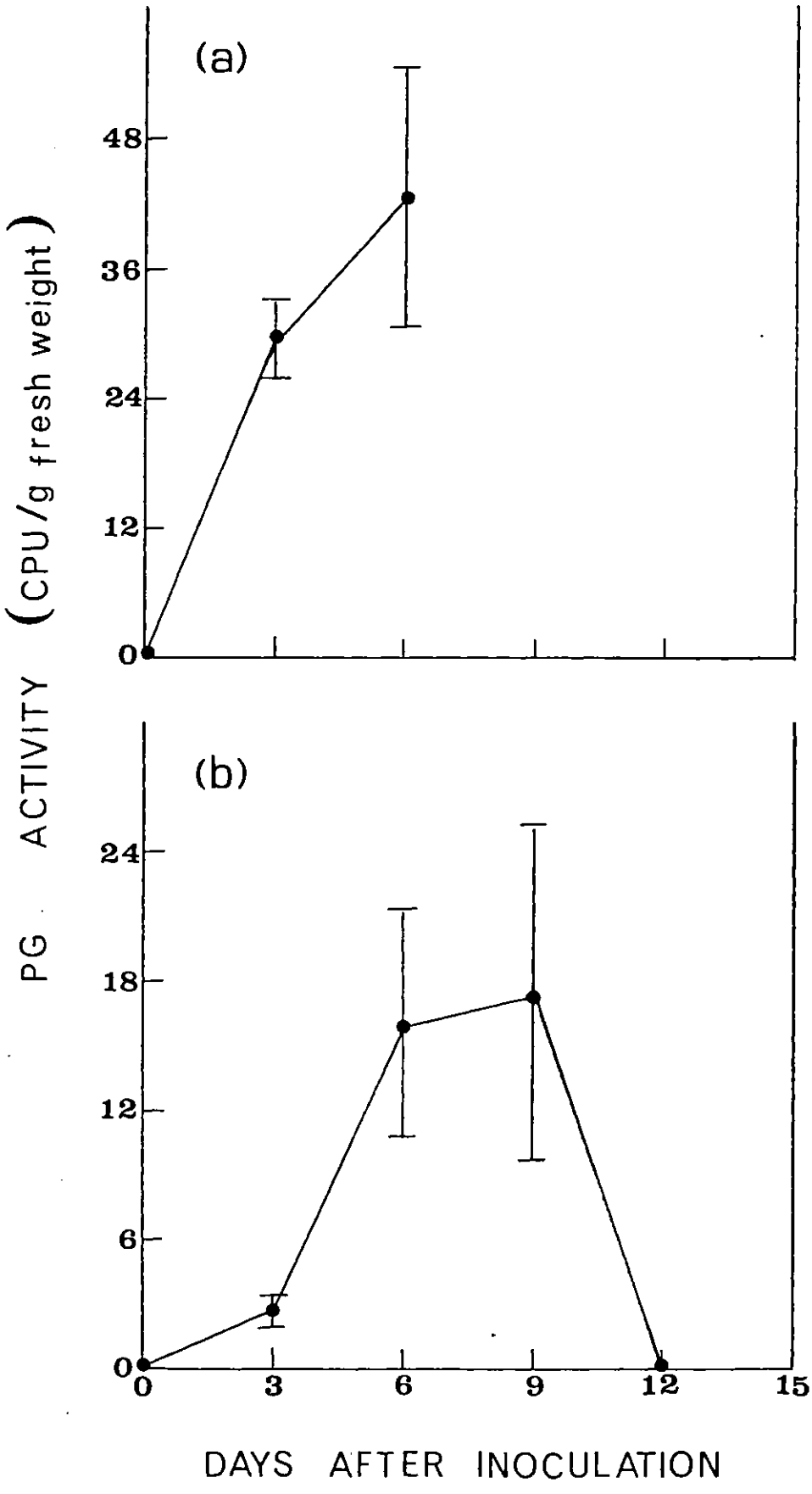
Activities of CWDE in healthy and diseased tomato stem tissue

Enzyme	Enzyme activity (mUnits/g fresh weight)	
	Healthy tissue	Diseased tissue
Polygalacturonase	0	8792
Carboxymethylcellulase	0	1932
Arabanase	0	1929
α-L arabinofuranosidase	0	1645
Xylanase	0	770
Pectinmethylesterase	17	59

Figures are the means of two determinations.

FIG. (22)

Accumulation of PG in infected stem tissue of (a) 4- and (b) 8-week old plants. PG activity was measured by cup-plate assay and expressed as cup-plate units (CPU) per gram fresh weight. Bars denote the standard deviations of the means for each value.


(a)
48
36
24
12
0
(b)
24
18
12
6
0
0
3
6
9
12
15
PG ACTIVITY (CPU/g fresh weight)
DAYS AFTER INOCULATION

Amounts of PG in young plants were higher than those in older plants, by a factor of approximately ten at three days after inoculation, and by three fold at six days. Enzyme activity continued to increase in 8-week old plants up to 9 days after inoculation but no PG activity was detected at 12 and 15 days. However, when polyclar AT was included in the extraction medium, enzyme activities of 3.8 and 2.5 units/ml of dialysed tissue extract were detected at 12 and 15 days respectively. On only one occasion were trace amounts of PG detected in extracts from healthy stem tissue.

5.2 Isoelectric points of CWDE produced *in vivo*

Tissue extracts were prepared from infected stems of 5-week old plants and dialysed extensively against distilled water or 5% (w/v) sucrose solution. The dialysed extract was concentrated 3 fold and then fractionated by isoelectric focusing in a pH 3 to 10 gradient. The results of a typical experiment are presented in Fig. (23).

PG was resolved into a single peak of activity of pI 4.7 which corresponded closely with that for PGI (pI 4.8) produced *in vitro*. No other peak of PG activity could be detected. Ar showed a wide poorly resolved peak in the acid area of the gradient with a pI around 4.3 and another minor peak at pI 7.6. AF occurred in a sharp peak at pI 7.6 coincident with the minor peak for Ar. The isoenzyme profile of Cx was similar to that of polypectate culture filtrates except that the major peak of Cx activity occurred at pH 2.8 instead of 3.5. Only two peaks of Xy activity were resolved, at pH 4.2 and 6.2. PME showed a broad peak of activity with a pI around 4.1.

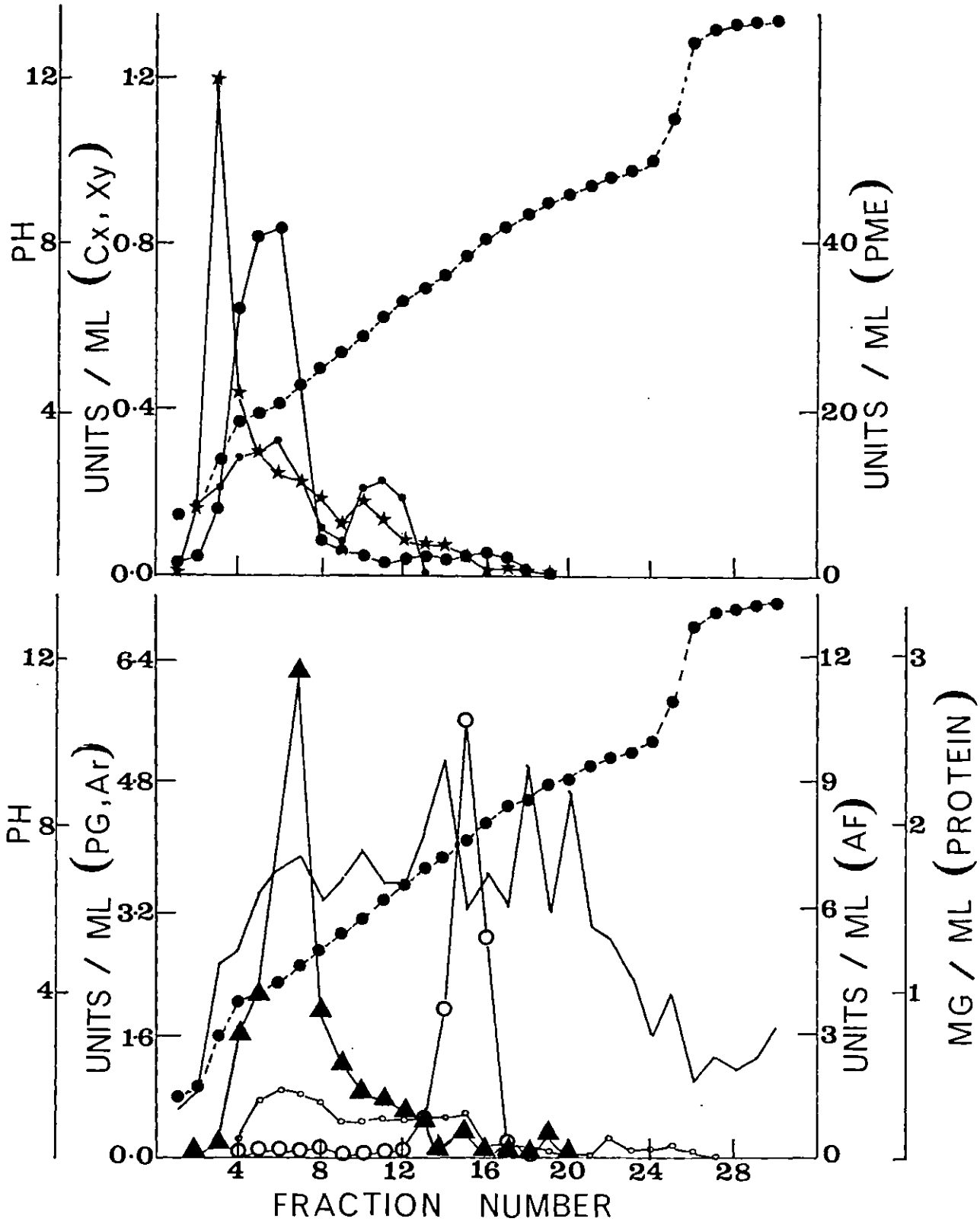
5.3 Molecular weights of the enzymes produced *in vivo*

Infected tissue extracts were electrofocused and the fractions corresponding to the peaks of PG, AF, PME and Cx were further purified by

FIG. (23)

Enzyme activity in fractions obtained by isoelectric focusing of diseased tomato stem extracts using Ampholine carriers pH 3 to 10.

(-●-) pH gradient; (——) Protein content; (★) Cx; (•) Xy; (-●-) PME; (▲) PG; (◦) Ar; (O) AF.


PH
UNITS / ML (Cx, Xy)
UNITS / ML (PME)
12
8
4
1·2
0·8
0·4
0·0
40
20
0
PH
UNITS / ML (PG, Ar)
UNITS / ML (AF)
MG / ML (PROTEIN)
12
8
4
6·4
4·8
3·2
1·6
0·0
12
9
6
3
0
3
2
1
4
8
12
16
20
24
28
FRACTION NUMBER

Sephadex G-100 gel filtration in order to measure molecular weight.

Each enzyme was eluted as a single peak. The activity profiles of the four enzymes were identical with those of the corresponding enzymes excreted by the fungus in the liquid culture. Based on their elution volumes the estimated molecular weight of PG and AF were in the range 40,000 to 41,000 while those of PME and Cx (pI 2.8) were in the ranges 37,000 to 38,000 and 22,000 to 23,000 respectively.

5.4 Plant age in relation to enzymic degradation of isolated stem cell walls

Extracts prepared from healthy and diseased stem tissues were tested for their abilities to degrade cell walls isolated from healthy stems of 4-, 8- and 12-week plants. Reaction mixtures, agitated on a wrist action shaker to keep the cell walls in suspension, contained 1 ml of 0.5% (w/v) cell wall suspension; 0.5 ml of 0.1M sodium acetate buffer (pH 4.6) and 0.5 ml of plant extract. After 1 hour incubation enzyme action was stopped by heating to 100°C and the cell walls were removed by centrifugation. The supernatant fluid was then analysed for reducing sugars and total carbohydrate. The extent of wall degradation after 1 hour incubation with extracts from healthy or diseased stems is recorded in Table (26).

Extracts from diseased plant tissue released soluble carbohydrates from insoluble cell walls from the four different sources. Tissue extracts from healthy stems degraded only walls from 4-week plants and only to a trivial extent. Susceptibility of cell walls to degradation decreased with increasing age of the plant from which they were extracted. The small amounts of soluble carbohydrates released from walls isolated from infected stems may be explained by the likely partial degradation of these walls during pathogenesis which has already consumed a proportion of the substrate.

TABLE (26)

Effect of plant extract from healthy and diseased tissues on cell walls from four different sources

Source of cell wall	Reducing sugars released by: *		Total carbohydrates released by: *		% of the wall solubilised	
	Healthy plant extract	Diseased plant extract	Healthy plant extract	Diseased plant extract	Healthy plant extract	Diseased plant extract
Healthy 4-week plants	0.3	0.99	0.36	2.5	0.014	0.1
Healthy 8-week plants	0.0	0.74	0.0	2.2	0	0.09
Healthy 12-week plants	0.0	0.60	0.0	1.4	0	0.06
Diseased 9-week plants	0.0	0.40	0.0	0.47	0	0.02

* Results are expressed in μM glucose equiv./ml of reaction mixture.

Each figure is the mean of two determinations.

5.4.1 Sugar composition of the solubilized fractions of cell walls treated with enzymes extracted from diseased stem tissue

Equal volumes of dialysed crude plant extract from infected tissue and a suspension of cell walls prepared from stems of 4-week plants (0.5% (w/v) final concentration) were incubated at 26°C on a wrist action shaker. Samples were removed after 0, 1, 3, 6, 12 and 24 hours, heated at 100°C for 5 minutes and 50 ml samples were applied at the origin of cellulose plates and Whatman No. 1 chromatography paper.

Galacturonic acid, galactose, glucose and arabinose together with oligogalacturonides were the initial reaction products visible after 1 hour incubation. A faster moving compound with Rg value of 2 was also detected at low levels on cellulose plates developed with butan-1-ol-acetic acid-water (4:1 : 1 by vol.) and its concentration increased during further incubation. Galacturonic acid was the main initial product increasing over the 24-hour period as the oligogalacturonides proportionaly decreased.

DISCUSSION

The range of CWDE produced *in vitro* by *S. sclerotiorum* were also obtained from stems infected by this fungus. Except for PME, none of these enzymes were detected in the healthy plant. Occurrence of PME in tomato and other higher plants is well documented (McColloch and Kertisz, 1947). The absorption of this enzyme by host tissue (Bateman and Basham, 1976) may explain the low level of enzyme activity found in healthy plant extracts. It is known to be absorbed by plant cell walls in an acid environment (Bateman and Basham, 1976), and since the pH of *S. sclerotiorum*-infected tomato tissue drops from *c* 6.2 to 4.5 (Hancock, 1966), the increased activity of this enzyme in the diseased tissue is likely to be of fungal origin. The recovery of enzymes from infected tissues was not greatly enhanced by any of the extractant additives used. However, in the absence of ascorbic acid, activity of PG was slightly lower. This was probably due to inactivation of this enzyme by phenols accumulated in the disrupted tissue, (Hunter, 1974).

Susceptibility of cell walls to degradations by the CWDE detected *in vivo* decreased with increasing age of the plant from which they were isolated. Similar results were also obtained with enzymes produced *in vitro*. Small amounts of soluble carbohydrates were released from walls isolated from stems of 4-week old plants when they were incubated with extracts from healthy stems. This result may be explained by the presence of some CWDE in healthy plant tissue (Goodenough and Kempton, 1976; Deese and Stahmann, 1962).

The absence of some forms of Ar, Xy and PG from the *in vivo* extract might be due to a poor resolution of the enzyme samples by electrofocusing in a wide pH range (3-10). However, the isoelectric points of those isoenzymes which were detected in infected tissue were nearly the same as those found in culture filtrates with the only exception of the major peak of Cx activity which exhibited a lower isoelectric point than that detected *in vitro*.

Results obtained from gel filtration, from paper and cellulose thin layer chromatography also revealed no differences in the molecular weights or the enzymological properties of the enzymes produced by the pathogen in culture and those obtained from diseased tissue. These results are in agreement with those of Hancock (1966) and support other workers' reports (Bateman, 1972; Archer and Fielding, 1979) about the similarity between the enzyme systems produced by phytopathogenic fungi in culture and in infected host tissues.

6.0 Accumulation of and some effects of phenols in relation to enzyme activity

6.1 Introduction

Results obtained from the experiments on the response of tomato plants at different stages of growth to infection by S. sclerotiorum showed that resistance increased with the age of the host plant. At flowering and fruiting stages the plants became more resistant compared with the early seedling stages. The lesions formed on infected stems usually ceased expanding a week after inoculation with the formation of a dark brown zone surrounding the infected tissue. The darkening of tissues suggested that phenolic compounds presumably produced by the plant in response to S. sclerotiorum infection, might be involved in the limitation of lesions. The involvement of phenols in resistance mechanisms has been reported for a range of plants by many authors, (Hunter, 1974; Byrde et al., 1960; Byrde, 1963; Deverall and Wood, 1961; Retig and Chet, 1974). Certain phenolic compounds and their oxidation products are effective inhibitors of fungal pectolytic enzymes.

The objective of the experiments in this section was to investigate the hypothesis that phenolic compounds are involved in limiting lesion expansion.

6.2 Phenolic content in relation to plant age and infection

The change in the concentration of phenolic compounds in healthy and diseased stems of 4- and 8-week old tomato plants was determined at 3-day intervals for a period of 15 days. Plants were inoculated with mycelial disks under greenhouse conditions. Ethanolic extracts were prepared from healthy and diseased tissues and then assayed for total phenols and orthodihydroxy phenols.

As shown in Tables 27;28, initially the stems of 8-week tomato plants contained more phenols than those of 4-week old plants. After infection, the concentration of phenolic compounds increased in the stem tissue of plants of both ages. The rate of phenolic accumulation increased sharply and reached a maximum of 0.76 mg/g fresh weight on the sixth day in stems of 8-week old plants. In 4-week old plants, the amount of phenols increased rapidly from 3 to 6 days where it reached a 0.63 mg/g fresh weight, by which time plants were moribund. Total alchohol soluble phenols and o-dihydroxy phenols showed, with few exceptions, similar trends in amounts in diseased stems. The total phenolic concentration in stems of healthy plants did not change significantly over the period of the experiment, and the amount of o-dihydroxyphenols in healthy plants remained low throughout (below 20 µg/g fresh weight).

6.2.1 Histochemical localization of phenols

Fresh, freehand cross-sections of healthy and diseased tomato stems were treated with aqueous 10% (w/v) $FeCl_3$ and 0.05% (w/v) methyl red to detect phenols. Sections were placed on microscope slides and flooded with water or reagent.

Histochemical tests for phenols gave positive reactions in the vascular tissues of healthy and diseased plants. The cortical tissue of healthy stems gave no colour reaction but those of infected stems showed high colour intensity which indicated marked accumulation of phenols in the disrupted tissue.

TABLE (27)

Total phenol and o-dihydroxy phenol contents of healthy and *S. sclerotiorum* infected stems of 4-week plants

Days after inoculation	Total phenols		O-dihydroxy phenols	
	Healthy	Diseased	Healthy	Diseased
0	0.161 ± 0.11	-	0	-
3	0.163 ± 0.015	0.295 ± 0.013	0.004 ± 0.000	0.011 ± 0.001
6	0.177 ± 0.015	0.632 ± 0.181	0.013 ± 0.012	0.025 ± 0.010

Results are expressed in mg chlorogenic acid equiv./g fresh weight.

Figures are the means and the standard deviations.

TABLE (28)

Total phenol and o-dihydroxy phenol contents of healthy and *S. sclerotiorum* infected stems of 8-week plants

Days after inoculation	Total phenols		O-dihydroxy phenols	
	Healthy	Diseased	Healthy	Diseased
0	0.26 ± 0.00	-	0.009 ± 0.007	-
3	0.48 ± 0.15	0.64 ± 0.04	0	0.021 ± 0.006
6	0.32 ± 0.08	0.76 ± 0.31	0	0.026 ± 0.017
9	0.25 ± 0.06	0.57 ± 0.23	0	0.02 ± 0.02
12	0.23 ± 0.05	0.49 ± 0.75	0	0.02 ± 0.02
15	0.34 ± 0.08	0.62 ± 0.10	0.010 ± 0.004	0.021 ± 0.01

Results are expressed in mg chlorogenic acid equiv./g fresh weight.

Figures are the means and the standard deviations.

6.2.2 Distribution of phenolic compounds in relation to lesion area

Tomato plants (8-week old) were inoculated with mycelial disks in the glass-house. At 7 days after inoculation, the infected plants and comparable healthy plants were harvested. Ethanolic extracts were prepared from the tissue of the apparently healthy part of the stem, tissue above (outside) and below (within) the lesion margin and from the tap root of the stem-infected plants. Extracts were also prepared from stem and root tissues of the healthy plants. All extracts were assayed for total phenols.

Infection of tomato stem with S. sclerotiorum resulted in the highest concentration of phenolic compounds in the diseased tissue at a level 5-fold greater than that found in healthy tissue, (Table29). Following stem inoculation of whole plants, the concentration of phenolic compounds in the tap root differed only marginally from similar tissue of healthy plants. The concentration of phenolics in tap roots of diseased plants was about 2-fold greater than that in the apparently healthy stem tissue, but in healthy plants the concentration of phenols in the tap root was approximately 3-fold greater than that of the stem.

6.3 Effect of phenolic extracts on growth of S. sclerotiorum and production of PG

Phenolic extract was prepared from infected 8-week old tomato plants at 6 days after inoculation. The extract was concentrated at 35°C under reduced pressure and its total phenol content was measured. As the highest concentration of phenolic compounds in the infected tissue was 2 mg/g fresh weight,(Table29), 0.2 ml of 100% ethanol containing 12 mg phenolic compounds was added aseptically to 6 ml of autoclaved sodium polypectate liquid medium to give a final concentration of 2 mg/ml. To each of three sterile 50 mm diameter Petri plates 2 ml of medium was added and then inoculated with a mycelial disk cut with a

TABLE (29)

Effect of S. sclerotiorum infection on the phenolic contents of tomato stem and root tissue

Tissue type	Total phenols (mg equiv. of chlorogenic acid/g fresh weight)
Lesion tissue	2
Peripheral tissue	1.5
Apparently healthy tissue	0.5
Root tissue from stem-infected plants	1.08
*Healthy stem tissue	0.35
*Healthy root tissue	0.98

Figures are the means of two determinations

* Uninoculated plants

No. 4 cork borer from the active margin of a 4-day old colony of *S. sclerotiorum* grown on sodium polypectate tartrate medium. As a control, 6 ml of the same medium containing 0.2 ml of 100% ethanol but no phenolic compounds was used. All cultures were incubated in the dark at 20 ± 1°C. Mean radial growth of the fungus was calculated from the edge of the inoculum disk 24 and 48 hours after inoculation. After four days, 3 ml of 0.05M sodium citrate buffer (pH 4.6) was added to each plate. The liquid from each replicate was mixed, collected, filtered and then dialysed against water. PG activity was measured using the reducing group release and cup-plate assay methods.

The effect of the phenolic extract on the radial mycelial growth was also tested using small filter paper disks (1 cm diam.) containing various amounts of phenolic compounds. Samples of the plant extract containing the equivalents of 2, 1 or 0.5 mg chlorogenic acid were placed on each filter paper disk. All disks were allowed to dry thoroughly and then two were placed on the surface of MA medium in each of three 9 cm diam. glass Petri dishes. Each plate was inoculated with a mycelial disk (5 mm diam.) placed 1.5 cm from the margin of the filter paper. In the control cultures the plant extract was replaced by 100% ethanol. Assay plates were incubated under the same conditions as for the liquid cultures above and the growth of the fungus was observed daily.

Results obtained from the first experiment (Table 30), showed that the mycelial dry weight and the PG activity - expressed per ml of dialysed culture filtrate - were greater in the presence of phenolic extract. However, when the enzyme activity was expressed per mg dry weight of the fungus, the PG activity was less in the presence of phenolics. Radial growth rate of the fungus was little affected by the phenolic extract (Table 30), a conclusion reinforced by the results obtained with filter paper disks, where linear growth rate remained approximately constant regardless of treatment.

TABLE (30)

Effect of phenolic extracts from diseased stems on growth of S. sclerotiorum and activity of PG produced by the fungus

Treatment	Rate of radial growth (mm/hr)		Mycelium dry weight (mg)	PG activity Assay method			
				Reducing sugars		Cup-plate	
	First day	Second day		mUnits/ml dialysed culture filtrate	mUnits/mg dry weight	Units/ml dialysed culture filtrate	Units/mg dry weight
Medium with phenol	0.28 ± 0.05	0.68 ± 0.03	34.97 ± 6.50	427.07 ± 46.91	38.45 ± 0.66	6.76 ± 0.38	0.61 ± 0.06
Medium without phenol (Control)	0.20 ± 0.05	0.78 ± 0.18	10.83 ± 0.93	264.33 ± 61.17	90.56 ± 9.04	2.98 ± 0.85	1.02 ± 0.15

6.4 Inactivation of PG by phenolic extract

The effect of the phenolic extracts on PG activity was determined according to Hunter (1974). The pH of each extract was adjusted to 4.5 and then water was added until the volume was equivalent to 10 ml/g fresh weight. After dilution, the extract was divided into two equal portions. To one portion an equal volume of water was added and to the other portion an equal volume of polyclar AT - water suspension (1:10 w/v) was added. The polyclar AT was purified according to Loomis and Battaile (1966). The portion of the extract without polyclar AT was kept at 4°C and the other portion was kept with slight stirring for 30 minutes at 24°C and then centrifuged at 7800 g for 15 minutes. The fine particles of polyclar AT were removed by passing the supernatant liquid through 0·45 millipore filter. Water was then added until the final volume was equivalent to 20 ml/g fresh weight. To 2 ml of PG containing culture filtrate (activity $\underline{c}$ 250 CPU/ml), 1 ml of the phenol extracts (with or without polyclar AT treatment) was added. The mixtures were kept at 24°C for 12 hours after which PG activity was measured using the cup-plate method. The percentage inhibition of PG was calculated as follows:

$$\text{Percent inhibition of PG activity} = 100 - \left| 100 \times \frac{\text{Mean activity of PG with untreated extract}}{\text{Mean activity of PG mixed with polyclar AT-treated extract}} \right|$$

Results obtained are shown in Tables 31;32 . Phenolic extracts from diseased stems of plants of both ages were more inhibitory to PG than those from stems of healthy plants although in no case did inhibition exceed 30%. Extracts prepared from infected tissue at 6 days after inoculation caused the

TABLE (31)

Inhibition of PG activity by extracts from healthy and diseased stems of 4-week old plants

Days after inoculation	% inhibition of PG activity	
	Healthy plants	Diseased plants
0	0	-
3	0.3	3.4
6	0.7	5.7

TABLE (32)

Inhibition of PG activity by extracts from healthy and diseased stems of 8-week old plants

Days after inoculation	% inhibition of PG activity	
	Healthy plants	Diseased plants
0	8.3	-
3	10.0	7.9
6	4.5	29.9
9	1.8	16.3
12	4.5	23.6
15	4.1	18.0

greatest inhibition. A correlation was found between the phenol content and the percent inhibition of PG activity. The calculated correlation coefficients indicated significant positive relationships (Table 33).

TABLE (33)

Correlation coefficients showing degree of association between percent inhibition of PG and phenolic content of plant extract

Plant age		Correlation coefficient with PG inhibition
4-week old	Total phenol	+ 0.975**
	o-dihydric phenol	+ 0.920*
8-week old	Total phenol	+ 0.787**
	o-dihydric phenol	+ 0.795**

* Significant at 5% level of probability

** Significant at 1% level of probability

DISCUSSION

Following infection of tomato plants by S. sclerotiorum, the total phenol content of the infected stem tissues increased. The low level of total phenol in the 4-week old plants might be one reason for their greater susceptibility to infection than older plants. In tomato plants susceptible to Fusarium oxysporum, infection incited a small increase in the phenolic content compared with resistant plants, (Matta et al., 1967). A correlation between the rate of production of phenols after infection and the degree of resistance to disease has also been reported by other investigators (Cruickshank and Perrin, 1964; Farkas and Kiraly, 1962; Hunter, 1974). The observed decline in the phenolic content of infected stems of 8-week old plants at 9 and 12 days after inoculation was accompanied by the formation and development of adventitious roots above lesions. There are several possible explanations for this decline. Phenolic compounds might be involved directly in the initiation of the adventitious roots as has been suggested by Bouilleme and Bouillene (1955). Bachelard and Stowe, (1963) found a positive correlation between the ability of Acer rubrum cuttings to root and the amount of anthocyanin present in the leaves. It is also possible that some of the phenolic compounds might be involved in the protection of IAA against IAA oxidizing systems (Matta, 1968). Zenk and Muller (1963) reported that the structural requirement for protection of IAA against IAA oxidizing systems is an ortho relationship of the hydroxyl groups. Degradation of phenolic compounds by S. sclerotiorum also seems possible since little fungitoxicity was detected in vitro.

Histochemical tests for phenols revealed high concentrations in the cortical tissue of the infected stems. This accumulation in the disrupted tissue may be due to liberation of these compounds by hydrolysis of conjugated precursors during pathogensis (Waggoner and Dimond, 1956; Davis and Dimond, 1954). Davis et al., (1953) hypothesized that a glycosidase released from the disorganized cells hydrolyzed the glucosides in infected areas thus enriching

them with phenolics. Phenolic compounds may be synthesized in the leaves and then translocated to the infected tissues following infection by the fungus. Although chlorogenic acid is found in easily detectable amounts in tomato leaves, Waggoner and Dimond (1956) , could not find it in the stems of three tomato varieties. On the other hand, phenolics in tomato stems were reported by Bragt et al., (1965). Beckman et al., (1972) found that the stored substance in the capitate hairs of tomato was probably a phenol which might have an ortho dihydroxyl structure. In the present work, only very small amount of o-dihydroxy phenols were detected in stem tissue of healthy 4- and 8-week old tomato plants.

Phenolic extracts from healthy and diseased stems of plants of both ages inhibited PG. Inactivation of the fungal pectolytic enzymes by certain phenolic compounds and their oxidation products has been reported on many occasions (Byrde et al., 1960; Williams, 1963; Kuc et al., 1967; Hunter, 1974). Deese and Stahman (1963) suggested that the increased polyphenol oxidase activity in resistant tomato plants after infection by Fusarium oxysporum resulted in a relatively higher concentration of quinones which inhibited the pectolytic enzymes.

In the present study, the positive correlation between the phenol content of the stems and the percent inhibition of PG activity suggested that enzyme inhibition might be causally related to the limitation of lesion expansion. Another point is that the amount of PG used in the inhibition tests was much higher than that detected in vivo (see Fig. 22). It can be argued that at more realistic physiological concentrations the degree of inhibition might be greater, but this presupposes that PG activity recovered in extracts reflects the amount active in vivo. Since an element of enzyme inhibition must already have occurred, it is effectively impossible to gain a reliable estimate of in vivo activity using such methods. Accordingly, an assay conducted with relatively high levels of PG will at least give a conservative estimate of inhibition under 'worst case' conditions.

A similar response to infection is probably exhibited by both 4- and 8-week old plants but the rate of synthesis or accumulation of phenols is too low in the younger plants to raise their concentration sufficiently to cause significant inhibition of PG activity found in infected tissues of 4-week old plants (see Table 27).

The results obtained from the experiment on the effect of phenolic extract on growth of S. sclerotiorum suggest that this fungus may have the ability to metabolize phenolic compounds. Such a property is a known attribute of many microorganisms operating under normal physiological conditions , (Fawcett and Spencer, 1968; Walker, 1975). The increase in the dry weight of the mycelium in the medium containing phenolic extract presumably resulted from the presence of sugars and other nutrients in the extract. The method used for extraction of phenolic compounds can also trap sugars, amino acids, etc.

The marked difference in PG activity between the medium containing phenols and the one without phenols when the enzyme activity was expressed per mg dry weight of the mycelium is probably only partly due to the direct inhibitory effect of the phenolics on PG activity. Hunter, (1978), found that catechin added to sodium polypectate medium reduced PG activity of the filtrates from cultures of two isolates of Rhizoctonia solani. It is also possible that PG synthesis by S. sclerotiorum was suppressed by the phenolic compounds, (Suresh, 1968) , and / or the sugars present in the plant extract. Glucose, for example, suppressed the production of PG and the other CWDE by S. sclerotiorum when it was used as the sole carbon source in the culture medium (see Results 3.2.6). A similar result has also been reported by Vidhyasekaran, (1974), for the production of pectolytic and cellulolytic enzymes by Helminthosporium tetramera.

GENERAL DISCUSSION

Infection of tomato by S. sclerotiorum results in maceration of infected tissues and development of a watery soft rot. The earliest symptom of the disease is the appearance of water-soaked lesions on the infected stems. In young plants in the greenhouse, infection causes a sudden wilting and collapse of the plants. The disease spreads rapidly through the young tissues, blocking the vessels and cutting off the water supply with the result that the plant wilts and soon dies. Older plants are more resistant to the disease. From fruit-set onwards infected plants exhibit yellowing of the leaves and epinasty of the petioles but they remain erect and exhibit little, if any, wilting. Expansion of lesions is checked by the formation of dark brown zones around the infected tissues. In contrast to these results, Chamberlain (1932) reported that infection of tomato plants by S. sclerotiorum, especially those grown out of doors, caused a sudden wilting of the plants when they were in the four or five truss stage - i.e. just as they were coming into bearing.

Penetration of tomato stem by S. sclerotiorum is mainly by mechanical rupture of the cuticle by means of simple or multicellular appressoria. This result is in agreement with those of other workers (Purdy, 1958; Prior and Owen, 1964; Lumsden and Dow, 1973; Abawi et al., 1975). However, entry of hyphae via stomata was also observed. Jones (1976) reported a similar result for penetration of potato leaf tissues by S. sclerotiorum. Having gained entrance to the stem the hyphae develop a mycelium which rapidly spreads both inter- and intracellularly.

Emergence of the fungus from host tissue is through breaks in the cuticle and also through the stomata on the upper surface of the leaves. Jones (1976) was unable to find hyphae emerging from stomata on the abaxial surface of potato leaves infected by S. sclerotiorum.

Ascospores released from apothecia produced under laboratory conditions failed to infect healthy tomato tissues. Several investigators have reported

the inability of ascospores to initiate infection of unwounded healthy tissues and they have demonstrated the importance of exogenous nutrients for successful infection, (Purdy and Bardin, 1953; Purdy, 1958; Natti, 1971). In the presence of glucose 1% (w/v) and sodium citrate 0.01% (w/v), water-soaked lesions were formed on detached leaves but no sign of penetration by the fungus was observed. Formation of such lesions might be due to the effect of enzymes or oxalic acid secreted by the fungus on the surface of the leaves.

Amorphous extracellular material accumulated on stomatal and other epidermal cells when leaves were inoculated with ascospores germinated in distilled water. A similar deposition of extracellular material was also reported by Lazarovits and Higgins (1976) in the response of a highly resistant (immune) tomato variety to infection with Cladosporium fulvum race1. According to these workers, the accumulations contained polyphenols.

The intercellular movement of the fungus within the infected tomato stem and the maceration of tissues during infection suggested the involvement of CWDE in pathogenesis. The action of these enzymes or particularly of the pectolytic enzymes should facilitate intercellular pathogen movement through the host tissue, i.e., in the area between cells rich in pectic materials, (Bateman, 1964).

When grown on pectin or sodium polypectate as the sole carbon source, S. sclerotiorum produced a wide range of pectolytic, hemicellulolytic and cellulolytic enzymes. Approximately the same enzymes were also recovered from infected tomato stems. The synthesis of enzymes was sensitive to catabolite repression by glucose and they had pH optima for activity in the range 3-5. The pH of the culture medium affected enzyme synthesis. At pH6 the production of all the enzymes was maximal when the fungus was grown on sodium polypectate as the sole carbon source. This pH value is close to that reported by Hancock (1966) for healthy tomato tissue. The difference in enzyme production found when S. sclerotiorum was grown on cell walls from 4- and 8-week plants and

the relatively higher susceptibility of the former walls to enzymic degradation compared with walls from 8- or 12-week plants may be explained by the difference in the cell wall composition between young and old tissues. In young tissues cell walls are composed primarily of polysaccharides and a structural protein rich in hydroxyproline (Lamport, 1970), but in older tissues, walls may also contain lignin (Bateman and Basham, 1976) which is a complex three dimensional polymer of phenlypropanoid (C-C) units (Walker, 1975). The polysaccharide constituents in cell walls are quite resistant to enzymic decomposition after the walls become lignified, since they are complexed with and masked by the lignin component (Dehority et al., 1962).

The increasing resistance with age of tomato stem cell walls to enzymic degradation may account for the greater resistance of older stem tissues to infection by S. sclerotiorum. A similar relationship has been observed in the case of Rhizoctonia infection of Phaseolus beans, and a causal connection was proposed, (Bateman, et al., 1969).

Studies in vitro and in vivo revealed PG to be the most active enzyme present. This, together with its ability to digest walls in vitro suggest that it is the most important CWDE in pathogenesis. Synthesis of PG was induced by various carbon sources with pectin being the best inducer. The peak of PG activity found 5 - 6 days after inoculation when S. sclerotiorum was grown on cell walls as the carbon source may explain the collapse of the infected 4-week old plants observed a week after inoculation. This peak of enzyme activity is comparable in timing with that reported by Lumsden (1976) for exo-PG produced by S. sclerotiorum in culture filtrate 3 - 4 days after inoculation.

PG was capable of affecting tissue maceration, one characteristic symptom of the disease incited by S. sclerotiorum. Maceration of plant tissue involves separation of cells along the line of the middle lamella. It is now known that the endopectolytic enzymes that spilt the α-1, 4 bonds between the galacturonosyl moieties of the pectic fraction can on their own account for

the process of tissue maceration (Dean and Wood, 1967; Hall and Wood, 1970; Bateman 1972; Cooper et al., 1978). Exo-acting enzymes have much less macerating potential (McClendon 1964; Hancock and Miller, 1965). The PG produced by S. sclerotiorum possessed both endo- and exo-properties. Although this enzyme is capable of rapid maceration of tomato stem tissue, it has the further advantage of releasing low molecular weight sugars suitable for fungal nutrition and induction of further enzyme synthesis. Enzymes having both properties which are capable of macerating plant tissues, have also been reported by Cooper et al., (1978) for Verticillium albo-atrum. The synergistic relationship found between PG and oxalic acid with respect to tissue maceration may contribute to the importance of PG in pathogenesis by S. sclerotiorum since this fungus was found to produce oxalic acid both in culture and infected tissues.

Cell death and cell leakage were also associated with PG activity. Several hypotheses have been proposed to explain cell injury by pectolytic enzymes. One hypothesis suggests that some specific feature of the pectic enzyme molecule apart from its catalytic activity, is responsible for the toxic interaction. None of the specific characteristics of pectolytic enzymes such as isoelectric points, pH optima and type of activity was found necessary for cellular injury (Basham and Bateman, 1975). Another hypothesis suggests that pectic enzymes might cause cellular injury by direct interaction with a substrate in or on the plasmalemma (Mount et al., 1970). In favour of this is the known association of polysaccharides with the plasmalemma (Roland, 1969; Roland and Vian, 1971). It has also been suggested that membrane damage during wall degradation is caused by failure of the enzyme-damaged wall to provide sufficient mechanical support to the plasmalemma (Hall and Wood, 1973).

The increase in the electrolyte loss observed when disks of tomato stem tissue were incubated with enzyme preparations containing PG indicates that the semi permeable nature of the host cell plasma membrane was impaired by PG action. According to Hall and Wood, (1970), a marked increase in permeability

to electrolytes was closely related to the activity of PG. Thatcher (1939) reported increases in permeability exhibited by cells adjacent to Sclerotinia lesions in excised celery petioles. In contrast, Hancock (1972) reported a decrease in permeability of sunflower hypocotyl cells adjacent to S. sclerotiorum lesions to water, urea and methylurea and a reduction in the electrolyte loss. A decrease in permeability to urea was also noted in tomato stems above Sclerotinia lesions. Hancock's results are at variance with those reported here and also with currently accepted theories as to the mechanisms of soft rot development.

In contrast to PG, other CWDE exhibited lower activity both in vitro and in vivo, suggesting that they are less important in the disease. However, Ar and/or AF could degrade isolated tomato stem cell walls as demonstrated by the release of arabinose when walls were treated with enzyme preparation containing high Ar and AF activities.

The specific role and significance of Ar or AF in plant pathogenesis by S. sclerotiorum and by other plant pathogens is obscure, although it is becoming increasingly evident that many plant pathogens are capable of producing enzymes capable of hydrolyzing the araban in plant cell walls (Akinrefon, 1968; Byrde and Fielding, 1965 and Fuchs, 1965). The araban main chain is believed to consist of α-1, 5 linked L-arabinofuranose residues. Arabinose side branches linked by 1, 3 bonds theoretically account for one-third of the total linkages within the molecule (Hirst and Jones, 1947). Arabans may serve as a bridge between the rhamnogalacturonan and the hemicellulosic wall components (Talmadge et al., 1973). Lamport (1967) has reported the occurrence in plant cell walls of an arabinose oligosaccharide glycosidicly linked with hydroxyproline through its hydroxyl group. This type of linkage may be important in cell wall structure in terms of carbohydrate-protein linkages. So, hydrolysis of arabans or of oligosaccarides containing arabinose in the carbohydrate-protein complexes could be of pathological significance. Knee (1975) detected in apple fruit the presence of wall glycoproteins with typical hydroxyproline-arabinoside

linkages that were susceptible to very slow attack by AF. Howell (1975), reported that virulence of isolates to S. fructigena was significantly correlated with AF. This correlation is surprising because AF of S. fructigena does not have readily detectable physiological effects, for it causes neither tissue maceration (Byrde and Fielding, 1968) nor cell leakage (Byrde et al., 1973). The most likely positive role for AF in pathogenesis is in fungal nutrition. Arabinose is readily assimilated by many fungi including the closely related Monilinia fructigena (Archer, 1973).

Inactivation of fungal enzymes by phenolic compounds produced by host plants in response to infection has been reported by several investigators and is shown to be related to resistance of plants to certain fungal pathogens, (Byrde, 1963; Patil and Dimond, 1967; Hunter, 1974). Byrde (1956) and Cole (1956) found that varietal resistance to brown rot in apples could be explained by inhibition of the pectolytic enzymes by tannins produced after infection or injury. In the present work, the relationship between phenol concentration and plant age has been studied in relation to the activity of S. sclerotiorum PG. Following infection of 4- and 8-week old tomato plants, the phenol content of the infected tissues increased. The increase was more marked in 8-week plants which were also more resistant to S. sclerotiorum. Similar results have been reported by Hunter (1974) for resistance of cotton seedlings to soreshin disease incited by Rhizoctonia solani.

The inhibitory effect of extracts from tomato stems on PG activity was positively correlated with their phenol content. The greatest inhibition of enzyme activity caused by the highest concentrations of phenolic compounds at 6 days after inoculation may contribute to the limitation of lesion expansion observed on stems of the 8-week old plants. The large difference between 4- and 8-week old plants in the levels of PG activity recovered probably results from enzyme inhibition because no such large difference was found in PG production when S. sclerotiorum was grown on cell walls from plants of the two

ages. Similarly, the drop of PG activity to undectable levels in 8-week plants at 12 and 15 days after inoculation might be due to the effect of phenolic compounds since enzyme activity could be recovered when polyclar AT was used in the extraction medium. Polyclar AT or insoluble polyvinypyrrolidone (PVP) was reported by Loomis and Battaile (1966), to be the most effective agent in removing phenols from H-bonded complexes with proteins. However, not all phenolic compounds absorb or form strong H-bonded complexes with polyclar AT (Gray, 1978) and not all the reactions of phenols and quinones with proteins are reversible (Loomis and Battaile, 1966).

The results obtained from this investigation indicate that the increase in resistance with age of tomato plants are due, at least in part, to the partial inactivation of the fungal PG by the increased concentrations of phenols and to the increase in resistance of tomato stem cell walls to degradation by the CWDE produced by the fungus during infection. Other resistance mechanisms such as the absorption of PG by cell walls or its inactivation by protein inhibitors appear not to be operating in tomato plants in the case of Sclerotinia disease.

APPENDIX I

Rglucose (Rg) values of various sugars in the systems used in t.l.c.

Sugar	System				
	A	B	C	D	E
Arabinose	1.28	1.03	1.20	1.12	1.09
Galactose	0.91	0.89	0.91	0.96	0.9
Galacturonic acid	0.77	0.83	0.86	0.89	0.26
Glucose	1	1	1	1	1
Xylose	1.42	-	-	1.17	-

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ACKNOWLEDGEMENTS

I sincerely thank my supervisor Dr. S. A. Archer for constant guidance, kind interest and constructive criticisms throughout this work and in the preparation of this manuscript. I wish to thank Professor R. K. S. Wood in whose department this study was undertaken.

I would also like to thank Drs. K. Price and R. Coulter for supplying cultures of the fungus.

I am grateful to Mr. E. E. Green, Mr. R. Mepsted, Mr. J. Smith and Mrs. P. Tyler of Imperial College at Silwood Park for technical assistance and Mrs. Julie Douglas for typing this thesis.

My grateful thanks are also extended to Mr. Abd El Majeed, M.A., and Mr. Adam, H.F., for their help in many different ways.

Finally, my sincere thanks go to my family for their help and encouragement.

The study was supported by grants awarded by Sudan Government and this is also gratefully acknowledged.

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