Studies on the Host-Parasite Relationships of Phytophthora cactorum (Leb. and Cohn) Schroet. and Apple Trees

A thesis presented in partial fulfilment of the requirements for the degree of Doctorate of Philosophy in Plant Pathology at Massey University .

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ABSTRACT

The present work was undertaken to gain a greater understanding of the nature and location of resistance mechanisms present in apple cultivars to Phytophthora cactorum . Artifical inoculation methods of evaluating resistance to this pathogen were also studied .

The amount and type of sporangial germination in the presence of root exudates was not related to the susceptibility of the apple cultivar involved .

The greatest accumulation of zoospores occurred at root tip regions and wound sites (the sites of maximal root exudation) . The numbers of zoospores which encysted at root tip regions was not related to cultivar resistance and the large variation suggested that the quantity and/or quality of exudation varies considerably from root to root .

The germination of zoospore cysts was markedly enhanced by root exudates of all cultivars tested . Ninety-five percent of the cysts germinated on the root surface while only 80% germinated on cellophane membranes laid over roots . Very few germ tubes formed appressoria upon membranes . More than 95% of the germ tubes grew towards the root and over 98% of them formed appressoria on the root surface . Appressorial formation appeared to be induced by specific surface configurations such as breaks in the outer wall and junctions between cells, on the root surface .

The severity of P. cactorum infection of the seedling unsuberised apple roots (cv. 'COP' and 'GS') was dependent upon the initial inoculum loading, up to a saturation point i.e. extensive infection; a larger inoculum loading was required to reach this point with the more

resistant 'GS' cultivar . The resistant cv. 'M 793' tended to have a lower amount of infection than the susceptible 'MM 106' at corresponding levels of inoculum . However, in general, mechanisms of resistance did not appear to be operating external to apple tissue .

Electron microscopy studies showed that the process of infection was similar in susceptible (MM 106) and resistant (M 793) cultivars . Hyphae grew both intracellularly and intercellularly within root cortical tissue . Penetration through cells appeared to be both mechanical and enzymatic, although penetration by mechanical pressure alone may sometimes occur . Cellular deterioration was frequently obvious 2-4 cells in advance of the mycelium .

The histochemical studies showed that in vivo wall degrading enzymes produced by P. cactorum appeared to have a significant role in assisting fungal growth through host tissue but were relatively insignificant in its destruction .

The lack of apparent structural wound associated responses (e.g. papilla formation) formed in cells of either tested cultivar in response to P. cactorum infection indicates that the mechanisms of resistance of the more resistant cultivars are primarily physiological .

Investigations of internal resistance were hampered by the lack of a fully reliable method of assessing resistance . The most convenient system, inoculation of excised twigs, was further characterised revealing that the type of tissue tested (cortex, phloem-cambium or unlignified xylem) and the basal-distal location of the sampled piece of the shoot needs to be specified for valid comparisons with other workers and correlations with other criteria .

A technique of assessing levels of infection by oospore numbers in unsuberised roots is described and would possibly be a better method for determining a cultivar's resistance to the root rot and perhaps the crown rot forms of the disease .

Endogenous levels of nutritional compounds: total nitrogen, soluble sugars and starch and of phenolic compounds did not appear to determine

the rate of apple tissue colonisation by *P. cactorum* . Phloridzin, the major phenolic of apple tissue, was toxic to *P. cactorum in vitro* yet mean pathogen growth rates of over 10 mm per day were measured within host tissue indicating that the hyphae are not physically encountering inhibitory amounts of phloridzin *in vivo* .

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GENERAL INTRODUCTION

Phytophthora cactorum is primarily known as the cause of crown and collar rot, the most important soil-borne disease of apple trees in the world . It was the second described species (1886) of the then newly established *Phytophthora* genus (de Bary, 1876) . Other *Phytophthora* species have since been described, to the total of 44 species currently known (Zentmyer, 1983) .

Phytophthora species are the causal agents of a wide range of diseases on a large number of plants, including major fruit crops, forest trees, subtropical and tropical fruit trees, berries, nut trees, and many ornamental plants .

The host specialization of the members within this genus varies tremendously from just a single host for species such as *P. castaneae* and *P. vignae* to over 900 different species for *P. cinnamomi* (Ribeiro, 1978) .

Morphological Characteristics

The mycelium is coenocytic but frequently becomes septate with age . Hyphae are hyaline in colour and typically branch at right angles with a constriction commonly present at the base .

Sporangia are formed at the end of hyphal-like sporangiophores . Sporangia are usually ovoid-ellipisoid to pyriform in shape and range in size from those of *P. botryosa* (14-16 µm wide x 26-31 µm long) to the large sporangia of *P. macrospora* (50-65 µm wide x 85-112 µm long) (Riberio, 1978) . They either germinate directly by one to several

germ tubes or indirectly by zoospore formation .

Zoospores are reniform in shape with two flagellae (one tinsel and one whiplash) attached at a common point in the centre of a shallow, longitudinal grove . After a motile period, zoospores encyst and germinate by germ tube or secondary zoospore formation .

Chlamydospores are formed by some Phytophthora species . They are produced either terminally or inter-calary, are spherical to ovoid in shape, hyaline to brown in colour, and may be thin or thick walled . They are non-papillate, and are distinguished from hyphal swellings by the presence of a septum and from oospores by the lack of a separate (oogonial) wall . Germination is by germ tubes which either continue growth or terminate in a sporangium .

Oospores are rarely formed by the heterothallic Phytophthora species, but are commonly produced by the homothallic ones . Interaction between an antheridium and oogonium results in the formation of an oospore . The single spherical, thick walled oospore is enclosed in a spherical to pyriform oogonium . Germination is as described for chlamydospores .

Survival

The genus includes saprophytes (P. bahamensis), necrotrophs (P. cactorum), and biotrophs (P. infestans) . The comparatively saprophytic members live on organic particles and on litter, which acts as a reservior of inoculum enabling their perpetual existance (Weste, 1983). The biotrophic parasites often achieve an ecological balance with their hosts, seldom killing them . Overwintering of these pathogens usually occurs within infected plant material . The necrotrophs survive for long periods in the absence of host plants as dormant resting propagules : oospores or thick walled chamydospores . Survival of these spore structures is frequently measured in terms of years . The mycelium, sporangia, zoospores, and zoospore cysts remain viable for a considerably shorter duration (from a few days to a few months) . The wide host range of necrotrophs further enhances their continuance (Cother and Griffin, 1973) .

Spread

Many Phytophthora species have a world-wide distribution . It appears that these fungi have been unnoticed passengers of the global movement of plant material and accompaning soil that has occurred over the more recent centuries (Gregory, 1983; Julis et. al., 1978; Zentmyer et. al., 1952; Zentmyer, 1983) . Local expansion from the new loci would have occurred by further dispersal of contaminated plants and soil and by wind and water (McIntosh, 1964; McIntosh, 1966; McIntosh and MacSwan, 1966) .

Control

Cultural practices such as good soil drainage, disease-free planting material and the use of resistant varieties and/or rootstocks are the main-stay of control programmes .

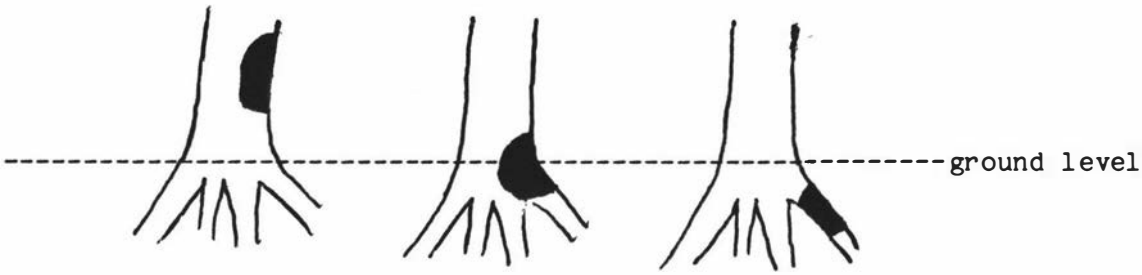
Fungicides have usually given only moderate control . Their present role, however, is likely to increase with the greater use of the effective recently introduced systemics : ethyl phosphonates and acylalanines .

Phytophthora cactorum (Leb. and Cohn) Schroet.

P. cactorum has a very wide host range, causing necrotic diseases on members of over 150 genera in 54 families (Waterhouse and Waterston, 1966) .

On apple trees, P. cactorum causes a rot of the trunk or roots near ground level . The location of the rot determines the disease name . If the canker occurs on the stem at or above ground level it is called a collar rot . Invasion of the root crown below ground level is known as crown rot and infection of the lateral or fibrous root system is called root rot (Figure 1) .

Figure 1. The Different Sites of P. cactorum Infection of Apple Trees



collar rot crown rot root rot

(from Robertson and Dance, 1971)

The canker can extend in any direction . Frequently, it progesses upward to the stock-scion union and downward along the main roots . It also widens to encircle the trunk . Enlargement of the lesion ceases during dry weather if it drys out, and in autumn with the onset of cooler temperatures . Pathogenic invasion is renewed at various places on the margins of most lesions the following spring .

The canker is brown in colour and is usually sharply delimited from the greenish-white healthy tissue . The bark tissue is killed to the cambium and unlignified xylem . This ring barking cuts the nutrient supply to the roots and eventually curtails their functioning . Saprophytic microorganisms quickly invade infected tissue and are probably responsible for the sour odour commonly associated with this disease .

Foliage symptoms appear late in the season or during the next . Terminal growth is poor and both leaves and fruit are smaller . The leaves become chlorotic, often with a red colouration of the veins and margins and fall prematurely . The fruit also becomes highly

coloured . Trees only partly girdled show these symptoms on the branches directly above the lesion . By the time these symptoms become obvious, much of the tree's circumference is diseased so that the tree either dies or has feeble growth for the next few years .

The pathogen does not usually cause spectacular epidemics but produces a steady attrition of trees . Over the expected life of a commercial orchard this can amount to a serious economic problem .

A number of size controlling rootstocks (e.g. 'MM 106') were released in the 1950's . Initially they were considered to possess good resistance to this disease but since that time some, especially 'MM 106', have been found to be very susceptible *. The 'MM 106' rootstock is widely used in New Zealand .

P. cactorum is considered to be of increasing importance, especially in the U.S.A. and Canada . For this reason, many of the current apple breeding programmes are seeking to incorporate Phytophthora field resistance in their final selections (McIntosh, 1975) . These programmes screen for such resistance by inoculating large numbers of plants .

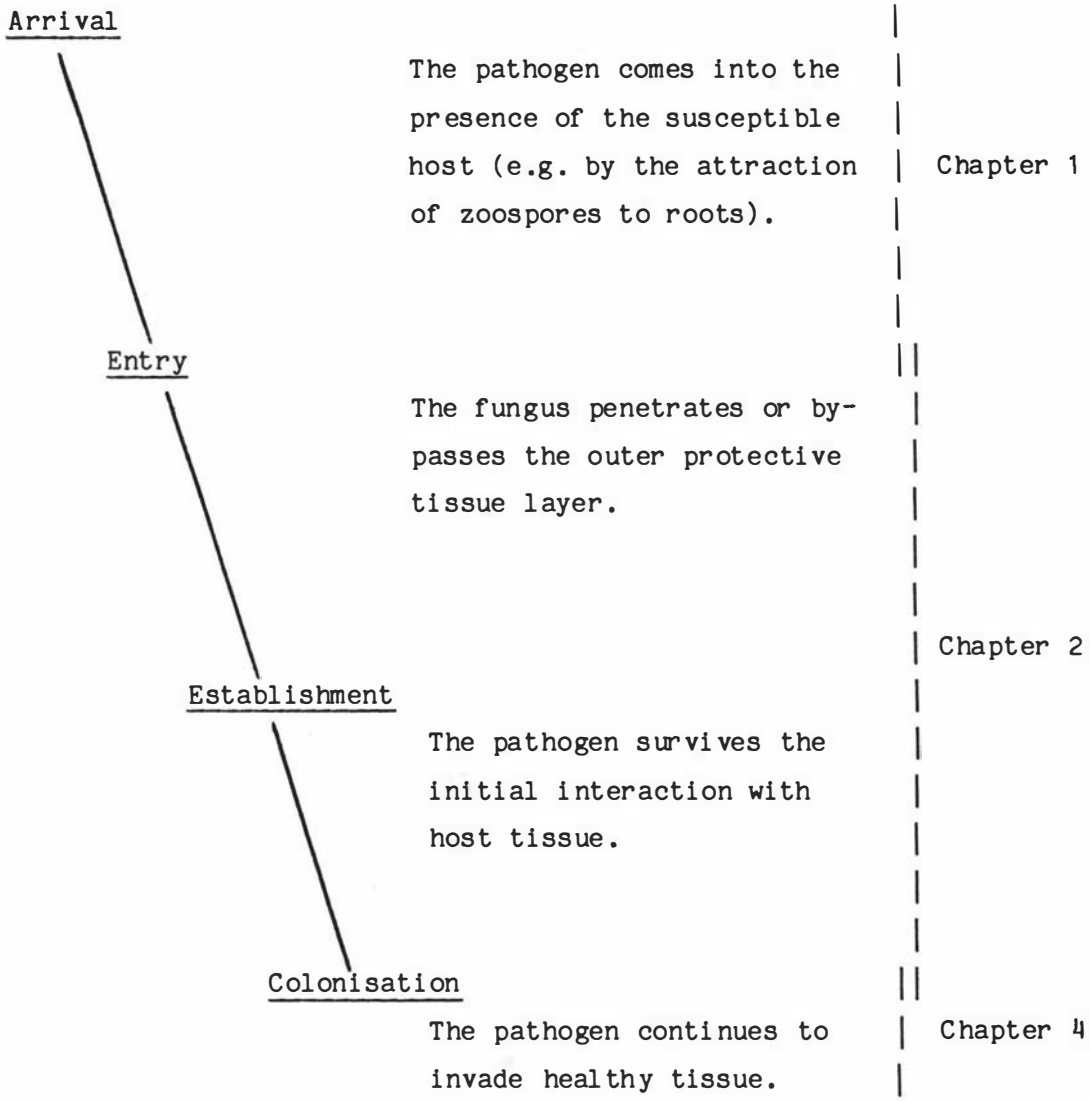
The present investigation had the objective of elucidating the mechanisms of resistance present in apple cultivars so that breeders may be able to make use of the information in their breeding programmes . A prerequisite to this work was a reliable method for evaluating resistance .

Thesis Outline

Studies on possible mechanisms of resistance were made at the following stages of the disease process .

* see Table 3-III, P.78

Figure 2. Sequences in the Infection Process



Chapter 3 deals with methods of evaluating resistance .

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** CHAPTER 1 **
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Studies of possible external mechanisms which may affect the host's susceptibility to *Phytophthora cactorum*.

1-1. INTRODUCTION

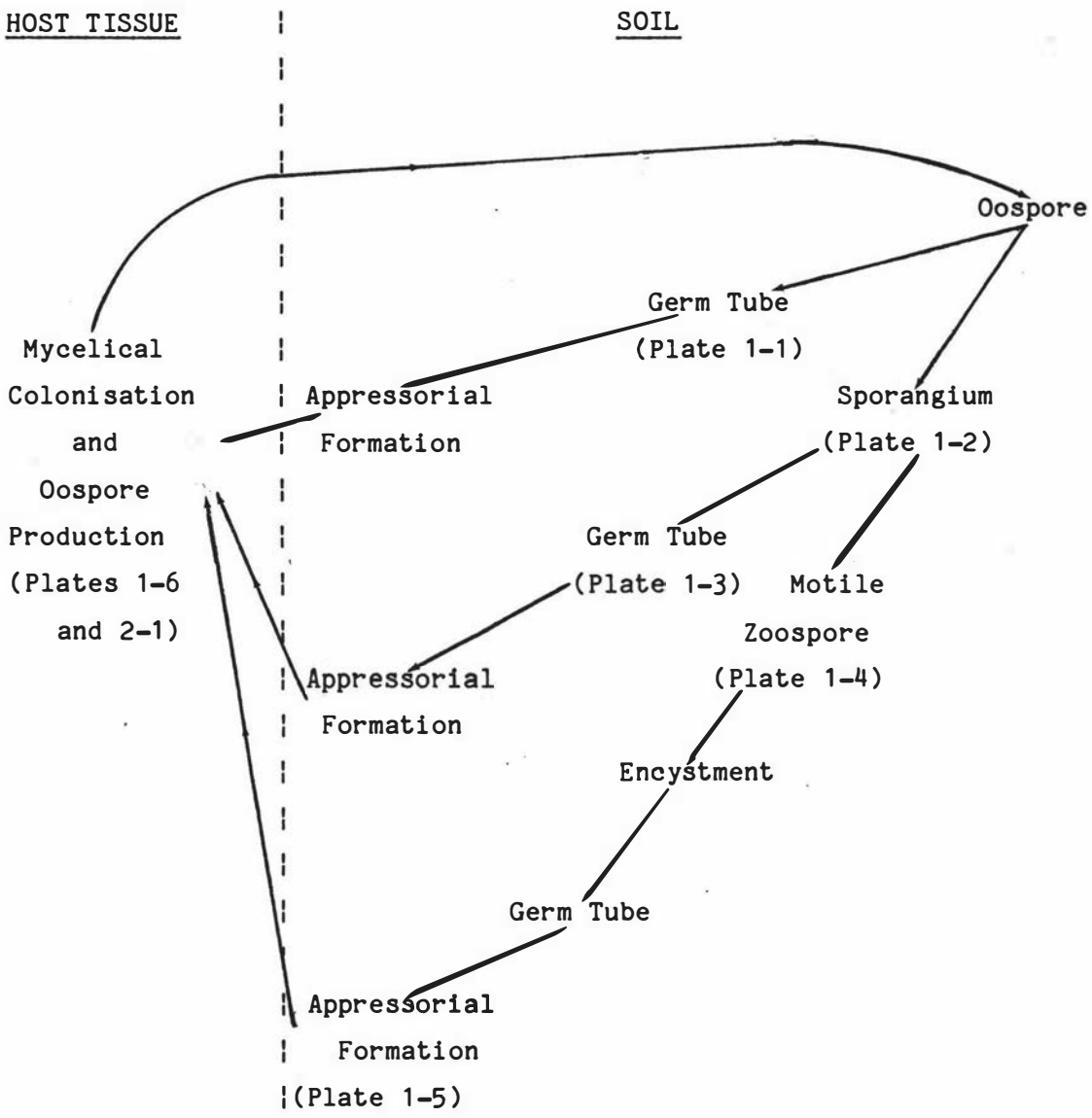
In nature, the predominant spore form produced by *Phytophthora* species varies . The mycelium of *P. infestans* do not usually produce spores within host tissue but under humid conditions forms external sporangiophores and sporangia (Roberts and Boothroyd, 1972) . *P. cinnamomi* largely forms chlamydospores within infected tissue (Kliejunas and Ko, 1973; Weste, 1983a) while species such as *P. cactorum* and *P. syringae* produce oospores (Brasier, 1969; Harris, 1979, Miller *et al.*, 1966; Tsao *et al.*, 1976) . Although, in practice, the life cycles of these fungi are similar , the importance of the details differ .

The life cycle of *P. cactorum* is shown in Figure 1-1 .

P. cactorum persists as oospores in decayed plant tissue or free in soil until influenced by roots or suitable plant tissue such as apple fruit wind falls (McIntosh, 1960; Sewell *et al.*, 1974) . The pathogen is relatively immobile compared to the host : root growth of an individual plant is measured in terms of kilometers while germ-tube growth of a fungal spore is measured in terms of micrometers and its zoospore movement through soil in centimeters (Huisman, 1982; Duniway,

1976) . Under conditions of flooding or irrigation, the pathogen may be more mobile as actively moving and passively moved zoospores (Kenerley et al., 1984; Klotz et al., 1959; McIntosh, 1966; Newhook et al, 1981) .

Figure 1-1. Life Cycle of P. cactorum



Chlamdospores are rarely formed by P. cactorum (Waterhouse and Waters on, 1966) .

The root system configuration of mature apple trees under orchard conditions consists of more or less horizontal main roots 25-50 cm below the soil surface, commonly spreading 1.5 to 3 times as far as the branches . Vertical sinker roots branch off from these to descend 2.75 m or more . Fine fibrous roots (>1 mm diameter) arise from this framework and are produced with a greater abundance in the more fertile top soil and in bands of richer soil at greater depths (Rogers and Head, 1969) . The root system extends into new areas of soil or reoccupies previously explored areas by fast growing roots . These white, 1-2 mm thick roots can elongate by as much as 4-5 cm/week (Rogers and Head, 1968) . The rooting density of woody plants may vary from 2-4 cm of root length per cubic centimeter of soil in the surface 10 cm (Bowen and Rovira, 1976) . Obviously, many of the susceptible host roots are in the upper portion of the soil where, in infested soil, a number of Phytophthora spores are present (Flowers and Hendrix, 1972; Hendrix and Campbell, 1983; Klotz et al., 1965; McIntosh, 1977; Sewell et al., 1974) .

The apple tree loses substantial amounts of organic material to the soil through root shedding and exudation . After a number of weeks (1-2 weeks in summer), the epidermis and cortex of the white fibrous roots often begin to degenerate, turn brown, and are shed completely . About half the dry weight of the root is shed . This tissue is subsequently broken down by the feeding of soil organisms such as small worms and arthropods (Rogers and Head, 1968) . One to ten percent and up to twenty-five percent of the weight of material translocated to the root from the shoot is lost through exudation (Bowen and Rovira, 1976; Drew and Lynch, 1980; Newman, 1978) .

Root exudates consist of simple sugars, amino acids, glucosides, organic acids, vitamins, enzymes, alkaloids, nucleotides, and inorganic ions (Pearson and Parkinson, 1961; Rovira, 1956; Schroth and Hildebrand, 1964) .

The effect of root exudates on soil microorganisms has been extensively covered in reviews by Bowen and Rovira (1976), Newman (1978), Rovira (1969), and Schroth and Hildebrand (1964) . Exudates are the main source of nutrients for the relatively large population of

microorganisms which are present in the immediate vicinity of the root (Bowen and Rovira, 1976; Curl, 1982; Newman, 1978) . They are known to stimulate the germination of dormant spores of many fungi (Schroth and Hildebrand, 1964) . A large number of studies (Allen, 1974; Allen and Newhook, 1973; Barash et al., 1965; Barber and Martin, 1976; Benjamin, 1979; Bimpong and Clerk, 1970; Brown and Brotzman, 1979; Cameron and Carlile, 1978; Chang-Ho and Hickman, 1970; Chee and Newhook, 1965; Chi and Sabo, 1978; Dix, 1964; Dukes and Apple, 1961; Duniway, 1976; Goode, 1956; Halsall, 1976, Halsall, 1978, Hickman, 1970; Hickman and Ho, 1966; Hinch and Clarke, 1980; Hinch and Weste, 1979; Ho, 1969; Ho and Hickman, 1967A and B; Ho and Zentmyer, 1977; Irwin, 1976; Khew and Zentmyer, 1973; Kim et al., 1974, Kliejunas and Ko, 1974; Kraft et al., 1967; Kuan and Erwin, 1980; Lipps and Bruehl, 1980; Malajczuk and McComb, 1977; Marks and Mitchell, 1971; Mehrotra, 1970; Milholland, 1975; Miller et al., 1966; Royle and Hickman, 1964A and B; Turner, 1963; Young et al., 1977; Zentmyer, 1960; Zentmyer, 1961; Zentmyer, 1966; Zentmyer, 1970) have shown germ tubes and zoospores of Pythiaceous fungi to be chemotrop ically and chemotaxically attracted towards the source of root exudates and to compounds found to be constituents of exudates, in particular, amino acids and simple sugars .

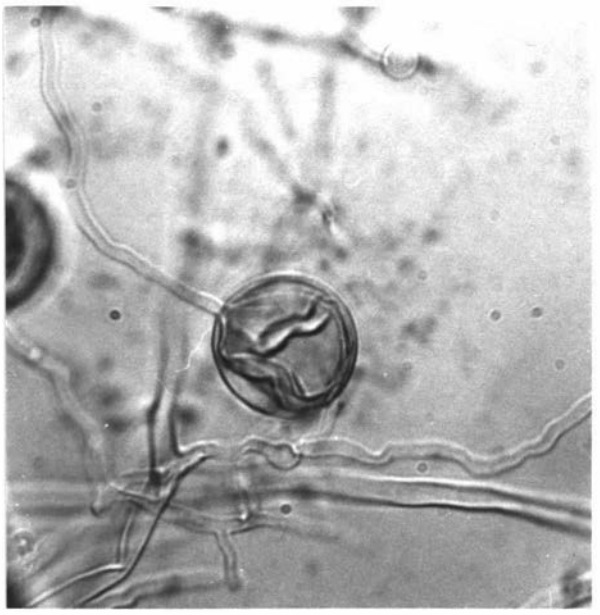
Obviously, the plant can have a marked influence via root exudates on the pathogen before physical contact and penetration occurs . Thus the aim of this portion of the work was to investigate those stages of P. cactorum's life cycle that occur external to host tissue, in order to determine whether resistant apple varieties are able to reduce pathogen arrival and penetration .

Plate 1-1

Oospore germination by germ tube formation. x700.

Plate 1-2

Oospore germination by a germ tube terminating in a sporangium. x450.



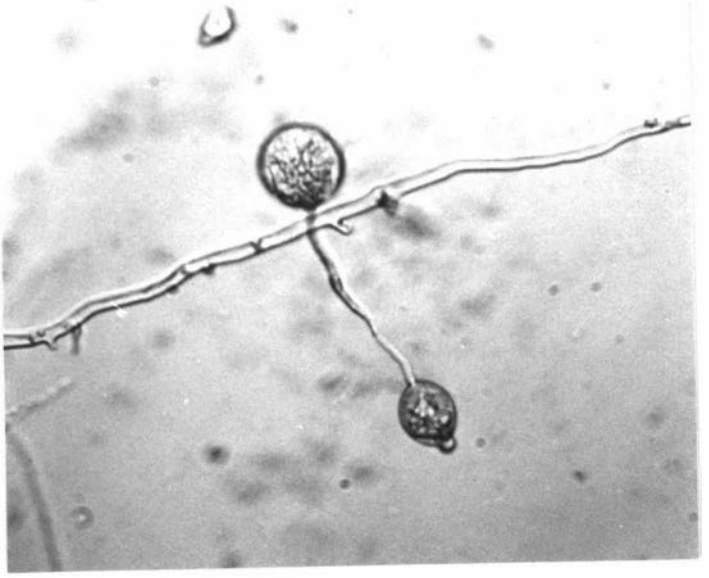


Plate 1-3

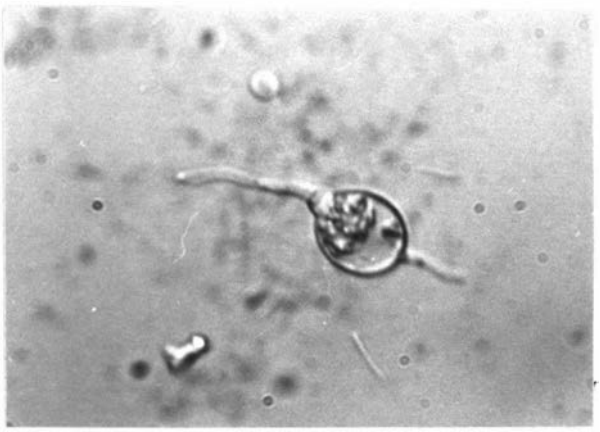
Direct sporangial germination. x700.

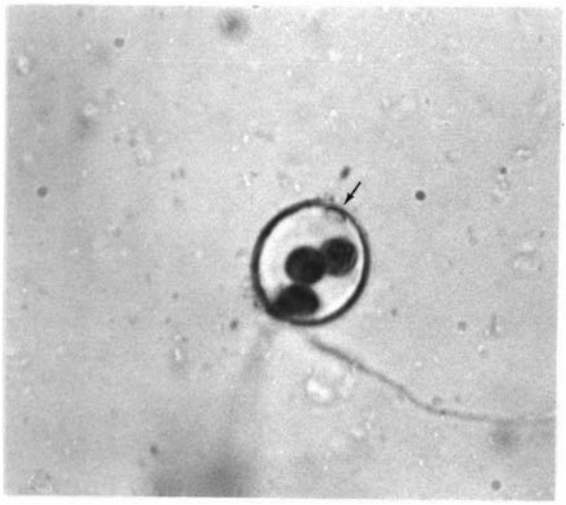
Plate 1-4

Indirect sporangial germination. Three zoospores have failed to escape through the germination pore (arrow) and have encysted within. x560.

Plate 1-5

Germinated zoospore cyst on a cv. 'M 793' root. Note the appressorium and penetration peg (arrow). x1100.





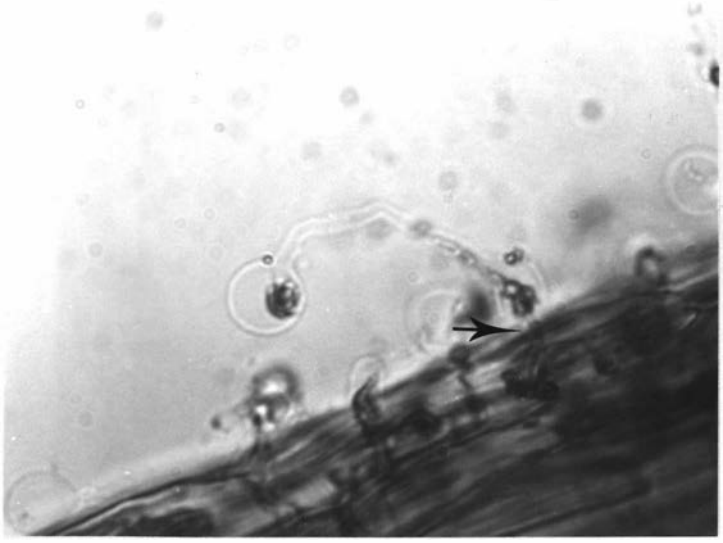
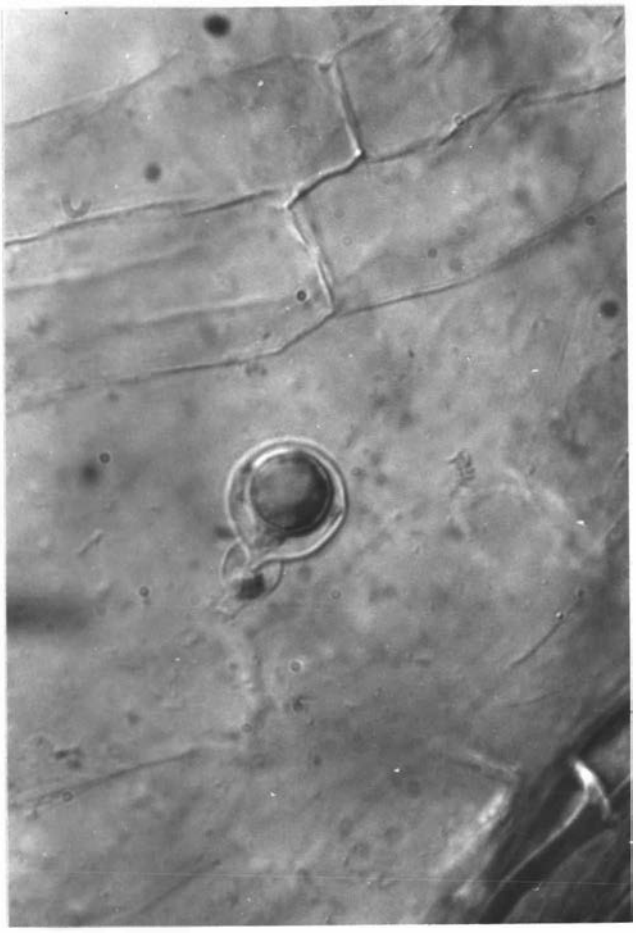


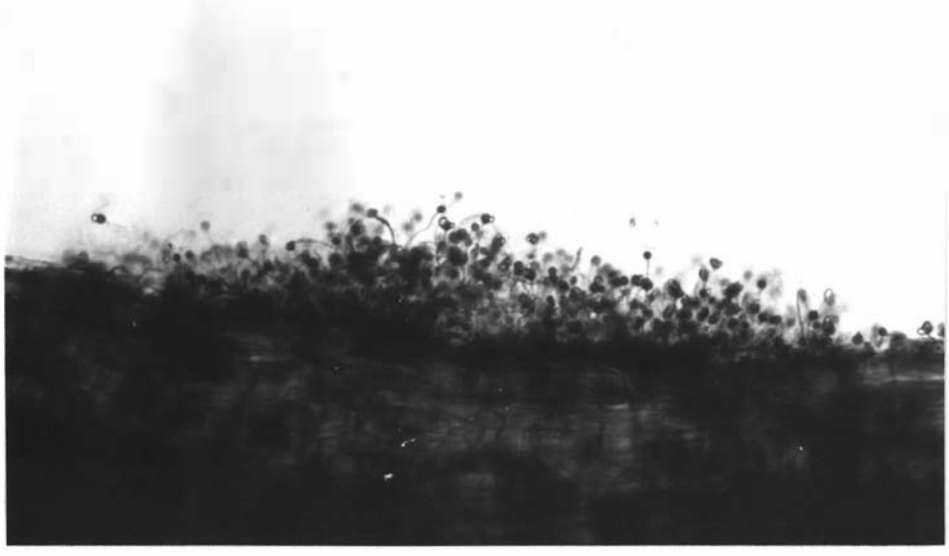
Plate 1-6

Ungerminated oospore with amphigynous antheridium within cleared cv. 'Cox's Orange Pippin' unsuberised root tissue. x700.

Plate 1-7

Zoospore attraction to a wound on a cv. 'M 793' root, 2 hours after inoculation. Note the orientiation of germ tubes towards the root. x280.





1-2. MATERIALS AND METHODS

Isolates 'MU1', 'MU2', (source: apple trees, Massey University), 'P149', and 'P10' (source: Plant Diseases Division, D.S.I.R., Auckland) were maintained on Difco Corn Meal Agar (DCMA, 17g/l) at 24°C in the dark .

1-2-1. Effect of Apple Root Exudates on the Germination of Sporangia

To produce the mycelial suspension, flasks containing 50 ml pea broth (160 g frozen peas homogenised and boiled 30 minutes in one litre of distilled water, strained through cheese cloth and autoclaved at 121°C for 15 minutes) were inoculated with 2 mm plugs of P. cactorum isolates 'MU1' and 'MU2' grown on DCMA . After 2 days incubation at 20°C with constant agitation, the mycelial balls were washed in deionised water and resuspended in soil water (100g Ashurst silt loam/litre deionised water, pH 5.5) or in deionised water (pH 5.5) and homogenised to 1-2 mm diameter pieces . The soil was collected from a grassed area which had never been under orchard .

Unsuberised apple roots were collected from at least five mature cv. 'MM 106' and five cv. 'M 793' trees and were washed to remove adhering soil particles . Similar root biomasses (one root of approximately 1 mm diameter and 2-3 cm length) were placed in small plastic containers (1 cm diameter) containing 1 ml of mycelial suspension . The mycelial fragments settled to the bottom of the plastic containers and sporangia developed on these fragments within 2 days .

The treatments were replicated three times and arranged as a 2x2x3 factorial with two isolates (MU1 and MU2) and two solutions (soil water and deionised water) and three root treatments (no roots, MM 106 and M 793) .

The plastic containers were placed into petri dishes and incubated at room temperature (15-21°C) for 7 days, before the material was fixed with a few drops of 0.1% trypan blue in lactophenol . The containers were left standing for several days at room temperature to allow most of the water to evapourate from them . The mycelial pieces on and within 2 mm of a root were then scraped together with a scalpel and the amount and type of germination of the sporangia which had formed on these mycelial pieces was recorded for one count of 50 sporangia from each of three replicate plastic containers .

Very few sporangia were observed to form from the mycelical pieces placed in deionised water. This difference was presumably due to the presence of soil bacteria such as Chromobacterium and Pseudomonas which stimulate sporangial production (Ayers, 1971; Marx and Haasis, 1965; Zentmyer and Erwin, 1970) . Assessment of sporangial germination in the deionised water treatments was not made because of their lack of sporangia .

1-2-2. Zoospore Attraction to Apple Roots

Zoospore Production

Cultures of P. cactorum were maintained on Dico Cornmeal Agar (DCMA, 17g/l) at 24°C . Flasks of pea broth were inoculated with agar plugs cut from fungal colonies and incubated for 2 days with constant agitation at 20°C . The resultant mycelial balls were washed with distilled water until most nutrients were removed and these were gently agitated in non-sterile soil water (100g Ashurst silt loam/litre distilled water) for a further 2 days at 20°C . A 15-60 minutes chilling period (5-8°C) followed by rewarming to room temperature resulted in an abundant zoospore release from the newly formed zoosporangia . Zoospore concentrations were measured with either a haemocytometer or 5µl microcaps (Drummond Scientific Company, U.S.A.) and adjusted to the required concentrations .

Experiment 1 : Open Pollinated Seedlings

Seed collections were made from 'Cox's Orange Pippin' (COP) and 'Granny Smith' (GS) apples grown in the Massey University Orchard . The seeds were soaked for 24 hours in 0.02% hydrogen peroxide and stratified in moist sand in polythene bags at 6°C in darkness until the radicle appeared through the seed coat (about 75 days) . The polyethene bags were left unsealed so that aeration could occur (Hansen and Hartmann, 1966) . The germinated seedlings were planted out into plastic pots containing sterilized vermiculite and watered with a nutrient solution or tap water .

After 8 weeks growth in the glasshouse, six seedlings of each cultivar (COP and GS) were gently removed and washed . The roots of the intact seedlings were placed in plastic petri dishes containing suspensions of 5000 'P149' zoospores/ml for 2 hours . The seedlings were fixed with 4% formaldehyde and the zoospore attraction was assessed by counting the number of cysts adhering to root tip regions of three roots of each seedling . The infected roots were cleared in hot 10% KOH at 70°C for 15 minutes, acidified, stained overnight in 0.1% trypan blue in lactophenol, and mounted in lactophenol .

Accurate counts of zoospore cysts were possible only with those on the edge of the root since they stood out clearly . The number of cysts along both root edges were recorded for the first 2.5 mm from the root tip . Wounds were not included in the assessment since considerable numbers of zoospores often encysted at these sites (Plate 1-7) .

Experiment 2 : Mature Trees

Young roots were collected from at least 12 trees each of cv. 'MM 106' (6 years old, scion = Oratia Beauty) and cv. 'M 793' (8 years old, scion = Cox's Orange Pippin) . The excised roots (20 per treatment) were washed, randomly assorted and each placed into zoospore suspensions in plastic petri dishes for 7 hours at room temperature .

The P. cactorum isolates 'MU2', 'P149', and 'P10' were used at concentrations of 100, 1000, and 10000 (5000 for 'P10') zoospores/ml . Ten roots were removed from each treatment for assessment (Section 1-2-6) . The remainder were fixed with 4% formaldehyde and cleared and stained as described in the previous experiment . Where there was a large aggregation of zoospores characteristic of a wound site, the aggregation was not included in the assessment whether the actual wound could be seen or not . The number of cysts along both root edges was recorded at 0.25 mm intervals from the root tip . Roots contaminated with more than a trace of other fungi (usually vesicular-arbuscular mycorrhizas) were recorded separately .

Although all isolates produced abundant numbers of zoospores, those of the 'P10' isolate encysted so soon after emergence that few reached the roots and hence this isolate was not included in the statistical analysis . The data were transformed (ln [x+1]) and statistically analysed as a 2x2x3x2 factorial with two isolates, two cultivars, three inoculum concentrations and plus or minus contamination .

1-2-3. Cyst Germination

Experiment 1 : Cellophane Membrane

A modification of the method of Mehrotra (1970) was used to more readily observe and measure the germination of cysts . Roots from cv. 'COP' and 'GS' seedlings were excised and placed into plastic petri dishes containing a shallow layer of distilled water . Five seedlings of each cultivar were used and five roots of one of the tested cultivars were placed in each petri dish (one root from each seedling) . A single layer of cellophane membrane (Jam and Preserve Covers, Kerr and Borthwich, Christchurch), cut to size, was placed on top of the roots of each treatment and 3 ml of P. cactorum isolate 'P149' zoospore suspension was poured on top . A control (no roots) was included with the treatments . After 2, 4, and 8 hours, the zoospore suspension was poured off and cyst germination was stopped by

the addition of 0.1% trypan blue in lactophenol . Sections were cut from those parts of the membranes lying over the roots and mounted on a slide and one count of 250 adhering cysts was made from each of two replicate petri dishes . Counts were made only at the tip region to minimise the effect of any compounds leaking from the excised end of the roots .

Experiment 2 : Root Surface

The infected plant material of Section 1-2-2, Experiment 2 was used . The percentage germination of clearly discernable cysts along the entire length of the uncontaminated 'MM 106' and 'M 793' roots of each inoculum concentration was recorded, giving three replicate counts for each of the tested isolates and cultivars . Generally, more than 50 cysts were counted per inoculum concentration .

1-2-4. Directional Growth of Germ Tubes

Experiment 1 : Cellophane Membrane

The experimental material used was as previously described in Section 1-2-3, Experiment 1 . The length of 25 germ tubes of cysts which had germinated over the root tip region was measured from each of two replicate petri dishes .

Experiment 2 : Root Surface

The material of Section 1-2-2, Experiment 1 was used . The orientiation of germ tubes in relation to the root was recorded for 300 germinated cysts per seedling for three seedlings of each cultivar ('COP' and 'GS') . Germ tubes growing parallel to the root were

classified with those that were growing away .

The experimental material of Section 1-2-2, Experiment 2 was also used . The percentage orientiation of the cyst germ tubes was recorded along the entire length of the uncontaminated 'MM 106' and 'M 793' roots of each inoculum concentration, giving three replicate counts for each of the tested isolates and cultivars . Generally, more than 50 germ tubes were counted per inoculum concentration .

1-2-5. Appressorial Formation

Experiment 1 : Cellophane Membranes

Section 1-2-3, Experiment 1 was also repeated with an extra (16 hour) treatment included . This experiment was not replicated . The number of appressoria formed by 500 germinated cysts per treatment was counted .

Experiment 2 : Root Surface

The experimental material used was as previously described in Section 1-2-2, Experiment 1 . The number of appressoria formed on 'COP' and 'GS' roots was recorded for 100 germinated cysts per seedling . Three seedlings were assessed per treatment .

Experiment 3 : Scanning Electron Microscopy (SEM)

At least six unsuberised roots (cv. 'MM 106') from field grown trees were excised, washed, and inoculated in a zoospore suspension (isolate 'P149') for 20 hours . The roots were fixed in 3% glutaraldehyde and 2% formaldehyde in 0.1M phosphate buffer pH 7.2 solution at 4°C for 2 hours, washed three times in 0.1M phosphate buffer pH 7.2, and post-fixed for 1 hour in 1% osmium tetroxide in 0.1M phosphate buffer

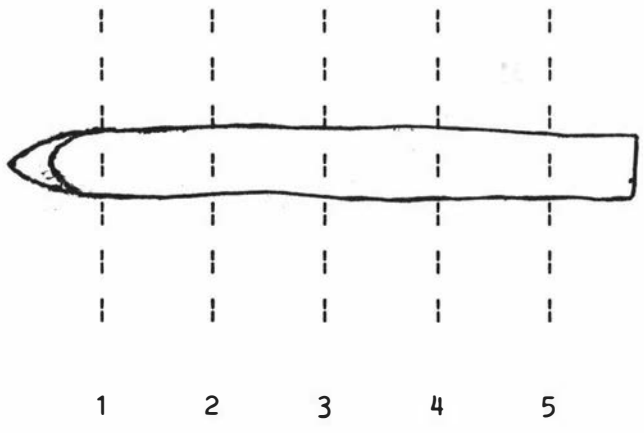
at 4°C . Following three buffer washes, the specimens were taken through a graded ethanol series (25%, 50%, 75%, 95%, 100%), treated in a Critical Point Drier (3 hours in liquid carbon dioxide), glued to a metal stud and coated with gold (100 - 200 Å thick) . Examination of the specimens was made with a Cwikscan 100 field emission scanning electron microsope .

1-2-6. Resistance of Roots of Open-Pollinated Apple Seedlings and Vegetatively Propagated Mature Trees

Experiment 1 : Seedling Roots

Eight week old glassshouse grown cv. 'COP' and 'GS' seedlings were positioned so that their roots were immersed in suspensions of 5, 50, 500, or 5000 'P149' zoospores/ml in plastic petri dishes for 2 hours . Four seedlings were used per treatment . The seedlings were placed into plastic containers lined with moist paper and incubated for 5 days at 20°C and then fixed in 4% formaldehyde, cleared and stained as previously described (Section 1-2-2) .

Figure 1-2. Assessment Positions along a Root



A grid-shaped eyepiece graticule was used to measure the number of oospores per 0.018 mm^2 at five equally spaced intervals along each root (Figure 1-2) . Ten roots were measured per seedling . Oospores were considerably more convenient to assess (Plate 1-8) than the amount of mycelium as P. cactorum mycelium was sparse in apple tissue and difficult to distinguish from that of other Phycomycete contaminants . Roots contaminated by other fungi were not included in the assessment . The data was transformed (square root) before analysis .

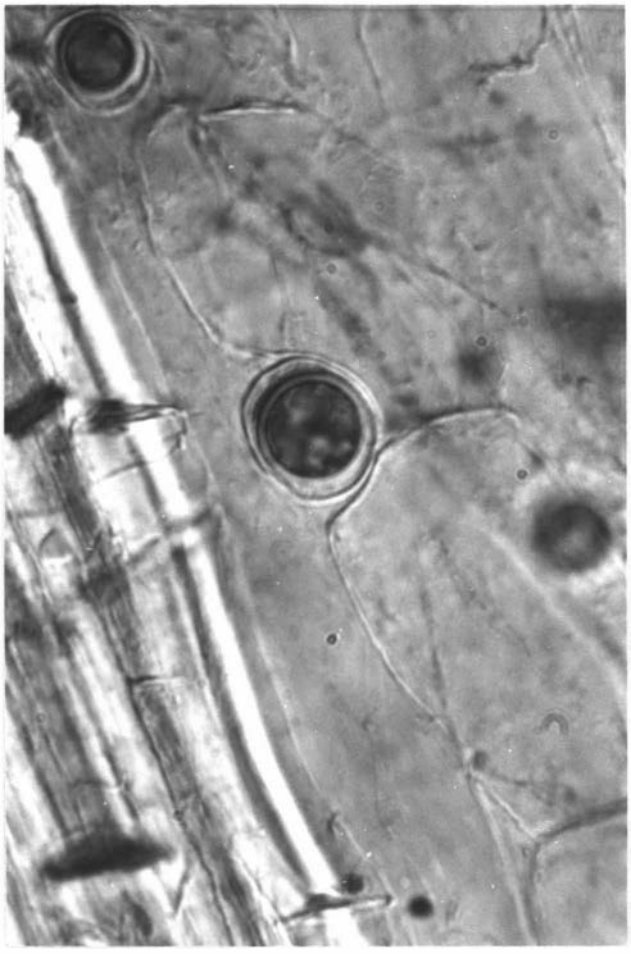
Experiment 2 : Roots from Mature Trees

Ten roots each of cv. 'MM 106' and 'M 793' which had been infected by isolates 'P149' and 'MU2' as described in Section 1-2-2, Experiment 2 were selected from each inoculum concentration . They were incubated, fixed, cleared, stained and assessed as described in Experiment 1 of this section . The data was transformed (square root) before analysis .

Plate 1-8

Oospores stained with trypan blue in cleared, cortical tissue of a cv. 'Cox's Orange Pippen' root. Oospores were not observed in the root stele (left-hand side). The area of the photograph is approximately equal to that of the individual area measurements (0.018 mm^2).

x700.



1-3. RESULTS

1-3-1. Effect of Apple Root Exudates on the Germination of Sporangia

Amount of Sporangial Germination

The mean sporangial germination of any tested isolate was 20% or less (Table 1-IA) .

Since the analysis of variance showed a significant isolate x treatment interaction (Table 1-IB), each isolate was examined separately . The sporangial germination of the 'MU1' isolate was similar in the absence and presence of root exudates, regardless of whether the exudates were from a susceptible# cultivar (MM 106) or a resistant one (M 793) (Table 1-IA) . The 'MU2' isolate had a significantly greater percentage sporangial germination in the absence of root exudates than in their presence (Table 1-IA) . The amount of germination was similar in the presence of roots of the 'MM 106' and the 'M 793' cultivars . The 'MU1' isolate had a significantly greater sporangial germination in the presence of apple roots than the 'MU2' isolate (Table 1-IA) .

Table 1-IA. Percentage P. cactorum Sporangial Germination in the Presence of Unsuberised Apple Roots

Isolates	Treatment			
	No Roots	MM 106	M 793	
MU1	19.3 ±2.9*	18.7 ±1.3	22.0 ±3.1	20.0
MU2	27.0 ±5.0	12.0 ±2.0	8.7 ±3.7	14.9

*Standard error

see Table 3-III, P. 78

Table 1-IB. Analysis of Variance of Table 1-IA

Source of Variance	Df	Mean square	F	
Blocks	2	48.03	3.68	NS
Isolates	1	124.10	9.52	*
Treatments	2	78.25	6.01	*
Isol x trt	2	163.04	12.51	**
Error	8#	13.03		

#Error Df=8 because of two missing values .
NS= not significant, *= P<0.05, **= P<0.01

Type of Sporangial Germination

Most of the sporangia germinated directly, forming germ tubes under these experimental conditions (Table 1-II) . In one block, however, sporangial germination was more commonly by zoospore formation . Overall, the type of sporangial germination was not significantly

Table 1-IIA. Percentage of Germinating Sporangia forming Germ Tubes in the Presence of Unsuberised Apple Roots

Isolates		Treatment			
		No Roots	MM 106	M 793	
	Block				
MU1	1,2	81.6 ±10.1*	100.0 ± 0.0	100.0 ± 0.0	
	3	30.0	30.0	69.2	
	$\bar{x}$	64.4 ±18.2	76.7 ±23.3	89.7 ±10.3	76.9
MU2	1,2	50.0	100.0	100.0 ± 0.0	
	3	72.7	40.0	100.0	
	$\bar{x}$	61.4 ±11.4	70.0 ±30.0	100.0 ± 0.0	80.4

*Standard error

Table 1-IIB. Analysis of Variance of Table 1-IIA

Source of Variance	Df	Mean square	F	
Blocks	2	0.3051	10.9	**
Isolates	1	0.0293	1.0	NS
Treatments	2	0.0907	3.2	NS
Isol x trt	2	0.0102	0.4	NS
Error	8#	0.0280		

#Error Df=8 because of two missing values .
NS= not significant, **= P<0.01

---

different between isolates or treatments (Table 1-II) . When the means of the first two blocks only are considered (Table 1-II), a greater amount of indirect sporangial germination occurred in the presence of roots . The large difference between blocks indicates that the type of germination is sensitive to some unknown factor . Obviously further work will be required to elucidate this .

1-3-2. Zoospore Attraction to Apple Roots

On the seedling roots, the region of greatest zoospore accumulation was at the root tips . Maximal attraction and encystment occurred at that part of the root encompassing the root cap, zone of elongation, and the beginning of the root hair zone . The number of cysts declined and then levelled off further from the root tip (Plate 1-9) . This pattern was also apparent on the unsuberized roots of mature apple trees when relatively large numbers of zoospores encysted on the root, otherwise a more random distribution occurred (Figure 1-3) .

Substantial zoospore accumulation was also found to occur at and just below the root-stem interface of the seedlings (Plate 1-10) .

Zoospores usually encysted over the entire root surface but on the stem surface only where the periderm was broken .

The amount of zoospore accumulation was greater on the susceptible (COP) roots but the difference was not significant (Table 1-III) .

Table 1-III. Mean Number of P.cactorum Zoospores Encysted per Root Tip Region

Unsuberised Apple Seedling Roots

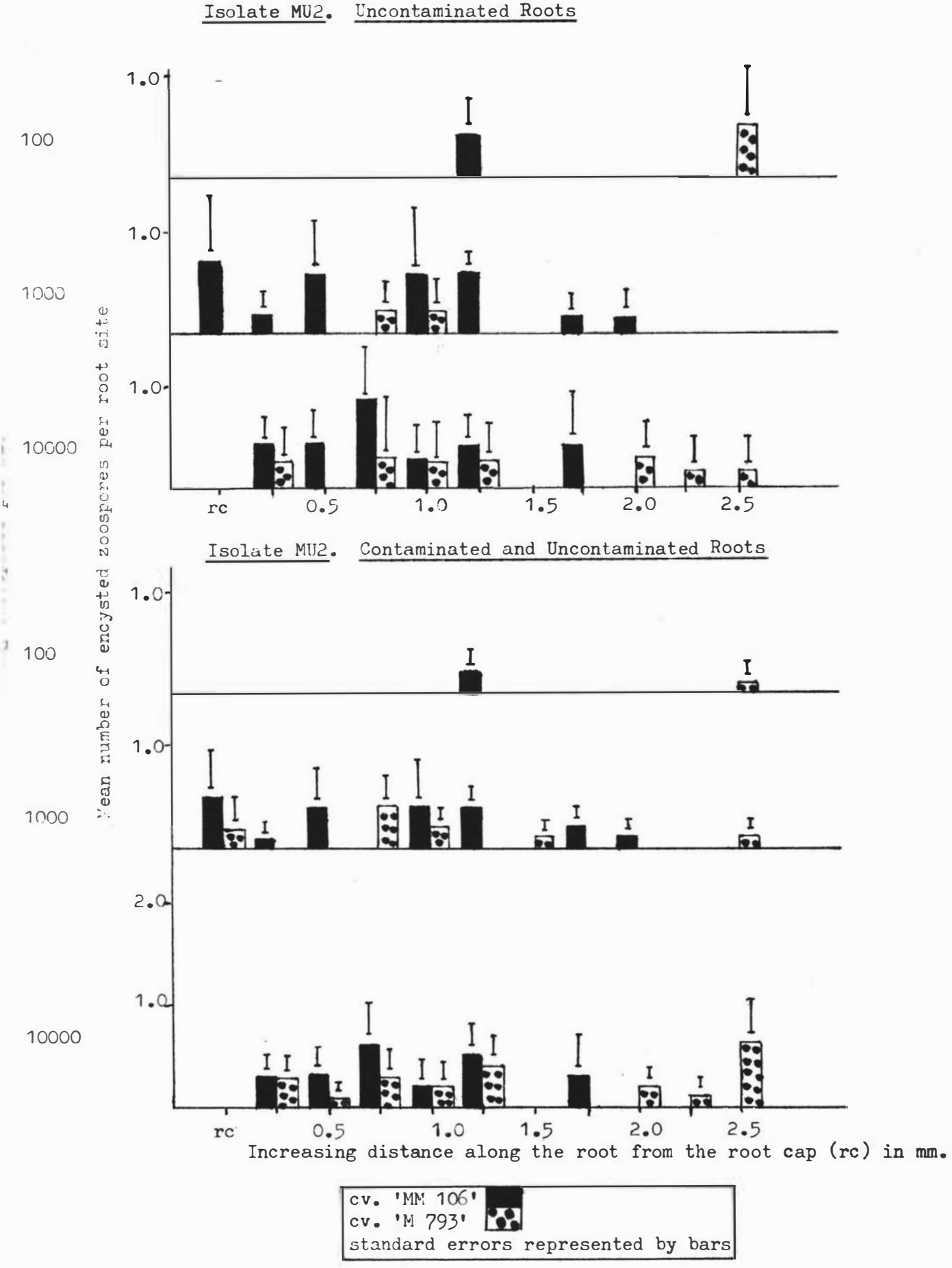
Isolate	Concentration (Zoospores/ml)	Cultivars	
		Cox's Orange Pippin	Granny Smith
P149	5000	272.5 a	182.5 a

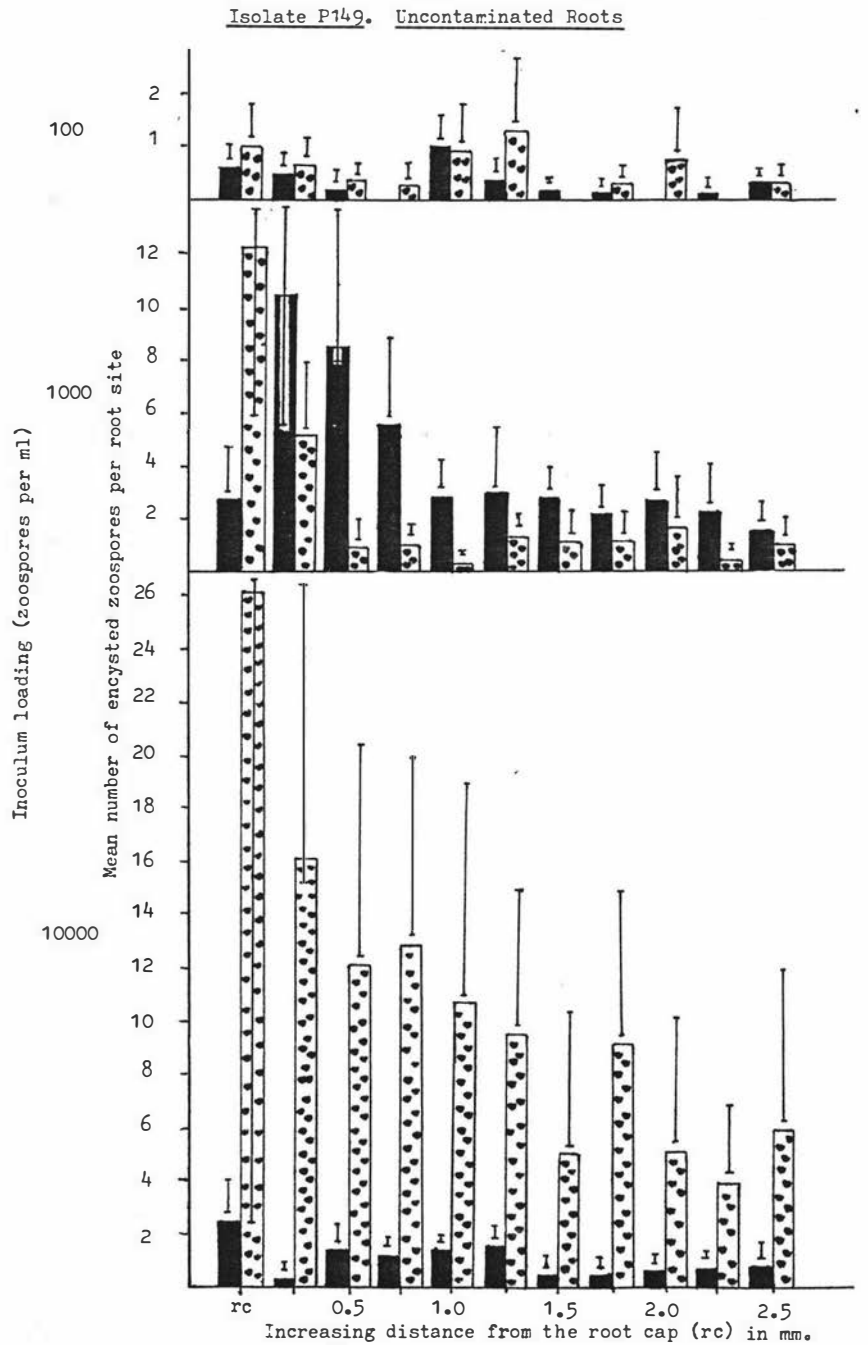
Mean values followed by same letter are not significantly different from each other at 5% level (Duncan's Mutiple Range Test) .

A similar amount of zoospore attraction occurred to roots of both resistant (M 793) and susceptible (MM 106) cultivars (Figure 1-4)*. Significantly more 'P149' zoospores were attracted to roots than were

*The 'P149' 10,000 zoospres/ml treatment had a low attraction to the cv. 'MM 106' roots. This is possibly due to a disturb nce of the zoospore suspension which could have caused them to rapidly encyst before many reached the root.

Figure 1-3. The Pattern of Zoospore Acculumation at the Root Tip Region of Unsuberised Roots of Mature Apple Trees.




Isolate P149. Uncontaminated Roots
Inoculum loading (zoospores per ml)
100
1000
10000
Mean number of encysted zoospores per root site
rc
0.5
1.0
1.5
2.0
2.5
Increasing distance from the root cap (rc) in mm.

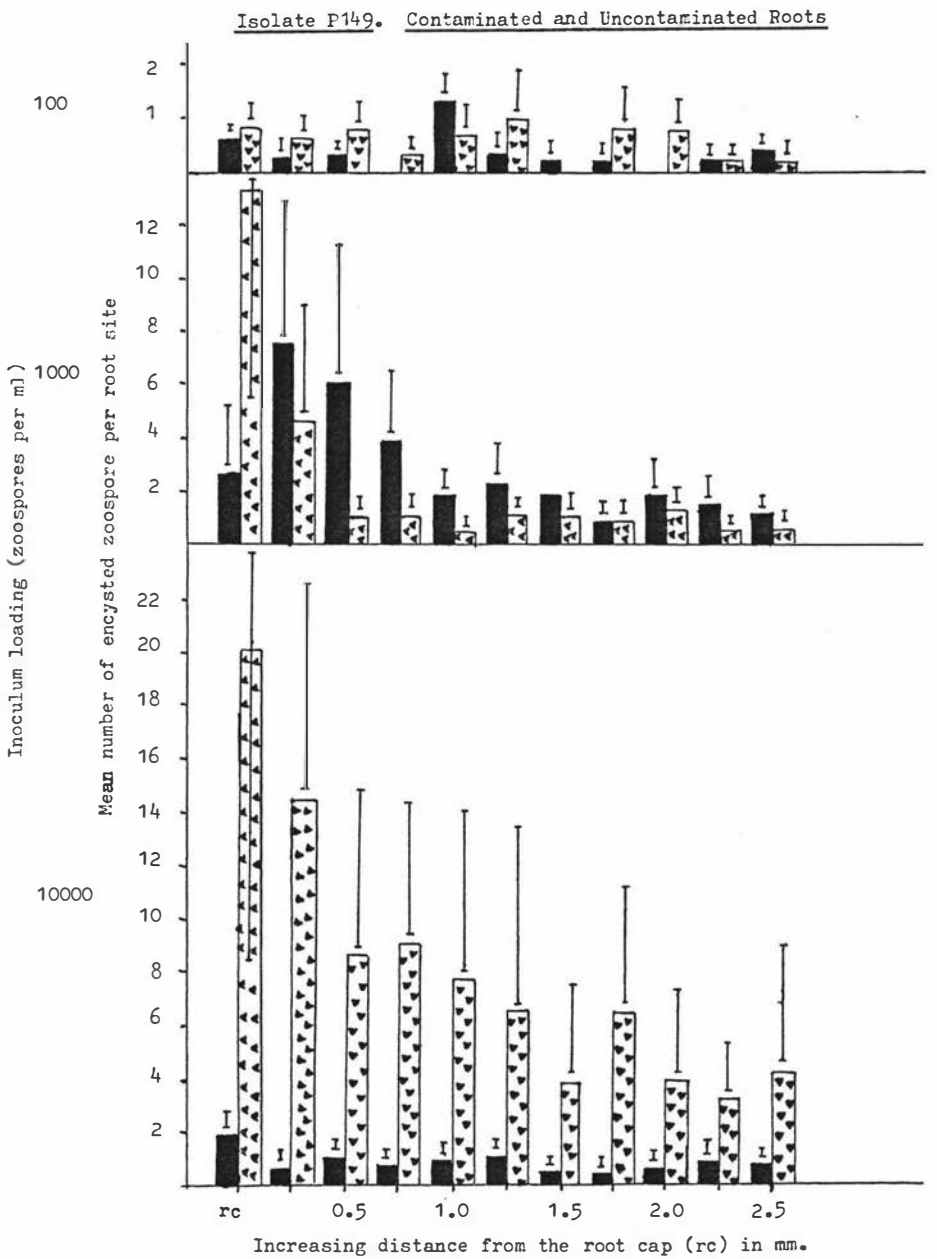
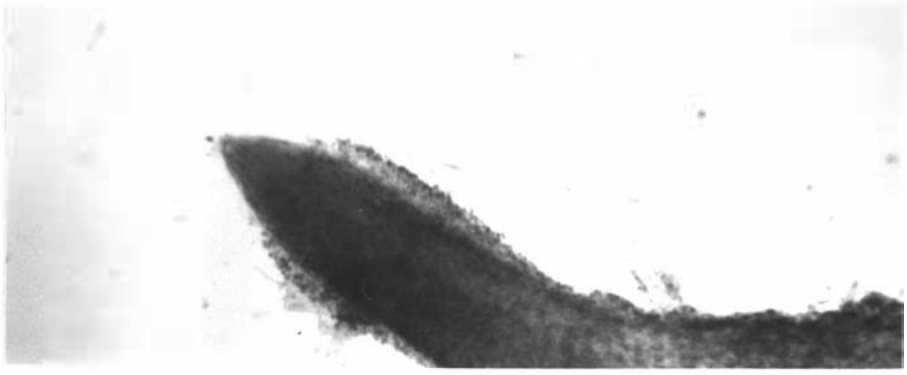

Isolate P149. Contaminated and Uncontaminated Roots
Inoculum loading (zoospores per ml)
100
1000
10000
Mean number of encysted zoospore per root site
rc
0.5
1.0
1.5
2.0
2.5
Increasing distance from the root cap (rc) in mm.

Plate 1-9

Zoospore attraction to an unsuberised cv. 'Cox's Orange Pippin' seedling root, 2 hours after inoculation. Considerable numbers have encysted at the zone of elongation, lesser amounts on parts of the root further from the tip and relatively few on the root cap. x70.

Plate 1-10

Zoospore acculumation at an apple seedling root-stem interface, 2 hours after inoculation. Few zoospores have been attracted to stem tissue (top half of plate) while many have encysted on the root surface. x175.



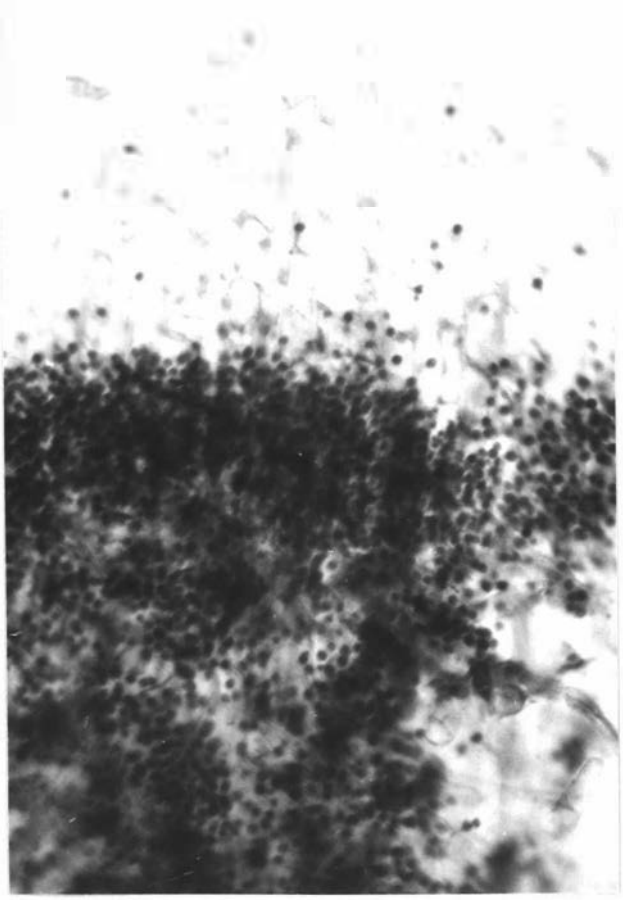
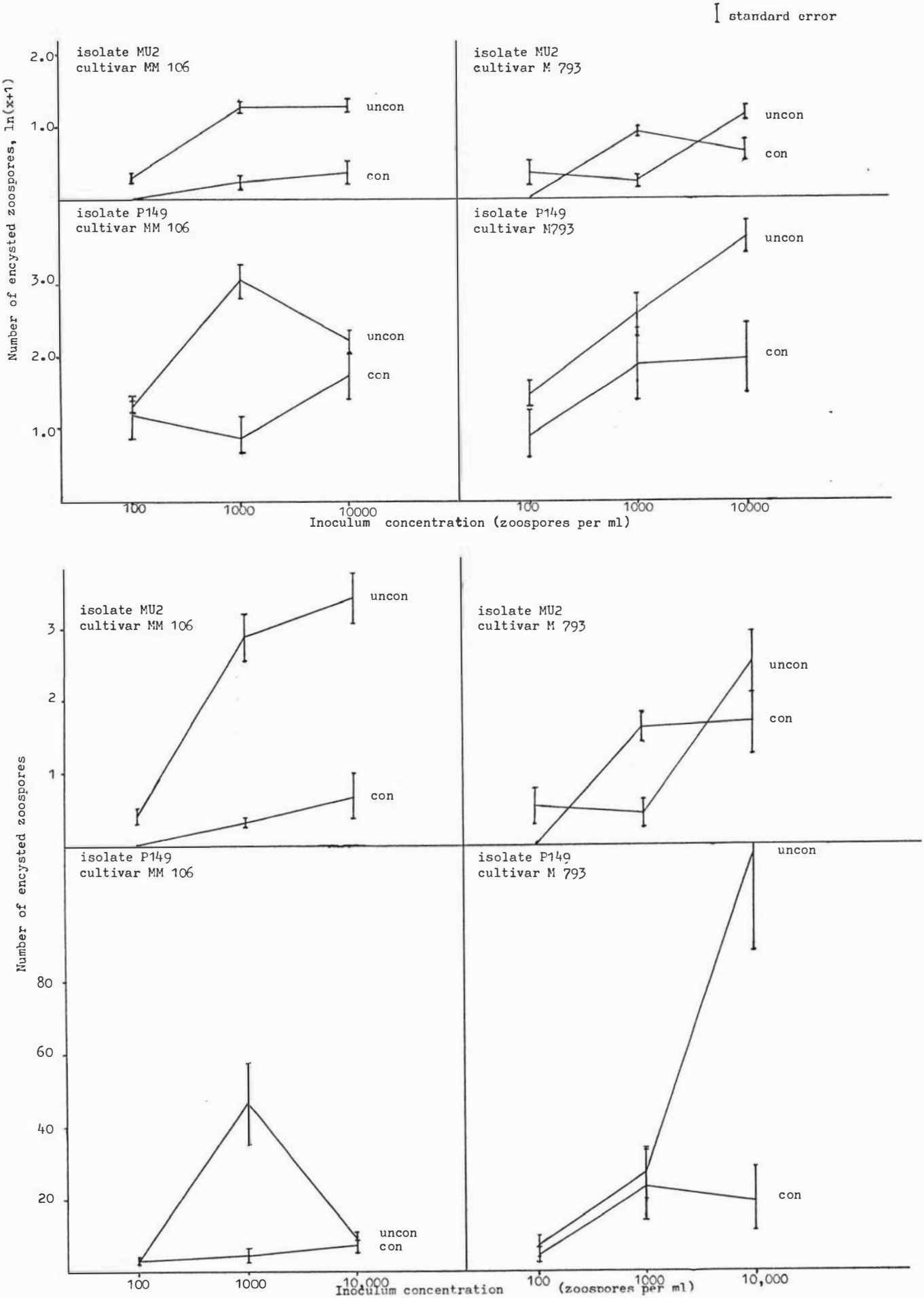


Table 1-IV. Analysis of Variance of Figure 1-4

Source of variation	Df	Mean Square	F	
Isolate	1	65.70	69.32	**
Variety	1	0.14	0.14	NS
Concentration	2	13.46	14.21	**
Contamination	1	11.54	12.17	**
Isol x Var	1	1.06	1.12	NS
Isol x Conc	2	1.31	1.38	NS
Isol x Cont	1	2.65	2.80	NS
Var x Conc	2	1.26	1.33	NS
Var x Cont	1	0.78	0.83	NS
Conc x Cont	2	1.01	1.06	NS
Isol x Var x Conc	2	0.50	0.53	NS
Isol x Var x Cont	1	1.12	1.18	NS
Isol x Conc x Cont	2	0.83	0.88	NS
Var x Conc x Cont	2	2.70	2.85	NS
Isol x Var x Conc x Cont	2	0.30	0.31	NS
Error	96	0.95		

Figure 1-4. Mean Number of P. cactorum Zoospores Encysted per Root Tip Region.



'MU2' ones . In most treatments uncontaminated roots were more attractive to zoospores than contaminated ones . Zoospore attraction to roots increased when inoculum concentration was increased from 100 to 1000 zoospores/ml, but generally little addition effect occurred when the inoculum loading was increased from 1000 to 10,000 zoospores/ml .

1-3-3. Cyst Germination

Zoospores accumulated on the cellophane membrane directly over the roots, especially over the tip region (Plate 1-11) . The pattern of zoospore accumulation appeared to be similar to that which occurred directly on the apple roots (Section 1-3-2) . No accumulation of zoospores occurred in the control treatment - zoospores were evenly spread over the membrane .

Table 1-V. Percentage Germination of P. cactorum Cysts on Cellophane Membranes

Time (hours) after inoculation	Control (No Roots)	COP	GS
2	16.3	69.6	46.3
	(4.0 ±0.1) f	(8.3 ±0.1) b	(6.8 ±0.2) c
4	25.6	83.2	78.4
	(5.1 ±0.3) e	(8.9 ±0.3) ab	(9.1 ±0.1) a
8	39.7	80.6	79.0
	(6.3 ±0.1) d	(8.9 ±0.1) a	(9.0 ±0.1) a

Mean values followed by same letter are not significantly different from each other at 5% level (Duncan's Mutiple Range Test) .
Transformed data means (square root) and standard errors in parentheses .

Approximately 80% of the cysts germinated in the presence of root exudates (Table 1-V) . Maximal cyst germination over the roots occurred within 4 hours of the zoospore suspension being placed on the cellophane membrane . No significant increase in germination occurred after this time when roots were present (Table 1-V) .

Root exudates appeared to stimulate cyst germination since it was significantly lower in the absence of roots (Table 1-V) . The effect of exudates from roots of the tested apple cultivars on cyst germination appeared to be nonspecific as differences between these treatments were not significant after 2 hours (Table 1-V) . A lower germination of cysts also occurred among those which had encysted more than one root width away from the excised roots .

Cyst germination on host roots 7 hours after placing roots into the zoospore suspension was greater than 90% for the tested isolates (Table 1-VI) . A similar amount of cyst germination occurred on the roots of both resistant (M 793) and susceptible (MM 106) varieties (Table 1-VI) .

Cyst germination was greater on roots than on cellophane membranes over roots (Tables 1-V and 1-VI) .

Table 1-VI. Percentage Germination of P. cactorum Cysts on Roots of cv. 'MM 106' and 'M 793'

Isolate	Cultivar	
	MM 106	M 793
MU2	94.8 ±1.9*	95.1 ±1.8
P149	96.7 ±1.2	99.0 ±0.4
P10	94.8 ±2.4	96.3 ±1.5

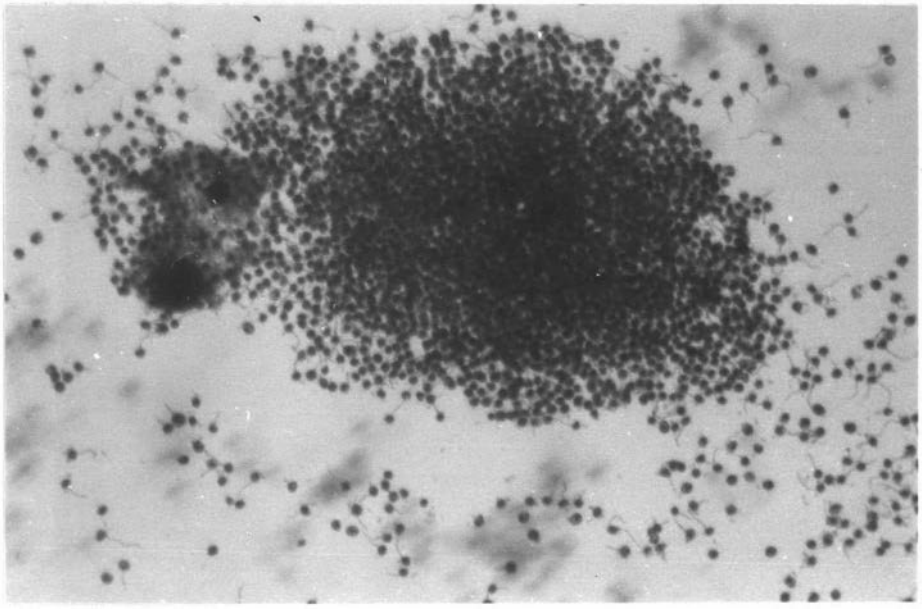
*Standard error

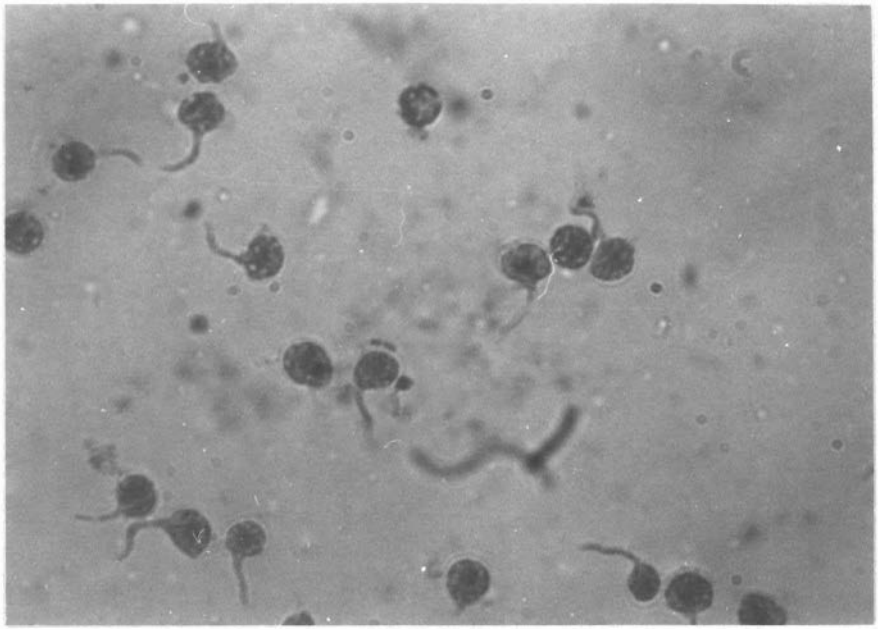
Plate 1-11

Zoospore accumulation on cellophane membrane over the tip region of an unsuberised cv. 'Cox's Orange Pippin' root, 2 hours after inoculation. Most of the zoospore cysts have germinated by germ tube formation. x175.

Plate 1-12

Germinated zoospore cyst on cellophane membrane over a cv. 'Granny Smith' root, 2 hours after inoculation. Zoospore contents have been stained with trypan blue. x875.





1-3-4. Directional Growth of Germ Tubes

The germ tubes produced by the cysts showed a strong chemotrop ic response towards the apple roots (Table 1-VII) . More than 95% of them grew towards apple roots for all tested isolates and with both the resistant ('GS' and 'M 793') and susceptible ('COP' and 'MM 106') cultivars (Table 1-VII) . The source of unsuberised apple roots, whether from glasshouse-grown seedlings or from field-grown trees, did not appear to affect the chemotrop ic response (Table 1-VII) .

Table 1-VII. Percentage of Zoospore Cyst Germ Tubes of P. cactorum orientiated towards Apple Roots

Seedling Roots		
Isolate	Cultivar	
	COP	GS
P149	95.6 ±0.9*	96.0 ±1.2

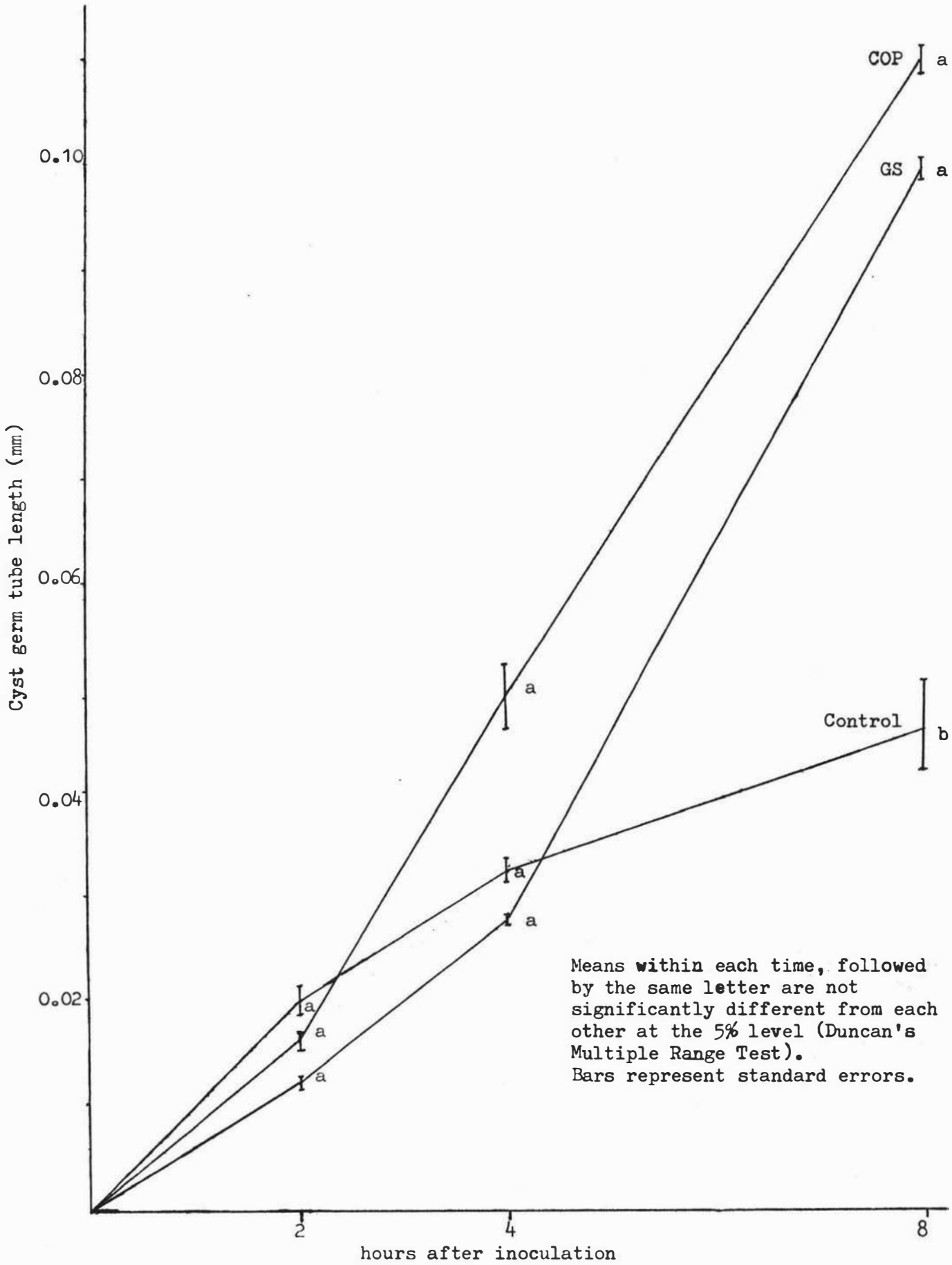
Mature Tree Roots		
	MM 106	M 793
MU2	98.6 ±0.8*	97.0 ±1.4
P149	97.3 ±2.2	98.5 ±0.5
P10	97.8 ±0.9	98.0 ±1.6

*Standard error

On the cellophane membranes, the germ tube grew without branching for the initial few hours, but after four hours branching occurred (Plates 1-12 and 1-13) .

Root exudates diffusing through the cellophane membrane caused a

Figure 1-5. Cyst germ tube elongation on cellophane membranes over apple roots.



non-specific promotion of the growth of the germ tubes which became apparent between 4 and 8 hours after inoculation (Figure 1-5) .

1-3-5. Appressorial Formation

Neither exogenous nutrients in the form of root exudates nor a hard smooth surface (cellophane membrane) stimulated the P. cactorum cyst germ tubes to form appressoria, as less than 5% were produced under these conditions (Table 1-VIII) . However it was noted that appressoria did form at sites where the cellophane membrane was crinkled . On the root surface of both tested cultivars about 99% of cyst germ tubes formed appressoria and penetrated the root within 2 hours of inoculation (Table 1-VIII and Plate 1-5) . This indicates that appressoria were formed in response to the root surface itself .

Table 1-VIII. Appressorial Formation of Germ Tubes produced by P. cactorum Zoospore Cysts

Cellophane Membrane

Time (hours) after inoculation	Control (No Roots)	COP	GS
2	1.0	0.5	2.6
4	4.2	3.0	1.8
8	1.0	2.8	2.6
16	0.9	2.5	1.8

Root Surface

2	-	98.7 ±0.3*	99.0 ±0.5

*Standard error

Plate 1-13

Germinated zoospore cyst on cellophane membrane over a cv. 'Granny Smith' root, 16 hours after inoculation. The cyst (arrow) is empty of contents and its germ tube has branched extensively. x875.

Plate 1-14

The germ tube produced by the encysted zoospore (20 hours after inoculation) has swollen before penetrating the unsuberised cv. 'MM 106' root at the junction of two epidermal cells. SEM x4000.

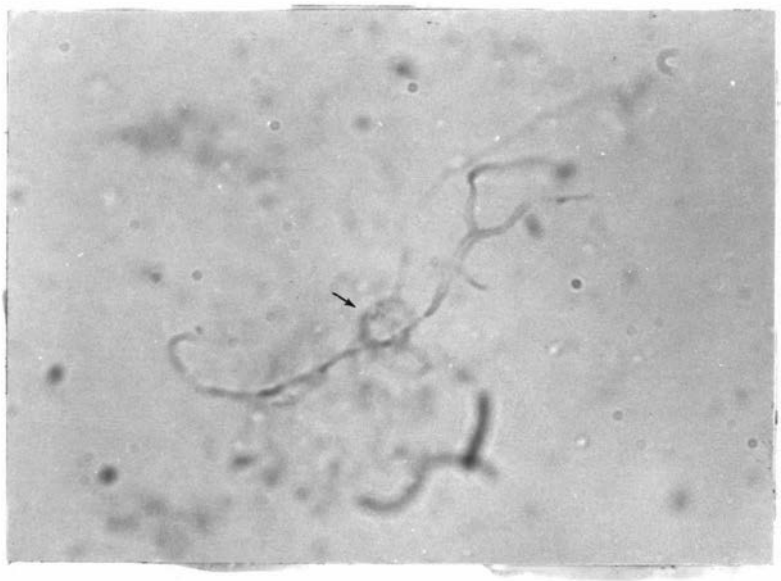


Plate 1-15

Penetration has occurred at the junction of the cv. 'MM 106' epidermal root cells, 20 hours after inoculation. Note the germ tube has not swollen. SEM x4000.

Plate 1-16

The germ tube of the encysted zoospore has produced a club-shaped appressorium over a break in the epidermal wall of a cv. 'MM 106' root cell. SEM x3400.

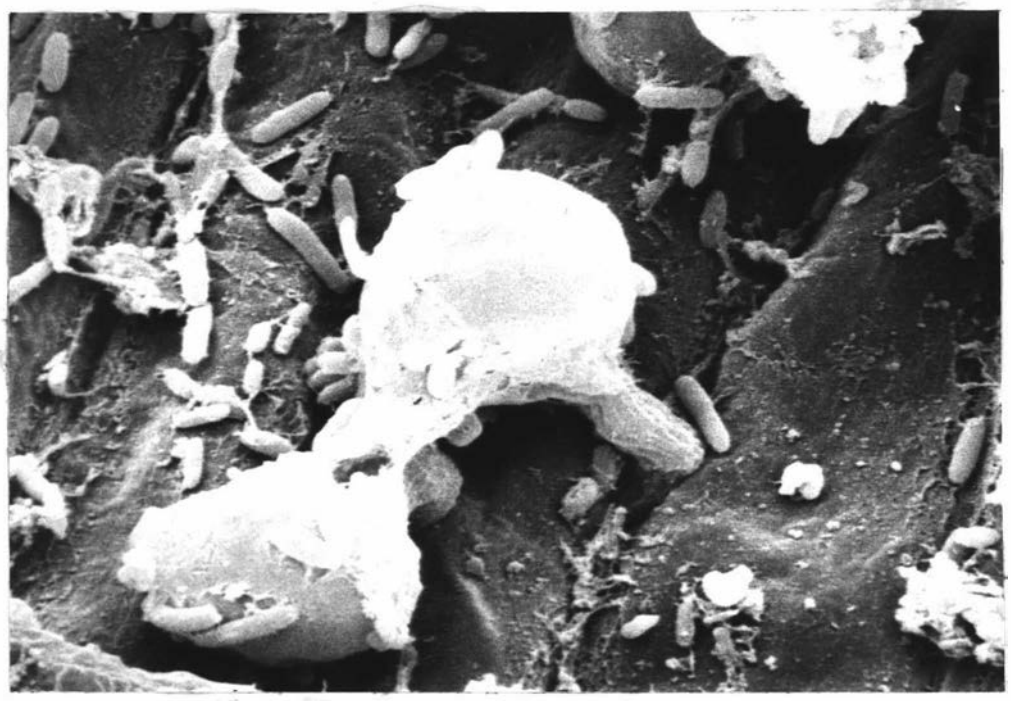
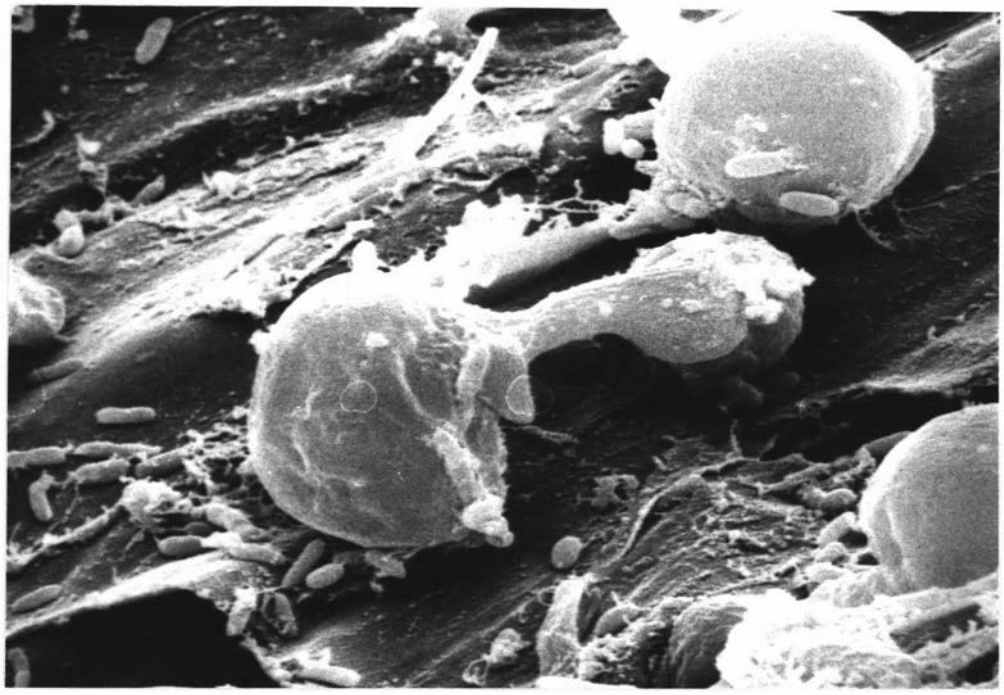


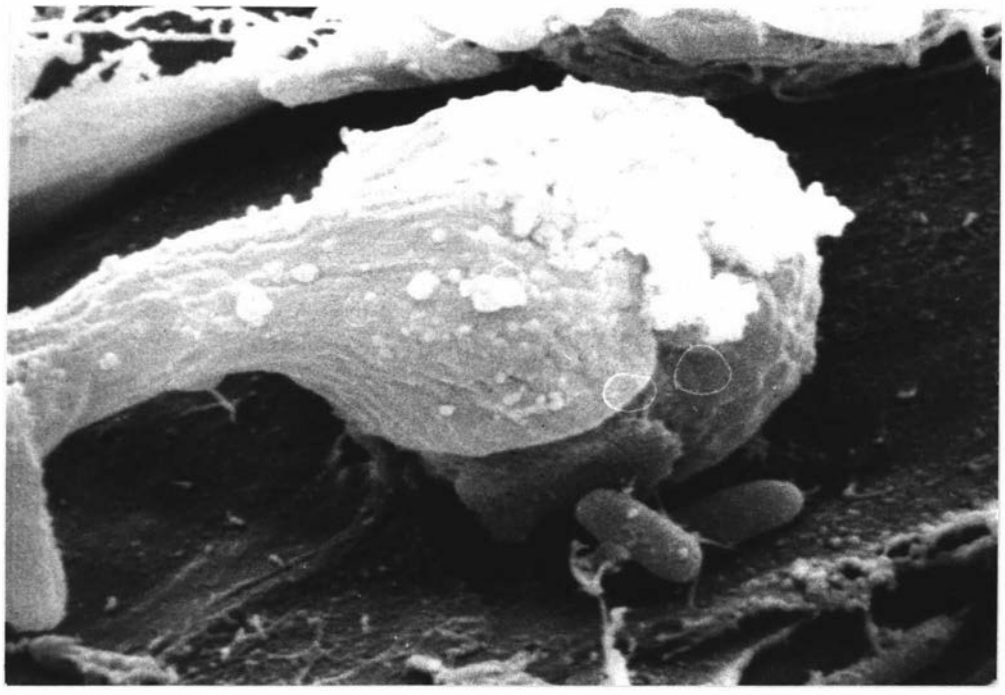
Plate 1-17

The germ tube of the cyst has terminated in a club-shaped appressorium over a break in the epidermal wall of a cv. 'MM 106' root cell. SEM x4000.

Plate 1-18

A close-up of the appressorium of Plate 1-17 showing the break in the epidermal cell wall. SEM x16,000.





Scanning Electron Microscopy

Germ tubes appeared to form appressoria in response to specific root surface configurations . The germ tubes grew towards and along the root surface until they encountered a junction between two epidermal cells (Plates 1-14 and 1-15) or a break in the epidermal cell wall (Plates 1-16 to 1-18) . At these sites, the germ tubes formed appressoria . The shape of the appressoria varied from a club-shaped body (Plates 1-16 to 1-18) to a pronounced swelling of the germ tube (Plate 1-14) . The former were produced on wall breaks and the latter at cell junctions .

1-3-6. Resistance of Roots of Open-Pollinated Apple Seedlings and Vegetatively Propagated Mature Trees

Experiment 1 : Seedling Roots

Increased inoculum loading gave increased infection of the seedling roots of both varieties (Figure 1-6A) . There was a steep increase of infection with increasing zoospore concentration up to 500 zoospores/ml for cv. 'COP' and a smaller increase up to 5000 zoospores/ml, possibly because of a saturation effect . There appeared to be a minimum threshold of 50 zoospores/ml for cv. 'GS' but thereafter the infection curve paralleled that of cv. 'COP' .

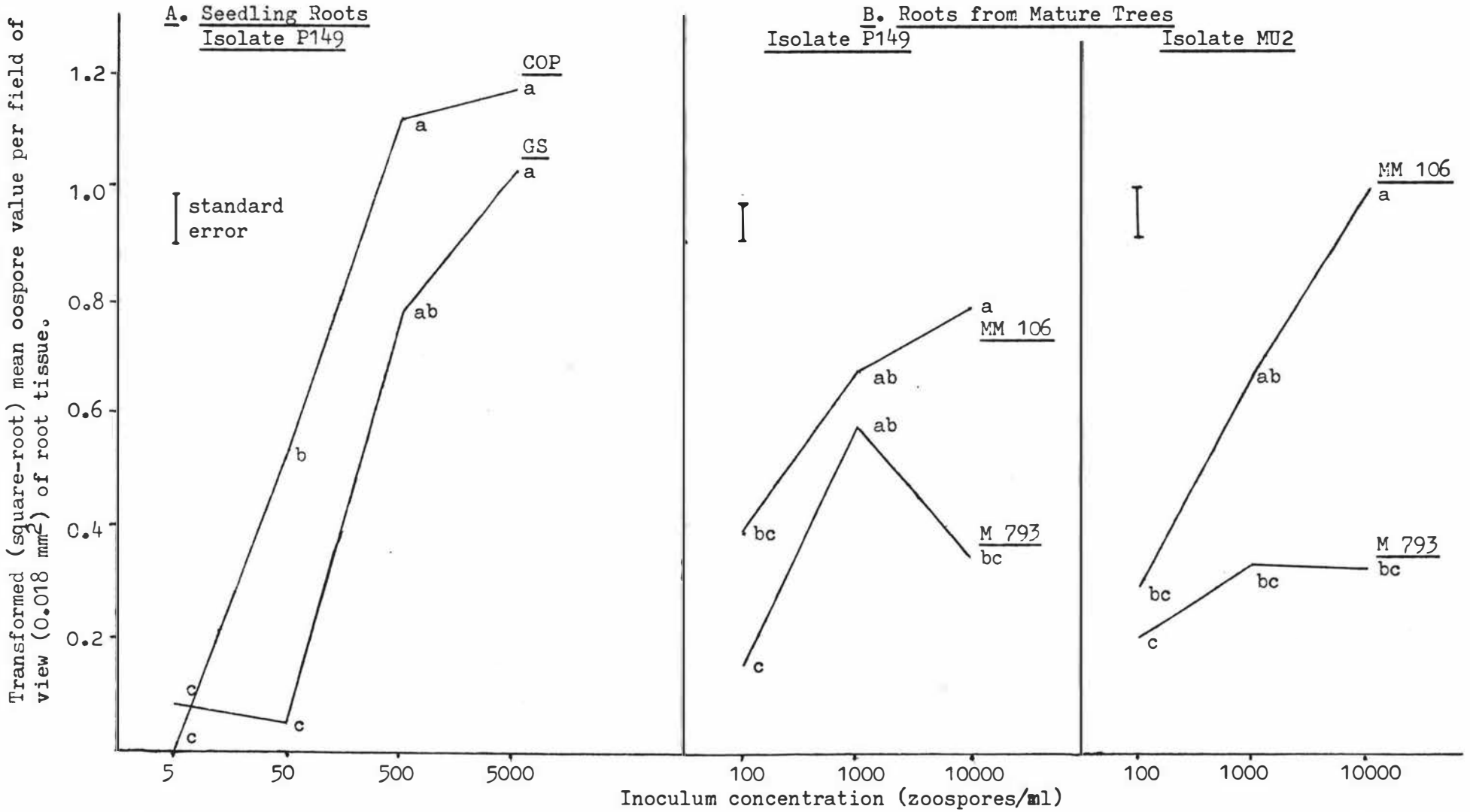
Experiment 2 : Mature Tree Roots

As with the seedling roots, increasing levels of inoculum caused significantly more root infection in the 'MM 106' roots with both isolates (Figure 1-6B) . Infection increased with inoculum loading from 100 to 1000 zoospores/ml for cv. 'M 793' but not with greater

zoospore concentrations . There appeared to be an abnormally large amount of infection with the 'P149' isolate in the 1000 zoospores/ml treatment .

The amount of established infection was generally lower in unsuberised roots from field grown mature trees than in those from glasshouse grown seedlings .

Figure 1-6. The Effect of Inoculum Levels on the Susceptibility of Unsuberised Apple Roots to P.cactorum



Means within each pair of graphs, followed by the same letter are not significantly different from each other at the 5% level (Duncan's Multiple Range Test).

1-4. DISCUSSION

The sporangial germination of P. cactorum isolate 'MU2' appeared to be inhibited by root exudates while that of the 'MU1' isolate was not (Table 1-I) . Sneh and McIntosh (1974) found the germination of P. cactorum sporangia in the rhizosphere of apple seedlings for 2-3 days was only slightly better than those in soil alone . They did, however, find that relatively high levels (0.5%) of glucose but not of asparagine resulted in germinations of 95% after 48 hours . P. palmivora sporangial germination was also inhibited in the presence of host exudates (Turner, 1963) and in the presence of amino acids and sugars (Clerk, 1972) . The degree of inhibition varied depending on the compound - inhibition was often greater with the amino acids (Clerk, 1972) . Thus it appears that the effect of root exudates on the sporangial germination of Phytophthora species varies according to the exudate composition and the sensitivity of the fungal isolate and species to those substances . In situ, the sporangial germination of various P. cactorum isolates do not appear to be stimulated to germinate by apple roots growing in their immediate vicinity (Table 1-IA).

The sporangia had a similar germination in the presence of roots of both tested apple cultivars indicating that the ability of apple root exudates to affect amount of P. cactorum sporangial germination was not related to the susceptibility of the apple cultivar . Other workers (Buxton, 1957; Kerr and Flentje, 1957; de Silva and Wood, 1964) have also found that the effect of exudates on fungal spore germination and growth may differ between host cultivars but not in accordance with cultivar resistance .

The type of P. cactorum sporangial germination seemed to be affected by the presence of root exudates (Table 1-IIA). Others have also found that the direct germination of sporangia of Phytophthora species appears to dominate in the presence of host roots (Ko, 1971) and nutrients such as amino acids and sugars (Aragaki et al., 1967; Clerk, 1972; Ho and Hickman, 1967A) . Such a mechanism would allow maximal use of the available resorces of the sporangia to achieve infection .

Although, the conditions of the experiment were favourable for sporangia to produce zoospores (Cook and Duniway, 1981; Duniway, 1975; Duniway, 1979; Fuller, 1977; Gisi et al., 1977; Gisi et al., 1979; McDonald and Duniway, 1978; Sugar et al., 1977) but even under such conditions, most of the germinating sporangia did not form zoospores . Phytophthora sporangia are less likely to germinate and to form zoospores as they age (Duniway, 1979; Gisi et al., 1977; King et al., 1968; Sneh and McIntosh, 1974; Zentmyer and Erwin, 1970) . Since an average of 20% or less of these young (>7 day old) P. cactorum sporangia germinated, then only a relatively small proportion of the sporangia would eventually germinate to produce zoospores, even under optimum conditions . This suggests that it is the germ tube which is the primary unit of inoculum rather than the zoospore as others (Eye et al., 1978; Gooding and Lucas, 1959) have suggested .

Maximal P. cactorum zoospore accumulation occurred at the root tip region and at wounds . This chemotaxic phenomenon has also been reported by many other workers (e.g. Carlile, 1983; Ho and Hickman, 1967A and B; Zentmyer, 1970) . Root tip regions, wounds and sites of lateral root emergence are sites of greatest exudation of amino acids and sugars (Rovira, 1965) . Such sites would provide the easiest route of entry for the pathogen into host tissue because at these places the outer protective layer is not yet suberised or is broken .

The P. cactorum zoospore attraction to the apple root tips was non-specific . The numbers of zoospores attracted to roots of cultivars of differing field resistance under the same experimental conditions was not related to their resistance . Studies by Goode (1956) and Zentmyer (1961) showed similar results . However, there are two reports in the literature of specificity of zoospore attraction with Phytophthora species . Chi and Sabo (1978) found the amount of zoospore attraction of P. megasperma to alfalfa seedlings to be related to the susceptibility of the cultivar involved . Zoospores of P. megasperma were also not attracted to some nonhost species (Zentmyer, 1966) .

The seedling roots were more attractive to zoospores than were those from mature trees suggesting that the former had a greater root

exudation . The seedlings were grown on vermiculite in a glasshouse, so the effect may have been due to plant age or growing conditions (Rovira, 1965) .

The number of zoospores attracted to uncontaminated roots was greater than to those contaminated by other fungi (Figure 14). Other workers have suggested that other microorganisms play a protective role in preventing zoospore arrival at the root infection court either directly by producing antibiotics or indirectly by utilising much of the host exudates (Barber and Lynch, 1977; Bowen and Rovira, 1976; Drew and Lynch, 1980; Malajczuk et al., 1977; Marx and Bryan, 1969; Rovira, 1965; Taylor and Parkinson, 1961; Utkhede, 1984; Waid, 1957) . Since a variety of contaminant fungi were present and these also varied in the extent to which they had colonised the unsuberised apple roots, the degree of protection confered by these micro-organisms would also vary considerably between roots .

More zoospores of the isolate 'P149' were attracted to roots tips than were those of the 'MU2' isolate implying that the chemotaxic response of P. cactorum zoospores varies in sensitivity between isolates .

Root exudates markedly stimulated the germination of P. cactorum cysts . Other workers have also reported that root exudates, sugars and amino acids increase cyst germination (Byrt et al., 1982; Gooding and Lucas, 1959B; Hemmes and Hohl, 1971; Ho and Hickman, 1967A and B; Ho and Zentmyer, 1977; Ko and Lockwood, 1967; Mehrotra, 1972; Turner, 1963) . During its motile phase, the zoospore utilises a portion of its energy reserves . Once germination of the encysted zoospore begins, the remaining energy reserves (mainly lipids) are progressively broken down and converted into carbohydrates and proteins (Bimpong, 1975; Bimpong and Hickman, 1975) . Since the cyst has a large demand for amino acids and sugars, it readily utilises any available exogenous ones prior to and during this period . Barash et al. (1965) found that Phytophthora drechsleri cysts metabolised ^{14}C - labelled sugars and amino acids . These exogenous nutrients thus appear to stimulate germination by ensuring that more cysts had sufficient energy requirements for germination .

The enhanced cyst germination by nutrients also indicates that P. cactorum zoospores may consume their energy reserves in an attempt to reach host roots in preference to retaining enough reserves for germination .

The amount of cyst germination on cellophane membranes over roots was similar between apple cultivars of differing susceptibility . Likewise, the amount of cyst germination on roots was similar between resistant and susceptible cultivars . Byrt et al. (1982) found that different sugars (50mM) stimulated P. cinnamomi cyst germination to varying degrees . The cyst germination of P. megasperma var. sojae and that of P. palmivora varied in the presence of root exudates from different host cultivars (Ho and Hickman, 1967A; Turner, 1963), indicating that cyst germination is influenced by the quality and/or quantity of the exudates . In the case of Turner's work (1963), there was greater germination of P. palmivora cysts in exudates from highly susceptible cacao cultivars than in those from more resistant ones .

Cyst germination was greater on the root surface (approximately 95%) than on cellophane membranes over roots (about 80%) (Tables I-V and I-VI). Ho and Zentmyer (1977) found that P. cinnamomi had a cyst germination of about 100% on the root surface of both resistant and susceptible avocado plants . This indicates that materials on the root surface itself appear to increase cyst germination .

The germ tubes arising from the zoospore cysts were stimulated by root exudates to grow toward the root in over 95% of the cases . The chemotrop ic response of the germ tubes was non-specific . This is in agreement with the studies of Bryt et al. (1982), Dukes and Apple (1961), Hickman (1970) and Hinch and Weste (1979) .

The reserves of the P. cactorum zoospore cysts appeared to be substantially depleted in the absence of roots after 4 to 8 hours by which time they had grown 30 to 50×10^{-3} mm (Figure 1-5) . The germ tubes of corresponding cysts on membranes over roots continued unimpeded indicating that the cysts were utilising the root exudates as an energy source for continued germ tube growth . Barash et al. (1965), Hemmes and Hohl (1971), Stanghellini (1974), Thomson and Allen

(1976) also found germ tube elongation to be greater in nutrient solutions . The germinating cyst's chemotrop ism and ability to utilise exogenous nutrients would enable cysts which were a relatively short distance from the root to make contact . Germ tubes of cysts on the root itself need only grow a little distance and so root exudates would probably be used to assist penetration (via appressorial formation) and subsequent growth .

Although appressorial formation by the Pythiaceae is variable (Agnihotri, 1969; Endo and Colt, 1974; Goode, 1956; Ho and Hickman, 1967A; Kim et al., 1974; Malajczuk and McComb, 1977; Marais and Harpe, 1982; Marks and Mitchell, 1971; Milholland, 1975; Miller and Maxwell, 1984; Thomson and Allen, 1976; Tippett et al., 1976, P. cactorum germ tubes produced them consistently (this study; Brown and Brotzman, 1979; Miller et al., 1966) . In contrast to the present study, nutrients and/or a flat hard surface has been found to induce appressorial formation in the Pythiaceae (Agnihotri, 1969; Ho and Hickman, 1967A; Thomson and Allen, 1976) . The rapid and almost total formation of appressoria (about 99%) by the germ tubes on the root surface and formation over membrane distortions suggests that P. cactorum appressorial formation is induced by a specific type of surface configuration . The SEM study shows that epidermal cell junctions and wall breaks are surface configurations which can induce P. cactorum zoospore germ tubes to form appressoria . The shape of the appressoria formed may either be related to the type of surface configuration or to the relative ease of penetration at these sites . The formation of appressoria by germ tubes in response to topographical features also occurs in rust fungi such as Uromyces phaseoli and Puccinia graminis (Staples and Macko, 1980; Wynn, 1976; Wynn, 1981) .

The severity of P. cactorum infection of the seedling unsuberised apple roots was dependent upon the initial inoculum loading, up to a saturation point i.e. extensive infection (Figure 1-6A) . A larger inoculum loading was required to reach this point with the more resistant 'GS' cultivar . A possible reason for the higher resistance of the cv. 'GS' is that it has a higher minimum threshold for more than slight infection than the cv. 'COP' . The more resistant cv. 'M 793' tended to have a lower amount of infection than the susceptible

'MM 106' at corresponding levels of inoculum . It appears to be resistant because it was able to reduce the amount of infection that occurs within its root tissues . The reduction of infection in the cv. 'M 793' roots was only significantly different at the highest inoculum level for both isolates (Figure 1-6B) . This appears to be a similar situation to what occurred with soybean hypocotyls infected with P. megasperma var. sojae (Stossel et al., 1980) . Although the pathogen generally colonised the susceptible tissue more extensively there were many abortive attempts to invade host cells in compatiable reactions and in incompati ble interactions individual hyphae spread deeply within tissue .

A greater degree of plant infection by Pythiacious fungi has been found to occur with increasing inoculum densities (Bhalla, 1968; Flowers and Hendrix, 1974; Gooding and Lucas, 1959A; Hwang and Ko, 1978; Kannwisher and Mitchell, 1981; Kendrick and Wilbur, 1965; Kerr, 1964; Kliejunas and Ko, 1974; Kraft et al., 1967; Kraft and Erwin, 1967; MacWithey, 1965; Mitchell, 1978; Mitchell et al., 1978; Ramirez and Mitchell, 1975; Shew and Benson, 1983; Tippett and Malajczuk, 1979) with the more resistant species and varieties being less infected than the susceptible ones at corresponding levels of inoculum (Flowers and Hendrix, 1974; Gooding and Lucas, 1959A; Halsall, 1978; Milholland, 1975; Stossel et al., 1980; Weste and Cahill, 1982) .

The amount of infection of apple roots was better related to the inoculum loading than to the number of zoospores which encysted at the root tip region . The zoospore attraction method assessed only a relatively small proportion of the total root (two edges of the first 2.5 mm of each root) and zoospore accumulation at wounds was also ignored . Since large numbers of zoospores were attracted to wounds, infection maybe a reflection of accumulation at these sites rather than at the tip . Part of the zoospore attraction variation may have been due to competitive attraction between root tips and wound sites, especially with wounds near or in the assessed region .

Since the amount of zoospore inoculum which arrived at the infection court was not related to the resistance of the tested apple cultivars, it would appear that resistance mechanisms were not operating

externally to the root but were within . These mechanisms appeared to be operating at the establishment stage of the infection process and effectively increased the inoculum level required for more than a low level of infection to occur in the more resistant cultivars .

This oospore assessment method has not been previously described . The pathogen forms oospores in infected tissue within 48 hours . Since the maximum number of oospores formed in colonised tissue per unit volume of unsuberised root tissue appeared to be similar for both resistant and susceptible cultivars, the number of oospores were considered to indicate the severity of disease in these roots . Other means of disease assessment were tried but were found to be unsuitable . Visual assessment of the lesion area on the excised unsuberised roots was not possible since the entire root turned brown within a day or so . The sparseness of mycelium and the occassional presence of other Phycomycete fungi made the use of standard area diagrams both laborious and inaccurate .

Results obtained by this method were consistent with the field resistance of the tested cultivars . In both experiments, the more susceptible cultivars ('COP' and 'MM 106') showed a greater degree of root infection than the more resistant ones ('GS' and 'M 793') . However, it has only been tested with a narrow range of apple cultivars (4) and so should be regarded with some caution until more extensive tests have been made .

The main advantages and disadvantages of this method are listed below :

1) It is simple .
2) The resistance of root tissue is directly assessed, thus the resistance is more likely to reflect a cultivar's resistance to the commonly occurring root and crown rot forms of the disease .
3) It assesses the most susceptible exposed tissue (unsuberised roots) of the apple tree - the easiest point of pathogen entry .
4) It assesses material grown under field conditions, i.e. the phenotypic resistance of the tree .
5) Sampling has little or no deleterious effect on the tree .
6) Collection of material is more difficult and measurement of infection is more time consuming compared with current techniques .

7) Young roots are not availaible throughout the year, although they are present throughout the entire period of tree susceptibility .

Some of these advantages are possessed by currently used methods of assessment (Chapter 3) but not all, especially advantages 2 and 3 .

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** CHAPTER 2 **
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Studies of possible internal mechanisms at the early stages of infection of apple tissue.

2-1. INTRODUCTION

Fungal Growth

Ultrastructural studies have been made of interactions of plants with a number of *Phytophthora* species : *P. capsici*, *P. cinnamomi*, *P. infestans*, *P. megasperma*, *P. palmivora*, and *P. parasitica* (Calonge, 1969; Calonge *et al.*, 1969; Ehrlich and Ehrlich, 1966; Hanchey and Wheeler, 1971; Hohl and Suter, 1976; Jones *et al.*, 1974; Klarman and Corbett, 1974; Malajczuk *et al.*, 1977; Marks and Mitchell, 1971; Miller and Maxwell, 1984; Shimony and Friend, 1975; Slusher *et al.*, 1974; Stossel *et al.*, 1980; Tippett *et al.*, 1977; Tippett *et al.*, 1976; Tippett and Malajczuk, 1979) . These fungi have necrotrophic, hemibiotrophic, and biotrophic modes of parasitism . Necrotrophism, hemibiotrophism, and biotrophism occurs when the pathogen derives its nutrients from dead cells, initially from living and then from dead cells, or only from living cells respectively (Hancock and Huisman, 1981) .

Phytophthora hyphae grow both intercellularly and intracellularly

through host tissue (Hohl and Stossel, 1976; Klarman and Corbett, 1974; Malajczuk et al., 1977) . Haustoria are produced by many but not all species (Blackwell, 1953; Ehrlich and Ehrlich, 1966) . A constriction of the intracellular hyphae and haustorium occurs at the site of cell wall penetration (Hohl and Stossel, 1976; Malajczuk et.al., 1977; Tippett et.al., 1976; Tippett et.al., 1977) .

Role of Fungal Enzymes in Pathogenesis

Various species of Phytophthora are able to produce cell degrading enzymes - pectic enzymes, protease, phospholipase, lipase, RNAse, DNAse, and cellulase - in culture (Akinrefon, 1968; Clarke, 1966; McIntyre and Hankin, 1978; Yoshikawa et al., 1977) . The cell wall degrading enzymes (pectic enzymes, macerating factors, and cellulase) do not usually appear to have a significant role in pathogenesis . Cell wall degradation, in vivo, is localised to the site of penetration (Calonge, 1969; Hanchey and Wheeler, 1971; Hohl and Stossel, 1976; Malajczuk et al., 1977; Slusher et al., 1974; Tippett et al., 1977) . An exception to this is P. megasperma var. sojae which caused a general degradation of cell walls of young (8 day old) soybean plants (Klarman and Corbett, 1974) . Protease, phospholipase, lipase, RNAse, DNAase, and lipase may play some role in the disorganisation and death of cellular contents that occurs just prior to or after hyphal or haustorial entry . Evidence to show that these enzymes contribute directly to the development of the fungus in the host is lacking .

Role of Fungal Toxins in Pathogenesis

Toxic metabolitics have been isolated from cultural filtrates of Phytophthora species (Ballio et al., 1972; Brieman and Barash, 1981; Keen et al., 1975; Paxton, 1972; Plich and Kudnicki, 1979; Woodward & Keen, 1980) . They, however, elicited only a few of the disease symptoms in bioassays with cuttings of several plants species (Ballio et al., 1972; Brieman and Barash, 1981; Keen et al., 1975; Plich and

Rudnicki, 1979; Woodward et al., 1980) .

In vivo, the presence of cellular alterations in advance but not directly in contact with the hyphae (Tippett et al., 1977; Calonge et al., 1969; Jones et al., 1974; Tippett and Maljczuk, 1979) supports the hypothesis that toxic diffusable substances are involved in the pathogenesis of the disease .

Host Reactions

In both resistant and susceptible tissues (root, stem, fruit and leaf) in many Phytophthora-host combinations, some disorganisation of cellular contents usually occurs in cells just in front of the mycelium (Hanchey and Wheeler, 1971; Jones et al., 1974; Marais and Harpe, 1982; Miller and Maxwell, 1984B; Slusher et al., 1974; Tippett et al., 1976) . In some cases, cellular damage is evident as much as 2-4 cells in advance of the hyphae (Hanchey and Wheeler, 1971; Tippett et al., 1979) . In other combinations, the cells in the immediate vicinity of the hyphae appear normal (Shimony and Friend, 1975) .

Usually the plasmalemma begins to separate from the cell wall and then other membrane systems and organelles such as ER, chloroplasts, mitochondria, and nuclear membranes are affected (Hanchey and Wheeler, 1971; Jones et al., 1974; Miller and Maxwell, 1984A and B; Shimony and Friend, 1975; Slusher et al., 1974; Tippett et al., 1977) . The cytoplasm is observed to increase in granularity or electron density (Miller and Maxwell, 1984B; Shimony and Friend, 1975) .

Cell necrosis is considered to occur when the cytoplasm and subcellular organelles have degenerated to an electron dense mass (Miller and Maxwell, 1984B) . It occurs in advance of the hyphae with the necrotrophic P. cinnamomi (Tippett and Malajczuk, 1979) and some time after cellular penetration for the hemibiotrophic and biotrophic P. capsici, P. infestans, P. megasperma and P. palmivora (Jones et al., 1974; Miller and Maxwell, 1984B; Shimony and Friend, 1976; Slusher et al., 1974) . The hemibiotrophic mode of parasitism was not able to

be distinguished from the biotrophic mode with P. parasitica var. nicotianae-tobacco roots and P. megasperma f.sp. medicginis-alfalfa roots (Hanchey and Wheeler, 1971; Miller and Maxwell, 1984B) .

Cell necrosis of resistant plants is often more rapid than in susceptible ones (Hanchey and Wheeler, 1971; Shimony and Friend, 1975; Stossel et al., 1980; Tippett and Malajczuk, 1979) .

Host cells often form wall appositions (papillae, encasements, wall lesions) in response to the presence of hyphae of necrotrophic (Cahill and Weste, 1983; Tippett and Malajczuk, 1979) and hemibiotrophic and biotrophic Phytophthoras (Hanchey and Wheeler, 1971; Hohl and Stossel, 1976; Shimony and Friend, 1975; Slusher et al., 1974), but not always (Malajczuk et al., 1977; Calonge, 1969; Shimony and Friend, 1975) . In some resistant plant species, this deposition of wall apposition material occurs with a greater frequency and to a greater extent (often completely encasing the haustoria) than in susceptible plants (Cahill and Weste, 1983; Hanchey and Wheeler, 1971; Hohl and Stossel, 1976; Miller and Maxwell, 1984B; Stossel et al., 1980) .

Mechanisms of Host Resistance

In some Phytophthora-host interactions, structural features such as wall appositions and periderm formation appear to function as resistance factors to these fungi (Cahill and Weste, 1983; Hohl and Stossel, 1976; Tippett et al., 1983) while phytoalexins may have a role in others (Keen, 1971; Keen and Yoshikawa, 1983; Sato et al., 1971; Yoshikawa et al., 1978) .

Hypersensitive reactions do not appear to directly arrest pathogen development since cell death occurs well before fungal death . The pathogen frequently continues to grow within and through these necrotic cells (Calonge, 1969; Klarman and Corbett, 1974; Maclean and Tommerup, 1979; Shimony and Friend, 1975; Slusher et al., 1974; Tippett and Malajczuk, 1979) .

The aim of this portion of work was to investigate the histopathology of the pathogen within apple tissue in order to determine the nature of plant-pathogen interaction and the type of resistance mechanisms .

2-2. MATERIALS AND METHODS

2-2-1. Light Microscopy Studies of P. cactorum in Apple Stem Tissue

Paraffin Embedding

The method of Stoughton (1930) was used . Apple stem tissue was infected as described in Section 3-2-1 and placed in formalin-acetic-alcohol (FAA) for at least 24 hours . The specimens were dehydrated by passing them through two changes of cellosolve (3 hours each), cleared in xylene (30 minutes), left overnight in a xylene paraffin mixture (1:1), immersed in molten paraffin (24 hours), and embedded in fresh paraffin . The resultant blocks were cut to size, attached to specimen holders, and sectioned on a Cambridge Rotary Microtome (Type 52164) into 15 µm sections .

Sections were hydrated by passage though xylene, cellosolve, and distilled water . They were stained in carbol-thionin solution (0.1g thionin and 5g phenol in 100 ml water) for 5 minutes, taken through a graded alcohol series, and counter-stained for 5 minutes in a saturated solution of Orange G in absolute ethanol . The specimens were washed in absolute ethanol, transfered through xylene, and mounted in D.P.X. mounting medium .

Tissue Clearing

Stem tissue was infected as described in Section 3-2-1 and immersed overnight in 0.1% aniline blue in a chloral hydrate-water solution (5:2) (Nicolson, 1959; Tuite, 1969), or boiled in 0.01% acid fuschin

in saturated choral hydrate (Gerdemann, 1955; Tuite, 1969) . Tissue was also cleared in hot KOH, acidified , and stained with 0.1% trypan blue in lactophenol (Philips and Hayman, 1970) .

2-2-2. Histochemistical Studies

2-2-2-1. Callose

Roots of 15 week old open-pollinated cv. 'COP' and 'GS' seedlings were infected with zoospores (isolate 'P149') and incubated for at least 3 days at 20 ^{o}C in humid conditions .

Cross sections of infected and healthy, suberised and unsuberised, fresh root material were stained for callose using the following histochemical tests :

Aniline Blue Fluoresence Test

Sections were mounted in 0.1% water soluble aniline blue in 0.1M K_3PO_4 and examined using a Reichert microscope equipped with a 100W quatz-halogen lamp, BG-12 excitation filter, and a Wratten No.12 barrier filter (O'Brien and McCully, 1981; Martin, 1959) .

Lacmoid Test

Sections were placed in a 0.1% lacmoid solution in 50% ethanol for 12 to 24 hours in darkness and mounted in fresh solution (Jensen, 1962; Sherwood and Vance, 1976) .

2-2-2-2. Pectic Substances

Previous seasons shoots of the cultivars 'COP' and 'GS' were collected from trees in the field and infected as described in Section 3-2-1 . The stem tissue was embedded in paraffin and sectioned as described above in Section 2-2-1 . Sections were cut with the lesion margin in the middle so that each section contained both healthy and infected tissue .

Ruthenium Red Test

Paraffin sections were stained with aqueous ruthenium red (0.02%) until the cells walls of the healthy tissue was bright red . The specimens were washed in distilled water and mounted in glycerol (Rawlins and Takahashi, 1952) .

Iron Absorption Method

Sections were placed in 10% $FeCl_3$ for 10-20 minutes and washed 4-5 times in distilled water over 1.5 hours to remove all uncombined ferric salt . One to two drops of 2% potassium ferrocyanide was added to each section and the 2% HCl after a further 2 minutes . They were then washed in distilled water and mounted in glycerol (Rawlins and Takahashi, 1952) .

2-2-2-3. Cellulose

Root material was treated as described above in Section 2-2-2-1 .

IKI - H_2SO_4 Test

Sections were stained 15 minutes in IKI solution (1g iodine and 3g KI in 300 ml distilled water)and mounted on a slide in fresh solution . A drop of 65% H_2SO_4 was then added to the edge of the coverslip (Jensen, 1962) .

Zinc-Chlor-Iodide Test

Sections were washed in water and mounted in the zinc-chlor-iodide solution (30g $ZnCl_2$, 5g KI, 1g I in 14ml of distilled water) (Rawlins and Takahashi, 1952) .

Photine Test

Sections were immersed in 0.1% Photine solution (Hickson and Welsh Ltd., Castleford, Yorkshire, U.K.) in 0.1M phosphate buffer pH 5.0 for 2 minutes, then washed and mounted in distilled water . The sections were examined using an Olypmus ultra high pressure mercury illumination apparatus equipped with a DV-1 excitation filter, BG-12 and L-42 filters (Peberdy and Buckley, 1973) .

Toluidine Blue O Test

Sections were stained in 0.05% toluidine blue O in 0.1M phosphate buffer pH 6.0 for 1 minute (O'Brien et al., 1964) .

2-2-2-4. Phenolics

Root material for tests (a) to (f) was collected and treated as described in Section 2-2-2-1 . Apple stem tissue for tests (g) and (h)

is as described in Section 2-2-2-2, except that fresh material was used .

(a) Diazotized Sulphanilic Acid Method

Sections were immersed in a freshly prepared 2.5mM solution of diazotized sulphanilic acid (Wistar and Bartlett, 1941) in 0.2M acetate buffer pH 4.7 (Mace, 1963) .

(b) Ferric Chloride Test

Sections were treated for 5 minutes with 2% $FeCl_3$ in 95% ethanol (Mace, 1963; Scott and Peterson, 1979) .

(c) Nitrous Acid Test

Sections were placed in a solution of 10% sodium nitrite, 20% urea, 10% acetic acid (1:1:1 v/v/v) for 3 minutes and mounted in 0.2M NaOH (Reeve, 1951) .

(d) Toluidine Blue O Test

As described in Section 2-2-2-3 .

(e) Sudan IV Test

Sections were stained for 20 minutes in an alcoholic Sudan IV solution (0.5g Sudan IV in 100ml 70% ethanol), rinsed in 50% ethanol, and mounted in glycerol (Rawlins and Takahashi, 1952) .

(f) Modified Chlorine-Sulphite Test

Sections were transfered to slides and suspended above a 1:1 mixture of 5% $KMnO_4$ and conc. HCl for 15 minutes before being mounted in 20% sodium sulphite (Pearce and Rutherford, 1981) .

(g) Maule Reaction

Sections were treated in 1% $KMnO_4$ for 10-20 minutes, water washed, immersed in conc. HCl (5 minutes), rinsed and mounted in 5% $NaHCO_3$ (Rawlins and Takahashi, 1952) .

(h) Phloroglucinol-HCl

Sections were mounted in a saturated solution of phloroglucinol in 18% HCl (Rawlins and Takahashi, 1952) .

2-2-3. Transmission Electron Microscopy

Experiment 1

Cultivar 'MM 106' root material was collected and infected as previously described in Section 1-2-6 .

Experiment 2

Young roots of cv. 'MM 106' (scion=Oratia Beauty, 6 years old) and 'M 793' (scion=Cox's Orange Pippin, 8 years old) were placed on agar colonies of P. cactorum 'MU2' for 5 and 24 hours .

Preparation of Material

Healthy and infected roots were fixed in primary and secondary fixatives as described in Section 1-2-6 . The fixed material was treated with two changes of propylene oxide (15 minutes), infiltrated and embedded in Polarbed 812 resin . Longitudinal sections were cut with a diamond knife on a LKB ultramicrotome and mounted on supported

grids . These sections were stained 5-8 minutes with saturated uranyl acetate in 50% ethanol, rinsed in 50% ethanol, then in distilled water, and stained 5-8 minutes with lead citrate (Venable and Coggeshall, 1965) . Sections were again washed in distilled water and examined with a Phillips EM 200 electron microscope .

2-3. RESULTS

2-3-1. LIGHT MICROSCOPY STUDIES OF P. CACTORUM IN APPLE STEM TISSUE

The carbol-thionin and Orange G stain gave a good contrast between plant and fungal tissue : host walls stained yellow-brown and the mycelium violet-blue . Unfortunately, this method was found to be inappropriate for histopathological observations as hyphae were seldom found in sections . Tissue clearing techniques were found to be more suitable and the best tested one - that of Philips and Hayman (1970) was used as the standard method for this Section and for that of Chapter 1 .

P. cactorum mycelium grew sparsely both intracellularly and intercellularly through the dead or dying stem tissue . Host cells a short distance in front of the hyphae and those behind the mycelial front were killed (Plate 2-1) indicating that the pathogen was secreting a considerable amount of toxin and/or enzyme in vivo .

Hyphal density was greater in phloem than in cortical tissue . Mycelical growth was greater around multiseriate ray cells as the hyphae often produced short lateral branches in their vicinity .

2-3-2. HISTOCHEMICAL STUDIES

2-3-2-1. CALLOSE

The lacmoid and aniline blue fluorochrome staining of the casperian strip (endodermis) and of the xylem vessels of the healthy and infected roots of the tested cultivars indicated that β-1,3 linked glucans were incorporated into the the walls of these apple root tissues (Table

2-I) . The aniline blue fluorochrome stained cortical cell walls whereas the lacmoid stain did not (Table 2-I), suggesting that β-1,4 linked glucans may be present in the walls of this tissue .

The cell deposition of callose (a β-1,3 glucan) in phloem tissue in response to fungal infection was not apparent with this host-pathogen combination .

Table 2-I. Callose Histochemical Reactions of Infected and Healthy Seedling Root Tissue

Tissue	Stain	
	Aniline Blue	Lacmoid
root hairs	none*	none
epidermis	none	none
cortex	dim to intense fluorescence	none
endodermis	intense fluorescence	light green-blue
pericycle	none	none
phloem	none	none
xylem	intense fluorescence	light green-blue
colour of stained callose	bright yellow-green fluorescence	blue

*Reactions of suberised and unsuberised, healthy and diseased root tissue are the same unless otherwise indicated .

2-3-2-2. Pectic Substances

Pectic substances in and between cells walls were not generally

degraded by the pathogen as they stained bright rose red with ruthenium red and deep green-blue with the iron stain . The intensity of staining was similar in both healthy and diseased tissue, even in tissue infected for 4 days . However, pectic degradation did occur in the immediate vicinity of the hyphae since cell walls were not stained with the ruthenium red in this region .

2-3-2-3. Cellulose

In this study, the colour of healthy cortical tissue was taken to be indicative of cellulose since cellulose is known to be a major component of cell walls . The staining response was always close to that expected for cellulose .

Although some positive reactions for cellulose occurred in the cortex, phloem and xylem tissues, only the former stained consistently (Table 2-II) . The IKI-H_2SO_4 test did not indicate cellulose to be present in any of the tissues (Table 2-II) . No differences in the staining response of the root tissue cell walls occurred between the two tested cultivars (Table 2-II) and only one difference was observed between infected and healthy material . The diseased phloem tissue stained a greener colour with toluidine blue than the healthy tissue suggesting that more phenolics were present in this tissue . The staining reactions between young unsuberised roots and older suberised ones were similar; although the photine fluorescence was more intense in the xylem vessels of the older roots and the epidermis of these roots failed to stain with Zn-Cl-I (Table 2-II) .

Much of the cellular contents of the cortical and xylem tissues of uninfected roots consisted of starch grains (stained black whenever iodine was present in a stain) . The pathogen obviously utilised the starch as little remained in infected cortex tissue .

Table 2-II. Cellulose Histochemical Reactions of Infected and Healthy Seedling Root Tissue

Tissue	Stain			
	IKI-H_2SO_4	Zn-Cl-I	Photine	Toluidine Blue O
epidermis	Y-O*+	yellow	dim or none (U)	green-blue
			none (S)	
cortex	Y-O	yellow ; blue	intense	blue-violet
endodermis	Y-O	bright yellow	none	green-blue
pericycle	Y-O	yellow	none	green-blue-black
phloem	Y-O	yellow	none	green-blue (I)
				blue-violet (H)
xylem	Y-R#	bright yellow	dim (U)	green-blue-black
			intense (S)	
colour of stained cellulose	blue	blue to blue-black	intense fluoresence	red or reddish-purple**

*Y-O = yellow or yellow-brown turning to amber within a few minutes of acid addition .

#Y-R = yellow turning to brick-red or brown .

**Due to the abundant presence of phenolics (green or green-blue), the blue-violet colour was considered to be indicative of cellulose .

+Reactions of suberised and unsuberised, healthy and diseased root tissue are the same unless otherwise indicated .

(U) = unsuberised roots ; (S) = suberised roots .

(I) = infected roots ; (H) = healthy roots .

2-3-2-4. Phenolics

Phenolic compounds were abundant in the cell walls of the root

Table 2-III. Phenolic Histochemical Reactions of Infected and Healthy Seedling Root Tissue

Tissue	Stain		
	Diazotized sulphanilic	$FeCl_3$	Nitrous
epidermis	light yellow-orange	unstained	red-brown
cortex	yellow-orange	unstained	unstained
endodermis	yellow-orange	unstained (I) green (H)	unstained
pericycle	yellow-orange	unstained	red-brown
phloem	yellow-orange-brown	unstained	unstained
xylem	light yellow	unstained	unstained
colour of stained phenolics	light yellow (free hydroxyl phenolics)	blue-green or ppt. (catechol type phenolics)	cherry-red (o-dihydric phenolics)
	Toluidine Blue O	$KMnO_4$-HCl	Sudan IV
epidermis	green-blue	unstained	red
cortex	blue-violet	unstained	unstained
endodermis	green-blue	orange-brown (I) orange-red (H)	unstained
pericycle	green-blue-black	red-brown	red
phloem	green-blue (I) blue-violet (H)	unstained	unstained
xylem	green-blue-black	brick-red	unstained
colour of stained phenolics	green or green-blue (lignin)	orange-red ; pink (lignin)	red (suberin)

*Reactions of suberised and unsuberised, healthy and diseased root tissue are the same unless otherwise indicated .

(I) = infected roots ; (H) = healthy roots .

epidermis, endodermis, pericycle, and xylem tissues but not in those of the cortex and phloem (Table 2-III) . The positive Sudan IV reaction showed suberin to be a major phenolic in both the epidermis and pericycle . The toluidine blue and $KMnO_4$-HCl stains indicated that lignin was important in the endodermis and pericycle, and dominant in the xylem . The toluidine blue lignin stain did not appear to be as specific as the $KMnO_4$-HCl one since the epidermis and phloem were unstained in the latter . These tissues were also not stained when stem tissues were tested with the $KMnO_4$-HCl and phloroglucinol-HCl stains .

In most cases, the root tissues had similar staining reactions regardless of root age, apple cultivar, and P. cactorum infection (Table 2-III) . Infection did alter the cell wall phenolic composition and/or chemical structure of phenolics in the root endodermis and phloem tissues (Table 2-III) . The stem tissue also showed a similar pattern to that of the root tissue with respect to the lignin stains - no staining differences occurred between cultivars or healthy and infected tissues .

2-3-4. Transmission Electron Microscopy

Ultrastructure of Healthy cv. 'MM 106' and 'M 793' Root Tissue

All sections were cut from the zone of elongation . In this region of the root, a layer of largely dead cells, derived from the flanks of the root cap, covered the epidermis . Cells wall thickness varied considerably and intercellular spaces were small and infrequent (Plate 2-2) .

Most cells of both tested cultivars possessed a large vacuole with a thin layer of cytoplasm lining the cell wall (Plate 2-2) . Mitochondria, endoplasmic reticulum (ER), vesicles and occassionally a nucleus was observed within sections of the cytoplasm of each cell (Plates 2-3 and 2-4) . The membranes of these subcellular organelles

and that of the tonoplast were intact (Plates 2-3 and 2-4) .

Fungal Penetration into cv. 'MM 106' Apple Root Tissue

The outer root cell walls were indented by the appressoria of the germ tubes at the site of penetration (Plates 2-5 to 2-7), indicating the involvement of mechanical forces . At this site, the cell wall became more diffuse (Plate 2-6 and 2-7), suggesting that some chemical alteration had occurred . At a more advanced stage (Plate 2-7), the cell wall had increased in electron density and some cell wall material was displaced by the appressoria suggesting that it had been softened . The diminution of wall material in front of one of the appressoria (Plate 2-7) indicated that some dissolution had occurred .

Fungal Growth through cv. 'MM 106' and 'M 793' Apple Root Tissue

The process of fungal growth through the root cortical tissue and the host cell degradation in advance of it was similar with both susceptible (MM 106) and resistant (M 793) cultivars .

Cellular deterioration was obvious 2-4 cells in advance of the hyphae (Plates 2-8 and 2-9) suggesting that the pathogen produced a diffusible toxin . The plasmalemma frequently separated from the cell wall and the cytoplasm increased in electron density (Plates 2-8 and 2-9) . The amount of larger spheriods (Plates 2-8 and 2-9) or small granular particles (Plates 2-10 to 2-12) in the tonoplast also increased . These particules and spheriods are probably phenolics (Scott and Peterson, 1979) . Membrane systems (plasmalemma, ER, nuclear membrane, and tonoplast) and membrane-bound organelles (vesicles and mitrochondria) were intact at this stage (Plates 2-8 and 2-9) .

The cytoplasm of those cells that were 1-2 cells in advance of hyphae had degenerated into a dense, amorphous or granular mass in which a few intact vesicles remained (Plates 2-10, 2-11 and 2-12) . The plasmalemma and tonoplast membranes were often still intact (Plates 2-10, 2-11 and 2-12) .

Most of the degenerated contents disappeared from the cells when the hyphae were in their immediate vicinity (Plates 2-13 to 2-23) . This was particularly noticeable in the cv. 'M 793' cells (Plates 2-14, -17, -20, -22, -23) . The cell wall remained unaltered except in the immediate vicinity of hyphal penetration (Plates 2-15 to 2-23) .

No morphological structures such as wall appositions were observed to be formed in cells of either tested apple cultivars in response to P. cactorum invasion .

The hyphae grew both intercellularly and intracellularly within dead or dying cells of root tissue (Plates 2-13 to 2-23) .

The intracellular hyphae swelled slightly before the cell wall, penetrated by means of a penetration peg, and immediately enlarged to normal size (Plates 2-15, 2-16, 2-17 and 2-18) . This process was repeated as the mycelium passed through successive cell walls . In most cases of intracellular penetration, the cell wall underwent some distortion (Plates 2-17 and 2-18) . However, the disappearance of the cell wall material in these instances suggests that penetration through cell walls was largely enzymatic . This is evident in Plate 2-16, where the fibrillar nature of the cell wall is almost intact up to the hyphae itself . The displacement and compaction of cell wall material by a hyphae (Plate 2-18) showed that mechanical pressure was sometimes used by the hyphae to puncture the cell wall .

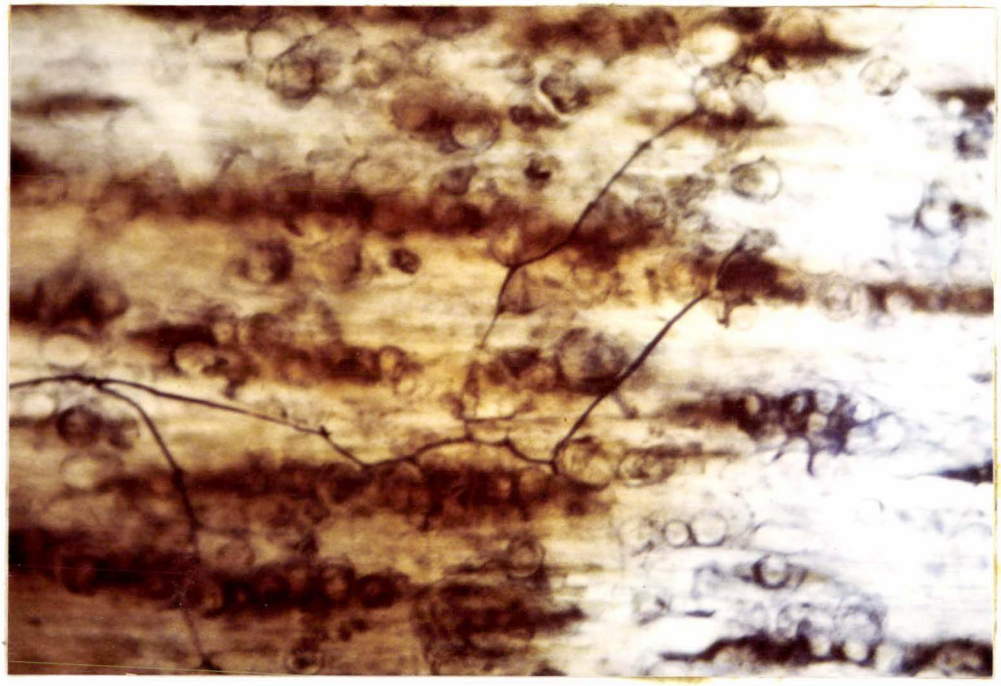
Intercellular growth of the pathogen occurred in the middle lamellae of the host cells (Plates 2-18, 2-20, 2-21, 2-22 and 2-23 . In many cases, a splitting apart of the cell walls was evident (Plates 2-19, 2-20, 2-22 and 2-23) . The wall splitting shown in Plate 2-19 and its breakage in Plate 2-20 indicates that the hyphae used some mechanical pressure as they grew between cells .

Plate 2-1

Cleared and stained (trypan blue) lesion margin of *P. cactorum*-infected apple phloem stem tissue. Infected tissue is brown and healthy tissue is blue. Note browning has occurred some distance from the hyphae. x200.

Plate 2-2

A healthy cortical cell of an unsuberised cv. 'M 793' root. x6,300.



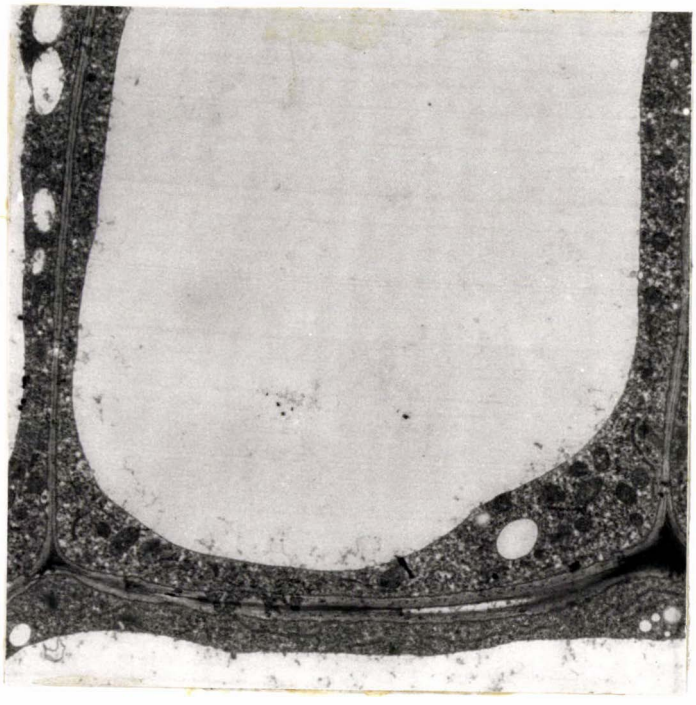
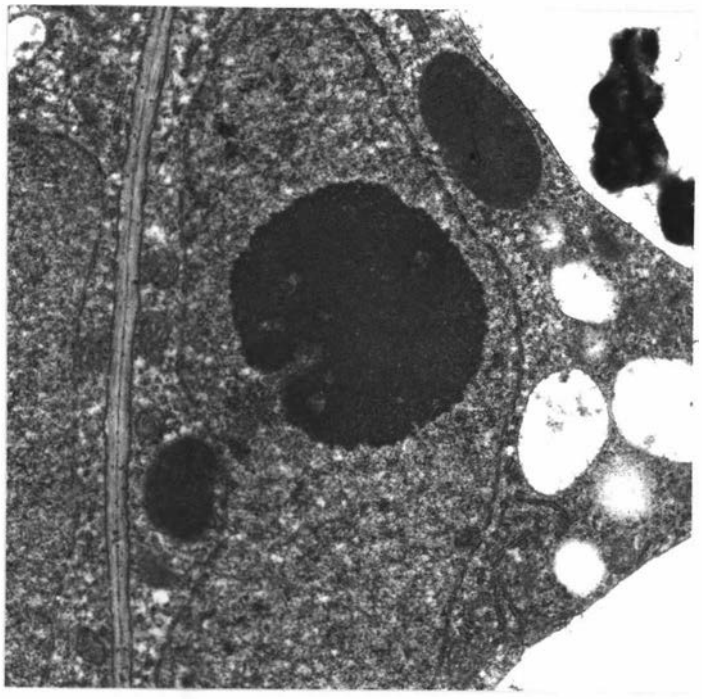


Plate 2-3

Nucleus of a healthy cv. 'M 793' root cortical cell. x10,500.

Plate 2-4

Cellular organelles of a healthy cv. 'M 793' root cortical cell. x13,600.



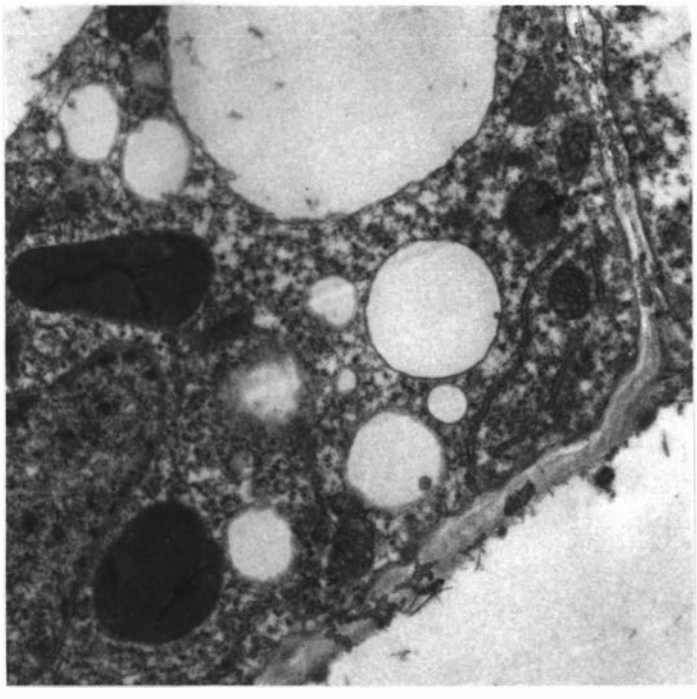
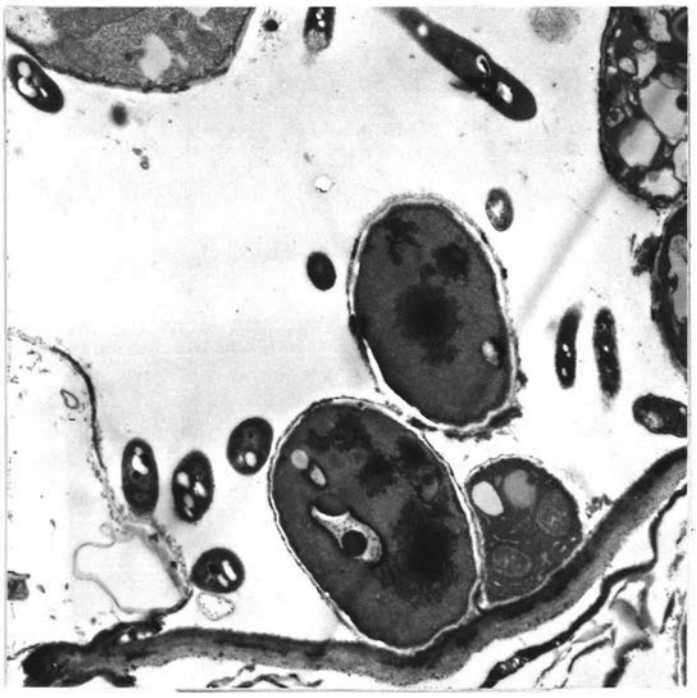


Plate 2-5

P. cactorum germ tubes from encysted zoospores have indented the outer epidermal cell wall of an unsuberised cv. 'MM 106' root, 20 hours after inoculation. x8,200.

Plate 2-6

The cv. 'MM 106' root cell wall in the immediate vicinity of one of the germ tubes (20 hours after inoculation) has become diffuse and some wall material has been displaced. x10,500.



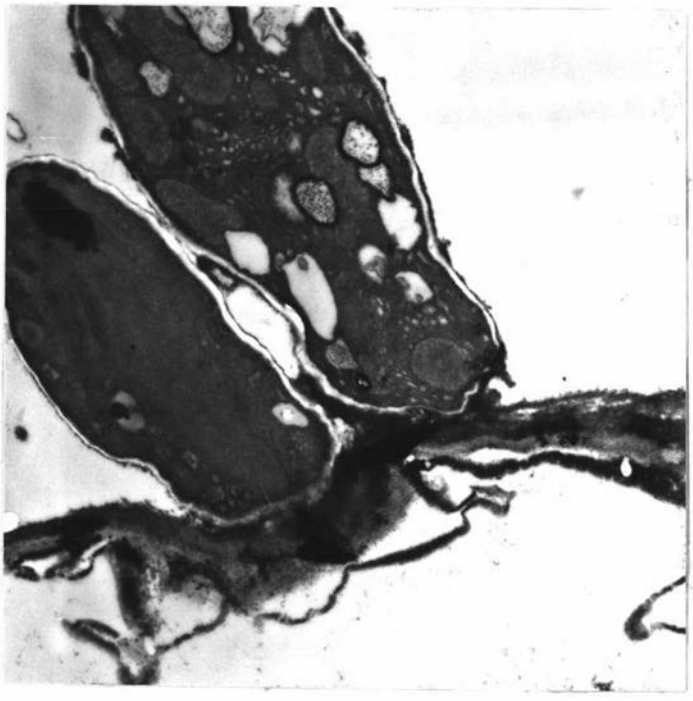
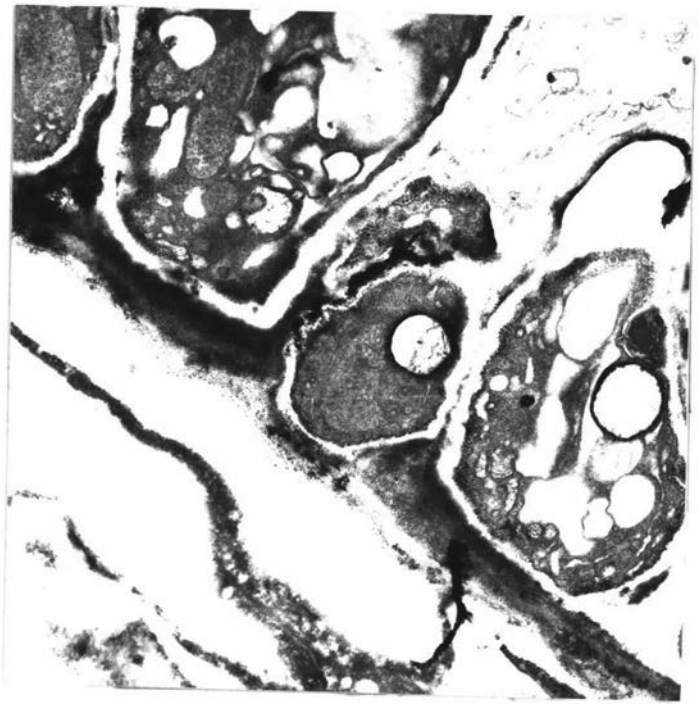


Plate 2-7

The cv. 'MM 106' root cell wall in the immediate vicinity of the germ tubes has become more electron-dense, 20 hours after inoculation. Some of the diffuse cell wall material has been displaced and some appears to have undergone dissolution. x13,600.

Plate 2-8

Plasmolysing cv. 'M 793' root cortical cell, 2-3 cells from the nearest hyphae, 5 hours after inoculation. The nucleus and cytoplasm have become more electron-dense and electron dense spheroids are present in the tonoplast. x19,200.



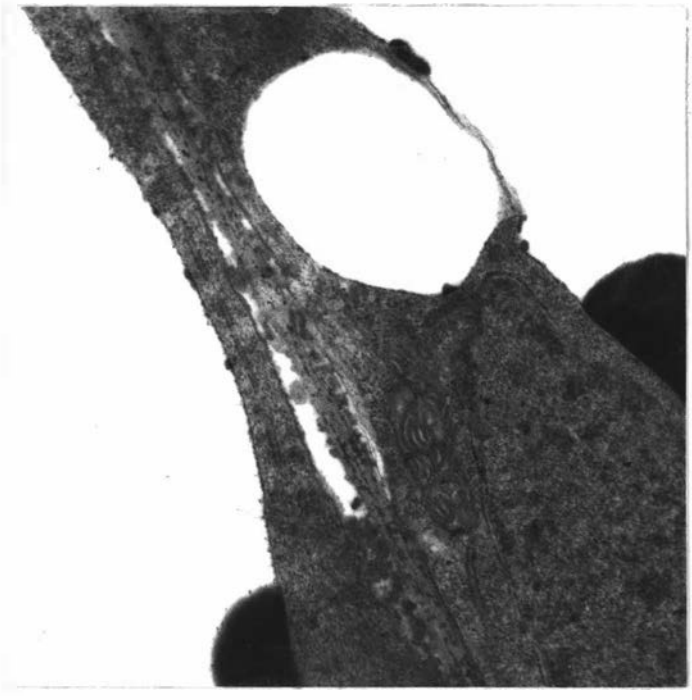
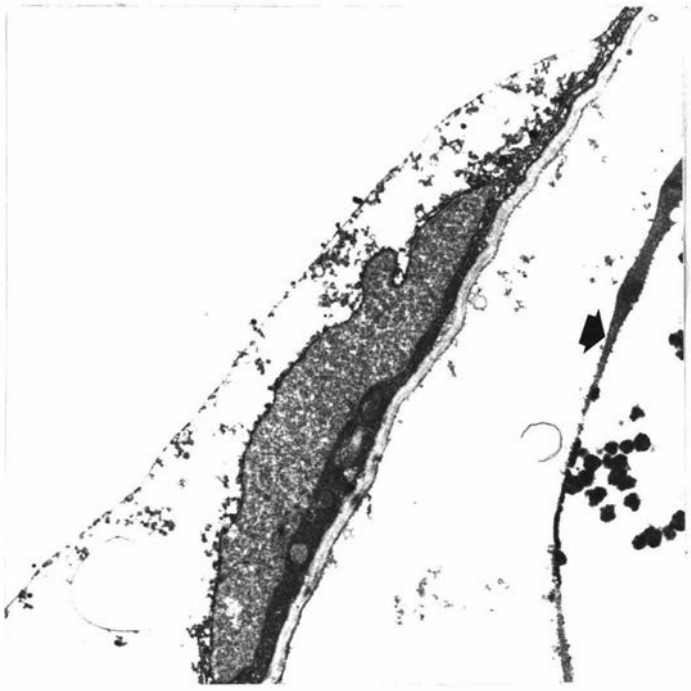


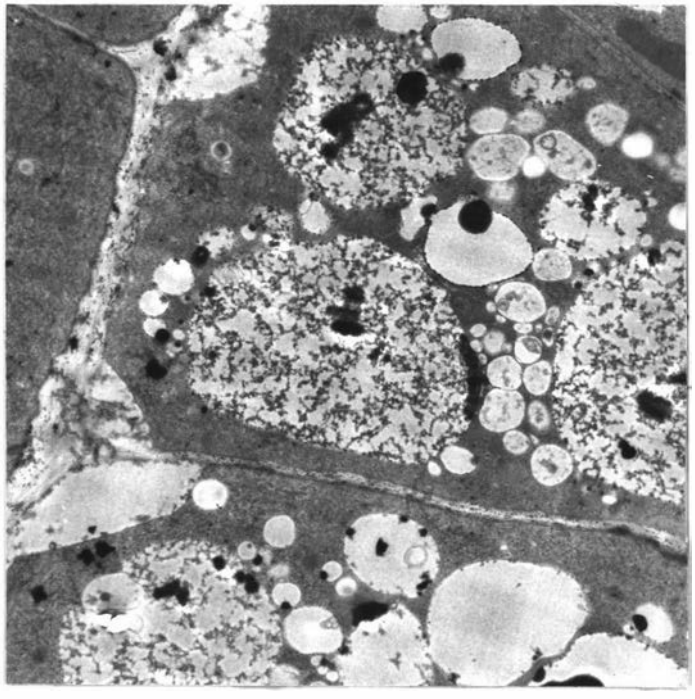
Plate 2-9

Cv. 'MM 106' root cortical cells 2-3 cells in advance of the mycelium. The plasmalemma (arrow) has separated from the cell wall and the nucleus and cytoplasm have become more electron-dense. Electron-dense spheriodal particles are present in the tonoplast. x5000.

Plate 2-10

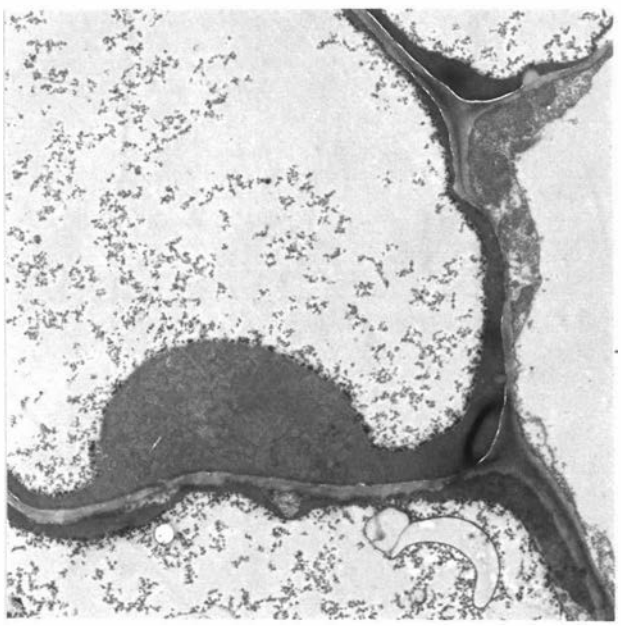
A cv. 'M 793' root cortical cell, 1-2 cells from the nearest hyphae, 5 hours after inoculation. Extensive breakdown of cellular organelles has occurred. x6,300.

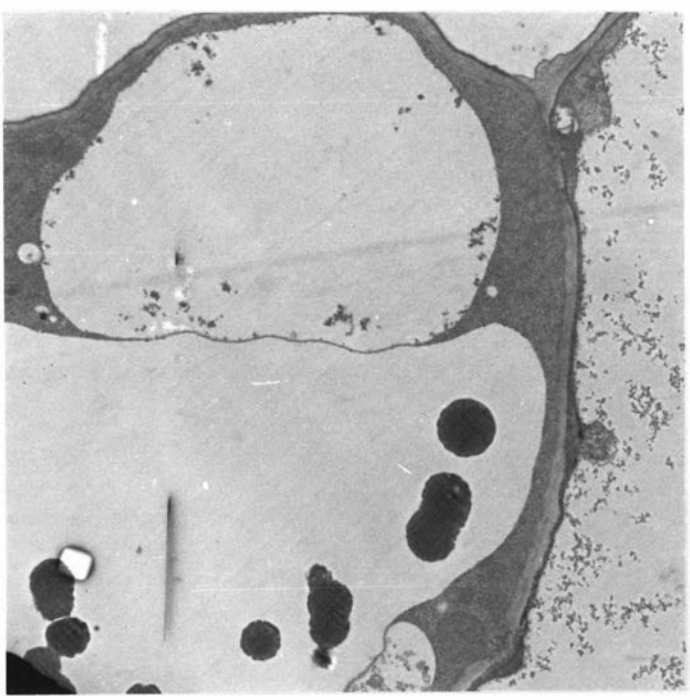




Plates 2-11 and 2-12

Cv. 'MM 106' root cortical cells, 1-2 cells in advance of hyphae, 5 hours after inoculation. The nucleus and almost all cellular organelles have been disrupted and electron dense material is present in the tonoplast. x5000.



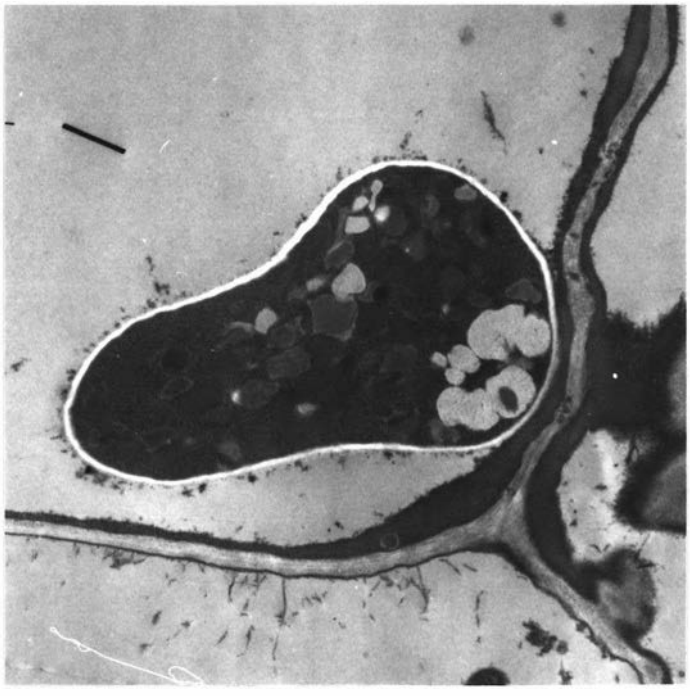


Plates 2-13 and 2-14

Intracellular mycelium in root cortical cells, 24 hours after inoculation.

Plate 2-13 Cv. 'MM 106'. x7,800.

Plate 2-14 Cv. 'M 793'. x5,000.



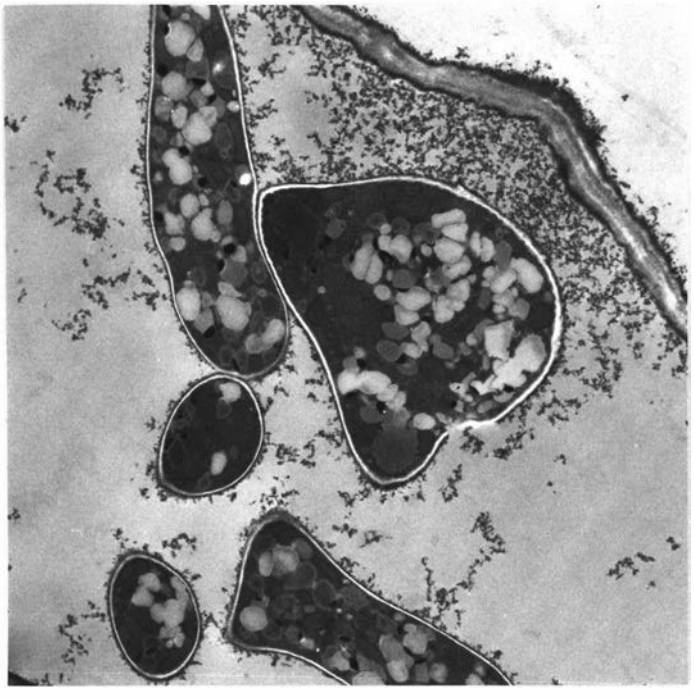
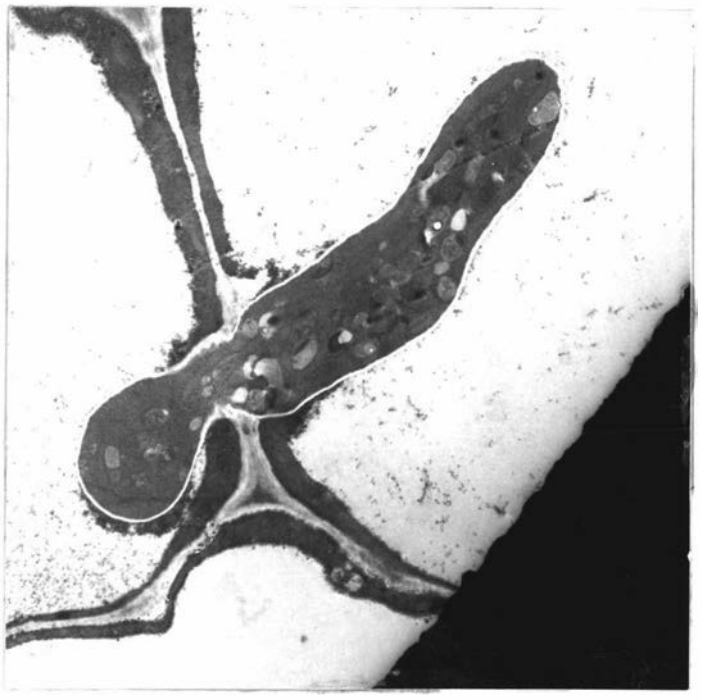


Plate 2-15

Intracellular penetration of a cv. 'MM 106' root cortical cell, 24 hours after inoculation. x5,200.

Plate 2-16

Close-up of Plate 2-15. The fibrillar nature of the cell wall is almost intact to the hypha itself. x15,300.



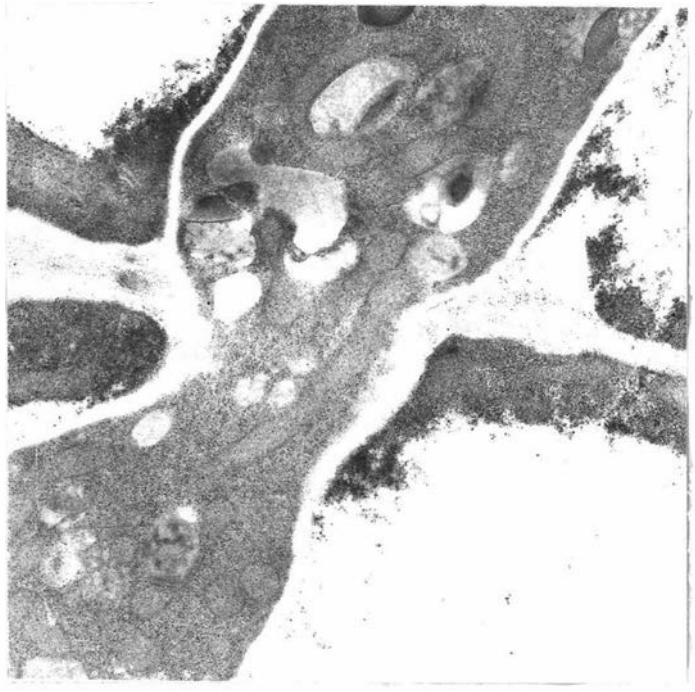
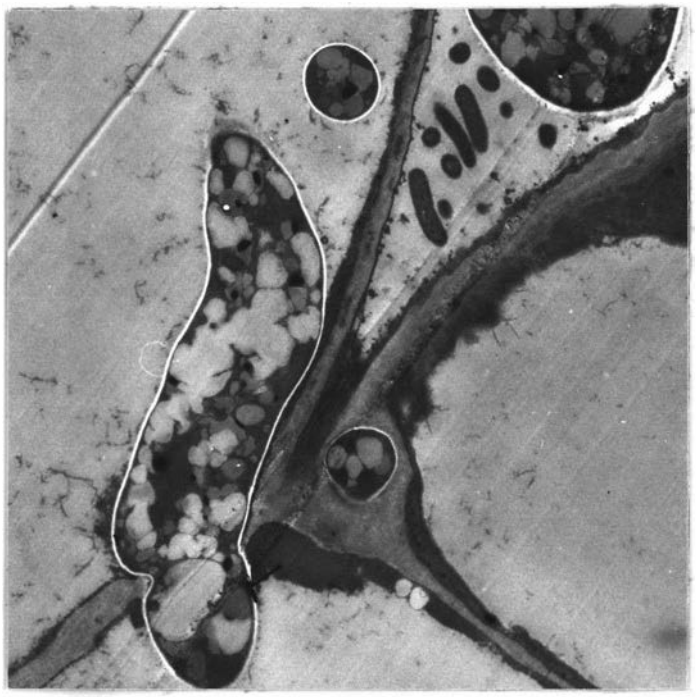


Plate 2-17

Intracellular penetration of a cv. 'M 793' root cortical cell, 24 hours after inoculation. x5,000.

Plate 2-18

Mechanical penetration of a cv. 'MM 106' root cortical cell wall, 24 hours after inoculation. Note the displaced cell wall material. x5000.



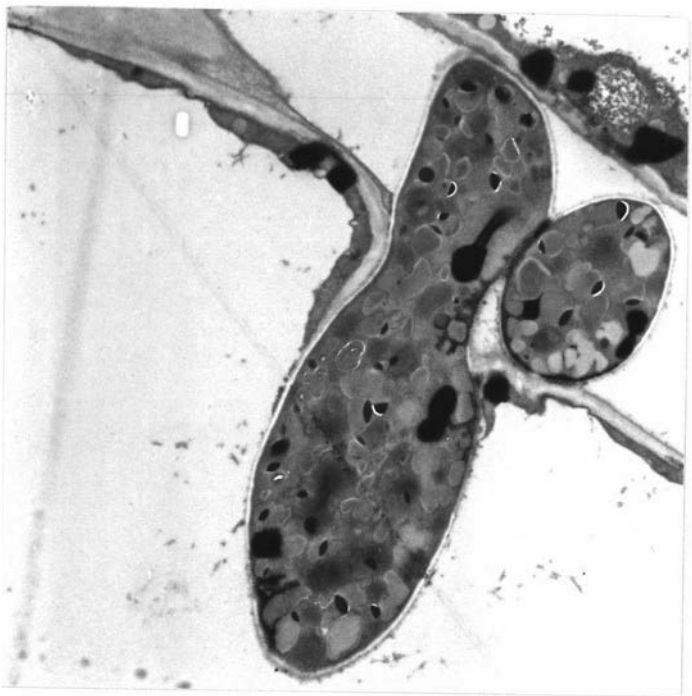
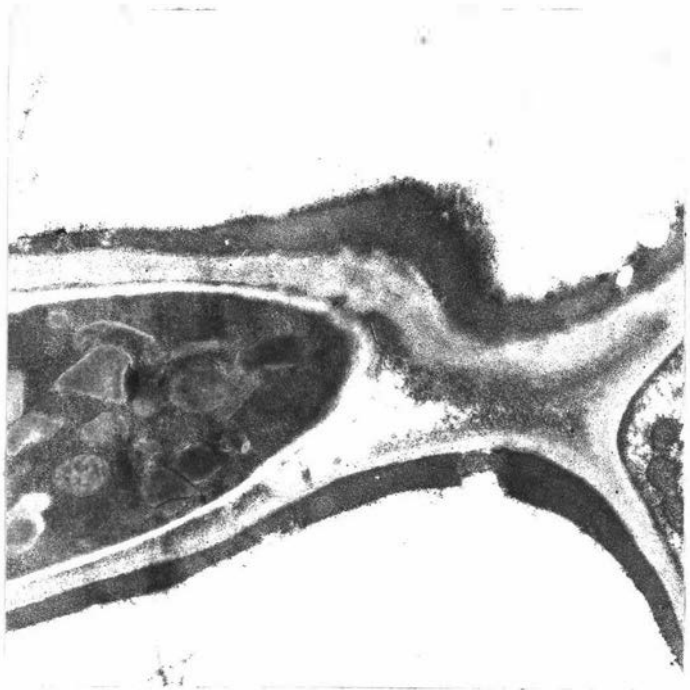


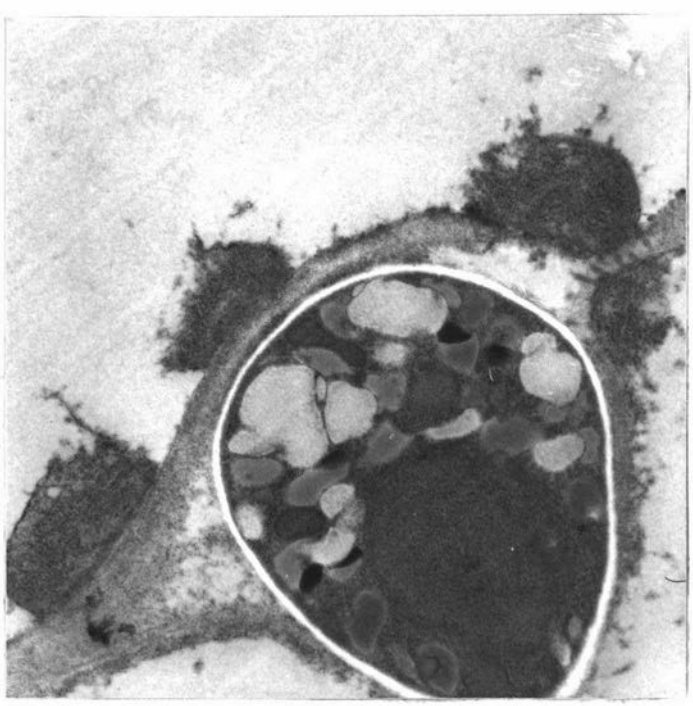
Plate 2-19

A hypha growing intercellularly between cv. 'MM 106' cortical root cells, 24 hours after inoculation. The cell wall has been distorted by the hypha and the middle lamella has been disrupted in advance of it. x11,200.

Plate 2-20

Mechanical penetration of a cv. 'M 793' root cortical cell wall, 24 hours after inoculation. The wall appears to have broken under stress. x19,200.



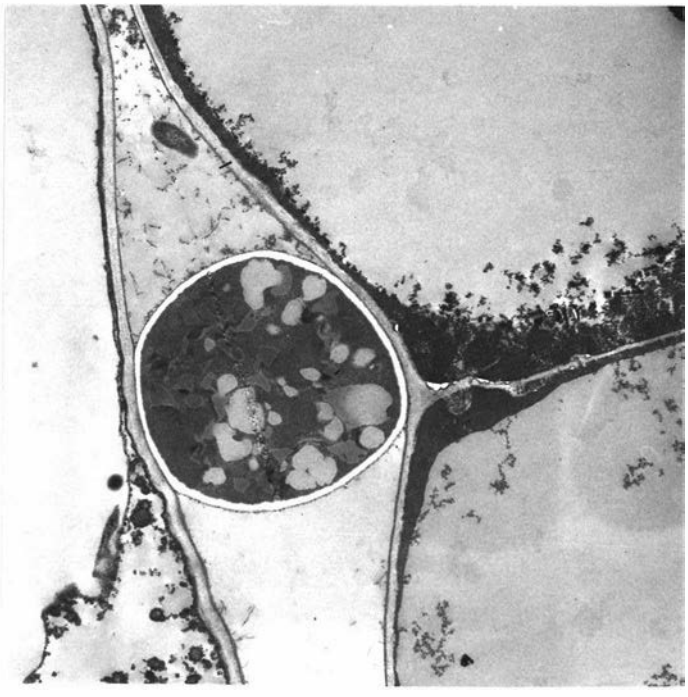


Plates 2-21 and 2-22

Intercellular mycelium between root cortical cells, 24 hours after inoculation.

Plate 2-21 Cv. 'MM 106'. x5,200.

Plate 2-22 Cv. 'M 793'. x8,200.



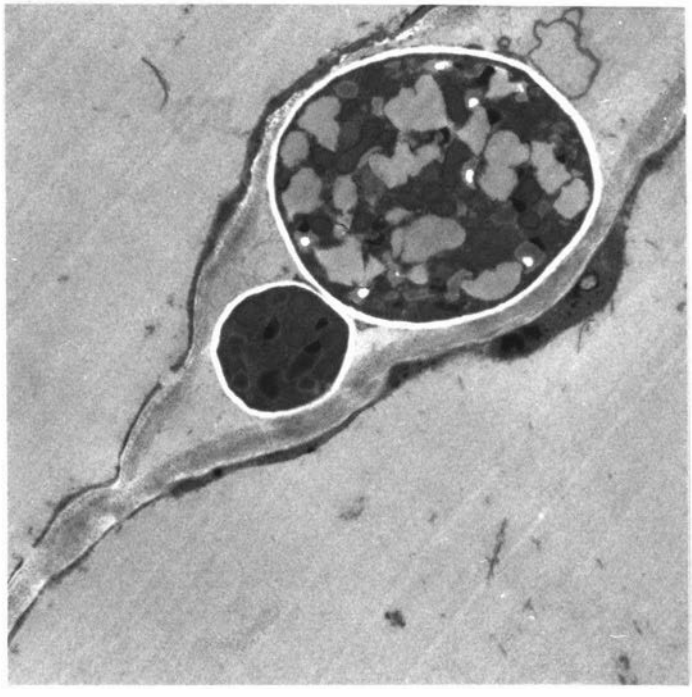
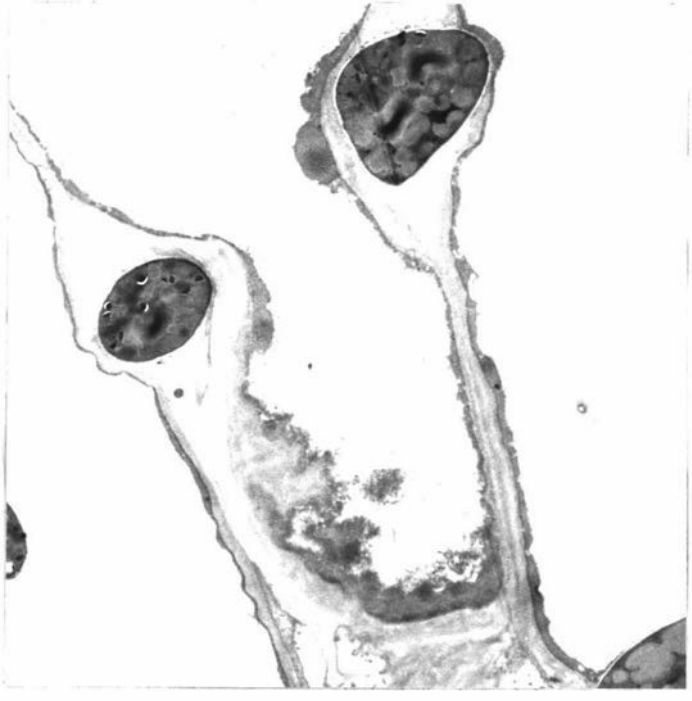


Plate 2-23

Intercellular mycelium in cv. 'M 793' root cortical tissue, 24 hours after inoculation. The cell walls appeared to have been forced apart along the middle lamella by the hyphae. x3700



2-4. DISCUSSION

P. cactorum has a necrotrophic mode of of parasitism . Host cells were visibly disrupted 2-4 cells in advance of hyphae and were necrotic when encountered by them . This indicates that comparatively powerful or abundant amounts of diffusive toxic metabolites were produced by the pathogen in apple tissue . These appear to play a major role in the pathogenesis of the disease .

P.cactorum is able to produce substances in liquid culture which cause apple twigs to wilt (Plich and Rudnicki, 1979) . Other *Phytophthora* species are also known to produce metabolites toxic to their host plants *in vitro* (Ballio *et al.*, 1972; Brieman and Barash, 1981; Keen *et al.*, 1975; Paxton, 1972; Woodward *et al.*, 1980) .

The histochemical studies showed that *in vivo* *P. cactorum* had little or no effect on major structural components of the cell wall i.e. cellulose, lignin and suberin . Degradation of some pectic substances in the immediate vicinity of the hyphae in apple stem tissue implies that the pathogen did produce pectic degrading enzymes in limited amounts (Section 2-3-2-2) . This would enable the intercellular hyphae to degrade the middle lamella and thus assist its growth between cells . The ultrastructural studies on root tissues showed that the middle lamella in this region of the root was thin and so it could not be determined whether the parting of cell walls by intercellular hyphae involved enzymes or not .

P. cactorum hyphae appeared to partially or fully hydrolyse cell walls at the site of wall penetration (Section 2-3-4) . This implies that the pathogen had a very limited production of a cell wall degrading enzyme(s) such as a cellulase or a hemicellulase at this site . The very localised nature of the wall hydrolysis suggests that the enzyme(s) may have been bound to the hyphal wall as suggested by Ingram *et al.* (1976) .

Wall degrading enzymes produced by *P. cactorum* thus appear to have a significant role in assisting fungal growth through apple tissue but

are relatively insignificant in the destruction of the host tissue . This is also noticable in the field, where disease lesions on either fruit, stem or root tissue remain firm and structurally intact .

Cells of the susceptible cultivar (MM 106) contained a greater amount of degenerated cytoplasmic contents than those of the resistant one (M 793) . Since the pathogen consumed little cell wall material, its main source of energy for growth *in vivo* appeared to be the cytoplasmic contents of the host cell . This indicates that the pathogen consumed more of the cytoplasmic contents in the cv. 'M 793' root tissue possibly because it had greater energy requirements .

The lack of apparent structural wound associated responses (e.g. papilla formation) to *P. cactorum* infection in apple tissue suggests that the mechanisms of resistance of the more resistant cultivars are primarily physiological .

Since the rate of cell death and fungal growth was similar between the apple cultivars tested, they do not appear to differ in their sensitivity to the fungal toxin(s) as has been found among some host cultivars of *Alternia* species (Nishimara and Kohmoto, 1983) .

The mechanism of resistance most likely to be operating in this case is a greater phytoalexin production in the more resistant apple cultivar . Studies have shown phytoalexins to have a role in host resistance to *Phytophthora* species in the *P.infestans* - potato leaf or tuber and in the *P. megasperma* var. *sojae* - soybean hypcotyl combinations (Keen, 1971; Sato *et al.*, 1971; Yoshikawa *et al.*, 1978) . The amount of phytoalexin produced in response to infection has been found to be greater in some resistant host cultivars than susceptible ones (Keen and Yoshikawa, 1983) . Resistance induced by phytoalexins is usually expressed within the first 24 hours after inoculation (Keen, 1971; Sato *et al.*, 1971; Yoshikawa *et al.*, 1978) . This is supported by morphological studies . Goode (1956), Halsall (1978) Malajczuk *et al.* (1977), Milholland (1975), Stossel *et al.* (1980) and Tippett *et al.* (1977) found that biotrophic, hemibiotrophic and necrotrophic *Phytophthora* fungi had a similar mode of penetration and early infection growth rate in both resistant and susceptible host

cultivars . After invasion of the first few cell layers, the hyphae became more abundant in susceptible tissue but decline in resistant tissue (Goode, 1956; Stossel *et al.*, 1980; Tippett and Malajczuk, 1979; Tippett *et al.*, 1977) . The pathogen probably grew through the first few cell layers because a short time was required to activate the host's defences .

Since a high inoculum loading was used in the ultrastructural studies to ensure that infection occurred in both cultivars, infection was probably greater than would have occurred in the field . Ultrastructural studies on apple roots infected with lower inoculum loadings and analytical studies on the phytoalexin production by the apple root (if any) at these levels of inoculum should provide further insight into the nature of resistance mechanisms in apple against *P. cactorum* .

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** CHAPTER 3 **
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Studies on resistance of apple shoots and roots to P. cactorum.

3-1. INTRODUCTION

3-1-1. Historical Development of Inoculation Methods of Assessing Resistance of Apple Trees to P. cactorum

Early workers (Baines, 1939 ; Buddenhagen, 1955; Fitzpatrick et al, 1944; Smith, 1955; Welsh, 1942) tested rootstocks and scions for varietal susceptiblity by artifical inoculation of apple trees in the field . Agar blocks containing P. cactorum mycelium were inserted under strips of bark which were then replaced and sealed to prevent desiccation of the inoculum . After a number of weeks the proportion of each tree girdled by the rot would be recorded . Other workers (Buddenhaggen, 1955; Sewell and Wilson, 1959; ten Houten, 1958) standardised the extent of bark wounding by using a cork borer to cut the bark .

This method required large numbers of trees and a considerable time before assessments could be made so cheaper, faster techiques were soon investigated . These faster methods consisted of the inoculation of excised pieces of the apple tree , usually twigs, but stem and roots (Buddenhagen ,1955; Sewell and Wilson, 1959; Smith, 1955), or apple seedlings (McIntosh and Mellor ,1953) were also used .

The excised twig method is based on the inoculation of wounded or exposed apple stem tissue using an agar plug containing fungal mycelium . The material is then incubated at a constant temperature (usually in the range 20-26 ^{o}C) and the lesion length or size is assessed after a selected time (4-45 days) . The trend has been towards shorter incubation times and smaller amounts of material .

The seedling method has not often been used to assess the resistance of scion varieties and rootstocks because of genetic heterogeneity among the seedlings . The method is usually used in breeding programmes to screen out plants with a high level of susceptibility to Phytophthora cactorum (Watkins and Werts, 1971) . The seedlings are inoculated by flooding seedling trays with a concentrated suspension of zoospores . The percentage of seedlings which survive are recorded and the plants retained for further evaluation .

3-1-2. Factors Affecting Results Obtained by the Excised Twig Method

(1) Isolate of the pathogen
(2) Tree age
(3) Type of tissue
(4) Seasonal susceptibility
(5) Environmental conditions

1. Isolate of the Pathogen

Isolates of P. cactorum differ both in their pathogenicity and in their specificity toward particular apple clones . Some isolates are more virulent than others and some grow relatively better or worse on the tissues of some varieties than of others (Aldwinkle et al, 1975; Bielenin, 1977A; Bielenin, 1977B ; Braun and Krober, 1958; Herb et al, 1975; ten Houten, 1958; McIntosh, 1975; Nienhaus, 1960; van der Scheer,1971; Sewell and Wilson, 1959) . Thus, the assessment of a particular variety's susceptibility can be influenced by the isolate

used .

2. Tree Age

Natural Infection

Natural infection of young apple trees in the field seldom occurs before they bear fruit . Once they commence cropping their susceptibility appears to increase markedly since the disease is found principally on bearing trees (Baines, 1939; Buddenhagen, 1955; ten Houten, 1958; McIntosh, 1968; McIntosh and MacSwan, 1966; Schwinn, 1965; Sewell and Wilson, 1973B; Smith, 1955) . Buddenhagen (1955) also noted that younger trees interplanted among older trees were not observed to become infected . This suggests that the bearing stress predisposes the tree to infection .

Artifical Infection

When trees of different varieties have been artificially infected in the field, frequently, no difference has been found between the susceptibility of young trees (1-6 years old) and that of more mature ones (10-35 years old) (Buddenhagen, 1955; ten Houten, 1958; Schwinn, 1965; Sewell and Wilson, 1973A) .

Bielinen (1977B) found that the tissue of younger trees was usually more rapidly colonised by the pathogen than that of older trees . Gupta and Singh (1979) also found the twigs of 5yr old trees to be more susceptible than their trunks .

In contrast, Krober and Karnatz (1979) and Gupta and Singh (1979) have found that younger trees (5 years or less) had a greater resistance to

the pathogen than older ones . Studies on susceptibility within the aerial parts of the tree showed that the trunks were consistently more susceptible than the younger branches (Baines 1939) . Schwinn (1965) found that 6 year old Cox trees were attacked only after wounding while older trees were attacked without wounding and concluded that bark conditions (eg. lack of cracking) rather than physiological factors were responsible for the field resistance of young trees .

The younger trees of some cultivars appear to be more resistant because of the structure of the protective outer tissues or altered physiological status due to bearing stress . The greater resistance of the older trees in other cultivars may be related to their vigour which is reduced with the onset of fruiting . The less vigourous rootstocks have been found to induce a greater resistance in the scion (ten Houten, 1958; Sewell and Wilson, 1973B) .

3. Type of Tissue

(a) Anatomy of Previous Season's Shoot

Previous season's shoots are those which were produced by the tree the previous year (one year old) . The outer layer of the shoot consists of a thin suberised periderm layer which is perforated by a number of lenticular openings . Under the periderm is the cortex the outer part of which is photosynthetic . The phloem is separated from the cortex by a ring of sclerenchyma fibres (phloem fibres) and from the xylem by a thin cambial layer .

Figure 3-1. The Anatomy of a Previous Season's Apple Shoot

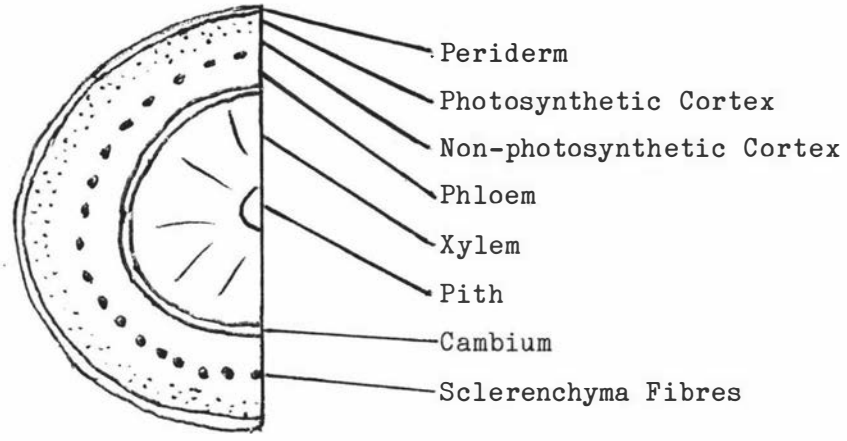


Diagram not to scale.

(b) Relative Susceptibility of Apple Stem Tissues

Table 3-I. Relative Susceptibilities of Different Apple Stem Tissues

Tissue	Susceptibility	references
Periderm	none	(c) (d)
Cortex	usually low	(a)
Phloem-cambium	low to high	(a) (b) (c)
Unlignified xylem	high	(c)
Lignified xylem	none	(c)

[(a) Borecki and Millikan, 1969; (b) Gates and Millikan, 1972; (c) Long, 1982; (d) Kober and Karnatz, 1979]

Borecki and Millikan (1969) were the first to test the susceptibilities

of different apple shoot tissues . They found the phloem-cambial tissue to be the most susceptible to the pathogen . Long (1982) noted that when unlignified xylem was present it had an even greater susceptibility .

4. Seasonal Variation in Susceptibility

Apple tissue does not show a uniform susceptibility towards P. cactorum throughout the year . Susceptibility is low when the tree is dormant but sharply increases in the period from early spring to bloom, the exact time depending on the variety and the climate .

In tests of rootstocks and varieties recorded in the literature, maximal susceptibility appears to occur at bloom (Bielenin, 1977 B; Borecki and Millikan, 1969; Gates and Millikan, 1972; Gupta and Singh, 1979; Harris and Tardivel, 1978; Harris et al., 1979; Kober and Karnatz, 1979; Long, 1982; Nienhaus, 1959; Sewell and Wilson, 1973A; Sewell and Wilson, 1973B) .

A number of workers have found several peaks of susceptibility with most of the varieties tested . The extent and duration of these peaks was usually greater in the more susceptible cultivars (Gates and Millikan, 1972; Bielenin, 1977B; Janisiewicz and Covey, 1982; Long, 1982; Sewell and Wilson, 1973A) .

The pattern of susceptibility varies somewhat from year to year (Bielenin, 1977B; Gates and Millikan, 1972) .

5. Environmental Conditions

(a) Temperature

In the field, the fungus is only active during the warmer months (Sewell and Wilson, 1973B; Sewell et. al., 1974) . The minimum

temperature for P. cactorum growth has been reported as being 2°C (Riberio, 1978) . Sneh and McIntosh (1974) found that P. cactorum mycelium formed sporangia in soil at 10°C and above but not at 4°C .

(b) Moisture

Good disease development in the field and laboratory requires moist conditions (McIntosh, 1964; Robertson and Dance, 1971; Schwinn, 1961; Sewell et al, 1974; Smith, 1955; van der Scheer, 1971; Welsh, 1942; Woodhead, 1957) . Fungal growth in infected apple twigs incubated at various humidities (32% to 90%) in the laboratory was greater at the higher air humidities . (Gupta and Singh, 1979) . Sewell et. al. (1978) found that the amount of moisture which accumulated at the trunk base was up to 40 times greater than that beneath the foliage canopy or in open ground . Such conditions could explain the prevalance of the crown rot form of the disease .

3-1-3. Evaluation of Assessment Techniques

The Accuracy of Cultivar Field Resistance Determination

The field resistance of the cultivars recorded in the literature were determined by observation and survey, especially during collar rot epidemics in or prior to the 1950's . Since then, extensive orchard plantings have been made with the graft-unions raised above soil level to avoid scion variety infection . Natural infection of the scion now seldom ocurrs and so the disease in the field sitation is largely confined to rootstock tissue and hence the majority of the later reports refer only to the field resistance of rootstocks .

Awareness of the importance of this pathogen has also increased and this has resulted in a greater detection of infected material, especially the less obvious crown rot and root rot forms of the disease .

The persistant difficulty in isolating and consequently identifying the

pathogen has probably caused inaccurate reports of disease occurrence when assessed by symptoms alone . Julis et. al. (1978) isolated Phytophthora cambivora as well as P. cactorum from lesions on Malling and Malling-Merton rootstocks . Robertson and Dance (1971) found Phytophthora megasperma to be the major causal organism of a crown rot epidemic of apple trees which occured in Hawkes Bay (N.Z.) in 1968-69 . The symptoms were similar to those of P. cactorum previously described by Welsh (1942) and Smith (1955) . Phytopthtora cryptogea, P. cinnamomi, P. drechsleri, P. citricola, and Pythium ultimum are also known to cause symptoms similar to those of P. cactorum (McIntosh, 1975) .

Even when the disease is caused by P. cactorum, the various isolates involved may not give a consistent reaction on any one individual variety . Bielenin (1977A), Herb et. al. (1975), and Sewell and Wilson (1959) found that differential interactions occured between cultivars and rootstocks and some isolates . Thus when a variety is grown in one particular place it may be considered to be resistant, but when exposed to a different isolate in another place the variety may be considered susceptible . This may be the situation with reports of the resistance of M7, MM 104, and MM 111 rootstocks (McIntosh, 1975) .

Another problem in the accurate determination of the field resistance of a specific rootstock relates to the form of the disease . Collar rot is a disease of stem tissue whilst crown and root rots are pathogenic invasions of root tissue . Artifical inoculations have shown that the susceptibility of the root and stem tissues of the same plant can differ markedly (cf. Section 3-7; Smith, 1955) . A further complication is that there is little information on the relative virulence of specific isolates on both stem and root tissue . It is possible that a given rootstock may vary in its susceptibility to collar and to crown rot .

Accuracy of the Artifical Inoculation Techniques

Artifical inoculation of apple stem tissue in the field and in the laboratory is often assumed to reflect the susceptibility of that

cultivar to infection in the field .

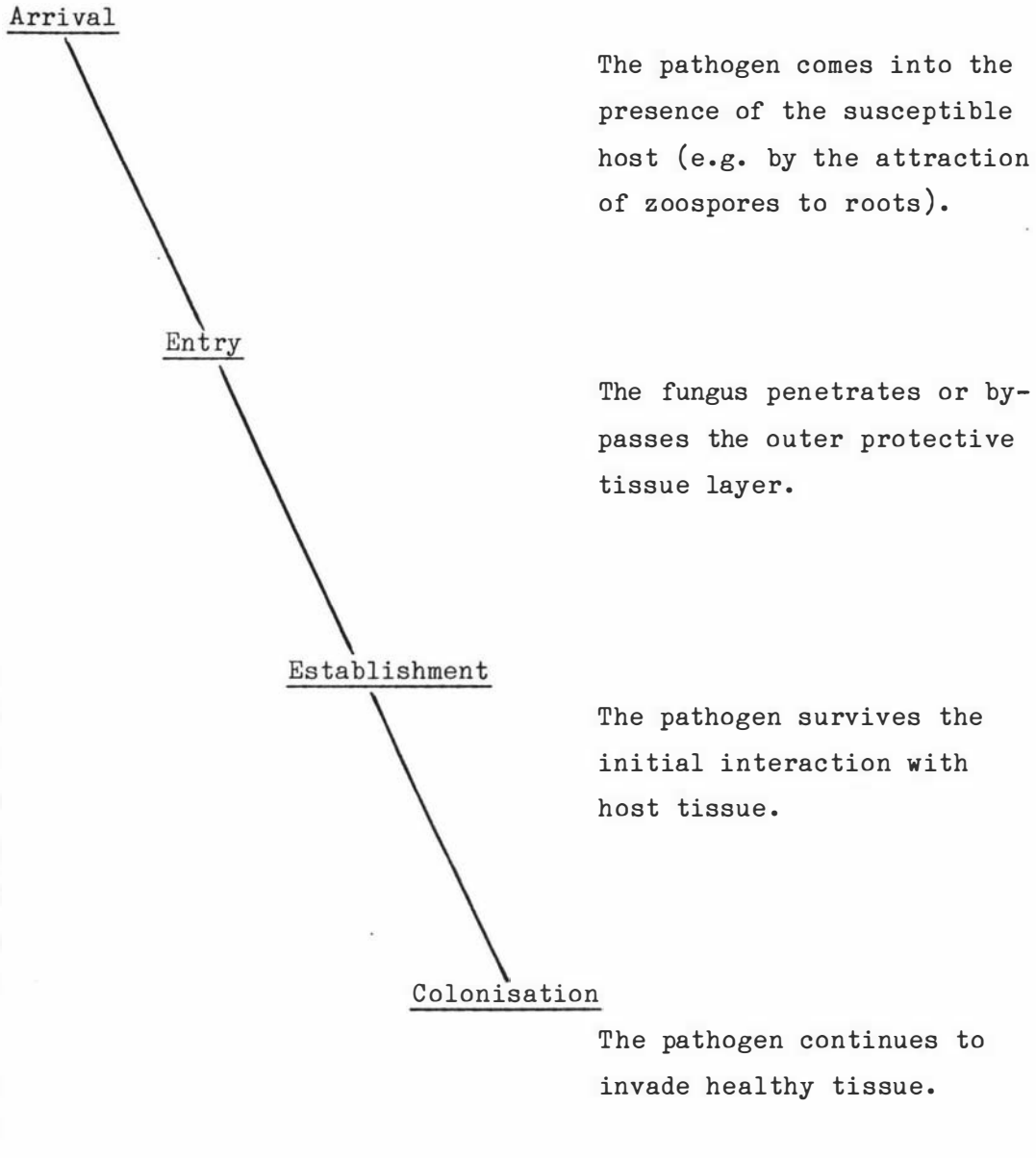
In the natural situation, the pathogen must first penetrate the periderm layer of either the root or trunk to encounter susceptible host tissue . Penetration may be direct or indirect via a wound or natural break in this tissue . The pathogen's ability to grow through the intact periderm or the frequency of wounding of this tissue will be related to its mechanical strength and chemical composition . The mechanical strength can vary from one variety to another (Alt and Schmidle, 1980; Schwinn, 1965).

This barrier is broken by artifical inoculation in the field or in the laboratory and the susceptible host tissue is directly exposed to the pathogen . The lesion which develops gives a measure of the inherent resistance of the exposed tissues . The testing of each specific stem tissue (cortex, phloem-cambium or xylem) gives an estimation of the inherent resistance of that particular tissue . The limitations of the artifical inoculation methods must be borne in mind as the periderm can provide an effective barrier to infection as shown by Schwinn (1965) and in Section 3-5 .

The sequences of the infection process which artifical and natural infection methods assess are illustrated in Figure 3-2.

Artifical inoculation techniques, especially the laboratory methods, have often been found to lack precision (Bielenin, 1977B; Herb et. al, 1975; Jeffers et al., 1981; Krober and Karnatz, 1979; Long, 1982) . A large variability in resistance occurs within each sample so that separation of cultivars becomes difficult . Thus artifical inoculations are only of value in classifying cultivars into broad resistance groupings .

Figure 3-2. Sequences in the Infection Process



* Natural infection assesses these stages.

\# Artifical infection assesses these stages.

Table 3-II. Summary of some Cultivar Resistances recorded in the Literature.

Cultivar	Natural Infection	Field Inoculation	Laboratory Inoculation
Baldwin	MR (4)	MR (4, 14)	HR (11)
Boskoop	# R (7)	MS (14)	
Cox's Orange Pippin	HS (8, 9, 16, 20, 21, 23)	HS (14, 20, 21)	HS (2, 10, 19)
Delicious	R (4, 18, 20) 23)	R (4, 20)	R (11, 15, 20)
Geheimrat Oldenburg	# R (7)	MS (14)	
Golden Delicious	R (4, 18)	R (4) MR (14)	MR (9, 11)
Goldparmane	# R (7)	MS (14)	
Granny Smith	R (23) HR (20)	R (20) HR (14)	R (14)
Grimes Golden	HS (4)	S (4)	HS (11) S (15)
James Grieve	@ R (21) HS (7, 8) S (9)	MR (5, 14) R (21)	MR (2, 5)
Jonathan	R (4, 18, 23)	MR (14, 21) R (4, 5)	MR (5, 9, 11)
Kidd's Orange	MR (20)	MR (14) MS (20)	
McIntosh	@ R (6, 9, 17, 18)	MR (4, 14) MS (5)	HS (11) MS (9, 20) R (15)
Northern Spy	R (4)	R (4, 5, 19, 20)	MR (2, 10, 11) R (22)
Oratia Beauty (Gravenstein)	R (23)	MR (14)	
Rome Beauty	@ S (4)	S (4, 20)	R (11)
Sturmer	# MR (18, 20, 23)	HS (20) MS (14)	
Winesap	R (4)	R (4)	R (15, 20)
Yellow Transparent	@ R (4) S (17)	MR (21) R (4) MS (5)	
Zuccalmaglious Renette	# R (7)	HS (14)	HS (14)

Table 3-III. Summary of some Rootstock Resistances recorded in the Literature.

Rootstock		Natural Infection	Field Inoculation	Laboratory Inoculation
M 2	@	MR (3, 8, 16, 18) R (19)	MR (20, 21) R (19)	MR (10, 19) S (15) MS (20)
M 7	@	MR (8, 13, 16, 18) S (16)	HR (19, 21) MR (1, 12)	HR (10, 19) S (15)
M 9		HR (3, 16, 18) MR (8, 13)	R (19)	MR (12, 15, 19)
M 26	@	HR (16) MR (16) S (8, 13, 18)	MS (19)	MS (1, 12, 19)
MM 104	@	MR (16) S (16, 18) HS (8)	MS (19)	MS (10, 19, 20) S (15)
MM 106**	*	S (3, 11, 13, 16) MS (8, 16)	HR (19)	MR (10, 19) MS (12, 15)
MM 109	@	HR (8) S (16, 18)	MR (19)	MR (10, 19) MS (20)
MM 111	@	HR (3, 8, 16) MS (13, 16, 18)	HR (19)	HR (10) MR (19, 20)
M 779	@	sS (22)	R (20)	MS (20, 22)
M 789		sS (22)	S (20)	S (20, 22)
M 793		sS (22)	HR (20)	HR (22)

S=susceptible, R=resistant, M=moderate, H=high, sS=some susceptiblity
Comparison of tests from natural infection to artifical inoculation~:
results consistent across tests.
* S -> R
# R -> S
@ no consistent trend.

(1) Aldwinkle et. al, 1972; (2) Altson, 1970; (3) Anomyous, 1978; (4) Baines, 1939; (5) Bielenin, 1977B; (6) Borecki et al., 1970 (7) Braun and Nienhaus, 1959; (8) Engel, 1977; (9) Gates and Millikan, 1972; (10) Harris et al., 1979; (11) Herb et. al., 1975; (12) Janisiewicz and Covey, 1982; (13) Julis et. al., 1978; (14) Krober and Karnatz, 1979; (15) McIntosh, 1963; (16) McIntosh, 1975; (17) McIntosh and Mellor, 1954; (18) Penn State Horticultural Reviews, 1982; (19) Sewell and Wilson, 1959; (20) Smith, 1955; (21) ten Houten, 1958; (22) van der Merwe and Matthee, 1973; (23) Woodhead, 1957.

**In 1979, over 60% of the trees on cv. 'MM 106' rootstock died of P. cactorum infection in one block of the Massey orchard but no loss occured with the adjacent trees on 'M 793'. Cv. 'M 793' was resistant and cv. 'MM 106' was susceptible to the isolates 'MU1' and 'MU2' which were isolated from these diseased 'MM 106' rootstocks.

In the table of cultivar resistances (Table 3-II) there is a lack of agreement between the natural situation and field inoculations and laboratory inoculations with cv. 'McIntosh', 'Rhode Island Greening' and 'Rome Beauty' . The cultivars 'Baldwin' and 'James Grieve' are reported to be susceptible in the field but show resistant reactions when artifically inoculated while cv. 'Boskoop', 'Geheimrat Oldenburg', 'Goldparmane', 'Sturmer', and 'Zuccalmaglious Renette' show the opposite trend . Disagreement between the two types of artifical inoculations techniques occurs with cv. 'McIntosh', 'Rhode Island Greening' and 'Rome Beauty' (Table 3-II) .

The table of rootstock resistances (Table 3-III) shows a less coherent picture . A confusion or lack of agreement occurs in 7/10 cases between the natural infection and laboratory inoculations (M 2, M 7, M 26, MM 104, MM 106, MM 109 and MM 111) . A considerable part of the confusion is due to the conflicting reports on the field resistance of the cultivar involved (i.e. M 7, M 26, MM 104, MM 109, and MM 111) .

Comparison between Natural Infection and Artifical Infection Techniques

Tables 3-II and 3-III show the resistances of some cultivars and rootstocks as recorded in the literature . They also show that artifical inoculation methods are more consistent between themselves than when compared to natural infection . The discrepancy suggests that environmental factors can have a substantial effect on varietial resistance at times .

No matter which artifical inoculation method was used a similar proportion of cases of assessed resistance (at least 40%) did not match that of natural infection . The existence of such a large discrepancy between the different types of assessment methods shows that it is dangerous to extrapolate the field resistance of any variety from results based on artifical inoculation methods alone .

It should be noted, however, that the varieties 'Baldwin', 'Cox's

Orange Pippin', 'Delicious', 'Golden Delicious', 'Grimes Golden', 'Jonathan', 'Northern Spy', 'Oratia Beauty' and 'M 9' displayed a consistent reaction for both natural infection and artifical inoculation methods .

Summary

It is obvious that the value of the present artifical inoculation methods in assessing varietial field resistance is limited . In at least 40% of comparable cases recorded in the literature, results obtained by these methods differ markedly from that observed in the field .

Field observations must also be treated with caution since recommendations of what rootstocks are resistant for one area may not apply in another .

Although the above constraints show that the value of artifical inoculation methods is limited in differentiating between cultivars, they were used in this portion of the work to determine the resistance of specific host tissues for the purpose of investigating possible correlations with levels of endogenous substances in these tissues and to understand host resistance on a whole plant basis by determining the resistance of various plant components and their inter-relationships .

3-2. MATERIALS AND METHODS

3-2-1. Seasonal Susceptibility of Artifically Inoculated Apple Stem Tissues to P. cactorum

Collection of Plant Material for Laboratory Inoculation or for Tissue Analysis

All trees used in this work were located in the Massey Orchard and were assessed by a laboratory inoculation test . Details of the varieties sampled during the 1980-81 growing season are given in Table 3-IV.

Table 3-IV. Apple Varieties Sampled in the 1980-81 Growing Season

Variety	Age (years)	Rootstock	Scion Susceptility
Cox's Orange Pippin	7	EM XII	susceptible
Oratia Beauty	21	M 779	moderately resistance
Oratia Beauty	3	MM 106	moderately resistance
Granny Smith	21	M 793	resistant

Samples were always collected between 8:00 a.m. and 10:30 a.m. to avoid variations in the composition of shoots due to diurnal fluctuations .

Previous season's shoots were taken from at least five trees of any one variety at each sampling date . Sampling was carried out at approximately fornightly intervals during the growing season .

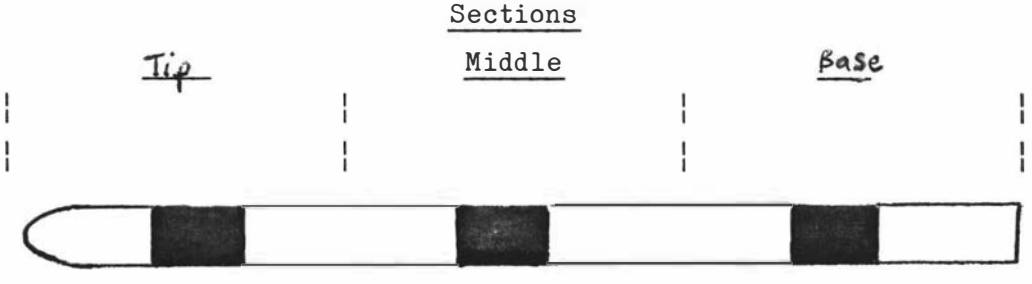
Assessment of Resistance

Shoots were cut into 8 cm lengths . Dormant shoots were prepared by the method of Borecki and Millikan (1969) except that the amount of stem pared off with a scapel was varied according to whether the xylem, phloem-cambium or cortex was to be tested .

When the trees became active in the spring, the cambial layer enlarged . A line of weakness freqently occurred between the unlignified xylem and cambium cells . The xylem and phloem-cambial tissues were thus easily peeled apart as described by Long (1982) . Although, the cambium proper is often regarded as a single layer of cells, it is impossible to distingish between the cambium and the young differentiating elements on each side of it (Catesson, 1974) . The cambial cells adjacent to the unlignified xylem were therefore considered to be cambium rather than undifferentiated xylem tissue .

During the 1981-82 growing season the testing procedure was slightly modified to investigate possible gradients of resistance present in apple shoots . Previous season shoots were divided into three sections as shown in Figure 3-3 .

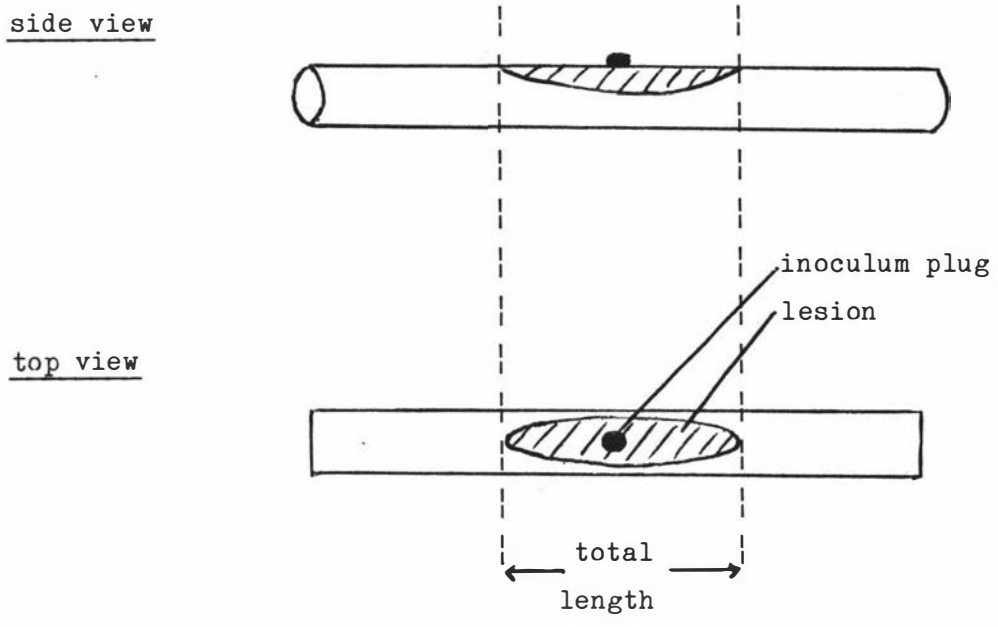
Figure 3-3. Diagram of the Subdivision of Apple Shoot Samples Collected during the 1981-82 Growing Season



Shaded areas represent pieces tested for resistance . Diagram not to scale .

Pieces of shoot with exposed cortex, phloem-cambium or xylem tissues were placed on polystrene supports in plastic boxes with air-tight lids (Clearseal No. 931, Downing Plastics Ltd.) . The bottom of each box was covered with a thin layer of deionised water to provide a saturated atmosphere (Plate 3-1) . The tissues were then inoculated with a five mm diameter plug cut from cultures of the pathogen (isolate P149) growing on Difco Cornmeal Agar (DCMA, 17g/l) . After four days incubation at 20 °C, the length of the disease lesion was measured to determine the pathogen's growth through host tissue (Figure 3-4) .

Figure 3-4. Diagram of Lesion Measurement on an Inoculated Shoot Piece



Length of growth of pathogen through host tissue = total length of the lesion minus the diameter of the inoculation plug .

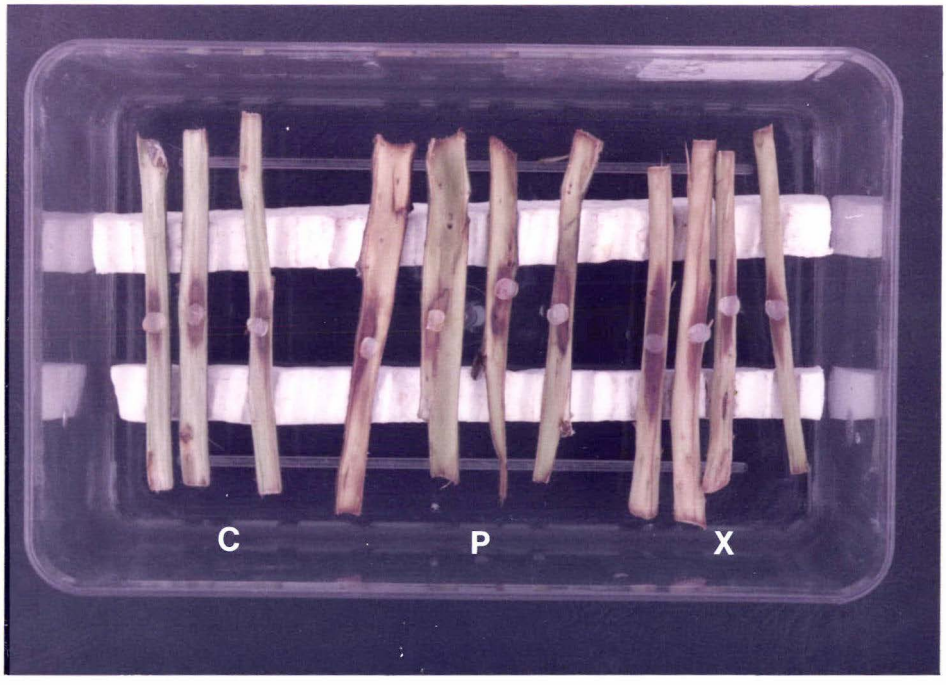
There were ten replicates per treatment .

Cortical and phloem tissues required for tissue analysis (Chapter 4) were removed from shoots, sliced up and immediately frozen in liquid air . If young, unlignified xylem was present, it was removed from the

Plate 3-1

Apple tissues 4 days after inoculation with *P. cactorum*.

C = cortex, P = phloem, X = xylem.


C
P
X

lignified xylem and immediately frozen . All the tissue samples were freeze-dried for 72 hours and stored in sealed containers at -15 °C until analysed .

3-2-2. Effect of Wounding on Apple Stem Tissue Susceptibility

In autumn, shoots from four different varieties of apple (Cox's Orange Pippin, Oratia Beauty, Granny Smith and Golden Delicious) were cut into 8 cm lengths and wounded to various depths using a scalpel . There were four methods of preparing the shoots :

1) intact shoots;
2) shoots with a small area of the periderm removed to expose the cortex;
3) shoots with a small area of the periderm and cortex removed to expose the phloem-cambium;
4) shoots with a small area of all bark tissues removed to expose the xylem .

These wounds were inoculated with 5 mm diameter plugs from cultured colonies of P. cactorum 'P149' growing on DCMA . Although the wound size was variable, it was always larger than the inoculum plug in order to contact the exposed tissue . After 8 days incubation at 20 °C in humid plastic boxes, the periderm was removed and the lesion size was recorded . There were ten replicates per treatment .

3-2-3. Relationship between Stem and Root Resistance of Rootstock Material

Two year old MM 106 rootstocks were grown outside in planter bags . Their stem and root resistance was assessed at fornightly intervals from bud burst . The rootstock stems were cut into 8 cm long pieces and tested as previously described (Section 3-2-1) . The largest, oldest roots were selected for assessment to give root material of a

similar age to that of the stem tissue . These woody roots had a similar structure to the stem . The outer suberised layer of the roots was pared off with a scapel and the underlying tissues were inoculated, incubated and assessed as described in Section 3-2-1 . The small size (usually >0.5 cm diameter) of the roots made it very difficult to determine whether it was the phloem or the cortical tissue which was exposed and infected . For this reason no distinction was made between these root tissues . Six plants were used per sampling date .

3-2-4. Stability of Pathogenicity of P. cactorum 'P149' in vitro

Stock cultures of P. cactorum were maintained on Difco Cornmeal Agar (DCMA, 17g/l) at 24 °C and subcultured at fornightly intervals . At the beginning of each growing season, isolate 'P149' was used to infect apple tissue and reisolated to culture via an apple fruit .

To test stability of pathogenicity in culture colonies of P. cactorum were prepared as follows :
Treatment 1 : a stock culture maintained and subcultured on DCMA for over 10 months .
Treatment 2 : an agar plug taken from treatment 1 and used to infect apple tissue and reisolated as described above .
Each treatment was subcultured to 5 plates of DCMA and the colony edge was marked on the bottom of the dish when the resultant colonies had grown halfway to the edge . Some inoculum plugs were taken from the marked zone immediately and some again after 16, 24, 28 and 30 days; and were used to further inoculate a series of dishes of DCMA (5 plates each) which were then incubated at 24 °C for 32, 16, 8, 4 and 2 days respectively . Plugs (5 mm diameter) were taken adjacent to the original inoculum plugs to give mycelial inoculum of the appropriate age . Previous season apple shoots were collected from a single 24 year old Richared (on EM XIII) apple tree and were cut into 8 cm pieces as previously described (Section 3-2-1) . The phloem-cambial tissue of ten randomly selected shoot pieces were infected and incubated at 20 °C . The lesions caused by the fungus were small after 4 days incubation (3 - 20 mm), so the phloem-cambium tissue was assessed at 11

days in order to accentuate any possible differences between treatments .

3-2-5. Mycelial Growth of P. cactorum, in culture, under Reduced Oxygen Conditions

Since disease development appeared to be more rapid in exposed apple tissues than in intact twigs where oxygen levels levels could be lower, the effect of reduced oxygen levels on the pathogen's growth was investigated in culture .

Agar strips colonised by the pathogen (isolate P149) were used to inoculate plates of DCMA (17g/l) (Figure 3-5) . After 5 days incubation at 24 ^{o}C, the colony edge was marked on the underside of the petri dish with a felt pen and a sterile coverslip was placed over it to reduce oxygen availability to the mycelium that was to grow beneath it . The growth of the mycelium was recorded 3 days later.

3-3. RESULTS

3-3-1. Seasonal Susceptibility of Artifically Inoculated Apple Stem Tissues to P. cactorum

During both growing seasons, in the phloem-cambial tissues a broad peak of susceptibility was formed either in late Janu ary (1980-81) or in late December - mid Janu ary (1981-82) (Figures 3-6 and 3-8) . Prior to this, other smaller peaks occurred in all but the resistant cv. 'GS' (Figure 3-6) . These peaks of susceptibility were more prominant during the 1981-82 season when the base level of susceptibility was higher for this tissue .

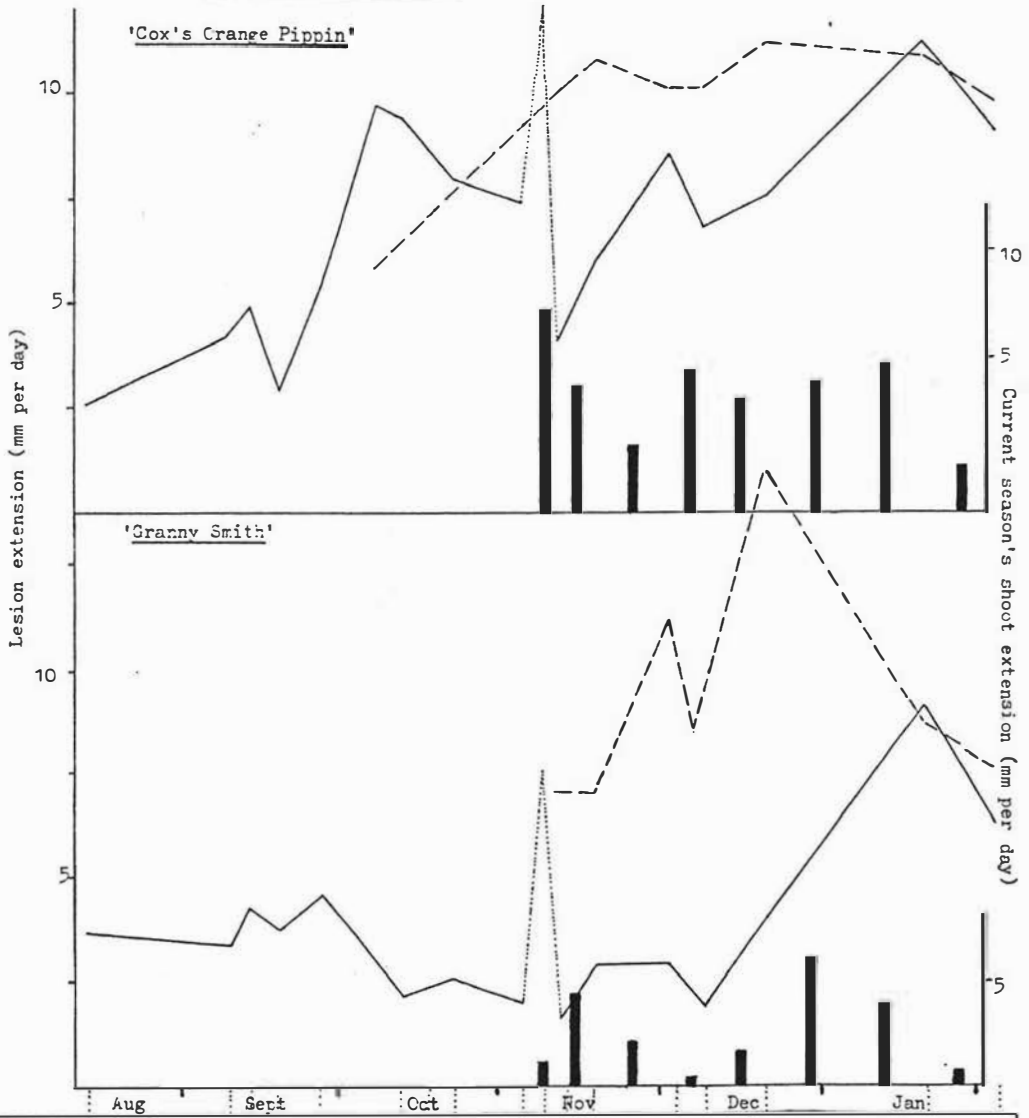
The peak in mid-November (Figure 3-6) for all the tested cultivars appeared to be anomalous since susceptibility rose and dropped rapidly and simultaneously for all tested cultivars . The reason for this is not known as no unusual weather conditions occurred at this time . A broader maximum did however occur at this period the following season for the 4 year old 'OB' and 'GS' cultivars (Figure 3-8) . This maximum is denoted on Figure 3-6 by points in the discontinuation of the line graph .

The seasonal pattern of susceptibility of the unlignified xylem tissue was the same between the seasons and the varieties tested (Figures 3-6 and 3-9), with the exception of the cv. 'GS' in 1980-81 (Figure 3-6) . Susceptibility increased sharply in October to a peak in mid-November which dipped noticeably in mid-December and then rose to another maximum before dropping to a very low level in late January - early February .

The seasonal susceptibility of the cortical tissue followed the same pattern for both tested cultivars (Figure 3-7) . In spring, minor peaks of susceptibility occurred before rising to a large peak (November to mid-December) . The susceptibility of the cv. 'OB' base and middle shoot sections remained low during this maximum . A steep decline in susceptibility followed reaching a minimum in mid-December

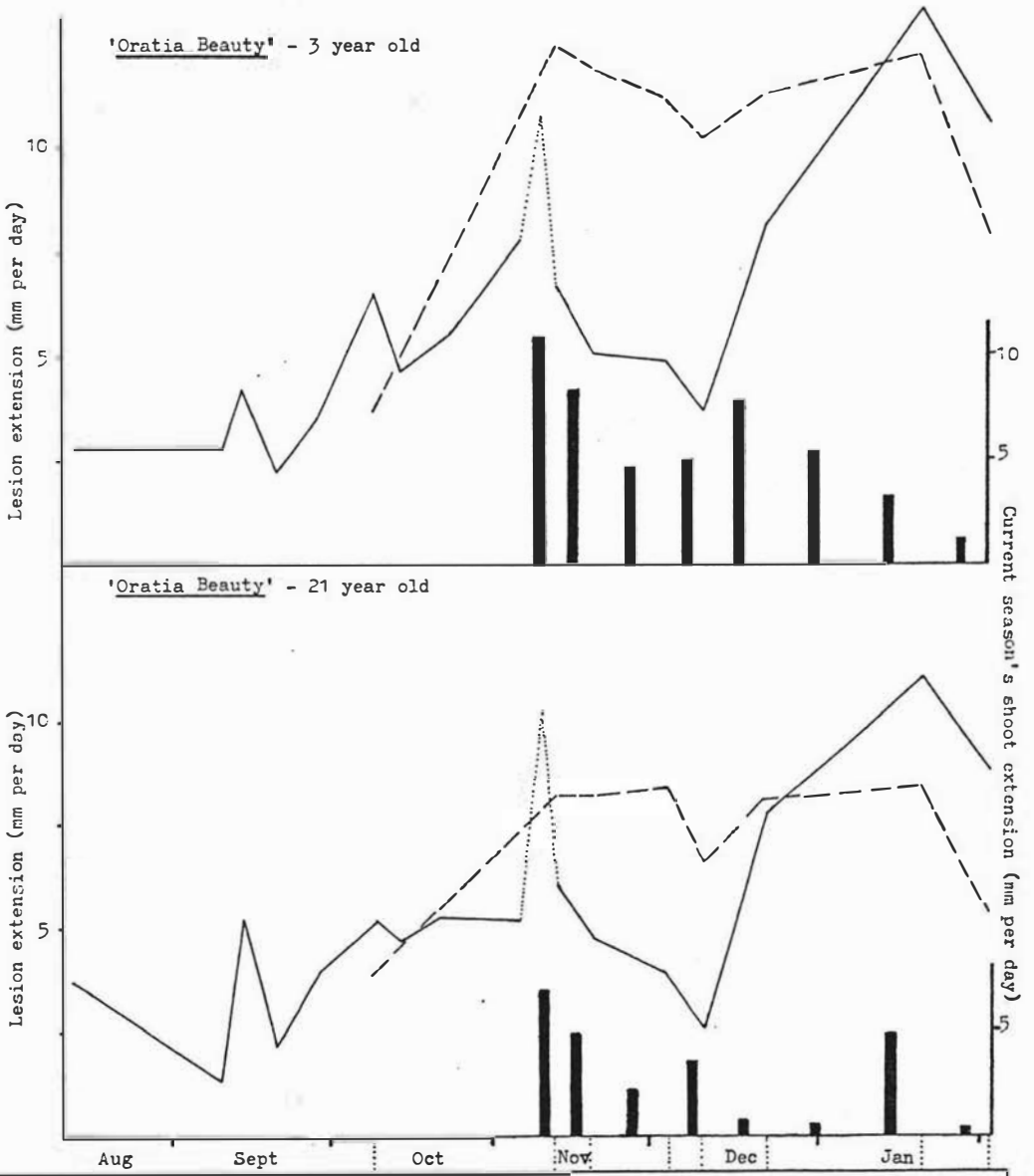
and increased to another maximum in late December to mid-January . The susceptibility then decreased almost to zero after harvest for the cv. 'OB' (5th. February) and to a little more for the later maturing cv. 'GS' . The susceptibility of the cortical tissues remained at these

Figure 3-6. The Susceptibility of the Phloem-cambial and Unlignified Xylem Stem Tissues of Three Apple Cultivars to P. cactorum 'P149' during the 1980-81 Growing Season.*



Phloem-cambial tissue																							
COP	b	a	a	a	a	a	a	a	a	b	b	a	a	a				b		b			
GS	a	b	b	a	b	d	c	c	c	c	c	a	c	c	b				c		c		
OB (3)	b	b	b	b	c	b	b	b	a	b	a	c	b	b	a				a		a		
OB (21)	a	c	a	b	c	c	b	c	b	b	a	c	b	bc	a				b		b		

Treatments within each column (sampling date) with the same letter are not significantly different from each other at the 5% level (Duncan's Multiple Range Test).

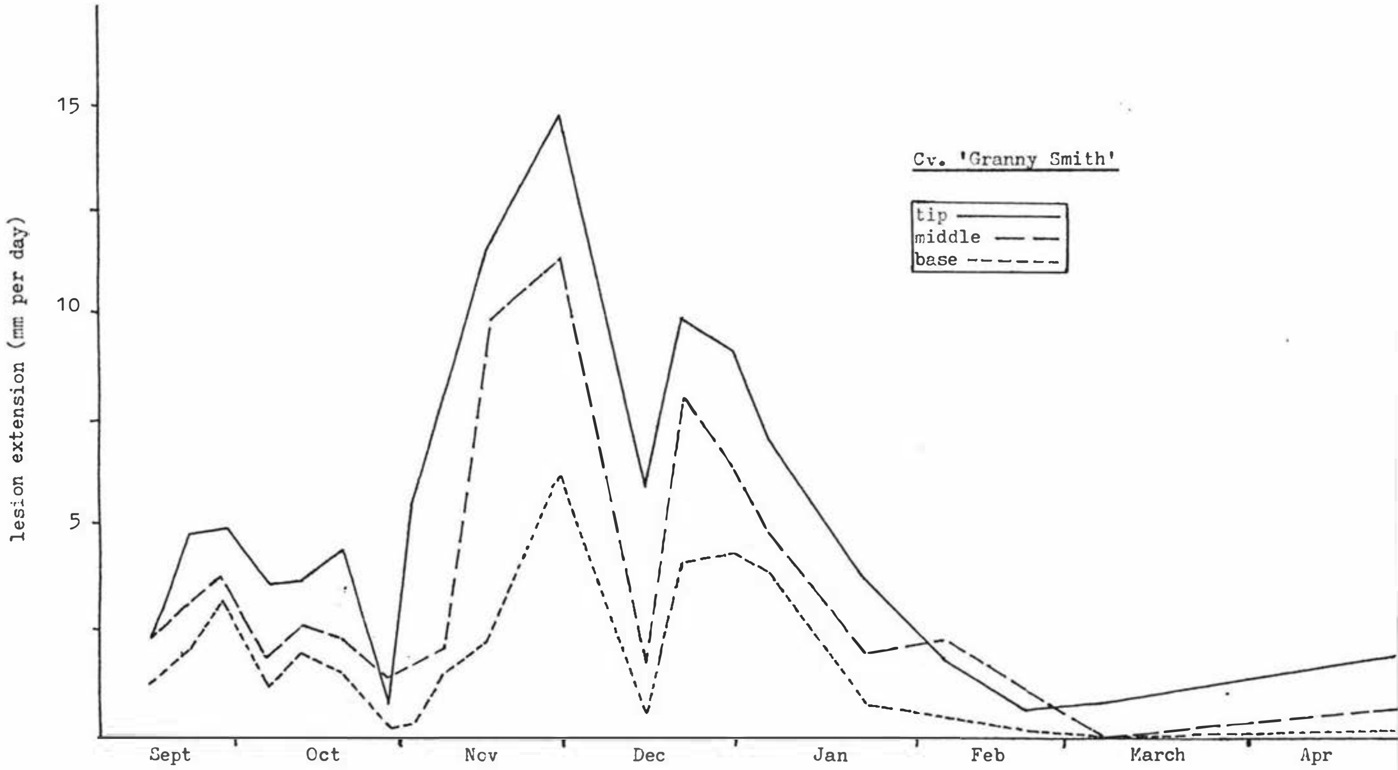


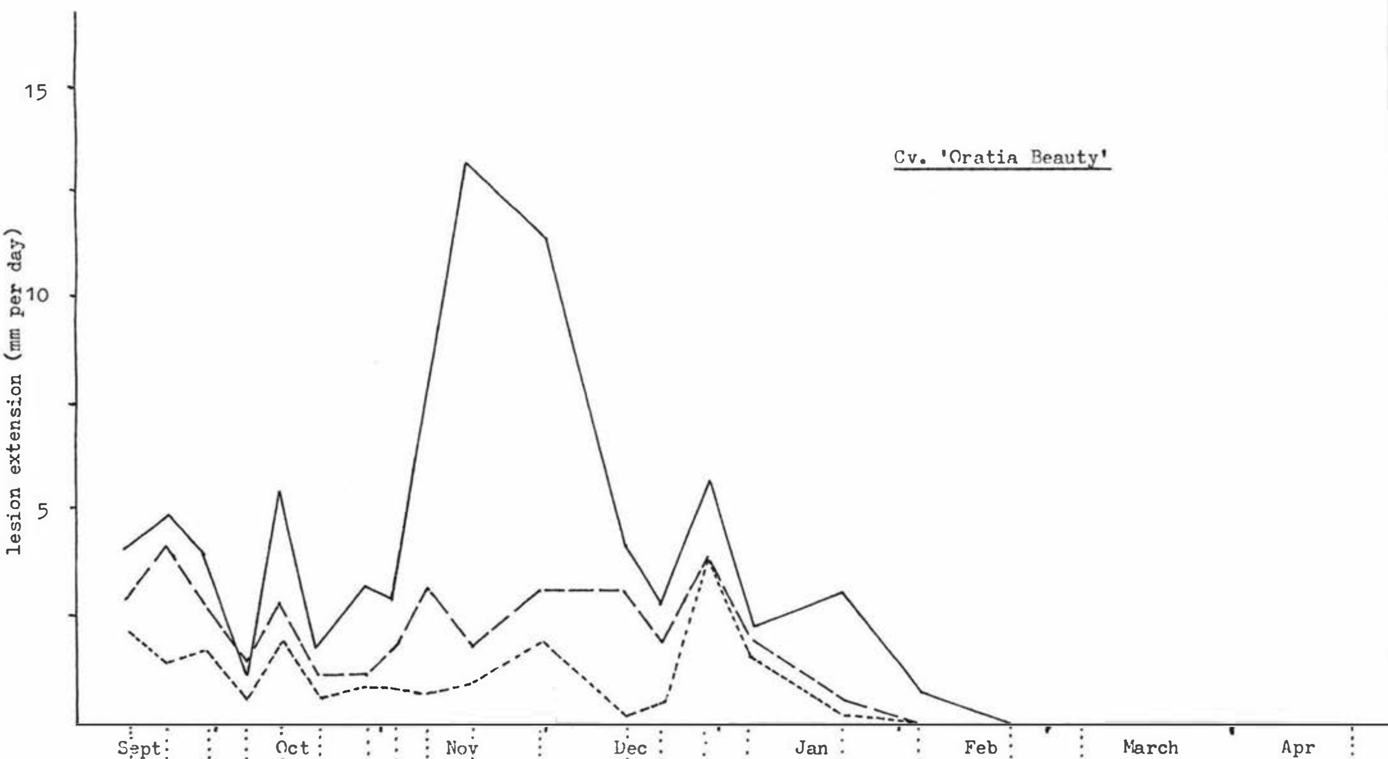
Unlignified xylem tissue	COP	a				b	a		a	a		b		b	a								
	GS	-				c	b		a	b		a		b	b								
	OB, 3 year old	b				a	a		a	a		b		a	b								
	OB, 21 year old	b				c	b		b	c		c		c	c								

Treatments within each column (sampling date) with the same letter are not significantly different from each other at the 5% level (Duncan's Multiple Range Test).

*Solid line= susceptibility of phloem-cambial tissue; broken line= susceptibility of unlignified xylem tissue.

Figure 3-7. The Susceptibility of Apple Cortical Stem Tissue to P. cactorum 'P149' during the 1981-82 Growing Season.

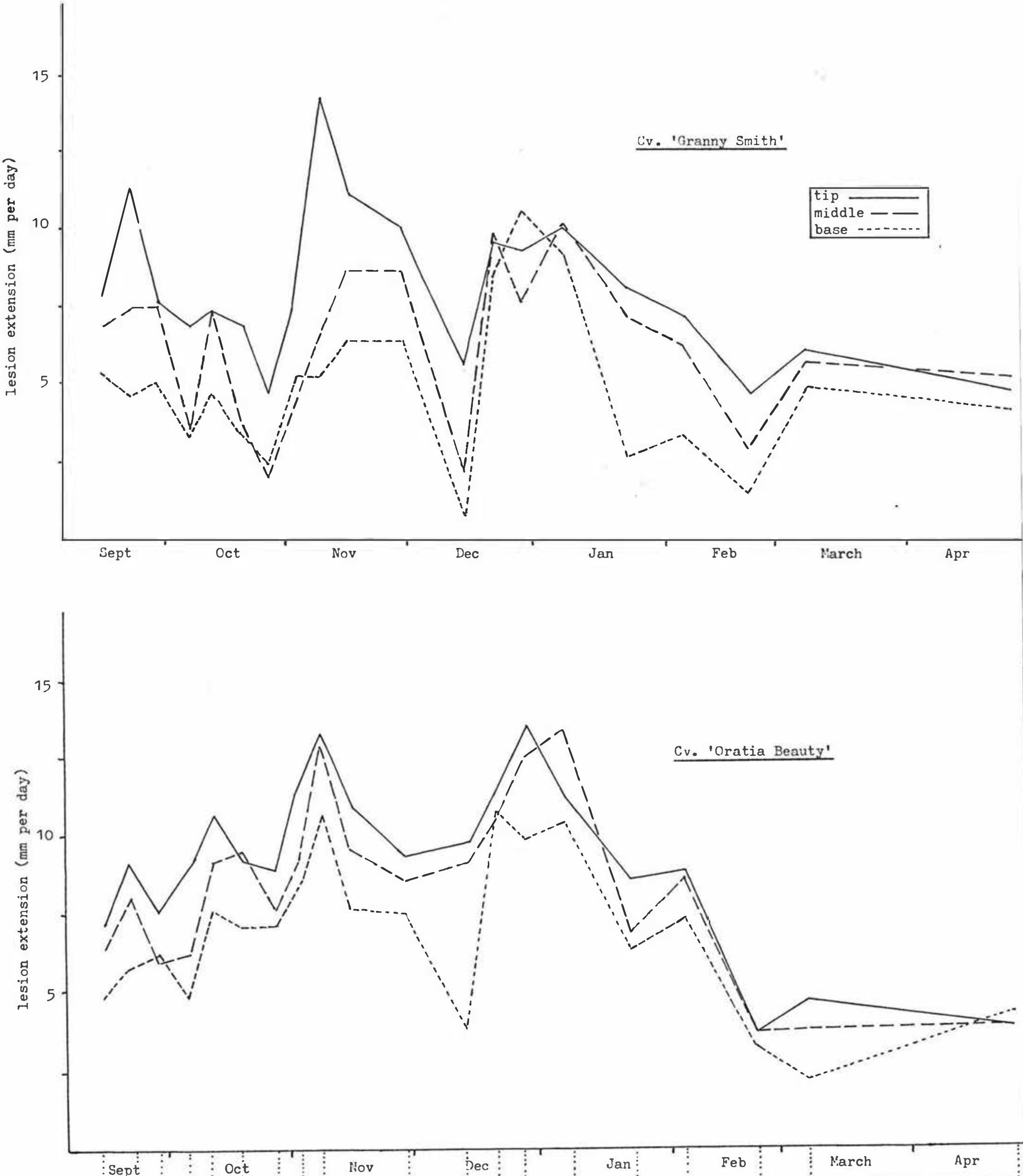




Oratia Beauty																				
tip	a	a	b	bc	a	bc	a	b	a	a	b	b	cd	b	c	a	c	c	b	c
middle	b	b	c	b	c	cd	b	bc	b	c	d	c	de	e	c	b	d	c	b	c
base	c	d	d	c	d	d	bc	cd	c	c	d	d	e	de	c	bc	d	c	b	c
Granny Smith																				
tip	c	a	a	a	b	a	bc	a	a	ab	a	a	a	a	a	a	a	b	a	a
middle	c	c	b	b	c	b	b	bc	bc	b	b	c	b	b	b	c	a	a	b	b
base	d	d	c	bc	d	c	c	d	bc	c	c	d	c	cd	b	c	b	c	b	bc

Treatments within each column (sampling date) with the same letter are not significantly different from each other at the 5% level (Duncan's Multiple Range Test).

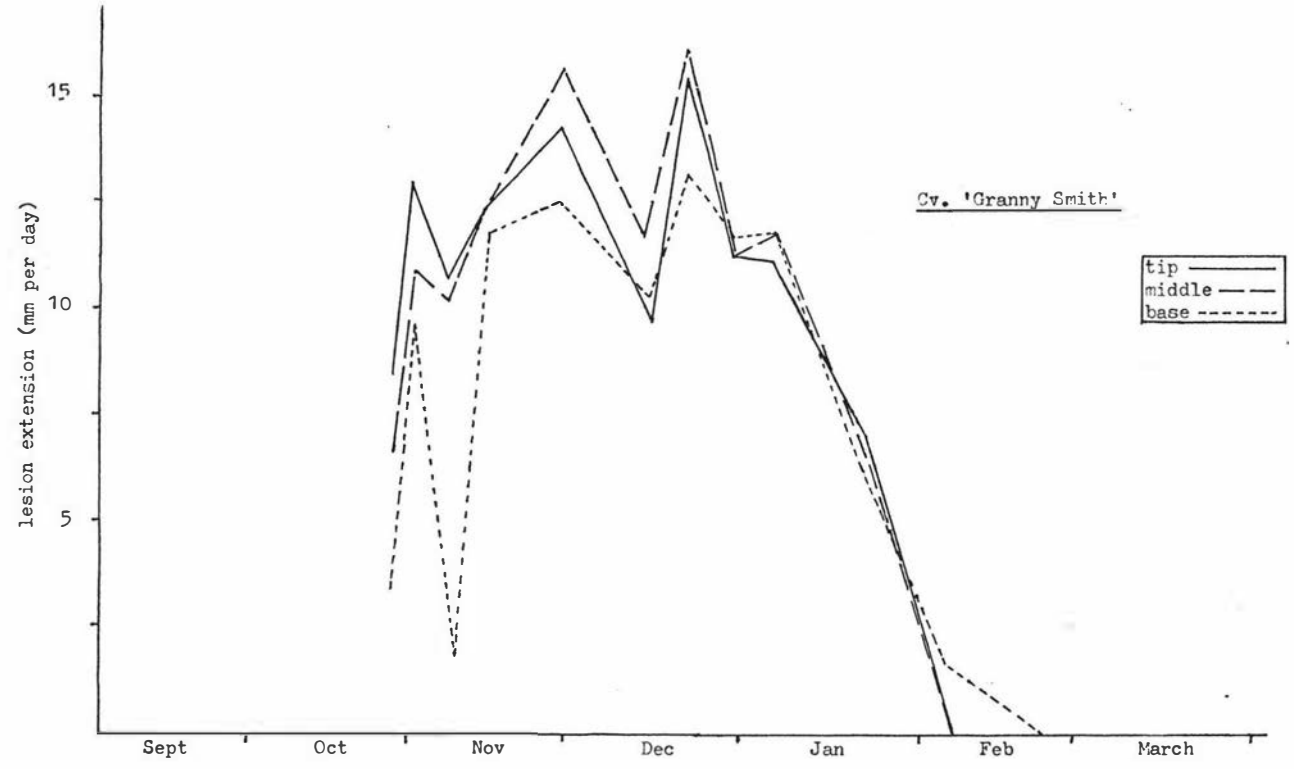
Figure 3-8. The Susceptibility of Apple Phloem-cambial Stem Tissue to P. cactorum 'P149' during the 1981-82 Growing Season.

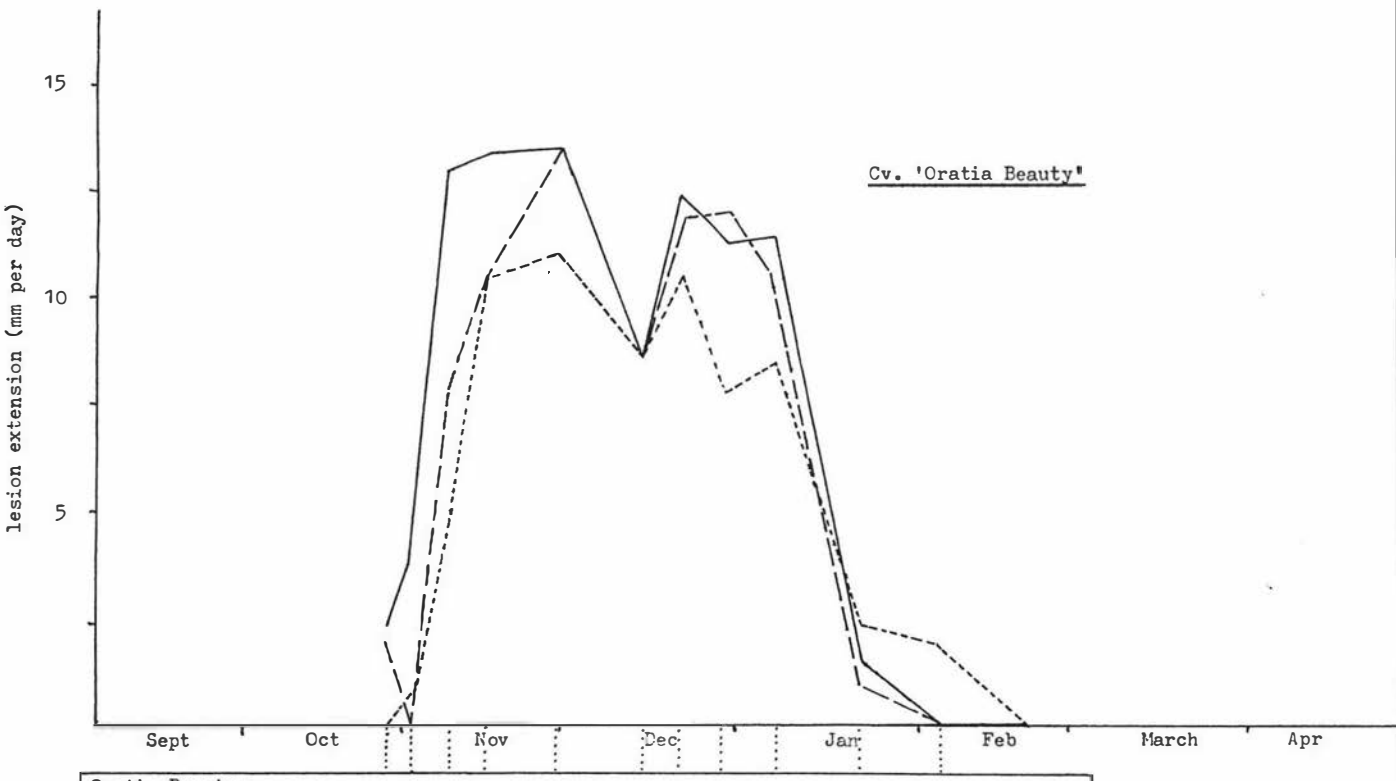


Oratia Beauty																				
tip	a	b	a	a	a	a	a	a	ab	a	ab	a	a	a	bc	a	a	b	c	c
middle	bc	bc	b	b	b	a	b	b	b	b	b	a	ab	a	a	b	a	b	d	c
base	d	d	b	c	c	b	b	b	c	cd	c	c	a	c	c	b	bc	bc	e	b
Granny Smith																				
tip	b	a	a	b	c	b	c	c	a	a	a	b	b	c	c	ab	b	a	a	a
middle	c	c	a	d	c	c	d	d	d	bc	b	d	ab	d	c	b	c	c	b	a
base	d	d	c	d	d	c	d	d	e	d	d	e	c	b	d	c	d	d	c	b

Treatments within each column (sampling date) with the same letter are not significantly different from each other at the 5 level (Duncan's Multiple Range Test).

Figure 3-9. The Susceptibility of Unlignified Xylem Stem Tissue to P. cactorum 'P149' during the 1981-82 Growing Season.





Oratia Beauty												
tip	c	c	a	a	bc	c	c	a	b	b	b	
middle	c	d	c	c	b	c	bc	a	b	b	b	
base	d	d	d	c	d	c	d	b	c	b	a	
Granny Smith												
tip	a	a	b	ab	b	b	a	a	b	a	b	
middle	b	ab	b	ab	a	a	a	a	a	a	b	
base	c	b	e	b	c	b	b	a	b	a	a	

Treatments within each column (sampling date) with the same letter are not significantly different from each other at the 5% level (Duncan's Multiple Range Test).

low levels as the season progressed into late autumn .

In the 1980-81 season, the order of phloem-cambial susceptibility from greatest to least based on the number of susceptibility maxima and the degree of susceptibility at each was : 'COP', 3 year old 'OB', 21 year old 'OB' and 'GS' while that of the unlignified xylem tissue was : 3 year old 'OB', 'COP', 'GS' and 21 year old 'OB' (Figure 3-6) . The following season, the difference between cultivars was less . The phloem-cambium of the 4 year old 'OB' was more susceptible while its cortical and unlignified xylem tissues were less susceptible than those of the cv. 'GS' .

At the beginning of the growing season, the phloem-cambium was the most susceptible tissue . By full bloom (end of October), the unlignified xylem tissue was formed and this was the most susceptible tissue while it was present . The other tissues did at times show a degree of susceptibility as great as that of the unlignified xylem (cortex in November and phloem-cambium in Janurary; Figures 3-7, 3-6 and 3-8) . The phloem-cambial and cortical tissues were most susceptible when the unlignified xylem was present .

The times of occurrence of susceptiblity peaks was the same for the cortex, phloem-cambium and unlignified xylem tissues within each season (Figures 3-6 to 3-9) . Their occurrence was also similar from one season to another, although the phloem-cambium Janurary peak (Figures 3-6 and 3-8) was offset by about a month between seasons .

A significant gradient of resistance was present along the cortex and phloem-cambial tissues of both tested varieties (Figures 3-7 and 3-8) . At peaks of susceptibility, susceptibility was significantly greater at the tip and decreased towards the base of the shoot, with the exception of the phloem-cambial tissues at the late December - early January maximum . This gradient of resistance along the shoot did not appear to occur in the unlignified xylem tissue (Figure 3-9) .

3-3-2. Effect of Wounding on Apple Stem Tissue Susceptibility

The thin periderm layer was a complete barrier to infection of this isolate of *P. cactorum* (Table 3-V) . Infection only occurred when the periderm layer was broken . A positive Sudan IV staining reaction showed the periderm to be highly suberised . Since lenticels were abundant on all the inoculated areas of the stem, *P. cactorum* appears to be unable to penetrate lenticels .

Table 3-V. The Lesion Length (mm) on Apple Twigs Wounded to Different Depths on 1/4/82 after 8 Days Incubation at 20°C

Cultivar	Tree Age (yrs)	Shoot Age (yrs)	Tissue Wounded to Pe	C	Ph
Cox's Orange Pippin	8	>1	0	25.9 a	20.2 b
Oratia Beauty	6	>1	0	12.9 c	6.4 e
Oratia Beauty	24	1-2	0	12.9 c	6.6 e
Granny Smith	24	>1	0	24.3 a	11.5 cd
Granny Smith	24	1-2	0	11.6 cd	12.3 cd
Golden Delicious	23	>1	0	13.4 c	8.4 de
mean				16.8	10.9

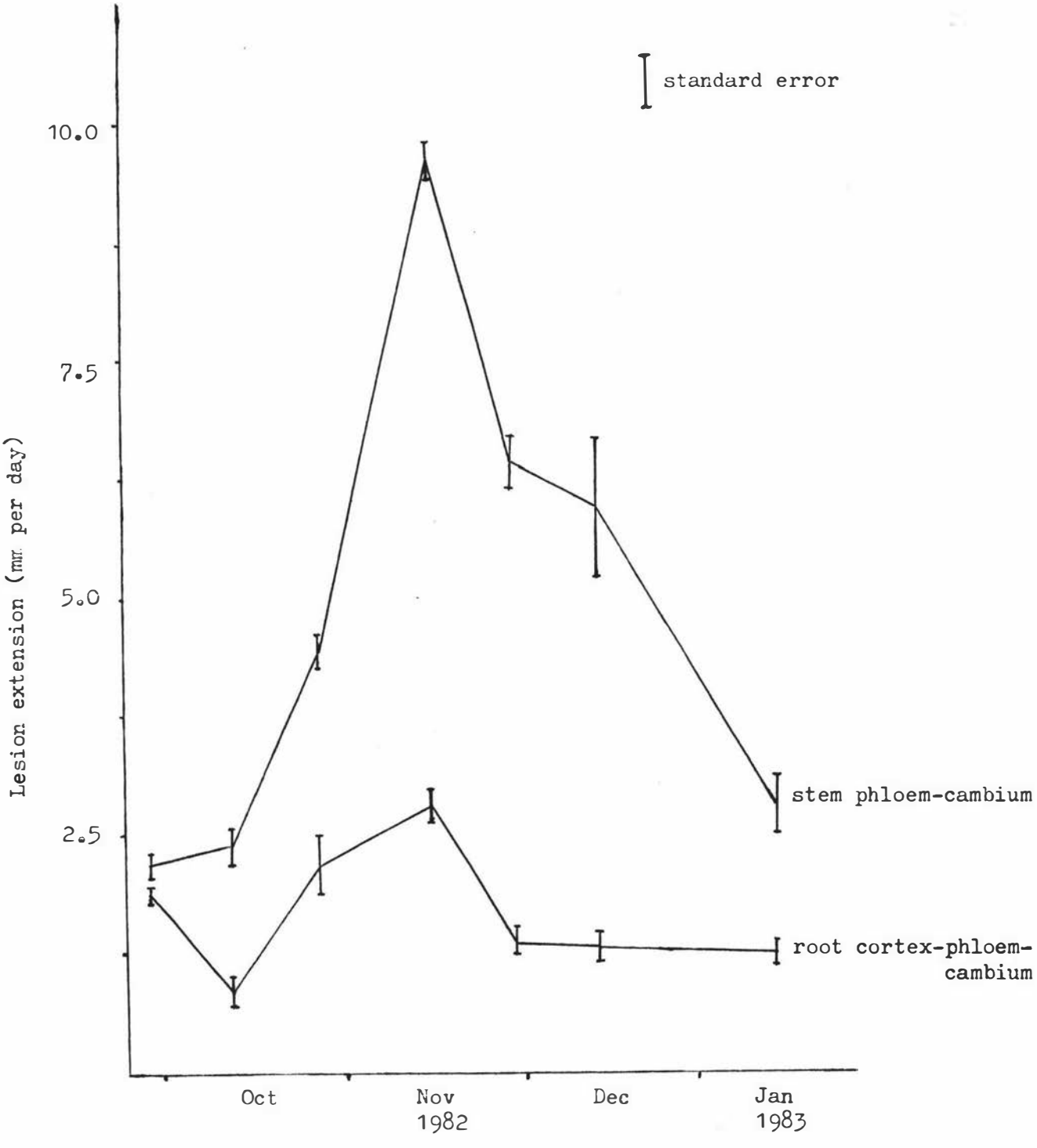
Pe= periderm , C= cortex , Ph= phloem-cambium

Mean values followed by the same letter in both columns are not significantly different at the 5% level (Duncan's Multiple Range Test).

The cortex and phloem-cambium tissues were both susceptible when inoculated via a wound . The pathogen grew significantly faster when inoculated in a wound to cortex tissue than in one to phloem-cambium tissue (Table 3-V) . The unexposed phloem-cambial tissue was less susceptible than the cortex - the reverse of the previous section (Section 3-3-1) . This indicates that the deeper *in situ* position of the phloem tissue affects its susceptibility . Xylem tissue at this time of the year was lignified and hence resistance to the pathogen .

When cutting away the periderm to assess the lesion length, it was noted that sometimes the cortex tissue was infected yet the pathogen did not grow in the phloem-cambium tissue . This indicates that the sclerenchyma bundles may act as a partial barrier to P. cactorum hyphae .

Figure 3-10. The Seasonal Susceptibility of Stem and Root Tissues of 2 year old MM 106 Apple Trees to P.cactorum.



3-3-3. Relationship between Stem and Root Resistance of Rootstock Material

The stem phloem-cambial and root cortex-phloem-cambial tissues both followed a similar seasonal pattern of susceptibility (Figure 3-10) . Susceptibility rose in early spring to a single maximum at the period of bloom and then declined . The root cortex-phloem-cambium had a considerably lower level susceptibility to P. cactorum 'P149' than the stem phloem-cambium .

Lesions were seldom found on the stem cortex and xylem and on the root xylem tissues . The stem cortex often felt quite dry compared to that from field grown trees (Section 3-3-1) and was probably the result of the ease with which water stress occurred in these planter-bag grown trees . The lack of moisture in the cortical tissues probably gave it a high resistance and also prevented the formation of much unlignified xylem .

3-3-4. Stability of Pathogenicity of P. cactorum 'P149' in vitro

On DCMA, P. cactorum 'P149' grew 2-5 mm/day and reached the petri dish edge (from inoculum placed in the centre) in about 10 days . The hyphae appeared to deplete the food reserves in the surrounding agar within a short time of colonisation since very little mycelial growth subsequently occurred and numerous spore structures (sporangia and oospores) were formed . Some of the mycelium disintergrated or became distorted but much remained intact often forming septations . Further aging did not alter the morphological appearance of the mycelium .

The pathogenicity of the fungus was not influenced significantly by which part of the colony the plug of inoculum came from (Table 3-VIB) . Thus the pathogenicity of vigourously growing mycelium (2 day old), mycelium with developing spore structures (4 day old) and non-growing mycelium of growing (8 day old) and non-growing colonies (16 and 32 day old) was similar to each other .

The pathogenicity of P. cactorum showed no significant deterioration

when absent from host tissue for periods of at least 10 months (Table 3-VIA and B) .

'P149' colonies with noticeably different growth rates or colony forms occurred infrequently .

Table 3-VIA. The Effect of the Length of Time and Mycelial Aging of P. cactorum in culture on the Susceptibility of Apple Phloem Tissue

	Mycelial Age in Days after Inoculation to Fresh Medium				
	2	4	8	16	32
continuously sub.	50.7±8.6	45.2±3.1	57.3±7.6	49.3±6.7	44.2±5.7
recently reisolated	44.6±7.4	43.9±6.1	56.4±5.7	46.7±6.0	42.2±5.7

Table 3-VIB. Analysis of Variance of Table 3-VIA

Source of Variance	Df	Mean square	F
Replicates	9	272.06	0.68 NS
Time in culture	1	162.65	0.40 NS
Mycelial age	4	548.68	1.36 NS
Time x age	4	20.72	0.05 NS
Error	89	402.30	

NS= not significant

3-3-5. Mycelial Growth of P. cactorum, in culture, under Reduced Oxygen Conditions

Hyphal growth produced under coverslips was morphologically similar but far less dense than that elsewhere on the agar (Figure 3-5). The hyphal growth rate was less under reduced oxygen conditions but the difference was not significant (Table 3-VII).

Figure 3-5. Diagram of P. cactorum mycelial growth on a Plate of DCMA under Conditions of Lowered Oxygen Availability

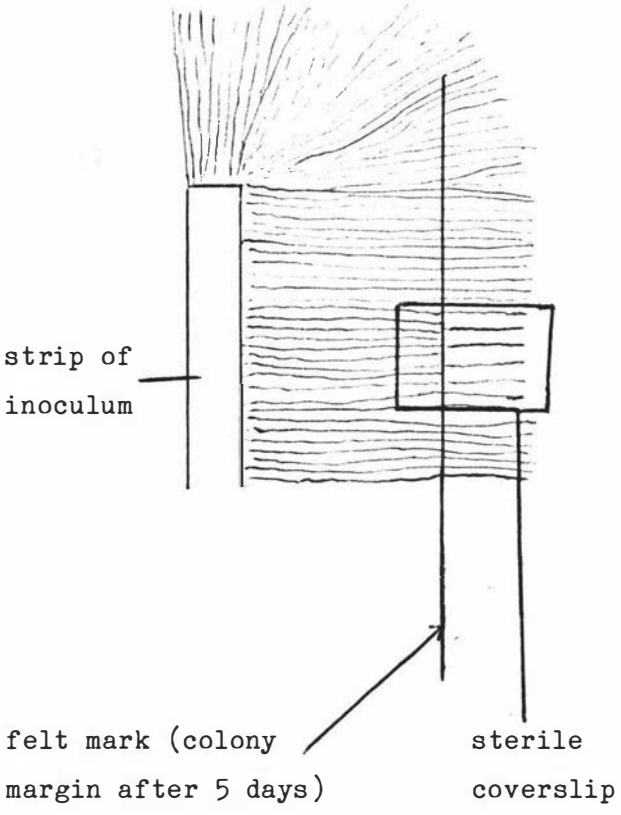


Table 3-VII. P. cactorum Hyphal Growth (in mm) on DCMA under Conditions of Varying Oxygen Availability

Control (outside coverslip)	Coverslip Edge (<1mm from edge)	Under Coverslip
8.25 ±0.33*	8.25 ±0.30	7.50 ±0.26

*Standard error

3-4. DISCUSSION

The pathogenicity of P. cactorum was stable in culture over the tested period of 10-11 months and was similar no matter which part of the colony the plug of inoculum came from . This indicates that the length of time which the pathogen remained in culture had no effect on virulence and hence would not have affected the seasonal susceptibility pattern of the tested apple cultivars . The stability of the pathogen's genetic composition in culture was further evidenced by the infrequent occurrence of 'P149' colonies with noticeably different growth rates or colony forms .

The different apple stem tissues when individually tested varied in their susceptibility to P. cactorum . The order of susceptibility from greatest to least was : unlignified xylem, phloem-cambium, and cortex . This order was also observed by Long (1982) . The base level of susceptibility of these tissues may be related to their mechanical toughness which may limit the rate that the pathogen can physically grow through that tissue . The unlignified xylem tissue consists of long slender tracheids and vessels aligned in tubes (Esau, 1977) . Hyphae growing along these cells would have comparatively few cell walls to penetrate and since this tissue was also easily crushed, its cell walls could be readily breached . The phloem is a mechanically stronger tissue, being far less easily crushed than the young xylem . The phloem consists of fibers, parenchyma cells and of long slender sieve elements which are continuous from one sieve element to another through perforations in the wall, while the cambium consists of small undifferentiated brick-like cells . Hyphae at times did grow through this tissue at rates similar to those found in unlignified xylem but this occurred infrequently . The cortex is composed mainly of closely packed, polyhedral parenchyma cells . Hyphae progressing through this tissue would either encounter more cell walls per unit length in comparison with other tissues or grow in a more twisting manner in the intercellular spaces .

In situ the pathogen grew faster in the cortex than in phloem-cambial tissue, the reversal of what has previously been found suggesting that

oxygen availability to the tissue may affect its susceptibility . The inner phloem and young xylem tissues showed a greater susceptibility only when they were well exposed to the atmosphere . When the amount of oxygen available to the pathogen in culture was reduced, a substantial decrease occurred in its biomass . Klotz et. al. (1962) also found that the mycelial dry weights of cultured P. parasitica and P. citrophthora were directly proportional to the concentration of oxygen, over the range of 0.04 - 21.0% . Histological observations (Section 2-2) showed that the mycelial density in infected apple phloem-cambial and cortical tissues is low . This suggests that the deeper and more susceptible host tissues (ie. phloem-cambium and unlignified xylem) lack sufficent oxygen to substain abundant mycelial production , especially since the hyphal growing tips would be competing with the oxidative reactions initiated by the disruption of host cells . These reactions occur just in advance of the hyphae (Plate 2-1) . The existence of gaseous gradients within the stem tissues is evidenced by the fact that the outer part of the cortex is photosynthetic while the inner part is not and that the periderm is penetrated by lenticels . These tissues, however, contain enough oxygen to support their own metabolic activities .

The root cortex-phloem-cambium had a considerably lower level of susceptibility to P. cactorum 'P149' than the stem phloem-cambium (Figure 3-10) . Other workers have also found and root tissue to be more resistant than stem tissue (Engel, 1977; Long, 1985; Smith, 1955), but not with every cultivar tested (Smith, 1955) . The parallel change in susceptibility between the root and stem phloem tissue showed that a relationship existed between the susceptibility of the root and stem .

Tree age and/or rootstock differences affected the susceptibility of the scion tissue - the one year old phloem-cambial tissue of the 3 year old (first year of bearing) 'OB' trees had a greater susceptibility than the 21 year old 'OB' ones . Such differences appear to be of a physiological nature .

It is important to note that a gradient of resistance occurred along the shoot in the cortex and phloem-cambium tissues of the 'OB' and 'GS'

cultivars but not in the unlignified xylem tissue (Figures 3-7 and 3-8) . Susceptibility was greatest at the distal end of the shoot and decreased towards the base . This suggests that the gradient may be caused by mobile compounds translocated from or to the apical meristem which are utilised during translocation . Compounds which are known to form such basipetal gradients in shoots are indole-3-acetic acid (IAA) (Digby and Wareing, 1966; Mullins and Rogers, 1971; Letham et al., 1978) and nitrogenous compounds in vacuum extracted xylem sap (Cooper et al., 1972) .

If the gradient of resistance is auxin determined, the auxin which largely moves in the phloem or associated tissues from the apex (Letham et al., 1978) probably diffuses laterally into the cortex . For the first two months of the growing season (until the beginning of November), the apical meristem is only a few centimeters from the previous season's growth . After this time, the current season's shoot rapidly extends increasing the distance between the apical meristem and previous season's growth . Shoot extension was considerablely greater in the cv. 'OB' than in 'GS' (Figure 3-6) . The gradient of resistance in the 'OB' cortical tissue is steeper in the distal portion of the previous season's shoot and flatter at the basal end than in the cv. 'GS' (Figure 3-7) . This gradient, however, is similar in the phloem-cambial tissues of both cultivars (Figure 3-8) . The gradient of resistance would be expected to be maintained throughout the season only if the IAA was metabolished or inactivated as it moved basipetally (Wareing and Phillips, 1978) .

Cooper et .al. (1972) found that the nitrogen gradient is maintained during the growing season - less nitrogen being in the distal portion of the previous season's shoot . The gradient of resistance may thus be related to the nutritional status of the tissue . This is further discussed in Chapter 4 .

It appears that this gradient of resistance has a greater effect on tissue susceptibility (of the phloem-cambium and cortex) than variety . This could account for a large proportion of the variation in the assessment method which others have found (Bielenin, 1977B; Herb et. al., 1975; Jeffers et al., 1981; Krober and Karnatz, 1979; Long,

1982) . Future testing of twig pieces should thus be at a standard location on the shoot in order to give sharper distinction in resistance between various cultivars .

A comparison of the seasonal pattern of susceptibility with that of cambial activity suggests that cambial activity may have a determinate role on apple tissue susceptibility to P. cactorum . Cambial activity commences early in spring in apple (Evert, 1963; Swarbrick, 1927) . During the first one and a half months of the growing season (mid-August to end of September), the rate of cambial cell division was low (Evert, 1963) . Susceptibility was also low over this time . At the beginning of October, the cambium enlarged (Long, 1982) and susceptibility increased . By full bloom (end of October) much of the cambial region had differentiated into unlignified xylem tissue (Long, 1982) . This period of active cambial activity and xylem production corresponds to the time of greatest susceptibility in all the tissues tested . In early autumn, the differentiation of new xylem tissue almost ceases (Long, 1982) and the tissue susceptiblity declines to a low level .

A decrease in susceptibility consistently occurs in mid-December . Lignification of the unlignified xylem commences and initially proceeds at a rapid rate at this time and thereafter continues only slightly faster than the production of new xylem (Appendix II) . The sudden heavy use of the shikimic acid pathway and phenylalanine ammonia-lyase (PAL) to produce lignin precursors (Barnett, 1981) would cause a change in the internal environment of the previous season's shoot and thus may indirectly decrease susceptibility at this time .

Susceptibility peaks of the cortical, phloem-cambial, and unlignified xylem tissues occurred at similar times and for similar durations throughout the season indicating that the resistance of the susceptible tissues are inter-related .

The pattern of susceptibility appeared to be relatively stable from one season to another with the tested cultivars . The susceptibility peaks occurred at approximately the same time in both the 1980-81 and 1981-82 season . This was particularly noticeable with the unlignified xylem

tissue, but was less obvious with the phloem-cambial tissue where the January peak of susceptibility was offset and the overall susceptibility varied somewhat between seasons . Gates and Millikan (1972) and Bielenin (1977B) both found that similar patterns of resistance frequently occurred between adjacent seasons for each variety . The occurrence and magnitude of susceptibility peaks varied between seasons, but the change was not consistent among their tested varieties (Gates and Millikan 1972) . Thus, environmental conditions and other factors such as biennial bearing appear to have a marked influence on the seasonal level of resistance but not the pattern .

The seasonal pattern of the cv. 'COP' susceptibility was similar to that found by Long (1982) . This is not surprising since the artifical inoculation method used, place and season were identical, although a different set of trees was assessed . Sewell and Wilson (1973A and B) found 'COP' to have only one peak of susceptibility (during flowering) in the first four months of the growing season . The rootstock cultivar 'MM 106' showed a single maximum which peaked in mid-November occurred (Figure 3-10) . Susceptibility then declined, perhaps because the plants (grown in planter bags) were subjected to water stress especially over the December-January period . This stress appeared to inhibit cambial activity since the phloem-cambial tissues were difficult to separate from the xylem and very little unlignified xylem was produced over this period . Gates and Millikan (1972) found that the susceptibility of 'MM 106' rose to a high level at blossom but thereafter declined to a considerably lower level where several peaks were formed while Janisiewicz and Covey (1982) found that it rose at the beginning of the growing season and remained at a high level for about the next 6 months . Comparison of seasonal susceptibility patterns with that obtained by others is difficult since they (Gates and Millikan, 1972; Janisiewicz and Covey, 1982; Sewell and Wilson, 1973A and B) used different isolates of the pathogen and made no distinction between the various stem tissues or whether their twig pieces had a basal or distal location on the shoot sampled .

In the varieties tested both the magnitude and number of the susceptibility peaks was frequently higher with the more susceptible varieties . Bielenin (1977B) also found that the susceptible varieties

Hibernal, Bancroft, Yellow Transparent and James Grieve frequently had larger susceptibility peaks than the resistant Antonovka, Wealthy and Red Delicious varieties when inoculated in the field ; but not when inoculated in the laboratory . Studies by Janisiewicz and Covey (1982) showed the susceptible 'MM 106' rootstock to have a larger peak of susceptibility than the more resistant 'M 7', 'M 26' and 'M 9' rootstocks . Gates and Millikan (1972) tested the seasonal susceptibility of 13 apple cultivars and found that the frequency, duration and magnitude of susceptibility peaks varied with the cultivar tested but not always in accordance to their field resistance . Obviously the cultivar itself has a large effect on the pattern of seasonal susceptibility . These varietial differences indicate that at least some rate reducing mechanisms of resistance were operative after fungal invasion .

The cv. 'GS' cortex was overall more susceptible than that of the cv. 'OB' while the phloem-cambial tissue had the opposite trend (Figures 3-7 and 3-8) . This shows that it is dangerous to assume that colonisation rates of the fungus through a specific tissue reflects the field resistance of that cultivar . It also highlights the importance of clearly identifying which particular stem tissue and which position of the shoot is inoculated by the pathogen if comparisons of relative cultivar resistances between workers are to be reliable .

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** CHAPTER 4 **
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Studies on constituents of apple stem tissues and their influence on the growth of P. cactorum.

4-1. INTRODUCTION

As the fungal mycelium progresses through host tissue, it encounters host substances which could markedly enhance or inhibit its growth .

Beever (1970) found that the amount of nutrients (carbohydrates and nitrogenous compounds) present in xylem sap extracts of fruit trees was greatest in late winter-early spring - the period of susceptibility to the silver leaf fungus (Stereum purpureum)*. Mycelial growth of this pathogen was best in extracts collected during this time and better in the nutrient richer sap from stone fruits . Beever suggested that the susceptibility of fruit trees to S. purpureum was related to the ability of the xylem sap to support the growth of this fungus . Thus one of the aims of this portion of work was to investigate the role that endogenous nutritional compounds (carbohydrates and nitrogenous compounds) may play in the resistance of apple trees to P. cactorum .

The role of phenolics as a defence mechanism has received considerable attention (Ingham, 1973; Kosuge, 1969; Lyr, 1965; Pridham, 1960; Wilson, 1967; Wood, 1967) since the infection of plants by virtually any microorganisms will lead to an accumulation of various phenolics both in and around the affected tissue (Ingham, 1973) .

* now Chondrostereum purpureum

Phenolics are usually lipophilic in character but possess hydrophilic groups and are therefore surface active toward membranes (Pridham, 1960) . Consequently, many phenolics and their metabolic products, especially the highly reactive quinones, are toxic to living organisms (Ingham, 1973; Kosuge, 1969; Loomis and Battaile, 1966; Lyr, 1965; Pridham, 1960) . Phenolics also have the ability to react with proteins and so inhibit the disease process by inactivating degrading enzymes produced by the pathogen (Byrde et al., 1960; Dias, 1965; Kosuge, 1965; Loomis and Battaile, 1966; Lyr, 1965) . Unfortunately, there are only a few instances where phenolics have been directly associated with disease resistance (Farkas and Kiraly, 1962; Ingham, 1973; Kosuge, 1969; Lyr, 1965) .

Phloridzin has also been considered to be a factor in the resistance of apple trees against P. cactorum by Bielenin et al. (1973) and Alt and Schmidle (1980) . Alt and Schmidle (1980) found higher levels of phenolics in the bark of the more resistance 'Maunzen' cultivar than in susceptible 'Cox's Orange Pippin' cultivar throughout the year . Bielenin et al. (1973) similarly found that the bark of the resistant 'Antonovka' cultivar contained more phloridzin than that of the more susceptible cv. 'Hibernal' . They however only sampled once . These workers (Alt and Schmidle, 1980; Bielenin et al., 1973; Schwinn, 1965) also found phloridzin and its oxidation products : phloretin and phloroglucinol to be toxic to P. cactorum in vitro . The role that phenolics may have in apple tree resistance to P. cactorum was also investigated .

4-1-1. Nutritional Compounds

4-1-1-1. Total Nitrogen

Seasonal Changes in the Total Nitrogen Content of Decidious Trees

In stem tissues of decidious trees, the concentration of total nitrogen

undergoes a pronounced annual cycle (Taylor, 1967A; Kench, 1939; Mason and Whitfield, 1960; Oland, 1959; Tromp and Ovaa, 1971A) . The endogenous level of nitrogen is high in autumn and winter, slowly decreases during the growing season when the plant's demand for nitrogen is great and builds up again after the cessation of shoot extension in late summer . The autumn build-up results not only from the lowered plant demand but also because of the increased nitrogen uptake due to the autumn root growth (Taylor, 1967A) and the translocation of nitrogen from the foliage prior to leaf-fall (Pate, 1980; Taylor, 1967A) .

The Main Nitrogenous Constituents of Apple Tissue

The total nitrogen in apple tissue can be divided into soluble and insoluble fractions . The insoluble fraction of the bark (phloem-cortex tissue) remains at a near constant level throughout the growing season (Oland, 1959) while the soluble fraction of the bark and xylem sap shows the same seasonal trend as the total nitrogen (Oland, 1959; Taylor, 1967A; Tromp and Ovaa, 1967) .

The main nitrogenous constituents of this soluble fraction are arginine, glutamine, asparagine, and aspartic acid (Bollard, 1957A, B and C; Hill-Cottingham and Bollard, 1965; Tromp and Ovaa, 1967; Tromp and Ovaa, 1971A; Tromp and Ovaa, 1971B) . Definite changes occur in the proportions of these amino acids : arginine dominates from autumn to early spring (up to 80-90% of soluble nitrogen) ; glutamine until shoot growth, asparagine and aspartic acid during shoot extension, and glutamine again until the onset of winter (Hill-Cottingham and Bollard, 1965; Hill-Cottingham and Lloyd-Jones, 1973; Tromp and Ovaa, 1967) .

4-1-1-2. Starch and Soluble Sugars

Seasonal Changes in the Starch and Soluble Sugar Content of Deciduous Trees

The pattern of seasonal variation of starch and soluble sugars in stem tissue appears to be similar in a large number of woody deciduous species (Bradfield and Flood, 1950; Kozlowski and Keller, 1966; Mochizuki and Hanada, 1957; Swarbrick, 1927) . The amount of these carbohydrates present in the tree at any one time is directly affected by the plant's energy requirements for growth .

After the cessation of shoot extension in late summer-early autumn, the total amount of these carbohydrates in the apple tree begins to slowly increase . Much of this material is translocated to the roots where it is either utilised for root growth or stored . The build up of photosynthates continues until leaf-fall . The onset of cooler temperatures in winter halts root activity and the starch level decreases or remains low as starch is converted to soluble sugars (Dowler and King, 1966; Gibbs, 1940; Hansen, 1967B; Hansen, 1971; Hansen and Grausland, 1973; Kozlowski and Keller, 1966; Mochizuki and Hanada, 1957; Murneek, 1933; Swarbrick, 1927) . The level of soluble sugars remains high throughout the winter but declines with the onset of warmer weather when much of the sugar is converted back to starch . The starch concentration consquently rises to a maximum in early spring, but decreases again as the carbohydrate reserves are utilised for growth . Levels of both starch and soluble sugars decline to a minimum during the period of leaf and flower development . Before long the young leaves are able to fix sufficient photosynthates for their own growth requirements, and as they mature they export carbohydrates to other actively growing plant parts (De Villiers et al., 1974A; Hansen, 1967A; Hansen, 1967B; Hansen, 1971; Quinlan, 1965; Quinlan, 1969) . After the period of maximal leaf expansion, levels of both starch and soluble sugars again peak and decline because of the large energy requirements of shoot extension and leaf expansion (Hansen, 1967C; Hansen and Ryugo, 1979; Mochizuki and Hanada, 1957; Priestly,

1960) . The amount of carbohydrates increases yet again with the reduction of shoot growth in autumn .

The Soluble Sugar Constituents of Apple Tissue

The soluble sugars known to occur in the apple phloem-cortical tissues (bark) are sorbitol, sucrose, glucose, fructose, raffinose, stachyose, and maltose (Bieleski, 1969; Bradfield and Flood, 1950; Hansen and Grausland, 1973; Sakai, 1966; Saniewski and Pieniazek, 1972; Webb and Burley, 1962; Whetter and Taper, 1963) . Sorbitol is the most abundant soluble sugar with sucrose next, and small amounts of glucose and fructose and lesser quantities of raffinose, stachyose, and maltose are also present . Only the first three of these sugars have been reported to be translocated in the xylem (Hansen and Grausland, 1973; Hansen and Ryugo, 1979; Lewis and Smith, 1967 ; Whetter and Taper, 1966; Williams et al., 1967) .

Apart from raffinose and stachyose which were only detected at the end of the growing season (Sakai, 1966), the amount of the different solubles sugars rise and fall together (Hansen and Grausland, 1973; Rohrbach and Luepschen, 1968; Sakai, 1966; Whetter and Taper, 1963; Whetter and Taper, 1966) .

4-1-2. Possible Inhibitory Compounds - Phenolics

The Phenolic Constituents of Malus pumila (Mill.)

Most commercial apple varieties belong to only one of the 25 species (Malus pumila) recorded in the genus Malus (Williams, 1960) . The main phenolic compounds in this species were identified by Williams (1960) and have since been confirmed by other workers (Alt and Schmidle, 1980; Schwinn, 1965; Williams and Kuc, 1969; Wilson, 1967) . In both bark and leaf tissue, the bulk of phenolics consist of a phenolic glycoside,

phloridzin . The amount of phenolics in bark tissue varies from 4-10% frozen weight and 6-12% dry weight (Alt and Schmimdle, 1980) and the amount of phloridzin has been found to consist of up to 11% dry weight of shoots (Hutchinson et al., 1959) and 2-4% dry weight of cortical tissue (Bielenin et al., 1973) . Similar amounts of phloridzin have been reported to be present in leaf and root tissue (Hutchinson et al., 1959; Noveroske, Kuc and Williams, 1964) . Phloridzin is accompanied by several 3-glycosides of quercetin and lesser amounts of the same 3-glycosides of kaempferol . The phenolic pattern of fruit tissues differs considerably from that of the bark and leaves . These compounds are listed in Table 4-I in decreasing order of amount present .

The phenolics present in leaf and bark tissue do not vary qualitatively among different apple varieties (Williams, 1960; Wilson, 1967), although some variation in the total and relative amounts of phenolics does occur (Alt and Schmidle, 1980; Flood and Kirkham, 1960; Wilson, 1967) .

Table 4-I. The Main Phenolic Compounds of Malus pumila

Bark	Leaves
phloridzin	phloridzin
phloretin arabinoglucoside	(3-galactoside
(3-arabinoside	(3-glucoside
quercetin (3-rhamnoside	quercetin (3-rhamnoside
kaempferol (3-glucoside	kaempferol (3-arabinoside
(3-galactoside	(3-rhamnoglucoside
(3-galactoside	(3-xyloside
(3-xyloside	phloretin
	chlorogenic acid

Fruit

chlorogenic acid
p-coumaryl-quinic acid
epi-catechin
catechin
leuco-anthocyanins
quercetin (3-galactoside
kaempferol (3-rhamnoside
(3-arabinoside
(3-glucoside
(3-xyloside
idaein
phloridzin (in seeds)

4-2. MATERIALS AND METHODS

4-2-1-1. Total Nitrogen

Digestion of Samples

The procedure used was that of Williams and Twine (1967) . Details of the reagents are given in Appendix 1 .

One millilitre of digestion mix was added to a series of digestion tubes containing 50 mg samples of dried, ground plant material . Hydrogen peroxide (0.5 ml) was added to assist digestion and the samples were digested at 320 ^{o}C on a heating block for 2.5 hours . During this time, care was taken to prevent particles of undigested carbon sticking to the sides of the tubes . After cooling, the digest was diluted to a final volume of 12.5 ml with distilled water . Nitrogen standards of 1,3, and 5% N (ammonium sulphate, AR) and a blank of distilled water were included with the treatments .

Distillation and Assay of the Ammonium-Nitrogen

The steam distillation apparatus used is shown in Figure 4-1 (Allen, 1974) . The steam required for distillation was generated by heating distilled water in a 5L flask which contained pummice to promote smooth boiling and a small amount of sulphuric acid to trap any ammonium ions .

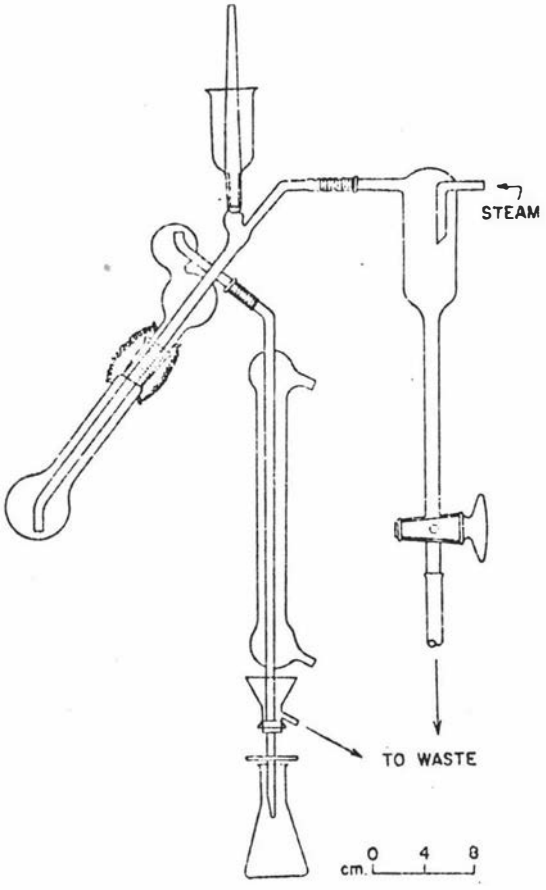
When the apparatus was ready for use, a 50 ml Erlenmyer flask containing 5 ml boric acid-indicator solution was placed under the condensor and a 100 ml Kjeldahl flask which contained an aliquot of sample digest (5 ml), 2 ml 10N NaOH, and 10 ml distilled water was fastened to the distillation apparatus . The ammonium-nitrogen was distilled and the boric acid-indicator solution back titrated to its equivalence point with 0.005N sulphuric acid .

The chemical reactions which occurred are presented below :

$$NH_3 + H_3BO_3 \text{ ---------> } NH_4^+ + H_2BO_3^-$$

$$H_2BO_3^- + H^+ \text{ ---------> } H_3BO_3$$

Figure 4-1. Steam-distillation apparatus



4-2-1-2. Starch and Soluble Sugars

Extraction of Soluble Sugars from Plant Material

Collection and storage of the plant material has already been described in Section 3-2-1 .

The method of Haslemore and Roughan (1976) was used . One hundred mg

samples of finely ground, freeze-dried tissue were each extracted with 10 ml 62.5% methanol for 15 minutes at 55°C in screw-capped culture tubes with teflon caps . Sucrose standards were prepared in 62.5% methanol and treated with the samples . The samples were centrifuged and 4 ml aliquots were transfered to a second series of capped culture tubes each of which contained 0.1 ml saturated lead acetate to remove non-carbohydrate material that could interfer with the test . Chloroform (5 ml) was added after 10 minutes to remove lipids and the tubes were securely capped, shaken vigorously, and centrifuged to hasten phase separation . Aliquots (50 µl) were taken from the upper aqueous phase and placed in test tubes containing 0.95 ml of distilled water each . One ml 5% phenol and 5 ml conc. sulphuric acid were added to each sample .

When the samples had cooled, the absorbances of the dark-coloured condensation products resulting from the interaction of phenol with furfural, hydroxymethyl furfural, and similar products formed from the sugars by the action of the acid (Noggle, 1957), were read at 490 nm .

Extraction of Starch from Plant Material

The method of Haslemore and Roughan (1976) was used . Following soluble sugar extraction, the residual plant material was treated with 4 ml methanol at 100°C for 5 minutes . The washings were aspirated and discarded after centrifugation and the process was repeated to remove soluble sugars and phenolics from the samples (Ebell, 1969) . Starch standards were prepared at this stage . Four ml of distilled water were added to each sample, the culture tubes were firmly capped, and samples were heated at 100°C for 60 minutes to gelatinise the starch . The samples were cooled and 0.25M sodium acetate buffer pH 4.5 (2 ml) and amyloglucosidase preparation (0.1 ml) was added to hydrolyse the the starch to glucose . They were then incubated at 55°C for 60 minutes before being diluted to 10 ml with distilled water, mixed, and centrifuged . Aliquots (0.10 to 0.50 ml) of the diluted hydrolysate were mixed with 2 ml glucose oxidase reagent and 1 ml distilled water ;

and incubated at 37°C for 60 minutes . A water blank and glucose standards underwent the same treatment .

The glucose oxidase oxidised β-glucose to gluconic acid with the release of hydrogen peroxide which, in the presence of the peroxidase, oxidised o-dianisidine to the corresponding imine . Addition of 5 ml 5M hydrochloric acid to each sample protonated the imine and this pink protonated form was measured colorimetrically at 540 nm .

Details of the enzyme reagents are given in Appendix 1.

Starch and Soluble Sugar Utilization In Vitro by P. cactorum

Soluble Sugars

Five mm diameter agar plugs from colonies of P. cactorum isolates 'P149' and 'MU2' grown on DCMA were used to inoculate solutions of basal medium containing individual soluble sugars as the sole carbon source . The solutions were incubated in darkness for 6 days at 24°C .

The basal medium had the following composition : $NaNO_3$ 2g, KH_2PO_4 2g, KCl 0.5g, $MgSO_4 .7H_2O$ 0.5g, thiamine hydrochloride 0.025g, soluble sugar 30g, Davis Agar 8g, and distilled water to a 1000 ml (Christie, 1958) . Glucose, fructose, and sorbitol were the soluble sugars tested .

Starch

Plates of Difco Starch Agar 25 g/l, pH 7.5 (starch 10 g/l) were inoculated with 5 mm diameter plugs of P. cactorum isolate 'P149' grown on DCMA and incubated at 24°C for 6 days in darkness . Colonies were flooded with an I-KI solution (1.5g KI, 0.3g I_2 in 100 ml distilled water) to stain the starch .

4-2-2. Phenolics

In Vitro Assay of Phenolics known to exist within Apple Tissue

Experiment 1

Varying amounts of phenolic compounds were added to autoclaved Difco Cornmeal agar (DCMA, 17g/L) which was then poured into petri dishes . Dissolving the phenolic compounds in the agar initially proved to be a problem . The phenolics were sparingly soluble in cold water . They were more soluble in 95% ethanol but amounts as low as 0.3 ml ethanol/100 ml DCMA substantially inhibited mycelial growth . Eventually, the compounds were dissolved in 50 ml of hot sterile water and added to 50 ml autoclaved DCMA (34g/L), mixed, and immediately poured into plates so that any breakdown of the phenolics was minimal . Very little contamination of the plates occurred using this method .

These phenolic enriched plates of DCMA were inoculated with five mm diameter plugs cut from a colony of P. cactorum 'P149' growing on DCMA and incubated in darkness at 24 ^{o}C for 5 days . The diameter of each of the resulting colonies was measured twice - one at right angles to the other and the inoculum plug's diameter was subtracted from that of the colony to give the distance of mycelial growth . Five petri dishes were inoculated per treatment .

Experiment 2

Four plates of the fungus growing on DCMA with (1.0mg/ml) and without phloridzin added were tested to assess the ability of P. cactorum to degrade phloridzin in vitro . Five mm disks were cut from the following zones of these petri dishes:

(a) DCMA , no phenolics added .
  (i) area colonised by P. cactorum for 3 days .
  (ii) area colonised and well in advance of the colony margin .

(b) DCMA , 1.0mg/ml phloridzin added .

(i) area colonised by P. cactorum for 3 days .

(ii) area just colonised , i.e. at colony edge .

(iii) area colonised and just outside colony margin .

(iv) area colonised and well in advance of margin .

Twelve disks were sampled per treatment (3 from each of the 4 plates) and pooled . These represented 1.06% of the total area of the 4 plates .

The disks were extracted in 5 ml of water at 2 ^{o}C in the dark for 14 days . Two ml aliquots were taken and phenolic content was determined with Folin-Ciocalteu reagent as described below .

The Determination of Endogenous Levels of Phenolics in Plant Tissue

Collection and Preparation of Material

Twigs were collected from four apple varieties over a period of two and a half months towards the end of the 1981-82 growing season (Table 4-II) . The following dormant season (at the end of June - beginning of July) and again in mid-January in the next growing season (1982-83), shoot collections were made from a larger number of apple varieties (Table 4-II) .

Table 4-II. Apple Varieties assessed for Total Phenolic Content of their Phloem-cambial Tissue

1981 -1982 Growing Season

Variety	Rootstock	Tree Age (yrs.)
Cox's Orange Pippin	M 793	6
Golden Delicious	EM XVI	21
Granny Smith	M 793	22
Oratia Beauty	MM 106	4

1982 -1983 Dormant and Growing Season

Variety	Rootstock	Tree Age (yrs.)
All Red Gravenstein	MM 106	7
Ames	MM 106	16
Cox's Orange Pippin	M 793	7
Early McIntosh	M IX	15
Ellison's Orange	EM XII	29
Golden Delicious	EM XVI	22
Granny Smith	M 793	23
Hawkes Bay Red Delicious	EM XIII	22
Hawkes Bay Red Delicious	M 793	22
Kempton	M 793	22
Kidd's Orange Pippin	EM XVI	22
Oratia Beauty	MM 106	5
Oratia Beauty	M 779	23
Red Dougherty	M 793	8
Rome Beauty	EM XVI	27
Spartan	M 793	22
Sturmer Pippin	EM XVI	23

The shoots were processed as previously described (Section 3-2-1) . The phloem-cambial tissues from twigs assessed for resistance was scraped from the cortex tissue using a scapel blade, sliced up and immediately frozen in liquid air to prevent oxidation of the phenolics . The tissues were freeze-dried for 72 hours and then stored at -15 ^{o}C until required for analysis .

Extraction and Analysis of Total Phenolics

The phenolic extraction procedure of Wilson (1967) and the analytical

procedure of Singleton and Rossi (1965) were followed . The polyphenoloxidase inhibitors sodium diethydithiocarbomate (DIECA) and sodium sulphite (Bielinin et al., 1973; Dias, 1965; Noordam, 1973) were initially used to inactivate the oxidative enzymes liberated by homogenisation . These compounds, however, reacted with the Folin-Ciocalteu reagent to give elevated absorbances . Since heating to 45 ^{o}C for at least 45 minutes in 70% ethanol inhibited the oxidative enzymes (Wilson 1967) without affecting colour, this was used as the extraction procedure .

Phloem-cambium samples (50 mg dry weight) were individually extracted in 10 ml of 70% ethanol (Wilson 1967) and homogenized for 3-5 minutes using a M.S.E. homogeniser . After extraction for a minimum of 45 minutes, the homogenate was filtered (Whatman No. 1) and the residue washed with ethanol in a Buchner funnel . The washings were combined with the rest of the filtrate . The ethanol was removed from the filtrate and its volume was reduced to approximately one-sixth using a rotary evaporator at 40 ^{o}C . The aqueous concentrate and the washings from the evaporator flask were then made up to 10ml with distilled water and left in the 45 ^{o}C water bath .

A 100 µl aliquot from each sample was placed into a test-tube containing 6.0 ml of distilled water . The samples were mixed with Folin-Ciocalteu reagent (0.5ml) and then 1.5ml of 20% Na_2CO_3 solution within the time limits of 30 seconds to 8 minutes . Samples were each diluted to 10ml with distilled water and maximum colour development was reached 2 hours later . The absorbance was read at 765 nm . Standards of phloroglucinol equivalent to 20, 40, 60 and 100 g/10ml and a blank were assessed along with the samples .

The test is based on the following reactions:

phenol $\xrightarrow[(Na_2CO_3)]{\text{alkaline conditions}}$ phenolate ion

Folin-Ciocalteu Reagent

A mixture of complex polymeric ions formed from phosphomolybdic and phosphototungstic heteropoly acids (+6 valence state) $\xrightarrow[\text{phenolate ion}]{\text{oxidation of}}$ complex molybdenum-tungsten blue (mixture of +5 and +6 valence states)

(Killeffer and Linz, 1952; Singleton and Rossi, 1965; Wu, 1920)

4-3. RESULTS

4-3-1-1. Total Nitrogen

The concentration of nitrogen in the phloem-cortical tissues was fairly constant from winter dormancy through to late summer when it declined (Figure 4-2) . In the unlignified xylem the concentration of nitrogen steadily decreased during the period it was measured ; i.e. from early summer to early autumn (Figure 4-3) . The pattern of variation in total nitrogen level of the phloem-cortex and unlignified xylem tissues was similar for all cultivars tested throughout the growing season .

During this time, the susceptibility of these tissues followed distinctly different patterns (Figure 3-6) . No significant correlations were found between the total nitrogen content of tested tissues and their susceptibility to P. cactorum .

4-3-1-2. Starch and Soluble Sugars

Soluble Sugars

The level of soluble sugars in the phloem-cortical tissues was maximal in early spring, declined during flowering, increased just after the period of greatest leaf expansion to a broad maximum in summer (December-January), and decreased as the summer lengthened (Figure 4-4) . The sugar concentration of the 'COP' and 21 year old 'OB' cultivars began to rise again in early autumn . Soluble sugars in the unlignified xylem tissue rose to a maximum in December, paralleling that of the phloem-cortex (Figure 4-5) . All tested cultivars showed a similar pattern and magnitude in their soluble sugar levels over the growing season . There were no significant correlations between tissue susceptibility (Figure 3-6) and soluble sugar levels with either phloem-cortical or unlignified xylem tissues .

Figure 4-2. The Seasonal Variation of Total Nitrogen Levels of the Phloem-cambial-cortical Tissues of Three Apple Cultivars.

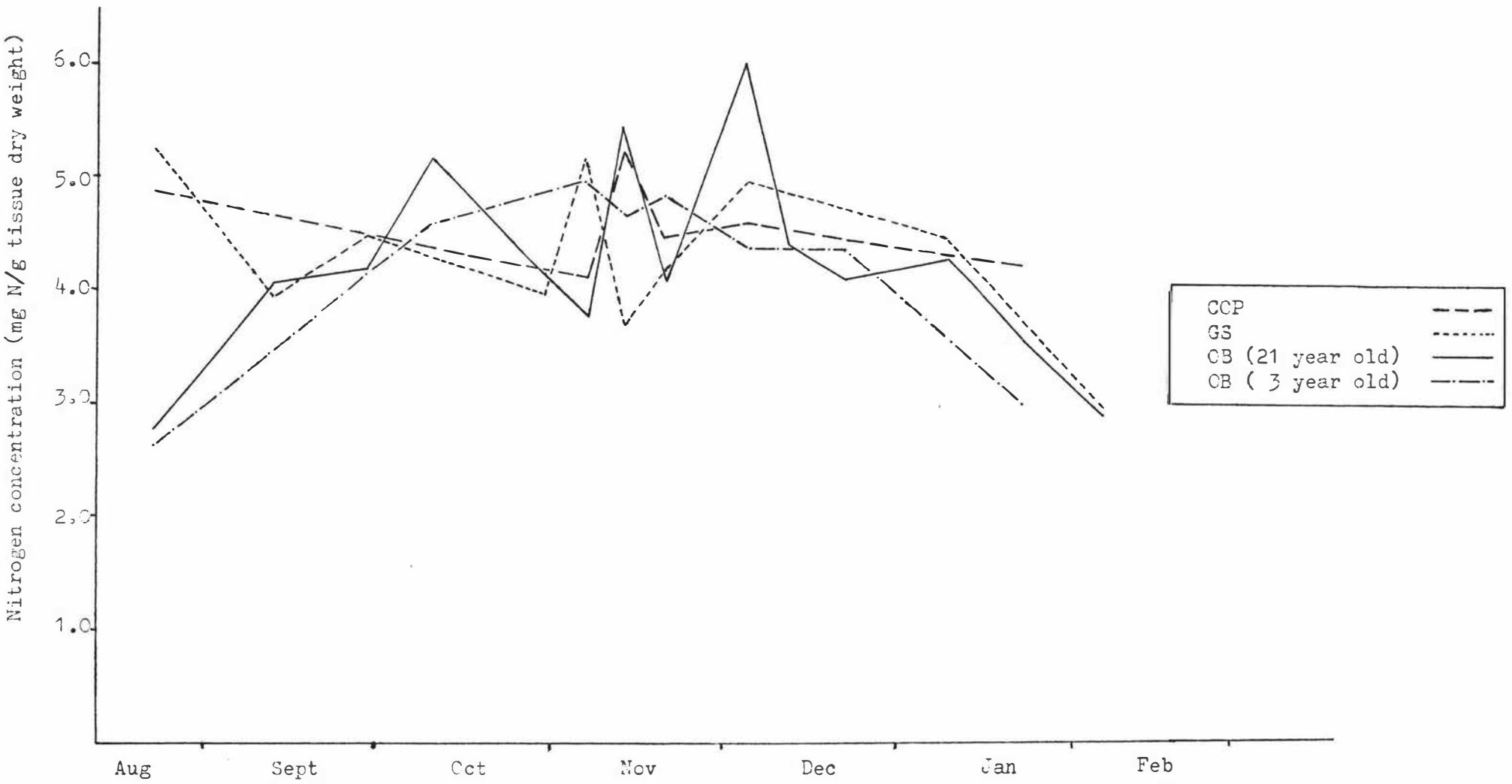


Figure 4-3. The Seasonal Variation of Total Nitrogen Levels of the Unlignified Xylem Tissue of Three Apple Cultivars.

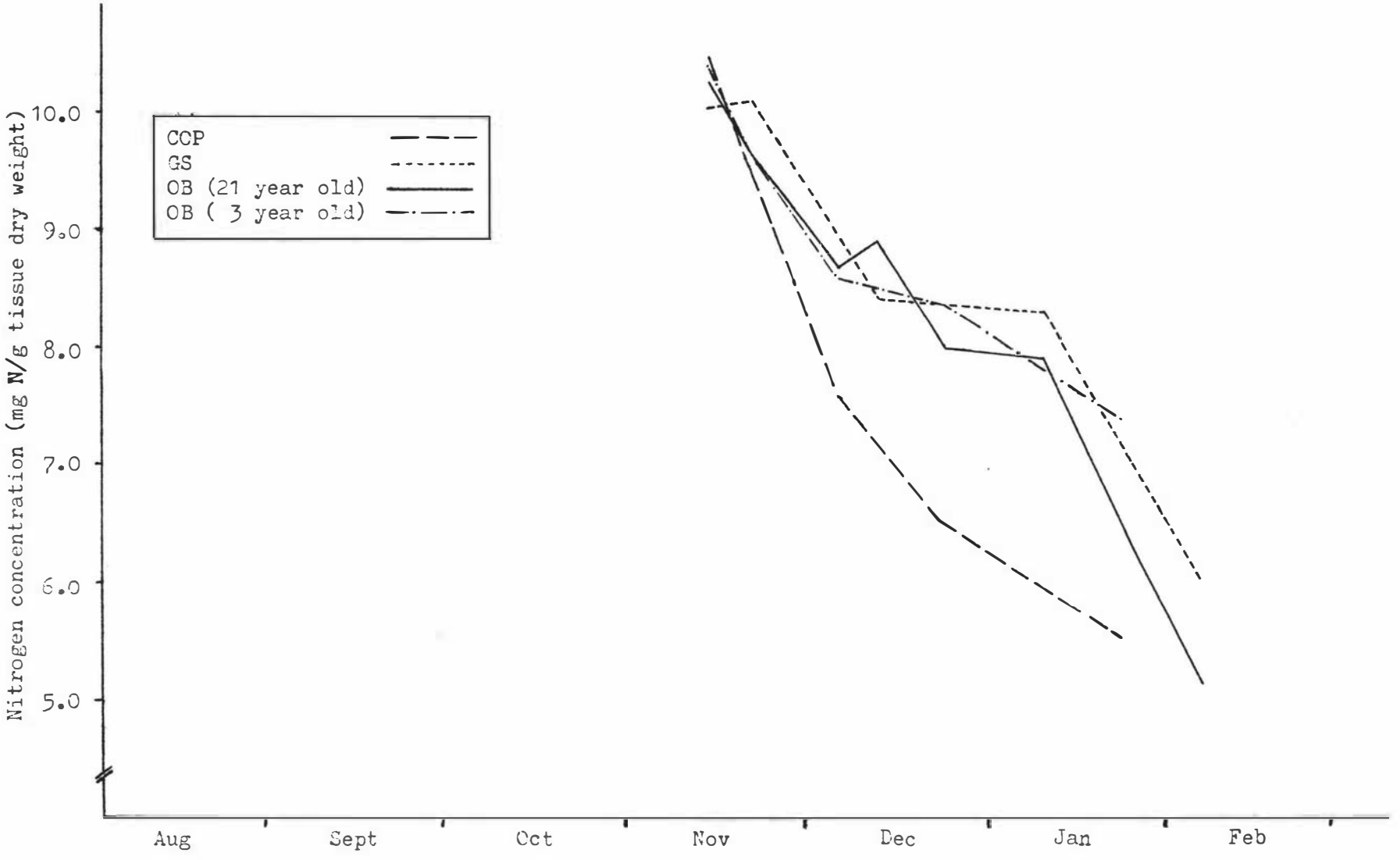


Figure 4-4. The Seasonal Variation of Soluble Sugar Levels of the Phloem-cambial-cortical Tissues of Three Apple Cultivars.

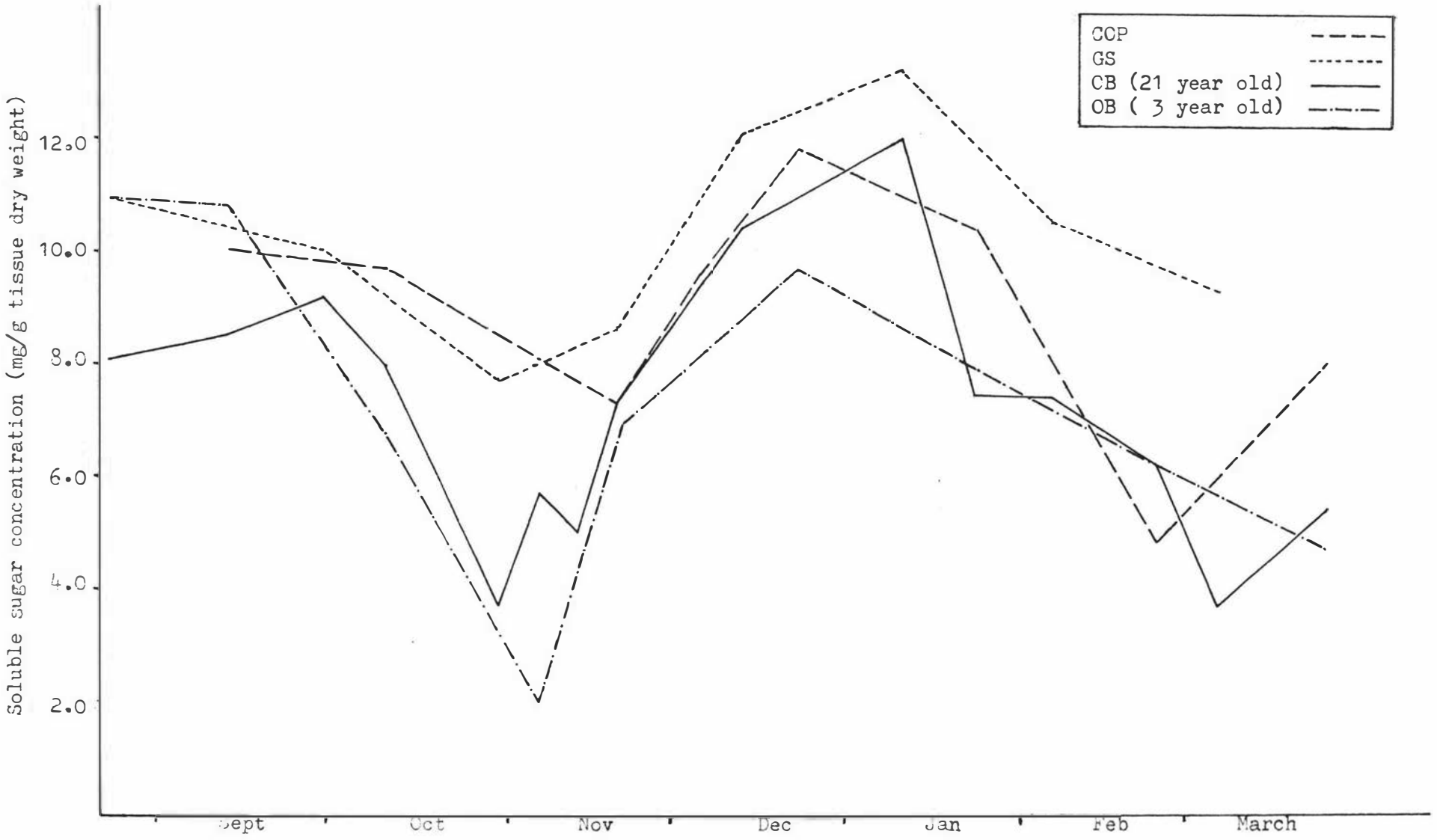


Figure 4-5. The Seasonal Variation of Soluble Sugar Levels of the Unlignified Xylem Tissue of Three Apple Cultivars.

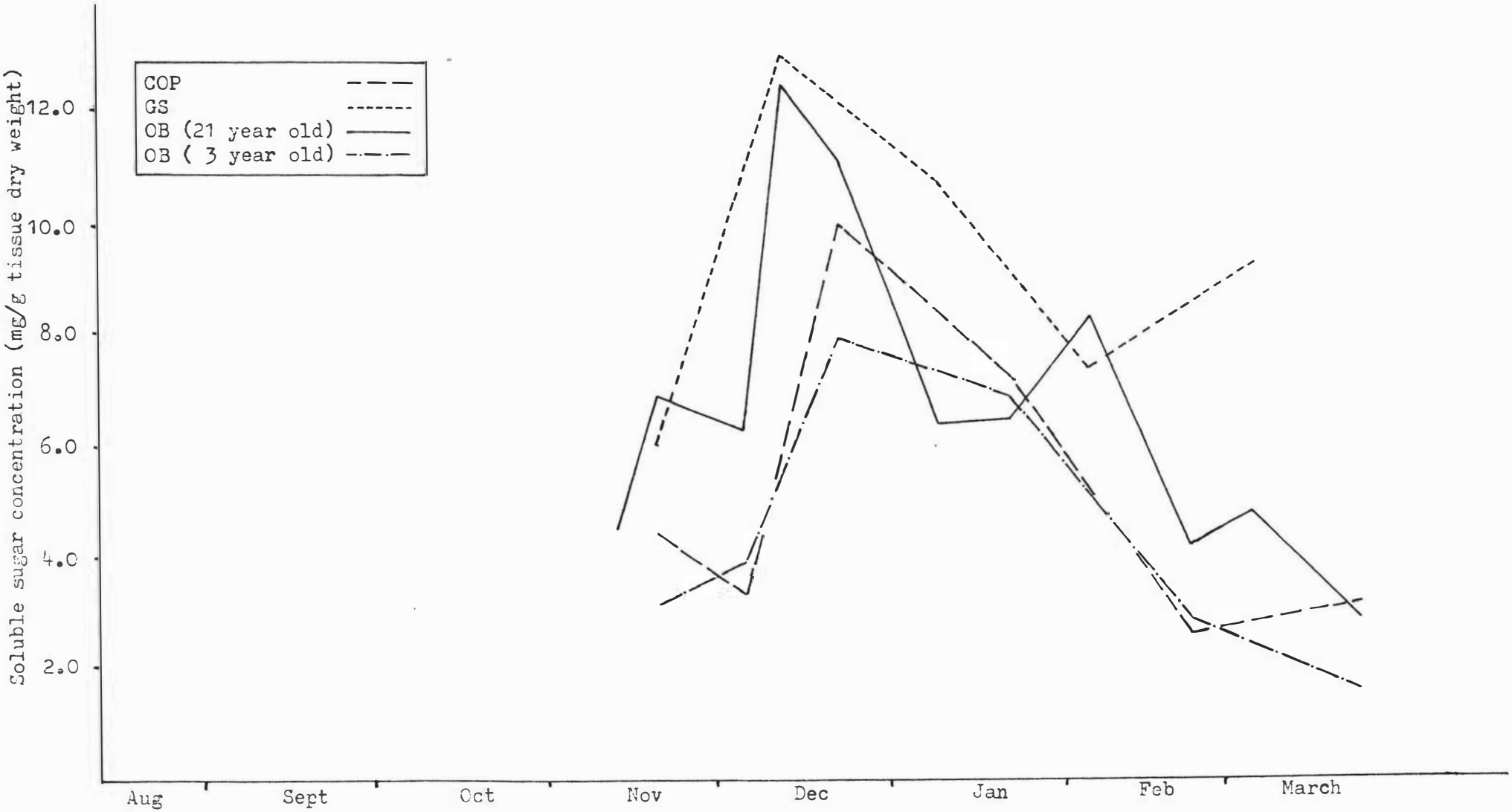
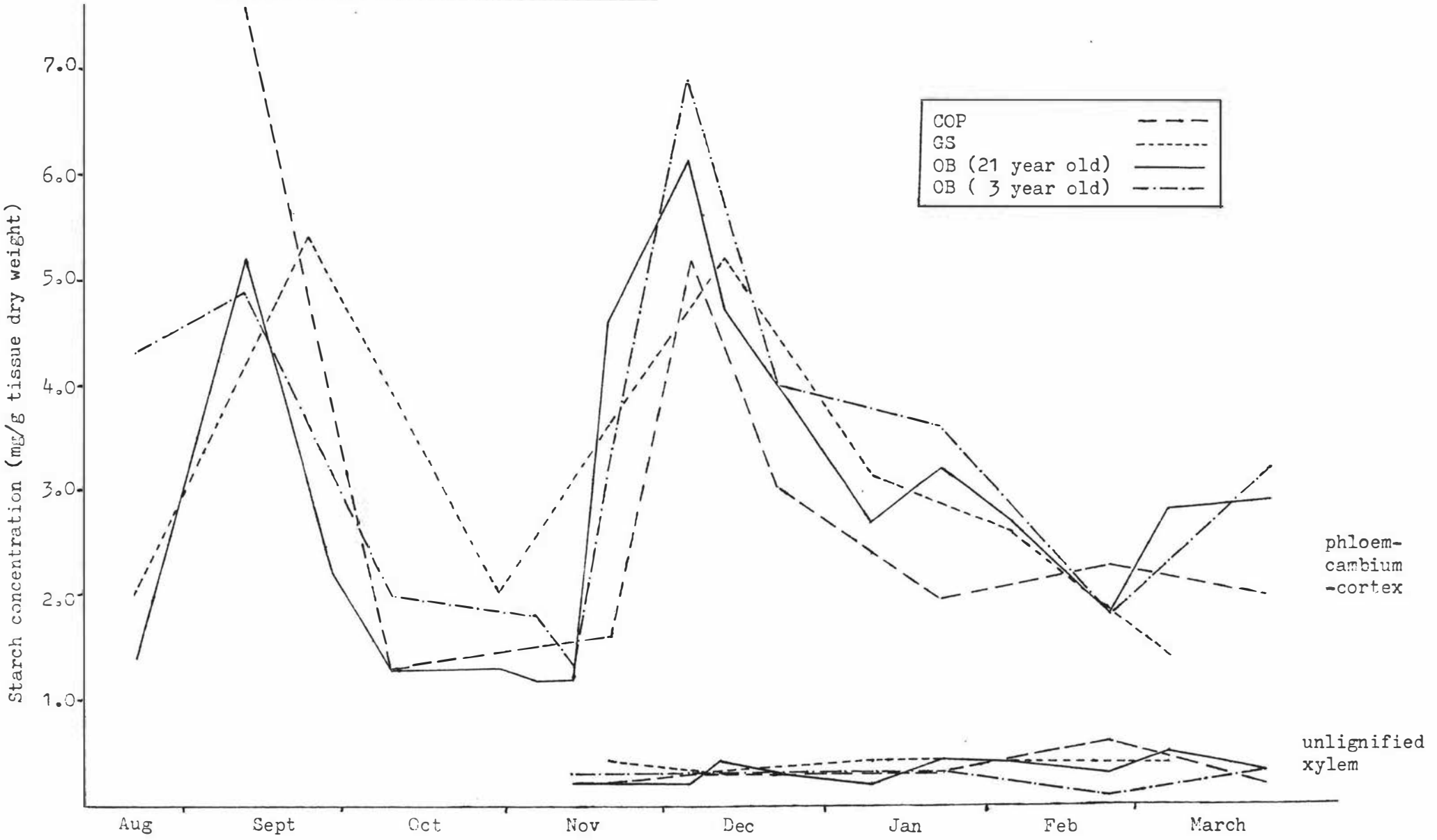


Figure 4-6. The Seasonal Variation of Starch Levels of the Phloem-cambial-cortical and Unlignified Xylem Tissues of Three Apple Cultivars.



Starch

The starch level was low in dormant cortex-phloem tissue, but increased prior to bud burst and subsquently declined during flowering (Figure 4-6) . It rose again in November, peaked in early December, and gradually decreased to a low level in early autumn (February - March) . The pattern of change was similar in all the tested cultivars . The amount of starch in the unlignified xylem was constantly very low (Figure 4-6) ; 0.01% to 0.06% dry weight compared with 0.1% to 0.8% for the phloem-cortical tissues . No significant correlations were found between starch content and tissue susceptibility .

Soluble Sugars and Starch, In Vitro Testing

Soluble Sugars

Table 4-III. Growth of P. cactorum on a Basal Medium Containing Different Sugars as the Carbon Source

Sugars tested	Isolate	
	P149	MU2
Glucose	25.4 ±0.7 a*	16.6 ±0.2 a
Sorbitol	7.8 ±0.6 b	4.8 ±0.3 b
Sucrose	4.9 ±0.5 c	3.0 ±0.4 c
Fructose	no growth	no growth
Control #	no growth	no growth

*Mean growth of 9 replicates after 6 days (colony diameter [mm] minus inoculum plug diameter) followed by standard error . Numbers in each column followed by the same letter are not significantly different from each other at 1% level (Duncan's Multiple Range Test) .

Basal medium with no sugar added .

Growth on the basal medium was poor . The P. cactorum growth rate was low (>1 mm/day for most treatments) when compared with its growth in apple tissue .

Glucose, sorbitol, and sucrose were all utilised as carbon sources but fructose was not (Table 4-III) . Glucose was the best carbon source for both isolates, sorbitol the second, and sucrose the third best .

Starch

Plates of starch agar flooded with I-KI solution were not stained under the colonies of P. cactorum or in a narrow zone (2-3 mm) just beyond the colonies's periphery . The remainder of the plate stained a dark purple black colour, typical of the reaction of starch and iodine .

4-3-2. Phenolics

In Vitro Assay of Phenolics known to exist within Apple Tissue

Experiment 1

The mycelial growth of P. cactorum was markedly inhibited by both phloridzin and its degradation product phloroglucinol (Table 4-IV) . The amount of inhibition increased with concentration so that by 8.0mg/ml (with phloroglucinol) the inhibition was total . Fifty percent inhibition occured at approximately 1.0mg/ml for phloridzin and 1.5mg/ml for phloroglucinol . The long runner hyphae became greatly shortened and the mycelium had a very branched appearance .

The related quercetin and rutin phenolics stimulated vegetative growth of the pathogen (Table 4-IV) . This stimulatory effect was significantly greater with quercetin indicating that the fungus metabolised these compound to a greater extent . At all tested

concentrations of rutin, a distinct browning occurred under P. cactorum colonies within 12-14mm of the colony's edge . The browning was faint on the 4 mg/ml and dark on the 8 mg/ml quercetin DCMA and it extended to within 7-8 mm of the colony margin . The mycelium growing in the presence of these phenolics was of normal morphology .

Table 4-IV. The Effect of Some Phenolics on the In Vitro Growth of P. cactorum

	Phenolics			
mg/ml	Phloridzin	Phloroglucinol	Quercetin*	Rutin*
0	46.3 ±0.4 a	46.4 ±1.0 a	46.4 ±1.0 d	46.4 ±1.0 b
0.5	35.9 ±0.9 b	38.9 ±0.2 b	51.5 ±1.1 c	51.8 ±1.2 a
1.0	23.8 ±1.0 c	30.1 ±0.5 c	53.8 ±0.8 b	49.0 ±1.3 a
2.0	12.9 ±0.9 d	16.5 ±0.5 d	56.3 ±0.5 a	51.3 ±1.2 a
4.0	-	3.5 ±0.2 e	58.4 ±0.3 a	52.0 ±0.4 a
8.0	-	0 f	58.0 ±0.6 a	49.9 ±0.8 a

* Quercetin = Quercetin-3-rhamnoside

* Rutin = Quercetin-3-rhamoglucoside

Means (colony diameter [mm] minus inoculum plug diameter) ± standard error followed by different letters are significantly different from each other , within each column , at the 5% level (Duncan's Multiple Range Test) .

Experiment 2

The DCMA contained phenolic compounds (the equivalent of 1.05 mg/mL of phloridzin) of which 20% were metabolized by P. cactorum (Table 4-V) . A similar amount of phenolics were utilised in the phloridzin-enriched DCMA, suggesting that P. cactorum can not degrade phloridzin in vitro . The phenolics that were degraded in culture appear to have been metabolised in advance of the hyphae (Table 4-V) .

Table 4-V. Ultilisation of Phenolics by P. cactorum In Vitro

Treatment	Amount of Phenolics (mg phloridzin/ml agar)
DCMA : control	
i) colonised by the fungus for 3 days	0.84
ii) uncolonised	1.05
DCMA : 1.0 mg/ml phloridzin added	
i) colonised by fungus for 3 days	1.60
ii) just inside colony margin	1.68
iii) just outside colony margin	1.65
iv) uncolonised, well in advance of margin	1.91

The Determination of Endogenous Levels of Phenolics in Plant Tissue

Experiment 1

Levels of extractables phenolics ranged from 0.65 to 1.6% dry weight within the phloem tissue of the four varieties tested over the late summer-autumn period (Table 4-VI) . During this time, the levels of phenolics generally increased, although cv. 'GD' and 'GS' (>1 year old shoots) peaked about the 19th of March .

The concentrations of these phenolics were usually greater in the phloem-cambium of the resistant 'Golden Delicious' (GD) and 'Granny Smith' (GS) varieties than in the more susceptible 'Oratia Beauty' (OB) and 'Cox's Orange Pippin' (COP) varieties . This trend was consistent with both the current and previous season's shoots, even though the phenolic levels were generally higher in the current season's shoots .

Table 4-VI. Levels of P. cactorum Susceptibility and Extractable Phenolics in the Phloem-cambial Tissue of Apple Shoots of Four Varieties during Autumn 1982

Table 4-VIA. Amount of Extractable Phenolics

Variety	Shoot age (years)	Date					
		11/2	15/2	16/3	19/3	29/3	23/4
COP	>1	-	-	8.4	11.7	-	-
	1-2	-	-	-	10.2	9.5	11.0
OB	>1	7.4*	6.5	-	10.8	11.8	14.0
	1-2	-	-	9.3	8.9	-	10.0
GD	>1	-	-	10.8	15.2	15.3	14.0
	1-2	-	-	-	12.4	14.3	-
GS	>1	10.8	10.8	11.4	16.2	12.6	13.5
	1-2	-	-	-	13.4	-	-

*Mean values in mg of phloroglucinol equivalents/g dry weight .

Table 4-VIB. Tissue Susceptibility to P. cactorum

Variety	Shoot age (years)	Date					
		11/2	15/2	16/3	19/3	29/3	23/4
COP	>1	-	-	7.0	8.3	-	-
	1-2	-	-	-	6.4	2.9	5.9
OB	>1	1.9#	5.8	-	7.8	3.6	4.8
	1-2	-	-	4.9	6.4	-	4.4
GD	>1	-	-	5.1	6.8	1.5	1.9
	1-2	-	-	-	4.4	1.2	-
GS	>1	1.6	5.6	11.3	6.4	4.5	2.8
	1-2	-	-	-	4.0	-	-

#Mean values in mm lesion extension/day .

The corresponding susceptibilities of the phloem-cambial tissues are shown in Table 4-VI) . During this two and half month period, a maximum of susceptibility occurred in mid-March and after which susceptibility usually decreased to a low level . The susceptibility of the 'OB' and 'COP' varieties was usually higher than that of the 'GD and 'GS' varieties .

No significant correlation was found within any variety between the endogenous levels of extractable phenolics and the degree of susceptibility of phloem-cambial tissue .

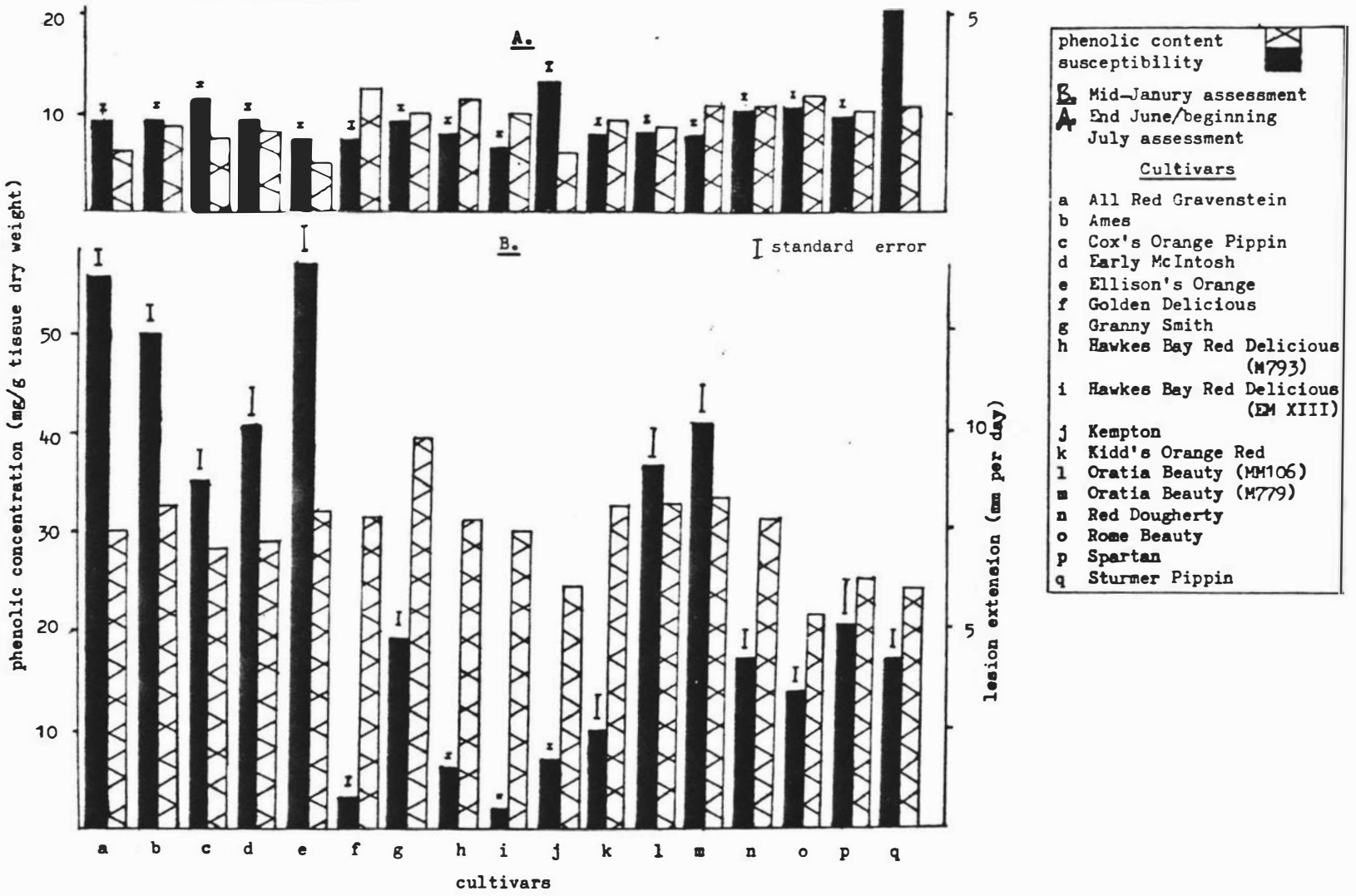
Experiment 2

Levels of extractable phenolics in the phloem-cambial tissue ranged from 0.45 to 1.29% dry weight when the plant was dormant in June/July and in mid-January when the plant was active, levels were greater - 2.18 to 3.96% (Figure 4-7) . The susceptibility of the phloem-cambial tissue was also lower during dormancy . In mid-January, the susceptibility was more variable and often considerably greater . The varieties which showed a large susceptibility at this time were 'All Red Gravenstein', 'Ames', 'Cox', 'Early McIntosh', 'Ellison's Orange' and 'OB' while the cultivars 'GD', 'HBRD', 'Kempton', 'Kidd's Orange Pippin' and 'Rome Beauty' showed a low susceptibility .

In mid-January 1983, higher levels of phenolics were measured in the resistant 'GS' cultivar than in the susceptible 'COP' one (Figure 4-7) . The cv. 'Early McIntosh' which recorded a large susceptible reaction at this time also had greater phenolic concentrations than the 'HBRD' and 'KOP' varieties which showed low susceptibility reactions . The endogenous levels of phenolics did not vary in accordance with the resistance of the cultivars tested .

The phenolic concentration in the scion variety did not seem to be affected by the type or age of the rootstock . 'HBRD' on different stocks of the same age and 'OB' on different stocks of varying ages had similar phenolic values within each variety .

Figure 4-7. Levels of Extractable Phenolics and Susceptibility to P.cactorum of the Phloem-cambial Tissue of Previous Season's Apple Shoots of 17 Cultivars.



4-4. DISCUSSION

4-4-1. Total Nitrogen

The total nitrogen level of the unlignified xylem decreased steadily during the period of fruit expansion . The nitrogen content of the unlignified xylem followed the same pattern as reported for the xylem sap by Bollard (1953), Cooper et al. (1972), Hill-Cottingham and Bollard (1965), Tromp and Ovaa (1967) and Tromp and Ovaa (1976) .

The total nitrogen content of the phloem-cambium-cortical tissues of previous season shoots was fairly constant throughout the growing season but lower in early spring and autumn . The tested trees had a low nitrogen status - no nitrogenous fertiliser had been applied to them in either the previous or current year - probably accounting for the small variation in nitrogen levels throughout the season . Other workers (Manson and Whitfield, 1960; Oland, 1959) have also found low nitrogen status apple trees to have a less marked seasonal variation in levels of bark tissue nitrogen .

Phytophthora species grow well in synthetic media with amino acids as the sole source of nitrogen (Cameron and Milbrath, 1965; Christie, 1958; Christie, 1959; Davies, 1959; Hohl, 1975; Leal et al., 1971; Leal et al., 1964; Lopatecki and Newton, 1956) . Arginine is one of the best sources of nitrogen for P. cactorum in vitro (Cameron and Milbrath, 1965; Christie, 1958; Leal et al., 1971) and is most abundant in apple tissue in winter when resistance is high . Therefore, the ability of P. cactorum to readily utilise the main soluble nitrogen compounds present in apple tissues implies that the type of nitrogen compound does not determine the colonisation rate of host tissue .

The lack of any significant correlation between the total nitrogen level and tissue susceptibility indicates that the amount of endogenous nitrogenous compounds do not appear to determine tissue susceptibility

to P. cactorum .

The lack of correlation between tissue susceptibility and endogenous levels of nitrogenous compounds does not necessarily mean that these substances had little or no effect of the pathogen's rate of spread through apple tissue . When the sampling was done, a large source of variation caused by the basipetal gradient of resistance along the previous season's shoots was not taken into account . Future work on whether endogenous levels of nitrogenous compounds play a determine role or a limiting role when levels are low must take this gradient into consideration .

4-4-2. Soluble Sugars and Starch

The starch present in the phloem-cambium-cortex of the previous season's shoots reached a maxima in early spring when soluble sugars were converted to starch, and during the period of temporary retardation of shoot elongation . The two minima occurred at times of intense plant growth : one at the flowering, fruit set, and leaf expansion stages ; and another at active shoot extension and fruit expansion (Figure 4-6) . The soluble sugar content showed a similar pattern, but the second peak rose to a maximum at a later date than the corresponding starch one (Figure 4-4). This was probably due to the conversion of starch into soluble sugars maintaining a high soluble sugar level even while the plant's utilization was great . This pattern and magnitude of the seasonal variation in soluble sugars and starch content has found by other workers (Bradfield and Flood, 1950; Kozlowski and Keller, 1966) .

The soluble sugar fluctuation within the unlignified xylem paralleled that of the phloem-cambium-cortical tissue suggesting that the amount of sugars in the former was in equilibrium with those in the latter . Little or no starch was present in the unlignified xylem . Swarbrick (1927) also observed an absence of starch in the cells of the unlignified xylem .

In vitro, P. cactorum utilised the three main endogenous soluble sugars present in apple tissue (sorbitol, sucrose, and glucose) to various extents, but not fructose . Other workers (Christie, 1958; Roncadori, 1965) have found isolates of P. cactorum to grow well on basal media containing fructose as the sole carbon source . The three isolates that Roncadori (1965) tested grew almost as well with fructose as they did with glucose . Christie (1958) fou nd that fructose utilisation appeared to depend on the pH of the medium ; P. cactorum grew well on fructose when initial pH was 4-5 but not at all when it was 6.5 . The growth rate of this fungus was lower on the basal medium than within apple tissue even though the amount of soluble sugar present was considerably greater in the basal medium, indicating that other factors were limiting fungal growth . It appears that the ability of P. cactorum to utilise a soluble sugar in culture depends on the medium used and its initial pH .

P. cactorum is also able to utilise starch . The iodine staining pattern of P. cactorum growing on the starch agar showed that the pathogen produced abundant amounts of α-amylase which diffused from mycelial tips and degraded the starch in advance of its mycelium . Ho and Foster (1972) also found P. cactorum to hydrolyse starch, but not beyond the colony edge .

P. cactorum is able to utilise the major carbohydrates, these do not appear to be determinant factors in the colonisation of apple tissue by the pathogen since correlations between tissue susceptibility and levels of soluble sugars and starch were not significant .

4-4-3. Phenolics

Alt and Schmidle (1980) and Bielenin et al. 1973 who each tested two varieties, found that the resistant apple variety had a greater endogenous level of phenolics than the susceptible one . This trend was also found when four varieties were tested in the late summer-autumn of 1982 (Table 4-VIA) but was not observed when 17 varieties were tested the following season .

The levels of phenolics in the tissue of the varieties collected in the late summer-autumn of 1982 after the cessation of cambial activity (Table 4-VIA) were of similar magnitude to the winter (June/july) levels (Figure 4-7) . Endogenous concentrations of extractable phenolics appear to be greater when the plant metabolism is most active . Howard and Bassuk (1980) found that the amount of phloridzin in vacuum extracted xylem sap increased substantially in 'M 26' shoots at the onset of rooting . Alt and Schmidle (1980) however found phenolic levels to be relatively consistent throughout the year .

The vast majority of the phenolics present in apple tissue consists of phloridzin (Wilson, 1967) . Phloridzin was toxic to P. cactorum in vitro . It exhibited strong fungistatic effect on the fungus by reducing the extension growth of the long runner hyphae, thus inhibiting vegetative growth on DCMA 49.5% and 72% with 1.0 (0.1%) and 2.0 mg/ml (0.2%) phloridzin, respectively . Concentrations of 0.1% phloridzin reduced zoospore germination while 1% phloridzin decreased mycelial growth (Schwinn 1965) . The colony growth of isolate 15 was inhibited 23.5% by 1mg/g (0.1%) phloridzin (Bielenin et al., 1973) and the same concentration prevented the growth of 'P18' (Alt and Schmidle, 1980) . The toxicity of phloridzin in vitro appears to be influenced by the isolate used and/or the medium on which it is assayed . Its degradation products, phloroglucinol and phloretin, were also toxic but to a lesser extent (Alt and Schmidle, 1980) .

The quercetin and rutin phenolics stimulated mycelial growth. This suggests that they are utilised as a carbon source (Lyre, 1965) with the former being the better energy source . Effects of these two phenolics in vivo are likely to be insignificant since they are present only in low concentrations and since the fungus will have more carbon sources available in apple tissue than it has on the nutrient-low DMCA .

P. cactorum did not appear to be able to degrade phloridzin in vitro (Table 4-V) suggesting it is unable to detoxify this phenolic in advance of its mycelium in apple tissue .

The pathogen however appeared to rapidly metabolise a portion of

the unidentified phenolics present in the DCMA in advance of the mycelium . Further degradation of the phenolics even after several days was slight, indicating that these phenolic-degrading enzymes were largely secreted from the hyphal tips . Kaosiri and Zentmyer (1980) have also found Phytophthora species to produce phenolic-oxidising enzymes in vitro . P. capsici, P. cinnamoni and 3 different morphological forms of P. palmivora all produced peroxidases, although the amount and type varied between the species and the morphological forms (Kaosiri and Zentmyer, 1980) .

Phenolics, especially phlordzin, are present in apple tissues in large quantities (1-11% dry weight of phloem-cambium-cortical tissue) and, theoretically, very inhibitory amounts (Alt and Schmidle, 1980; Barnes and Williams, 1961; Bielenin et al., 1973; Hutchinson et al., 1959; Schwinn, 1965; Wilson, 1967) . Yet mean pathogen growth rates of over 10 mm per day have been measured within host tissue (Section 3-2-1) indicating that the hyphae are not physically encountering inhibitory amounts of phloridzin in vivo . Histopatholgical studies (Section 2-3) have shown that P. cactorum secretes toxins in advance of the mycelium . These toxins cause the disruption of the host's cytoplasm and membrane systems allowing the mixing of formerly compartmentalised cellular components . The liberated host polyphenol oxidases rapidly convert phloridzin and other phenols into non-toxic polyphenolics (Dias, 1965; Bassuk and Howard, 1980; Kosuge, 1965; Loomis and Battaile, 1966; Raa, 1968; Raa and Overseem, 1968) so that it is possible that by the time the hyphae reach the host cells, phloridzin is no longer present in toxic levels . In the presence of apple enzymes phloridzin is rapidly oxidised to polymeric material . These transformation reactions proceed very rapidly so that the majority of phloridzin present is oxidised within an hour (Hunter, 1975; Noveroske, Kuc and Williams, 1964; Noveroske, Williams and Kuc, 1964; Podstolski and Lewak, 1970; Raa, 1968; Raa and Overeem, 1968) .

The amount of phenolics directly contacted by the pathogen in vivo will depend not only on the endogenous quantity of phenolics but also upon the activity of the phenolic oxidising enzymes (Byrde, Fielding and Williams, 1960; Ingham, 1973) . It is likely that the balance between the concentration of phenolics and the production and activity of host

polyphenol oxidases upon infection may have a major determining influence upon the seasonal susceptibility of the apple tissue . The establishment phase of infection may be the critical stage . At this stage, hyphae will be directly encountering host cells at the same time or only shortly after their toxins .

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** GENERAL DISCUSSION **
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Apple trees have not one but many mechanisms of resistance to P. cactorum . These mechanisms of resistance are morphological and physiological in nature and can be either preformed or formed after infection by P. cactorum .

In the soil, before the pathogen contacts the host tissue it encounters root exudates and the micro-organisms of the rhizosphere . Root exudates play an important role in chemotaxically and chemotrop ically attracting motile zoospores and germ tubes to the root . Exudation is greatest from the unsuberised roots and wound sites where the outer protective suberised layer has not yet formed or is broken . The fungal-attractive properties of roots exudates of unsuberised apple roots appeared to be similar for cultivars of varying resistance .

At the unsuberised zone, the rhizosphere is least developed since roots often out-grow the fungi and new colonisation is mainly from the surrounding soil (Bowen and Rovira, 1976) . Competitive or antagnostic effects from other micro-organisms would thus be less at this site . In the current study, micro-organisms on apple roots appeared to have some inhibitory effect on P. cactorum . Certain soil micro-organisms have been shown to inhibit Phytophthora growth in vitro (Janisiewicz and Covery, 1982; Utkhede, 1984), to parasitise Phytophthora spores in soil (Duniway, 1979; Sneh et al., 1977) and to reduce Phytophthora infection of host plants (Bowen and Rovira, 1976; Malajczuk et al., 1977); these experiments show that soil micro-organisms have some role in below ground Phytophthora disease situations but have not been able to assess the relative importance of competitive or antagonistic micro-organisms under various field conditions . This is due to the complexity of the situation which includes factors such as the number

of different competitive and antagonistic micro-organisms and their abundance, the presence of other micro-organisms, the sequence of infection, the degree of colonisation of the root, soil conditions especially when wet, and the stage(s) of P. cactorum life-cycle which are affected . This is an area for future work .

In this work, cultivar specific mechanisms of resistance in apple roots did not appear to be acting external to host tissue to prevent P. cactorum inoculum arriving at the infection court or at the host-environment interface to prevent penetration of host tissue . Resistance was expressed after pathogen entry . This fits in with the findings of most workers on zoospore attraction to resistant and susceptible host and non-host plants as discussed in Chapter 1 .

Investigations of internal resistance were hampered by the lack of a fully reliable method of assessing resistance . Further characterisation of the excised twig method revealed the importance of the basal-distal location of the sampled shoot piece . Future testing using this method to reveal susceptibility differences between cultivars should be done on twig pieces taken from a standard position on the shoot . Since gradients of auxin are known to exist in stem tissue, future work could be undertaken to determine if IAA does cause this gradient of resistance along previous season's apple shoots . For example, IAA could be infiltrated into shoots and susceptibility could be assessed immediately, or sometime after, to verify whether IAA has a direct or indirect effect on resistance .

The order of susceptibility of the exposed stem tissues was not the same as that in situ . Whether or not this is due to oxygen availability or other factors needs to be verifed . It however means that this modification of the excised twig method is limited in it application and the method of Jeffers et al. (1981) in which one end of the intact twig piece is inserted into P. cactorum colonised agar would obviously be the more sensible one to use to test between cultivars .

The technique of assessing oospore numbers in unsuberised roots requires further evaluation but possibly would be a better method for determining a cultivar's resistance to the root rot and perhaps the crown rot forms of the disease .

In all but the youngest stem and root tissue, the apple tree presents an outer protective suberised layer to the environment which the pathogen must penetrate . Schwinn (1965) found that the bark tissues of the field resistant cv. 'Goldparmane' had a similar susceptibility to P. cactorum as the highly susceptible cv. 'Cox's Orange Pippin' . He suggested that the field resistance of cv. 'Goldparmane' was due to the greater structural integrity of its periderm .

The production of wound barriers to contain or impede the pathogen after entry, occurs in a number of plant-Phytophthora combinations such as P. megasperma infection of alfalfa roots (Marks and Mitchell, 1970) and P. cinnamomi infection of the roots and stem of Eucalptus marginata (Tippett et al., 1983) . In this work, apple tissue was not observed to produce wound periderm in response to infection . Since inoculations were always carried out in the laboratory in this work, the faster growth rates of the pathogen may have given the host insufficient reaction time . Alt (1980) found that the highly resistant cv. 'Maunzen' formed a well developed periderm in its bark tissue in response to infection while the cv. 'Cox's Orange Pippin' formed a poorly developed one which failed to contain the fungus .

Apple shoot and root tissue did not produce morphological barriers to P. cactorum at a cellular level . Callose-like deposits are formed in the cells adjacent to hyphae or around haustoria in a number of plant-Phytophthora combinations . In some of these combinations, these have been found to occur more frequently in resistant cultivars and species (Cahill and Weste, 1983; Hohl and Stossel, 1976) but have not been considered to be of any significance in the resistance of the species (Cahill and Weste, 1983) .

Since morphological structures such as wall depositions were not observed to be formed within apple root cells in response to the fungus in this work, it appears that some of the cultivar specfic mechanisms of resistance operating were physiological . These would either consist of substances produced by the host before infection which confers a degree of resistance or susceptibility to that host (preformed substances) or inhibitory substances formed in response to invasion by the pathogen (phytoalexins) or a combination of both .

Endogenous levels of some nutritional compounds (carbohydrates and nitrogenous compounds) did not appear to determine the rate of pathogen spread through apple tissue . Phenolics may have a role in restricting fungal growth . The most likely phenolic to do so is phloridzin . This is present in apple tissue in considerable amounts and it and its degradation products inhibited P. cactorum in culture while the other tested phenolics did not . The levels of phloridzin which the hyphae encounter may determine the rate of pathogen spread through apple tissue . The amount of phloridzin present in the dead cell zone and the activity of host polyphenol oxidases in different cultivars could be a topic for future investigation . If polyphenol oxidase activity is lower in the more resistant cultivars, phloridzin would be less rapidly degraded and so the hypha would encounter higher levels of it .

In these plant-Phytophthora systems where phytoalexin production has been studied, phytoalexins appear to be triggered either by fungal products or by host substances produced by adjacent dead or dying cells (Daly, 1984) . Phytoalexins are also produced more rapidly in the host cultivars or species that are resistant to various Phytophthora species than in susceptible ones (Keen and Yoshikawa, 1983) . If phytoalexins form part of the defenses of apple tissue to P. cactorum then one can postulate that if the healthy apple cells are killed fast enough, these cells will have insufficent time to produce fungitoxic levels of phytoalexin . Thus the expression of resistance will depend on both the speed with which the host can produce these phytoalexins and upon the speed with which the pathogen can kill the healthy host cells and thus prevent further phytoalexin production .

Plant defense mechanisms do not consist of any single mechanism but are a composite . The research to date has left many gaps in our understanding of what they are, how they are formed or triggered and how they function to prevent or reduce infection . Ii is unlikely that this information will be easily or quickly obtained in the future due to the diversity of the defence mechanisms involved and the vastness of the number of plant-pathogen interactions . A full elucidation of the relative importance of various mechanisms under various environme ntal conditions for any single plant-pathogen interaction will probably require a multi-discipline approach . However for sometime to come we will probably continue to focus on single mechanism studies and use

them as our bases to explain how a specific host stops or reduces infection caused by a specific pathogen .

APPENDIX 1 - Composition of Reagents

Enzyme Reagents used for Starch Extraction from Plant Material

Refer to Section 4-2-1-2.

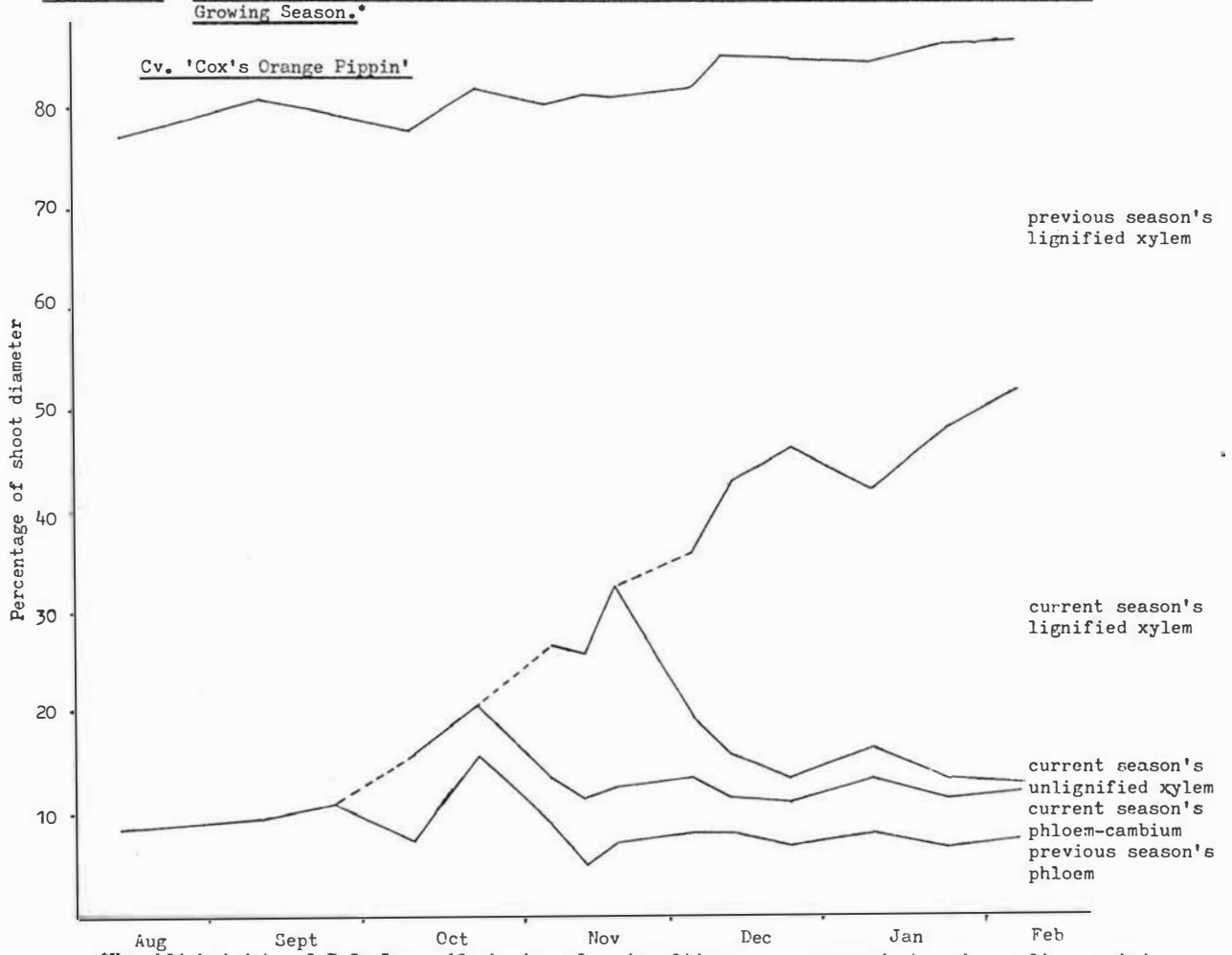
Amyloglucosidase Preparation

Fifty ml of amyloglucosidase (Glaxo Laboratories Ltd., Greenford, Middlesex, England) was centrifuged at 13,000 *g* for 10 minutes and the supernaturant passed through a 5.7 x 20 cm column of Sephadex G-25 pre-equilibrated with 25mM sodium citrate pH 6.0 . The protein eluted after the void volume was collected and stored at -20 C . When required 1 ml of this stored solution was diluted to 10 ml with 25mM sodium citrate pH 6.0 .

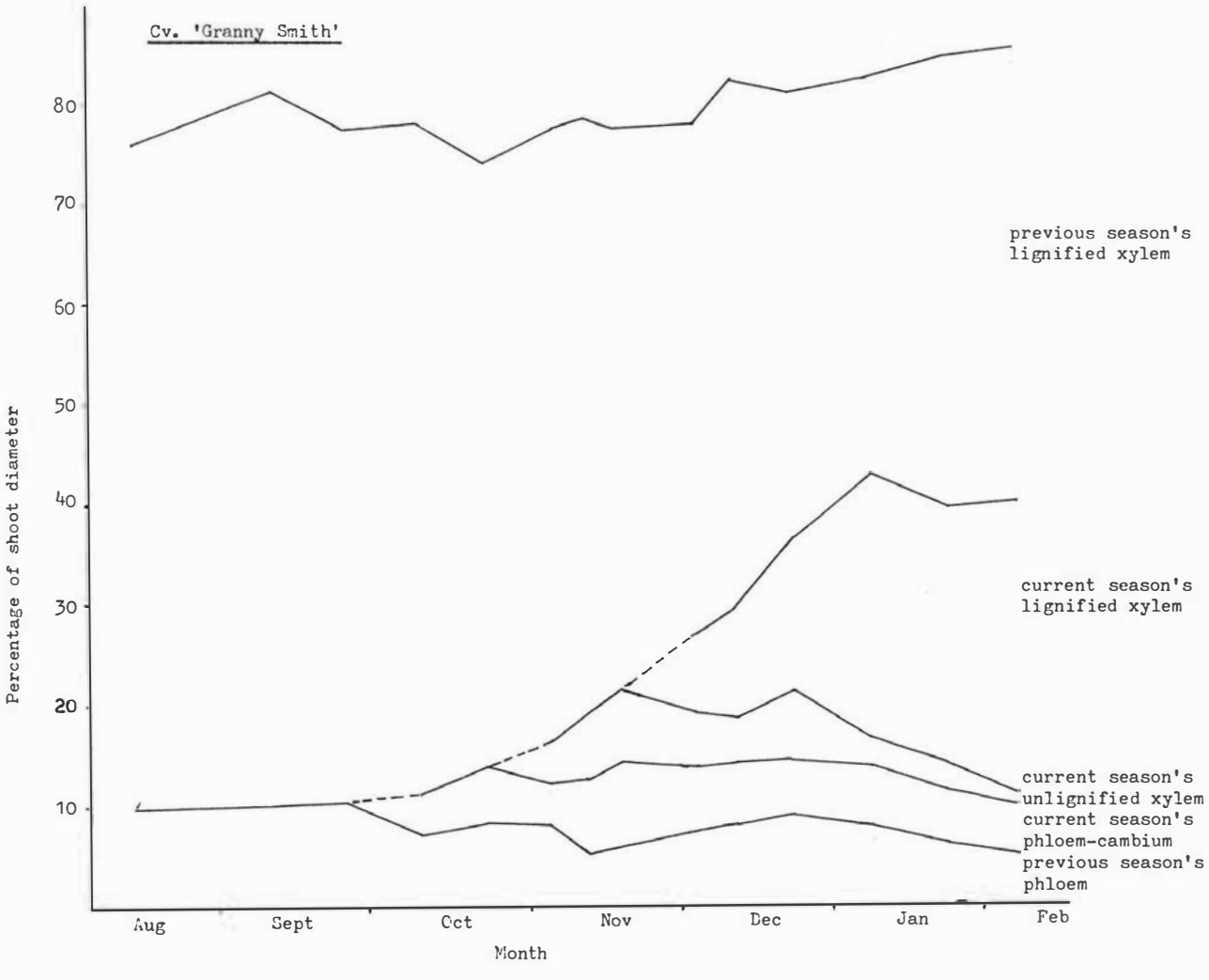
Glucose Oxidase Reagent

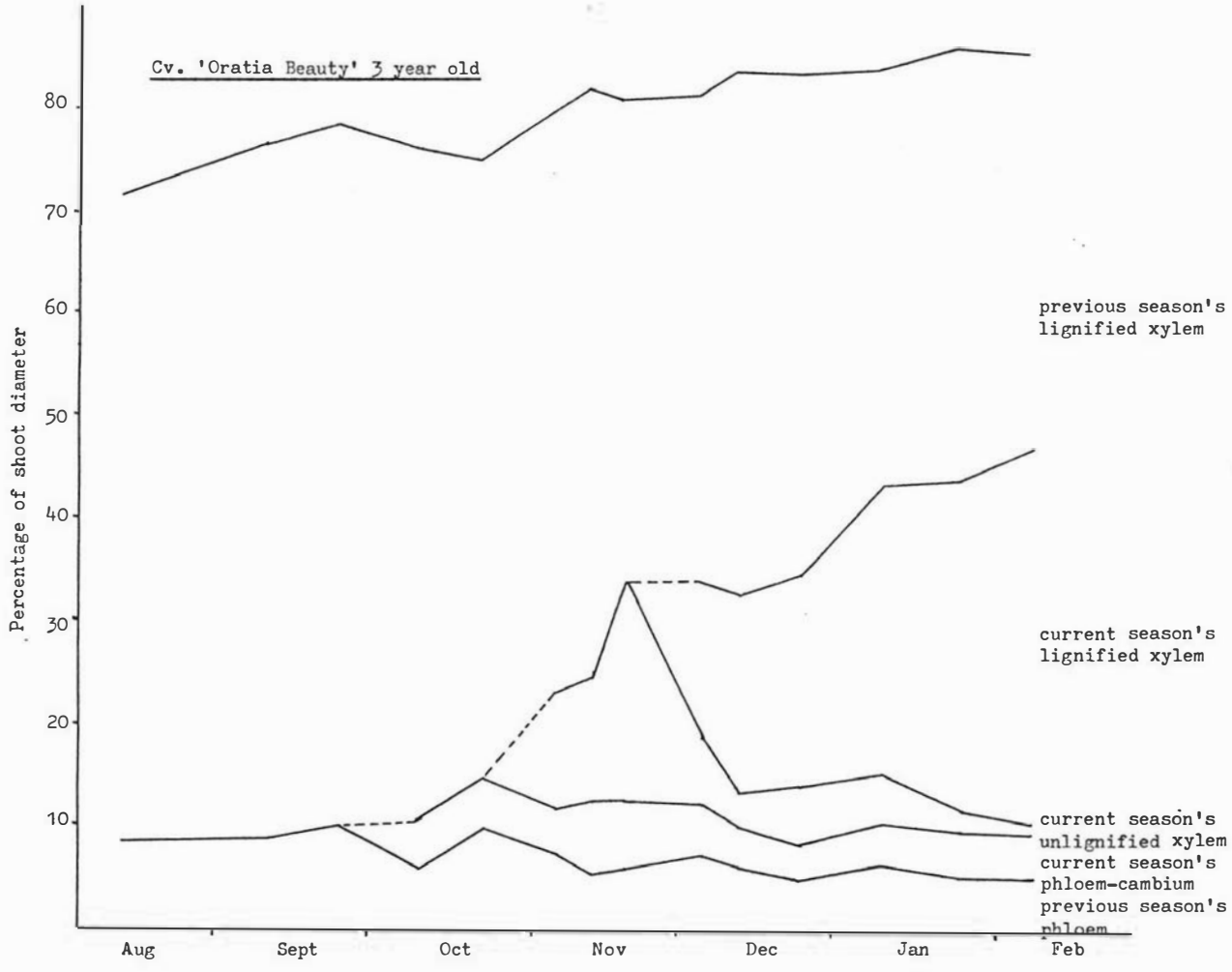
Glucose oxidase (Sigma, *Aspergillus niger*) 180 mg, peroxidase (Sigma, Horseraish) 18 mg, and o-dianisidine hydrochloride 120 mg (Sigma) were dissolved in 600 ml Tris-glycerol buffer pH 7.0 . The Tris-glycerol buffer was made with 30.2 *g* Tris in 500 ml distilled water (adjusted to pH 7.0 with conc. hydrochloric acid) and 330 ml glycerol.

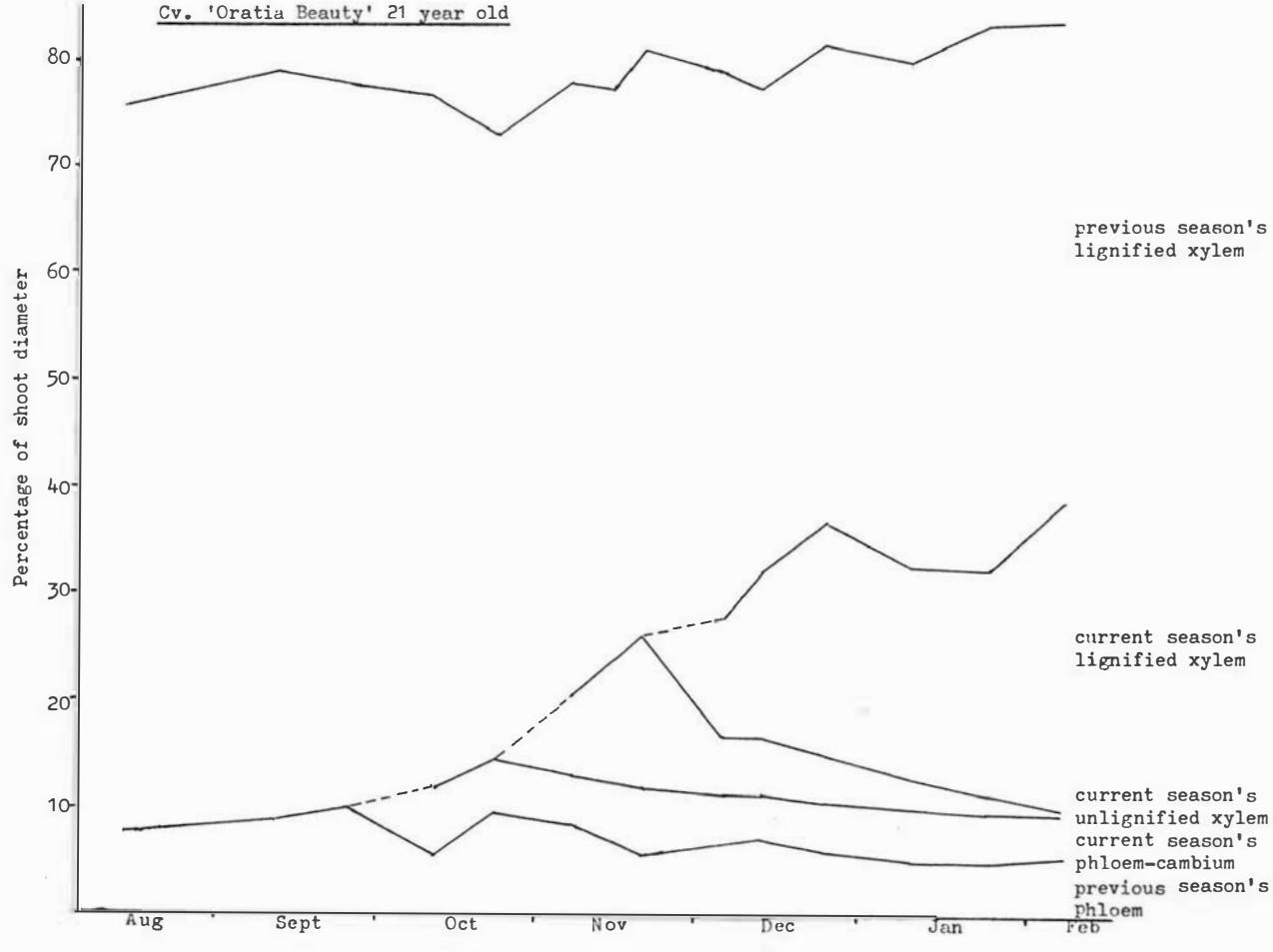
Appendix II. Proportion of Stem Tissues in Previous Season's Shoots Collected during the 1980-81 Growing Season.*



*Unpublished data of P.G. Long; 10 shoots of each cultivar were measured at each sampling period.




Cv. 'Oratia Beauty' 3 year old
Percentage of shoot diameter
80
70
60
50
40
30
20
10
previous season's lignified xylem
current season's lignified xylem
current season's unlignified xylem
current season's phloem-cambium
previous season's phloem
Aug
Sept
Oct
Nov
Dec
Jan
Feb


Cv. 'Oratia Beauty' 21 year old
Percentage of shoot diameter
80
70
60
50
40
30
20
10
previous season's lignified xylem
current season's lignified xylem
current season's unlignified xylem
current season's phloem-cambium
previous season's phloem
Aug
Sept
Oct
Nov
Dec
Jan
Feb

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