

PYTHIUM SPECIES IN APPLE ORCHARD SOILS AND
THEIR PATHOGENICITY TO APPLE SEEDLINGS

BY

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ABSTRACT

In assays of Pythium populations in 137 English apple orchard soils the most frequent and abundant species were P. heterothallicum and P. sylvaticum (both heterothallic), and a presumed heterothallic species designated Pythium x. P. intermedium (heterothallic) and P. irregulare were less frequent but abundant when present. Other Pythium species included: acanthophoron, coloratum, dissimile, paroecandrum, rostratum, salpingophorum, torulosum, ultimum and vanterpoolii. The incidences of the most frequently occurring species were significantly associated with region and with soil texture, pH and phosphorus content.

Growth of apple seedlings in small volumes of orchard soils was associated with increased abundance of P. sylvaticum, P. heterothallicum and P. intermedium, and with greatly increased frequency of occurrence of P. sylvaticum. The incidence of apple replant disease (ARD), reflected by apple seedling growth increases after chloropicrin soil fumigation, was significantly associated with the incidence of P. sylvaticum. P. sylvaticum, however, was not the sole cause of ARD, and its presence was not invariably accompanied by ARD.

In laboratory tests with apple seedlings, most Pythium isolates caused partial or complete inhibition of root growth and diffusible toxins were demonstrated. Many isolates were parasitic. P. sylvaticum, P. irregulare, and to a lesser extent, P. ultimum, (all parasitic) and one non-parasitic isolate of P. heterothallicum caused significantly diminished apple growth in pot-plant tests.

Orchard soils varied greatly in their fungistatic effects on the growth of Pythium spp. Strong fungistasis (to P. sylvaticum) was associated with high soil pH, best apple growth, and with diminished incidence of P. sylvaticum and ARD.

Cumulatively the results provided strong circumstantial evidence that P. sylvaticum is importantly implicated in the poor growth disease of apple in England. The involvement of other Pythium spp., both parasitic and non-parasitic, was also indicated.

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INTRODUCTION

Investigations of Pythium spp. in soil and as plant root pathogens (other than agents of seedling damping-off diseases) have become possible only with the development of new highly selective isolation media. Studies during the last two decades have demonstrated the widespread distribution of Pythium spp. in natural and cultivated soils and have also indicated their possible importance as major determinants of plant growth. Pythium spp. have been implicated in decline or replant diseases of several annual and perennial crop plants including apple.

The investigations presented in this thesis are concerned with the replant, or poor early growth, disease of apple in England. They are directed at providing information on the distribution of Pythium spp. in orchard soils, on factors influencing this distribution, and on possible associations between Pythium spp. and disease severity. They also include pathological studies to augment available knowledge, and examine the potential pathogenicity of newly-encountered species.

Historical and Literature Review

A. The genus *Pythium*

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A. The genus *Pythium*

Taxonomy of the genus

Pythium species are oomycetous Phycomycetes, sharing the family *Pythiaceae* with the genus *Phytophthora*. The distinction between these genera is based on differences in the manner of zoospore emission from the sporangium. In *Phytophthora* zoospores are formed within the sporangium, and in *Pythium* they are formed after the sporangial protoplast is protruded from the sporangium in a thin-walled vesicle. Some species of '*Phytophthora*' and many species of '*Pythium*' however, (including *P. sylvaticum*) do not form zoospores and taxonomically, the distinction between these genera is imprecise and vague.

The first taxonomic systems for the genus *Pythium* were proposed by Sideris (1931; 1932) and Matthews (1931). Sideris' treatment was based on his studies of species isolated from pineapple and other plants in Hawaii. Matthews' treatment was a more broadly-based classification of known *Pythium* species and included a workable key. A number of new species were subsequently described and Middleton (1943) compiled an extensive monograph of the genus, with complete mycological treatment and host records of 66 species. His extensive studies on the morphology of the species indicated that the characters of the sporangium, oogonium, antheridium and oospore could be used as reliable criteria for the separation of species. Middleton also observed that the growth response to temperature was a consistent feature and could be used to supplement morphological characters in the identification of species.

Middleton (1943) strongly suggested that failure to produce the sexual stage in some species was due to factors other than heterothallism. Isolates that produced only 'hyphal swellings' or 'chlamydospores' in culture were grouped together as 'unidentifiable' in a later key to the genus by Waterhouse (1967). Waterhouse (1968) also published a most useful compilation of the original descriptions of *Pythium* spp.

In 1967, Campbell & F.F. Hendrix demonstrated the heterothallic condition in a newly described *Pythium* species (*P. sylvaticum* Campbell & F.F. Hendrix) by pairing compatible strains of this fungus on hempseed agar. The recognition of heterothallism in *P. sylvaticum* brought much attention to the hitherto 'unidentifiable' *Pythium* species that produced only asexual stages in culture.

F.F. Hendrix & Campbell (1968) subsequently discovered another heterothallic species, P. heterothallicum. Compatible strains of P. sylvaticum were later recorded in Canada (Pratt & Green, 1971), the Netherlands (van der Plaats-Niterink, 1968), and England (Sewell, 1979).

Pythium intermedium de Bary, a species long-regarded as a non-oosporic species was shown to be heterothallic by van der Plaats-Niterink (1968). Other studies, with species that rarely or reluctantly formed oospores in culture, demonstrated the heterothallic condition of P. catenulatum Matthews (Hendrix & Campbell 1969) and P. splendens Braun (van der Plaats-Niterink 1969).

F.F. Hendrix & Campbell (1973) attributed failure to recognise heterothallism in Pythium by earlier workers to the cultural media then in general use (Corn-meal agar or potato-dextrose agar) as the mating reaction rarely occurred on them. Hempseed agar or others rich in sterols were needed to stimulate mating. Van der Plaats-Niterink (1968) used potato-carrot agar (PCA), and a mixture of PCA and corn-meal agar successfully to induce mating in P. heterothallicum and P. intermedium.

Papa et al (1967) showed that distinct male and female strains of P. sylvaticum existed and described the heterothallism as 'morphological': antheridia and oogonia were restricted to distinct thalli. This view was supported by van der Plaats-Niterink (1968). However, Pratt & Green (1971) provided evidence that the heterothallic mating system of P. sylvaticum did not represent sexual dimorphism. They reported that several male isolates produced compatibility reactions in paired matings, but stronger reactions developed when they were paired with females. Child & Haskins (1971) induced oospore formation in some strains of heterothallic Pythium species by an amendment of media with cholesterol. They suggested that the isolates display a physiological heterothallism in which the genetic determinants of sexuality are not separated in thalli which differ in mating type.

The discovery of heterothallism in some Pythium species further complicated the taxonomy of the genus. Waterhouse (1967) did not include P. sylvaticum in her key, and suggested that it needed comparison with P. debaryanum auct. non Hesse. The original description of P. debaryanum Hesse was probably based on a culture contaminated with P. intermedium (Waterhouse, 1968). Middleton (1943) interpreted P. debaryanum differently from Hesse and he did not mention zoospore

production in this species. The main feature that separated P. debaryanum sensu Middleton from P. sylvaticum was that the latter is heterothallic. Pratt & Green (1971) argued that because an isolate is homothallic does not preclude its possessing a strong heterothallic character or vice-versa. F.F. Hendrix & Campbell (1974) after examining hundreds of isolates considered that P. debaryanum sensu Middleton; P. debaryanum var. pelargonii and P. debaryanum var. viticola were all synonymous with P. sylvaticum Campbell & Hendrix. F.F. Hendrix & Campbell (1973; 1974) concluded that the name 'P. debaryanum Hesse' had, in the past, been commonly applied to P. sylvaticum, P. irregulare and, to a lesser extent, to P. ultimum. The status, therefore, of one of the most commonly described Pythium species has to be considered doubtful (Domsch et al., 1980).

F.F. Hendrix & Campbell (1973) considered that several groups of species could not reliably be separated by the morphology of their reproductive structures. They proposed a system of 'species complexes' based on broad morphological characteristics. This system was later amended by F.F. Hendrix & Papa (1974) who grouped all known Pythium species into 15 complexes. The 'P. sylvaticum complex' included all the known heterothallic species that produce spherical sporangia or 'hyphal swellings'. These broad classifications may simplify taxonomy of the genus but are not likely to be of value to detailed pathological or ecological studies.

Pythium species as root pathogens

The importance of Pythium as a destroyer of primary roots of non-seedling plants was not recognised until recently, although Pythium species were reported to be pathogenic to tea bushes 40 years ago (Pfaltzer, 1940). The scarcity of information on Pythium as a pathogen of non-seedling plants was probably due to difficulties in isolating these fungi before the development of selective isolation techniques (Eckert & Tsao, 1962; F.F. Hendrix & Kuhlmann, 1965).

In recent years, a great deal of attention has been given to decline and replant diseases especially of perennial plants, such as peach, citrus, apple and cherry (F.F. Hendrix & Campbell, 1973). In these diseases the symptoms were typical of those occurring on plants with diminished root systems, often caused by 'fine' or 'feeder' root necrosis (Campbell & Copeland, 1954; F.F. Hendrix &

Campbell, 1966). Decline diseases of citrus (Sleeth, 1953), pecan (F.F. Hendrix & Powell, 1968), peach (F.F. Hendrix et al 1966) and loblolly pine (Lorio, 1966) have been associated with species of Pythium and Phytophthora. P. ultimum was the only pathogen recovered from the roots of citrus trees affected by decline disease in Texas (Sleeth, 1953). Only Pythium species were recovered regularly from the roots of several hundred peach trees with typical decline symptoms. A lack of fine roots was observed in all affected trees and shoot and root symptoms were reproduced following inoculation of peach trees with P. vexans, 'P. irregulare complex' or P. dissotocum (Hendrix et al, 1966).

Wilhelm (1965) reported that P. ultimum reduced growth of established plants of strawberry, cauliflower and tobacco, and was a primary factor in the strawberry replant problem. Great increases in the growth of these crops were achieved after fumigating field soils in which P. ultimum was the only known pathogen. Scarcity of root hairs in infested soil provided evidence for their importance as infection sites. Watanabe et al (1977) confirmed the pathogenicity of P. ultimum to strawberry plants and suggested that this fungus may be largely responsible for the 'stunt' disease, under low temperatures, in Japan. However, they also isolated heterothallic Pythium species (including P. sylvaticum) frequently from strawberry roots. All isolates of 'P. sylvaticum complex' and single isolates of six other species of Pythium were found to be pathogenic on strawberry roots on the basis of the severity of root discoloration. The growth of plants inoculated with these species was poorer than in non-inoculated controls.

Other studies, mostly with annual plants and often with seedlings, indicated mechanisms of non-parasitic pathogenicity by Pythium species (Woltz, 1978). Wilhelm questioned whether roots must be actually invaded by Pythium species for poor growth to occur. Hoppe (1949), in reporting Pythium-induced stunting of corn did not consider root invasion necessary. Brandenburg (1950), however, indicated that rootlets must be invaded to bring about stunting and chlorosis in sugar beet. His evidence was not histological, but was based on the instability, in the presence of soil micro-organisms, of the growth inhibiting toxins produced by P. irregulare.

Blok (1970) reported that autoclaved culture filtrates of P. sylvaticum caused considerable reductions in root length in flax seedlings in a liquid medium. A linear relationship was found between the dilution of a culture filtrate of

P. sylvaticum and the inhibition of root growth (Blok, 1973). It was also reported that P. sylvaticum produced auxin (IAA) (Posthumus, 1973) under laboratory conditions and that Pythium species differed in their ability to produce auxin. P. irregulare and P. paroecandrum also produced auxin while P. ultimum and P. torulosum did not (Blok, 1973).

Various authors have provided evidence for the fact that the Pythiaceae have mechanisms which are independent of parasitism (J.W. Hendrix, 1976; Orlikowski & J.W. Hendrix, 1977; Csinos & J.W. Hendrix, 1977; Kanlong & J.W. Hendrix, 1977; Csinos, 1979). J.W. Hendrix (1976) reported the ability of Phytophthora cryptogea to inhibit growth of Burley tobacco without killing the plants. The populations of P. cryptogea were not affected even though the plants were severely stunted by this pathogen (Orlikowski & J.W. Hendrix, 1977). Earlier, Flowers & J.W. Hendrix (1972, 1974) had found that the soil populations of P. parasitica var. nicotianae increased in the presence of only those plants that the fungus was capable of parasitizing. Kanlong & J.W. Hendrix (1977) found no relationship between the abilities of Pythium or Phytophthora species to parasitize plants, to cause necrosis in stems without killing plants and the ability to stunt growth of plants transplanted into infested soil.

Csinos & J.W. Hendrix (1977) provided direct evidence to show that growth reduction of Burley tobacco caused by P. cryptogea is not due to parasitism. Soils that were infested with the fungus and then sterilised by autoclaving or methyl bromide fumigation stunted tobacco planted into them. Mycelium produced on a defined medium and lyophilized, powdered and mixed with soil was toxic to plants at between 0.5 and 1.0 g per 400 gms of soil. Csinos (1979) also showed that powdered, lyophilized mycelium of P. myriotylum (at 250 mg/ml) caused necrosis and root discoloration in tomato seedlings. The effect of the dead mycelium, however, was reported to diminish at pH values at or above 6.0.

Pythium species have also been reported to be important in 'disease complexes' with other micro-organisms, mainly fungi and nematodes. Kerr (1963) found that P. ultimum and F. oxysporum caused more severe damage to peas than either fungus alone. Severity of root rot of chrysanthemum, caused by P. aphanidermatum, increased when the nematodes Belonolaimus longicaudatus or Meloidogyne incognita were present (Johnson & Littrell (1970).

Occurrence and distribution of *Pythium* species in soil

Virtually all references to *Pythium* species before 1960 were based on isolations from diseased plant tissues. These isolations indicated the wide host ranges of most species (e.g. 147 hosts of *P. ultimum*; Middleton, 1943), but did not indicate distinction with reference to soil types or locality. Such isolations also usually involved the use of surface sterilising agents which may have greatly diminished the chances of isolating certain species. The use of alcohol compared with washing, for instance, reduced isolations of *P. sylvaticum* from apple roots from 75 to 15% (Sewell, 1981).

Prior to 1960, Remy (1949), Warcup (1952), and Barton (1958) concluded that although *Pythium* species were abundant in cultivated and nursery soils, they were either absent or of very sparse occurrence in non-cultivated and forest soils. Highly selective isolation methods and media for pythiaceous fungi, mostly involving use of the antibiotic pimarin, were developed during the decade 1960 - 70 (Vaartaja, 1960; Singh & Mitchell, 1961; Schmitthenner, 1962; Eckert & Tsao, 1962; Stanghellini & Hancock, 1970; Tsao, 1970). These permitted a greater appreciation of the occurrence of *Pythium* species in soils, but ecological studies are few and mostly restricted to North America (and even to the South East States of N. America). Vaartaja & Bumbieris (1964) studied the abundance of *Pythium* species in South Australian forest nursery soils following severe damping-off epidemics. *Pythium* species were isolated from all plots tested and the number of propagules/g varied from 29 to 316. *P. irregulare* was the dominant species in all plots. Forest soils had however low *Pythium* populations. *P. irregulare* (followed by *P. rostratum* and *P. ultimum*) was also the dominant species in Ontario forest nursery soils, where maximum populations were 95 propagules/g (Vaartaja, 1968). (Heterothallic species were not described at the time of these studies).

All other ecological studies originate from the laboratory of F.F. Hendrix (University of Georgia, U.S.A). Campbell & F.F. Hendrix (1967 b), using three isolation methods, found the abundance of *Pythium* propagules in forest soils to range from 0 - 185 p/g. They reported that certain forest soils contained greater populations of *Pythium* than were found under declining peach trees.

P. sylvaticum was the fungus most commonly isolated from the hardwood and mixed pine hardwood stands in the mountain areas, but it was not found in soils of pure

pine stands of the Piedmont and coastal plain. The other frequently isolated Pythium species, P. irregulare, P. vexans, P. paroecandrum and P. rostratum, were not restricted to any particular tree association or locality.

F.F. Hendrix et al (1971) isolated and identified 11 species of Pythium from four old growth mixed forest sites in Kentucky, North Carolina and Tennessee in the United States. Heterothallic species (P. sylvaticum, P. heterothallicum and P. intermedium) were found to be the commonest species occurring in these soils. P. irregulare and P. vexans were the other frequently isolated species. Heterothallic species were isolated more frequently from forest than from agricultural soils, while P. irregulare was found to be the most common species in agricultural soils. P. ultimum var. sporangiferum and P. vexans were found in both locations. F.F. Hendrix et al (1971) therefore suggested that species indigenous in old growth forests evidently were able to adapt to agricultural habitats.

In a study of the Pythium populations in soils under various crops, F.F. Hendrix & Campbell (1970) found 'P.irregulare - debaryanum complex' to be dominant in almost all soil samples (more than two thousand were studied) except those from Tennessee which had a higher proportion of heterothallic species. Nearly all (90%) soil samples from pine and 69% of soils under apple contained dominant populations of 'P. irregulare - debaryanum complex'. In this study, heterothallic species were recorded from only 3% of (38) apple soils studied. 'P.irregulare - debaryanum complex' was also detected from a large percentage of samples of soil cropped with pecan, citrus and peach.

In another study of turf grass soils F.F. Hendrix et al (1970) assayed 157 samples. Pythium populations varied from 0-50 propagules/g with most samples in the range of 1-20 p/g. Although 20 species were identified nine were more frequently isolated than others. These included 'P.irregulare - debaryanum complex' and many non-spherical sporangial forms. Grasses have been recorded as relatively common hosts for non-spherical sporangial forms of Pythium. P.aphanidermatum is the most frequently cited cause of turf blight (Wells & Robinson 1954; Freeman & Horn 1963). The studies by F.F. Hendrix and colleagues, thus showed P. sylvaticum to be the dominant species in hardwood and mixed forest soils, but not in pure pine stands. In all studies (of Australian, Canadian, and USA soils) total Pythium populations rarely exceeded 300 p/g.

In apple soils of the SE States of N. America P. sylvaticum was of infrequent incidence (3%), and Pythium populations were dominated by species of the 'P. irregulare - debaryanum complex'. In root isolation studies of the rosaceous crop strawberry, P. sylvaticum was the most frequently isolated Pythium species in Illinois (Nemec & Sanders, 1970) and in Japan (Watanabe et al 1977).

Soil factors affecting activity of Pythium species

Barton (1958) reported that P. mamillatum failed to colonize maize meal in acid woodland soil having a dominant population of Trichoderma viride. Hancock (1977) reported that P. ultimum did not develop or survive well on cotton leaves in soils held above 30° C although in culture the optimum temperature for its growth and sporangial germination was found to be between 22° and 30° C. Hancock suggested that increased microbial competition at high temperature might be one of the soil factors causing the difference in behaviour of P. ultimum in soil and in culture. Hancock (1977) also observed that P. ultimum developed well on cotton leaves when incorporated into soils collected from two field sites in the San Joaquin Valley of California but developed poorly when incorporated into soils from another two sites.

The inability of fungus spores to germinate in soil under conditions considered to be favourable for germination, was described by Dobbs & Hinson (1953) as 'soil fungistasis'. Weltzien (1963) and Lockwood (1964) favoured rival theories for the cause of the fungistasis: inhibitors and lack of available nutrients respectively. Most investigations of soil fungistasis have employed diffusion methods in which soil and test organisms are separated by a membrane such as cellophane (Dobbs & Hinson, 1953; Griffin, 1962). Spores of many fungi are inhibited from germinating either when toxins are allowed to diffuse towards, or nutrients away from them. The diffusion method has been criticised because it cannot separate toxic effects from nutrient deficiency, and because it may create artefacts, for example by allowing bacteria to concentrate in the water films on the membrane or on the assay organism (Lockwood, 1964).

Luna & Hine (1964), while investigating the effects of temperature, nutrients and competitive activity of other soil micro-organisms on the saprophytic growth of P. aphanidermatum, found that the optimum temperature for the mycelial growth of this fungus was the same (28 - 30° C) in both sterile and non-sterile soil.

However, mycelial growth occurred only when the fungus was growing from a suitable energy source. Germination of zoospores occurred in non-sterile soil only when aqueous solutions of available energy sources were added at the time of infestation. No extensive mycelial growth from zoospores added to the non-sterile soil took place either in the presence or absence of added nutrients. However, heat treatment of this soil resulted in more luxuriant growth and production of more sporangia than in non-treated soil. Although the soil used in this study was a 'foreign' soil to the test-organism, the findings of Luna and Hine indicated that both nutrients and competitive microbial activity are important in determining the fungistatic properties of a soil.

Soils 'suppressive' or 'conductive' to Pythium-induced diseases have been reported by Vaartaja & Bumbieris (1964), Hancock (1977) and Bouhot & Perrin (1980). Vaartaja & Bumbieris (1964) found that in South Australian nurseries, susceptible conifer seedlings often remained healthy in spite of high populations of propagules of pathogenic Pythium species. They concluded that natural biological control had been operating in such instances. Evidence in support of 'soil fungistasis' as one of the mechanisms of natural biological control was later provided by Vaartaja & Agnihotri (1967). Soil leachates collected from a sandy nursery soil in Ontario, were found to cause effects varying from considerable stimulation to strong inhibition of the mycelial growth of P. ultimum, depending on the time of soil sampling and on leachate concentration. The inhibition was seen as a reduction of growth rate in the test-medium containing the leachate, compared to that in the distilled water controls. P. ultimum was inhibited by a lower concentration of the leachate than was the other test organism, Thanetophorus praticolus. Vaartaja & Agnihotri (1967) also reported that the differences in responses of Pythium and Thanetophorus matched those in pathogenicity tests where soil exerted greater biological control against Pythium inoculum.

The soil leachates remained potent after pasteurisation, autoclaving and storage at 0° C for 6 months. Addition of nutrients to the test medium counteracted the inhibition but not always completely. Vaartaja & Agnihotri (1967) pointed out that this fact and the increase of inhibition with increasing concentration of soil leachate could not be explained by the nutrient theory of fungistasis but indicated the presence of biologically produced inhibitors.

In a later study the same authors (Vaartaja & Agnihotri, 1969) showed that the

germ tubes of some Pythium species were lysed in a natural soil and in soils amended with antibiotics. No resistant bodies were formed prior to the initiation of lysis of germ tubes. P. ultimum and P. irregulare were inhibited by low concentrations of antibiotics such as griseofulvin, streptomycin, aureomycin and actidione, while P. rostratum was more resistant. Nutrients (such as sucrose or yeast extract) in general, prevented early lysis at low concentrations of antibiotics. Yeast extract was found to be superior to sucrose or asparagine in counteracting the toxicity of antibiotics.

Growth and survival of Pythium species in soil

The germination of spores, and subsequent mycelial growth of Pythium species in natural soils is generally greatly stimulated by the presence of traces of nutrients which may be complex (root and seed exudates) or simple (glucose). The influence of root exudates on the germination of oospores (of P. mamillatum Meurs) was first demonstrated by Barton (1957). Barton (1958) also showed that oospore germination was stimulated by exudates from germinating seeds, and that the effect occurred over greater distances as soil moisture content increased. Seed exudates of Pinus resinosa stimulated the germination in soil of encysted zoospores of P. afertile and sporangia of P. irregulare (Agnihotri & Vaartaja, 1968; 1970), as did soil amendment with yeast extract or sucrose (Vaartaja & Agnihotri, 1969). Singh (1965) found P. ultimum populations, in soil adjacent to various seeds, increased on average by $\times 2.4$ after 48 H and by $\times 7.7$ after 96 H. Stanghellini & Hancock (1971 a) recorded, by direct observation, 78% germination of P. ultimum sporangia in soil adjacent to bean seeds sown only 3-4 H earlier: germination and growth were greatly stimulated up to 10 mm from seed in 24 H. In other studies (Stanghellini & Hancock, 1971 b) P. ultimum sporangial germination was greatly increased in soils amended with traces of glucose solution: the amount of germ tube growth was directly related to the glucose concentration applied: after stimulation by exogenous nutrients secondary sporangia were formed associated with retraction of the mycelial protoplast into the sporangium.

Pythium species have been reported to survive in air-dry soils for periods of 2-12 years (Hoppe 1966; Vaartaja 1967). Sewell (pers. comm) observed that P. sylvaticum, which probably forms oospores infrequently in nature, was readily eliminated from soils by air-drying. Persistence, as mycelia, was considered

unlikely by Warcup (1957) and Lockwood (1960) because of its ephemeral nature : Stanghellini & Hancock's (1971 b) observations, however, suggest that mycelium plays an important role in survival.

The long-term survival structures are generally considered to be sporangia (or conidia) and oospores (Warcup, 1952; Bainbridge, 1966; Stanghellini & Hancock, 1971 a; Lumsden & Ayers, 1975; Hancock, 1981). The relative importance of the asexual and sexual spores may vary greatly with different Pythium spp. In general, the sporangium is considered the more important structure in sphaerosporangial species (Hendrix & Campbell, 1973) and particularly so in P. ultimum (Hancock, 1977). The oospore appears to be of primary importance in lobate sporangial species (Burr & Stanghellini, 1973).

Seasonal variation in the activity of Pythium species

Studies on seasonal variation of Pythium species are few. Some are of doubtful value because of problems associated with simultaneous quantitative and qualitative assessment of species, and their varying competitiveness. Agnihotri & Vaartaja (1967) doubted whether frequency of isolation, or abundance of propagules were necessarily related to mycelial activity in soil. In general, results indicate that the lowest levels of activity are in the hot, dry, summer months.

Total Pythium populations were assessed by McLaughlin (1947) in an unspecified location and by Robertson (1973) in New Zealand. McLaughlin assayed soils by placing soil crumbs beneath plain agar slabs, and Robertson used an in situ bait (filter paper treated with pimaricin). Both workers reported isolations were most frequent when soil temperatures were low, and soil moisture high (winter, spring, autumn).

F.F. Hendrix et al (1966) studying peach root necrosis in Georgia, isolated P. irregulare from rootlets in the winter and early spring, but not in the summer (even though it could be detected then in the soil) while P. vexans was isolated from roots in the summer. These results indicated that P. irregulare and P. vexans were more damaging at low temperatures and at high temperatures respectively.

Hancock (1977), in a detailed investigation, found higher P. ultimum soil populations during the autumn, winter and spring than during August or early September in the San Joaquin Valley of California. Crop residues, especially cotton leaf litter, supported population increases in late September through

November (in the Autumn) in many, but not all, fields provided that moisture was available. Hancock (1977) concluded that seasonal fluctuations in P. ultimum populations were related not only to climatic conditions and the presence of organic residues but also to unidentified soil factors.

B. Apple Replant Disease

Symptoms and characteristics

The main symptom of apple replant disease (ARD) is a non-lethal growth reduction of above and underground parts of both seedlings and trees (Savory, 1966; Hoestra, 1968). Savory (1966) reported that apple planted on some sites makes as little as one-fifth of 'normal' growth, and Hoestra (1968) observed apple growth reduction of up to 90% in apple soil compared with non-apple soil. Replant disease appears to affect tree growth mainly in the first year after planting; thereafter, replants and healthy trees have similar relative growth rates (Savory, 1967). Replant-affected trees also have considerably fewer growing shoots (Savory, 1966). Savory (1966) reported no specific leaf symptoms (other than rosetting), but Hoestra (1968) observed the leaves of severely affected trees to be small and cupped.

Underground root laboratory observations (Savory, 1966) showed root growth to be immediately affected following planting while shoot growth was normal in spring but subsequently was greatly diminished. Trees planted into 'old' apple soil (surrounded by non-apple soil) had weak, necrotic, sparsely branched roots, but these assumed normal growth patterns on reaching non-apple soil.

Studies on seedlings growing in apple soil (Hoestra, 1968) revealed rapid discolouration of root hairs followed, after a few days, by the development of root lesions. Root hairs were reduced in size and number. Rotting was confined to the epidermis and primary cortex. The roots, however, often survived and after formation of secondary cortical layers had a normal healthy appearance. New lateral rootlets either rotted completely when 5-20 mm long, or did not grow more than a few mm and became distinctly swollen.

In his review Savory (1966) listed four 'characteristics' of ARD and these are discussed below ((a) — (d)).

(a) Persistence - Apple replant disease persists in the soil in the absence of apple or related crops for very long periods. ARD remained severe after eight years of grass or arable cropping (Thompson, 1959; Savory, 1967).

(b) Recovery on transfer to non-apple soil -

R.S. Pitcher (fide Savory 1966) showed that severely stunted one-year-old apple

made normal growth when transferred to fresh soil. Recovery of vigour when trees were transferred to a fresh soil has been reported by several workers (Savory, 1967).

In pot experiments with mixtures of apple and non-apple soils the amount of growth reduction was directly, linearly related to the proportion of apple soil in the mixture (Schanders, 1955; Savory, 1967; Hoestra, 1968).

(c) Specificity - An important, but controversial feature of ARD is its 'specificity' (Savory, 1966; Hoestra, 1968; Sewell, 1981). Savory's concept of 'specific' replant diseases was derived from observations that growth of apple planted after plum or cherry was not greatly diminished and that growth of cherry planted after apple or pear was slightly diminished.

However, various reports of increased apple growth after the fumigation of some cherry and other non-apple soils have placed the 'specificity' of apple replant disease in doubt (Pitcher *et al* 1966; Hoestra, 1968; Benson *et al* 1978; Mai and Abawi 1978; Sewell, 1979). Sewell (1979) thought that the concept of apple replant as a 'specific' disease would be a possible barrier to the identification of cause: the cause might be a common, widespread but variable soil malaise affecting many plants (though not strongly affecting cherry and plum).

Some workers have suggested that the ARD problem can be split into 'specific' and 'non-specific' categories (Savory 1967; Hoestra 1968). Savory (1966) believed that non-specific responses to fumigation (i.e. improved apple growth in old cherry soil) could be due to the control of minor pathogens which are probably present in all fruit soils. Savory claimed that such non-specific effects were small compared with the effects of specific replant diseases. Hoestra (1968) also attributed fumigation responses in 'non-host' soils to effects such as the control of nematodes and regarded it as a separate and distinct problem. The concept of specificity in ARD, however, was dismissed by Mai and Abawi (1978).

(d) Diminished severity in acid soils

Savory (1967) and Hoestra (1968) reported that low pH soils tended to be less affected by apple replant disease than near-neutral soils. Acidification with sulphuric acid or sulphur of near-neutral soils in pot experiments resulted in improved growth of apple seedlings which was similar to that caused by chloropicrin fumigation. (Hoestra & Kleijburg, 1967; Hoestra 1968, 1973; Hein, 1972). However, the reports while indicating ARD to be frequently severe in

near-neutral soils do not give sufficient information about soils at the extremes of pH level and no statistically valid information has been presented. Data recorded by Hoestra (1968) and Upstone (1975) indicate tendencies only for ARD to occur less frequently in both very acid and very alkaline soils (within the limitations of apple growth soils). Jonkers *et al* (1980) failed to control ARD by soil acidification with sulphur in a field trial extending over seven years.

Disease control

Experiments conducted by research workers in various fruit growing regions of the world clearly indicate that soil disinfestation by broad-spectrum biocides (chloropicrin, methyl bromide, propylene oxide) or heat (either in the form of 'pasteurization' at 60° C or steaming at 100° C) resulted in greatly improved growth of replanted apple (Pitcher *et al* 1966; Hoestra, 1968; Ross & Crowe, 1976; Gilles, 1974; Mai and Abawi, 1978; Sewell & Wilson, 1979; O'Kennedy & Kavanagh, 1980). Many narrow-spectrum pesticides and fungicides have also been tested but found not to be effective (Pitcher *et al* 1966; Hoestra, 1968).

The most satisfactory disease control was obtained with chloropicrin and this has been the standard soil treatment for the past 20 years. Pot-plant and field experiments have recently shown formalin to be an effective, cheaper alternative to chloropicrin (Sewell & White, 1979), and is being increasingly used in the United Kingdom.

Possible causes of apple replant disease

The possible causes of replant diseases in general and of ARD in particular were extensively reviewed by Savory (1966) and Hoestra (1968). These included nutritional disorders or imbalances, physical site deterioration, toxins and pathogens. Much early work (Savory listed nearly 200 publications) concentrated, without success, on the first two listed factors. Toxins, especially in relation to ARD and phloridzin, were the subject of many papers during the 1950's and 1960's. However, the consistently successful disease control achieved by the use of broad-spectrum biocides had led virtually all investigators to consider the cause(s) of ARD (and most other RD) to be pathogenic. Further evidence for pathogenic cause was provided by the transferable nature of the disease from 'sick' to 'healthy' soils (Otto, 1972).

Among potential pathogens, viruses were discounted because of recovery on

transplanting, and nematodes were generally discounted after exhaustive, detailed investigations by Pitcher et al (1960), Oostenbrink & Hoestra (1961), Hoestra et al (1964), Hoestra (1968), and Ross & Crowe (1976). Winkler & Otto (1972) further showed that soil heating to 60 ° C was necessary to eliminate ARD while migratory soil nematodes were eradicated by 45-50 ° C. Some workers, however, maintain that nematodes are an important cause of ARD (Lalatta, 1966; Mai & Abawi, 1978). Evidence for effects of nematodes on tree growth is strong on certain atypically very light-textured soils (Colbran, 1979).

Certain evidence is considered to indicate the involvement of bacteria or actinomycetes: effects of soil acidification (Hoestra, 1968); disease control with 1500 ppm Agrimycin in soil (Mulder, 1971); effects of soil heating (Otto, 1972); failure to observe fungal pathogens in roots (Savory, 1967). No direct evidence for effects of bacteria or actinomycetes on tree growth, however, has been presented.

Among the soil microflora only Pythium spp. have been shown to cause diminished growth of apple (Mulder, 1969; Sewell, 1981). Mulder (1969) isolated P. sylvaticum from an apple root and tested its pathogenicity to apple seedlings, together with five other Pythium spp. from diverse hosts. All isolates appreciably reduced apple growth, but Mulder did not extend the investigation because of the 'non-specificity' of pathogenic effects (pers comm).

Sewell (1981) listed circumstantial evidence indicating the possible involvement of Pythium spp. in ARD. This included: disease enhancement after soil treatment with benomyl, PCNB, or maize-meal; disease control by application of low concentrations of formalin, by soil acidification, and by high doses of Agrimycin (containing largely a tetracycline antibiotic to which Pythium spp. are sensitive). Sewell found Pythium spp. to be abundant in 10 orchard soils, and all populations were dominated by P. sylvaticum. In pot-plant pathogenicity tests, all (10) isolates tested of P. sylvaticum and certain isolates of seven other Pythium spp. significantly reduced growth of apple seedlings. These growth reductions were comparable in magnitude to the growth increases resulting from the chloropicrin-fumigation of orchard soils. The Pythium isolates most pathogenic to apple were not pathogenic to cherry. Sewell reported that standard bulk tissue clearing techniques also cleared non-oospore forming Pythium spp. (P. sylvaticum rarely forms oospores), and therefore that they would not readily

have been observed in earlier investigations.

1. The soil dilution plate method and media formulations

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1.1. The soil dilution plate

The main isolation method used in these studies was based on that described by Mircetich (1969) and subsequently modified (Mircetich, 1971). The method was found, in comparative tests, to be more highly selective than others and to permit the isolation of a wider range of Pythium spp. (Mircetich & Kraft, 1973). The method was common to studies described in sections 4, 6 and 7 of this thesis and is therefore described here in detail separately.

Soil samples were sieved (1.7 mm) and 10 g aliquots were suspended in 100 ml of 0.2% plain water agar (PWA), in medicine flats of 250 ml capacity. Suspensions were shaken mechanically for 30 min. Further dilutions (1/100 and 1/1000) in 0.2% PWA were then prepared. Each dilution was spread evenly over the surface of solidified selective medium in each of three replicate petri-dishes (1 ml dilution/9 cm diam plate). The formulation of the selective medium (MPV) is given in Ss 1.2.

Soil dilution plates were incubated (22-25 ° C) in the dark for 48 h and then washed under running tap-water to remove soil particles and agar gel. Fungal colonies were observed (directly and under a low-power microscope) and delimited with a wax pencil. Colony counts (given as propagules/g) were mean number of colonies for three replicate plates per g soil, on the basis of oven-dry wt (105 ° C for 24 h).

In general, orchard soil Pythium populations were too large to permit accurate colony counting with the lowest dilution and too small to permit detection of Pythium at the greatest dilution. Most assays, therefore, were based on counts from 1/100 dilution. Pythium spp. rarely sporulated on soil dilution plates within the brief (48 h) assessment period. For qualitative assessment of Pythium spp., therefore, generally ten colonies were randomly selected for further culturing and identification. Where possible individual hyphae were transferred (aseptically) to one end of MPV strips (c 3 x 1 cm) in petri-dishes and reisolated free of bacteria, after growth along the length of the strip. Cultures were routinely maintained on slopes of 'Difco' corn-meal agar (CMA).

1.2. Formulations of media

The formulations and abbreviated designations of the media used in these studies are given below.

Mircetich's pimarinic - vancomycin agar (MPV)

(A)	CMA (Difco)		17 g
	Agar (B.D.H)		23 g
	Sucrose		20 g
	ZnCl ₂	(100 mg) a	1 ml
	CuSO ₄ · 5H ₂ O	(8 mg) a	1 ml
	MnCl ₂	(7 mg) a	1 ml
	FeSO ₄ · 7H ₂ O	(10 mg) a	1 ml
	MgSO ₄ · 7H ₂ O	(1.0 g) a	1 ml
	Thiamine HCl	(10 mg) b	1 ml
	Distilled water		1000 ml
(B)	CaCl ₂	(1.0 g) a	1 ml
	Pimaricin, 5% Soln	(1 ml) c	10 ml
	Vancomycin		300 mg
	PCNB, 72% w.p		140 mg

a. Sterile, stock solution (wt/100 ml DW)

b. Stock solution stored frozen (wt/100 ml DW)

c. Stock solution stored in dark, refrigerated (ml/100 ml DW).

Ingredients in group (A) mixed and autoclaved.

Ingredients in group (B) added to sterilised medium, cooled to 50° C, before pouring plates. Poured plates stored in dark (pimaricin light-sensitive) at 5° C.

Pimaricin as 'Delvocid-S' (Gistes - Brocades n v),

Vancomycin (Eli Lilly), PCNB as 'Terrachlor' (Olin Mattieson).

This formulation differs from Mircetich (1971) in the omission of rose bengal and molybdenum trioxide.

Gallic Acid Agar (G A A)

(Flowers & Hendrix, 1969)

Sodium Nitrate (NaNO_3)	2.0 g
Magnesium sulphate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$)	0.5 g
Potassium dihydrogen phosphate (KH_2PO_4)	1.0 g
Yeast extract	0.5 g
Thiamine hydrochloride	2.0 g
Gallic acid	425 mg
Rose bengal	0.5 mg
Sucrose	30.0 g
Agar (Plain, B.D.H)	20.0 g
Distilled water	1000 ml

These ingredients were mixed, autoclaved and cooled to 50°C before adding:-

PCNB	25.0 mg
Penicillin G	80,000 units
Nystatin	100,000 units

The pH of the medium was adjusted to pH 4.5 with HCl or KOH after cooling to 50°C . When the agar had solidified in poured plates, the plates were placed into paper bags and chilled to 3°C before use.

V-8 juice agar (Miller, 1955)

V-8 juice	200 ml
CaCO_3	2.5 mg
Agar (Plain, B.D.H)	20 g
Distilled water	800 ml

V-8 juice and CaCO_3 were added to the water and vacuum filtered through

Whatman no 1 filter paper and the medium autoclaved.

Carrot agar (Kaosiri *et al* , 1980)

Clean, fresh carrots were thinly sliced and placed in a 'Kenwood' blender with distilled water (200 g/ 500 ml). The carrot tissues were macerated for one minute each at low (12,600 r.p.m) and high (20,500 r.p.m) speed. The juice was squeezed from the macerate through four layers of cheese cloth and the volume brought to one litre with additional distilled water. Agar (Plain, B.D.H) (15 g) was added, and the medium autoclaved.

Schmittthener's medium (cited by Waterhouse, 1967)

Distilled water	1000 ml
Sucrose	2.75 g
NaNO_3	0.35 g
KH_2PO_4	0.15 g
K_2HPO_4	0.15 g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.10 g
B sitosterol	0.01 g
Agar (Plain, B.D.H)	20.0 g

The above ingredients were added in the order in which listed and the medium autoclaved.

Corn-meal agar (CMA)

'Difco' - Laboratories, Detroit, Michigan, U.S.A. Prepared to manufacturers recommendation (17 g/h). This medium was, at times, amended with B-sitosterol to stimulate sexual reproduction (J.W. Hendrix, 1965, 1970). B-sitosterol (20 mg) was dissolved in chloroform (10 ml) and the solution added to molten CMA (1 litre). The sterol flocculated when mixed with CMA, but the level of dispersion was the best that could be achieved.

Corn-meal / hemp seed agar (CMHSA)

Cracked hempseeds (20) were boiled in 600 ml distilled water (DW) for 10 minutes. The concoction was then filtered (Boots milk filters). The filtrate was made up to a litre and used to prepare CMA. This medium was used to stimulate development of reproductive stages (F. F. Hendrix & Papa, 1974).

2. Cultural procedures for species identification

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2. Cultural procedures for species identification

Very few isolates could be identified from examination of cultures maintained on CMA or CMHSA slopes after their initial isolation. Different methods were required to induce (or attempt to induce) either the zoospore or oospore formation on which identification depends.

2.1. Induction of zoospore formation

Asexual reproductive structures were often sparsely produced, and no isolate produced zoospores, on solid media. A few species formed zoospores in water cultures, and these cultures always greatly enhanced production and observation of asexual stages (and occasionally of sexual stages also). Water cultures were left on the laboratory bench, subject to fluctuating light and temperature conditions, for 1-2 wk. If zoospores were not observed during this period cultures were examined after various periods of refrigeration.

Simply placing agar culture blocks in shallow layers of distilled water (or filtered pond water) was often helpful, but cultures on organic tissues were preferable. Boiled hemp seeds or pieces of grass leaf were placed on agar cultures for 3-4 days to become colonised, and then these were placed in shallow water.

Despite repeated attempts it was concluded that the great majority of orchard soil isolates were non-zoosporic. Zoospore production was only observed with three infrequently isolated spp. (P. coloratum, P. salpingophorum and P. vanterpoolii).

2.2. Induction of oospore formation in homothallic spp.

Some isolates produced oospores on CMA or CMHSA or V-8 juice agar but the spores were often sparse or poorly developed. The incorporation of B-sitosterol into CMA greatly increased oospore production in these isolates. Very abundant oospores often developed on and around sterol particles in the medium (Plate 2.1). Certain species only formed oospores on carrot agar: this medium, however, was not preferred having the disadvantage that it was dark and particulate and made detailed observations of antheridial/oogonial relationships difficult. Isolates producing neither oospores (nor zoospores) were suspected to be heterothallic.

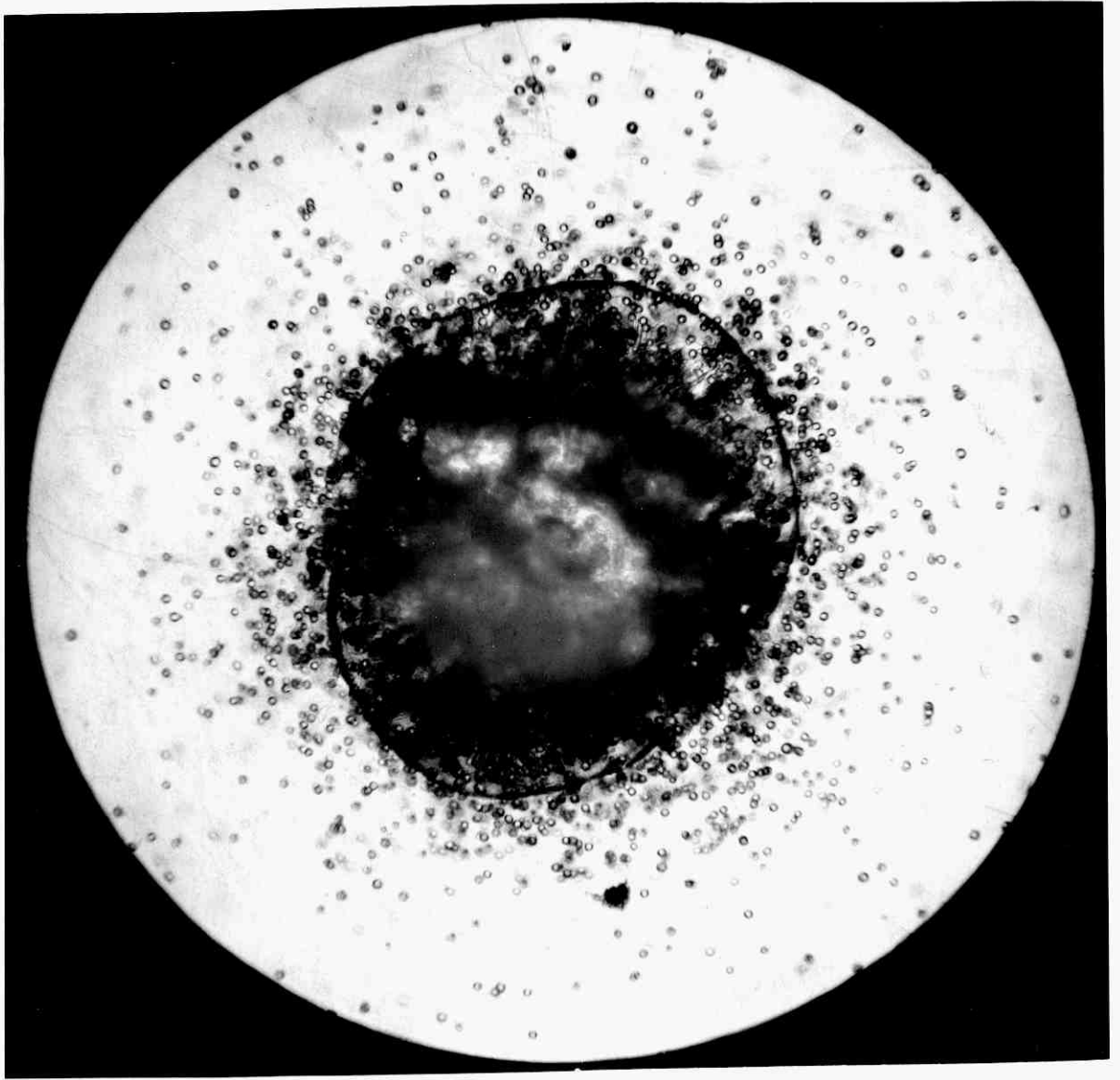


Plate 2.1 Abundant development of oospores of P. ultimum on and around a particle of B-sitosterol in CMA.

2.3. Induction of oospore formation in heterothallic spp.

Isolates producing only sphaeroconidia could be separated into groups based on colony form and growth rate (Ss 2.4). It was also expected that (at least one) heterothallic species would be dominant in orchard soils (Sewell, 1981). For certain identification, all isolates had to be mated. Authenticated male and female (or + and —) strains of P. sylvaticum, P. heterothallicum and P. intermedium were kindly supplied by Dr. J.A. Van der Plaats-Niterink (CBS Baarn).

Mating reactions were tested by inoculating agar plates, at distant points on the plate diameter, with two isolates. (Occasionally three isolates were tested together). With compatible reactions a distinct, sharp line of dense mycelium developed at the line of contact of the colonies. Within this agar zone sex organs formed, often in very great abundance. Subsequently, the zone of dense mycelium (and oospores) extended, unevenly, towards the female strain inoculum but remained straight and clearly defined on the male side (2.2a, b). On suitable media oogonial and antheridial initials generally developed within three days of inoculation.

The mating reaction of P. sylvaticum was strong on all media including CMA. However, carrot agar was found to be the only reliable medium for successful mating between isolates of P. heterothallicum.

Attempts at mating within one group of isolates (Pythium x) were unsuccessful on all media.

2.4. Colony growth rate and morphology

Most isolates required to be grown in plate culture for identification, either for mating studies or observations of colony morphology and relative growth rate. The colony morphology of many of the faster growing species was initially strongly arachnoid (rather than diffuse) on MPV. Subsequently, in 2–3 days, some became uniformly or patchily densely floccose without colony zonation: others developed distinct or less distinct (chrysanthemum-like) zonation patterns (Plates 2.3 A, B).

Growth rates of different species differed greatly and virtually all species grew less rapidly on MPV than on CMA (Table 2.1). Reduction of growth rate on MPV was particularly marked with P. ultimum.

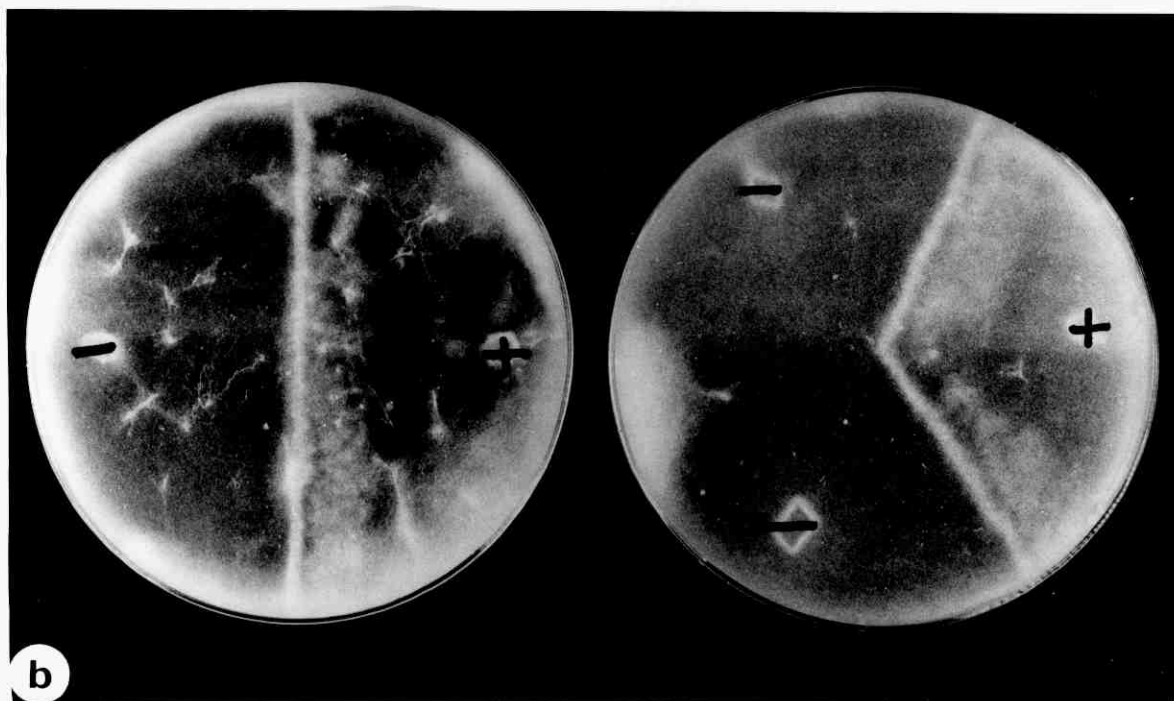


Plate 2.2.a Mating reaction on carrot agar between male and female isolates of P. heterothallicum.

Plate 2.2.b Mating reaction on carrot agar between (+) and (-) isolates of P. intermedium.

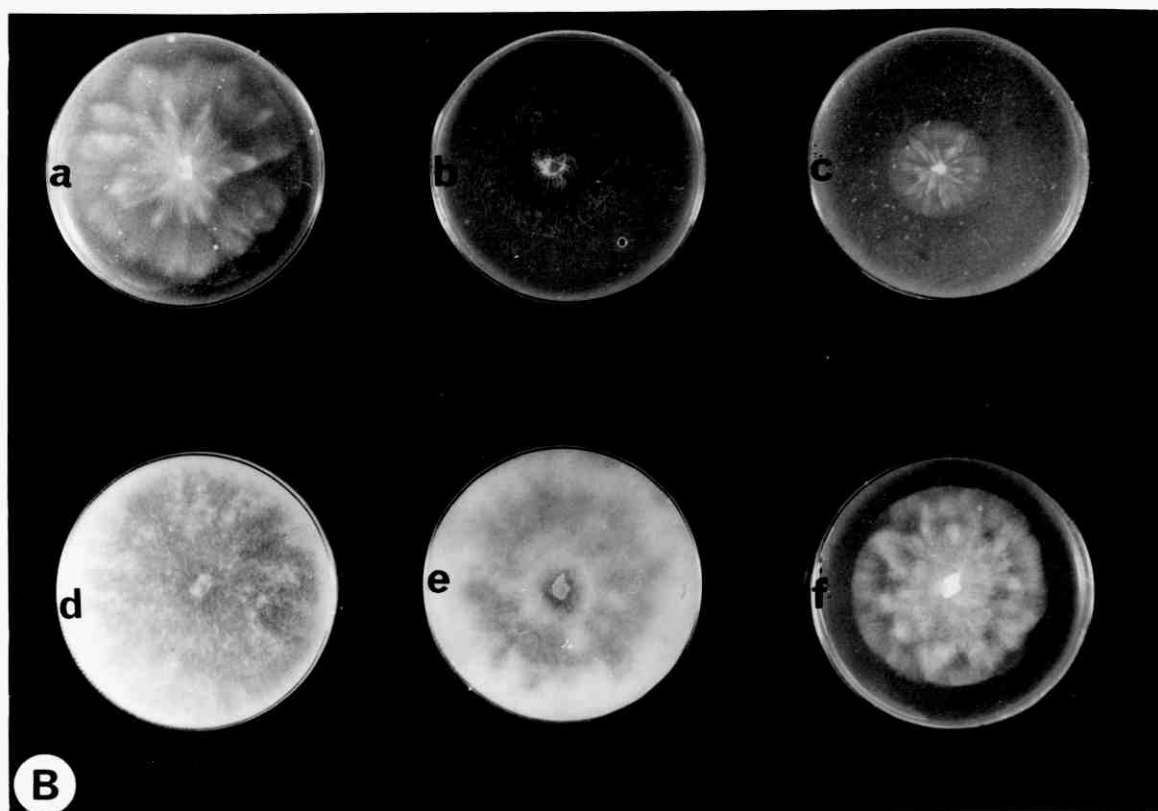
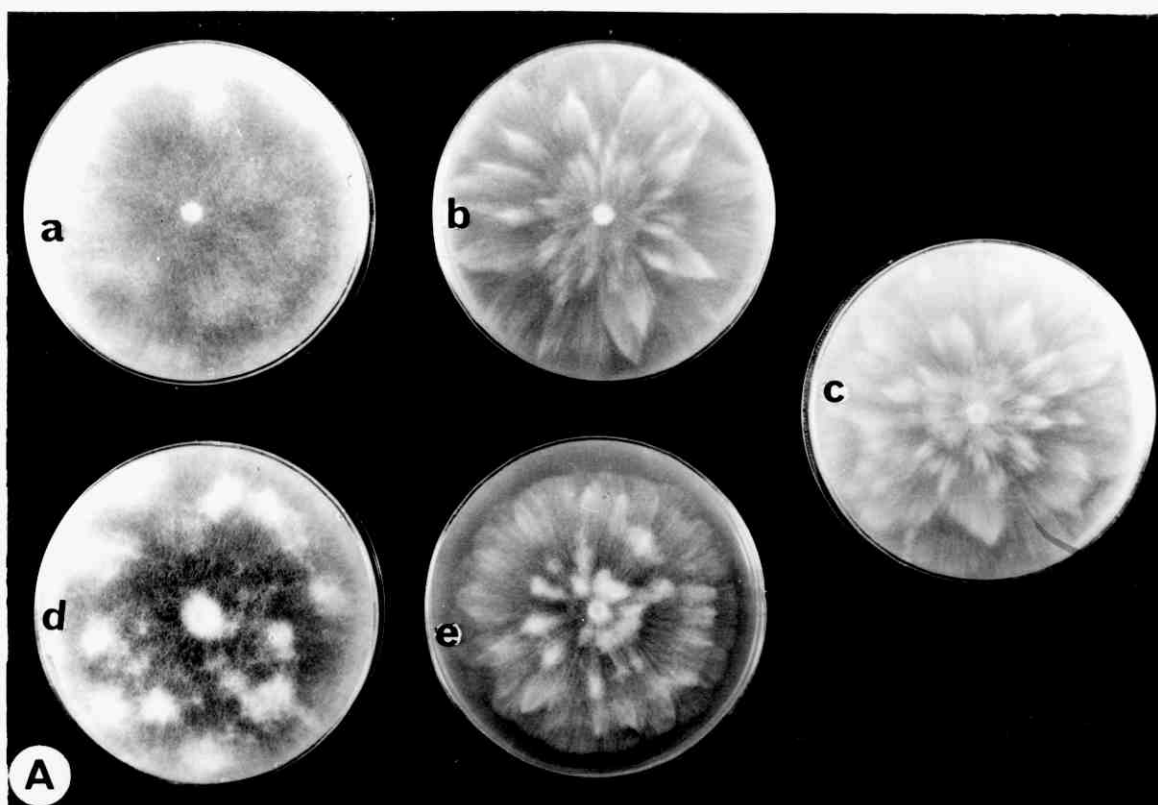


Plate 2.3.A Colony morphology of heterothallic Pythium species on MPV (a) P. intermedium (b) P. x (type A) (c) P. x (type B) (d) P. sylvaticum (e) P. heterothallicum.

Plate 2.3.B Colony morphology of homothallic Pythium species on MPV (a) P. sp 998 (b) P. sp 1089 (c) P. rostratum (d) P. irregulare (e) P. ultimum (f) P. torulosum.

Table 2.1. Mean colony diameters (mm) of Pythium spp. after 48 h incubation (22° C).

Pythium sp.	Medium	
	MPV	CMA
<i>P. sylvaticum</i>	82	87+
<i>P. intermedium</i>	66	87+
<i>P. irregulare</i>	65	83
<i>P. ultimum</i>	58	87+
<i>P. x</i>	51	60
<i>P. heterothallicum</i>	45	55
<i>P. coloratum</i>	45	53
<i>P. sp. (no. 998)</i>	34	36
<i>P. rostratum</i>	14	17

2.5. Summary of Section 2

Varied procedures were needed for the identification of Pythium spp. and virtually all isolates had to be plate-cultured individually or in pairs.

Culturing on grass leaves (or hemp seeds) in water, as recommended by Waterhouse (1967), was a valuable method. However Schmitthenner's medium (also recommended by Waterhouse), CMA and V-8 juice agar were found to be inferior to CMA/B-sitosterol for sexual production in homothallic spp. The sterol requirement for sexual reproduction was reported by J.W. Hendrix (1965, 1970, 1974). Kaosiri's carrot agar was superior even to the sitosterol medium in inducing sexual stages, but was far more difficult for observational purposes and was only used as a last resort.

3. The Pythium species isolated

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3. The Pythium species isolated

3.1. Taxonomic literature

The monograph of the genus Pythium by Middleton (1943) and the compilation of original species descriptions by Waterhouse (1968) were of great value to this study. Some dominant species isolated from orchard soils, however, were included in neither of these works and for these reference was made to the publications by Campbell & Hendrix (1967) and Van der Plaats-Niterink (1968). The 'species complex' system of Hendrix and Papa (1974) was not followed, or found acceptable, but their synoptic key was a valuable addition to the works of Middleton and Waterhouse.

3.2. List of species isolated

Homothallic spp.

P. acanthophoron Sideris

P. coloratum Vaartaja (z)

P. dissimile Vaartaja (z)

P. irregulare Buisman (z rare)

P. paroecandrum Drechsler

P. rostratum Butler (z)

P. salpingophorum Drechsler (z)

P. torulosum Coker & Patterson (z)

P. ultimum Trow

P. vanterpoolii Kouyeas & Kouyeas (z)

Heterothallic spp.

P. heterothallicum Campbell & Hendrix

P. intermedium de Bary

P. sylvaticum Campbell & Hendrix

(z) : Reported zoosporic spp.

3.3. Notes on the homothallic species

Six of the ten spp. listed were reported to be zoosporic in the original descriptions: zoospore formation, however, was not observed in three of these spp. (P. dissimile, P. rostratum and P. torulosum).

There is controversy over the taxonomy of P. irregulare and P. debaryanum (Biesbrock & Hendrix 1967; Hendrix & Campbell 1970) and it is considered by some (Domsch et al 1980) that 'P. debaryanum' should be considered a nomen dubium. In this study all isolates with intercalary oogonia, stalked antheridia making apical contact, and strongly aplerotic oospores were referred to P. irregulare (irrespective of the absence of oogonial protruberances).

Four homothallic, non-zoosporic, isolates could not be identified with any described species (isolates nos 491, 998, 1038, 1089).

The reproductive stages of some of these species are illustrated in plates 3.1 and 3.2.

3.4. Notes on the heterothallic species

The great majority of isolates from orchard soils did not form oospores in single cultures, but only spherical or sub-spherical, terminal or intercalary hyphal swellings. They did not produce zoospores in water cultures. These isolates could be separated into groups on the basis of certain cultural characters (Table 3.1). The formation of appropriately-sized, catenulate conidia identified one group as P. intermedium. This species was reported to be zoosporic in the original description and by Sewell (1981): none of the isolates studied in this investigation formed zoospores in water culture.

Mating on CMA between certain isolates of the fast-growing, large-spored group resulted in profuse formation of sexual organs (Plate 3.3 a). All isolates also mated with male or female strains of authenticated isolates of P. sylvaticum, and were therefore identified with certainty.

Isolates in the P. heterothallicum and P. x groups presented considerable problems of identity until a medium (carrot agar) was found which stimulated mating of certain isolates with authenticated strains of P. heterothallicum. Carrot agar also stimulated mating and confirmed the identity of P. intermedium isolates.

A large group of isolates resembling, but not mating with, authenticated male or female strains of P. heterothallicum were designated Pythium x. Within this group two extreme types (A and B) could be distinguished among certain isolates, but the group included many isolates with characters varying between these extremes. It seems probable that P. x isolates are heterothallic spp. (or one heterothallic sp.) with undiscovered requirements for sexual reproduction.

Plate 3.1 Reproductive stages of Pythium species.

- (a) Conidia - P. sp 1089 (b) Plerotic, spiny oogonium with one antheridium - P. acanthophoron (c) Sessile, single antheridium and intercalary oogonium - P. ultimum (d) Sessile single antheridium and terminal oogonium - P. ultimum (e) hypogynous antheridium - P. sp 1038 (f) Parthenogenic oospores in chain - P. salpingophorum (g) intercalary oogonium and sessile antheridia - P. rostratum (h) aplerotic oospores - P. ultimum (i) lobate sporangium - P. torulosum.

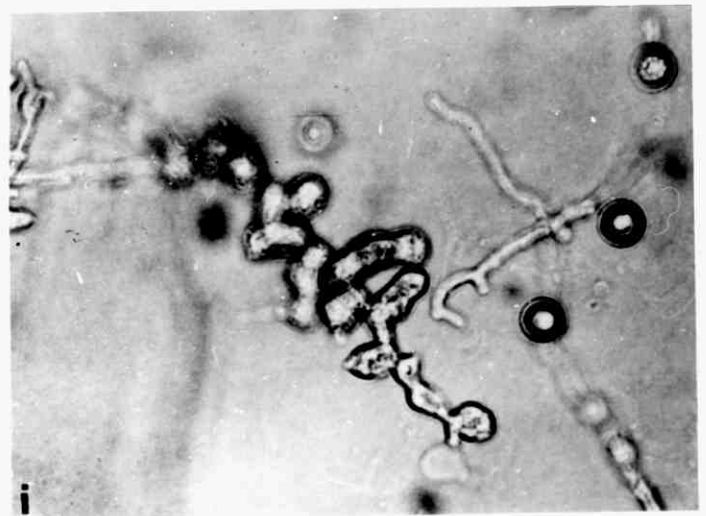
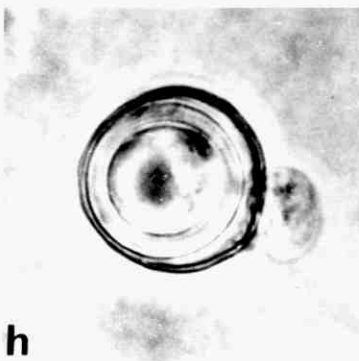
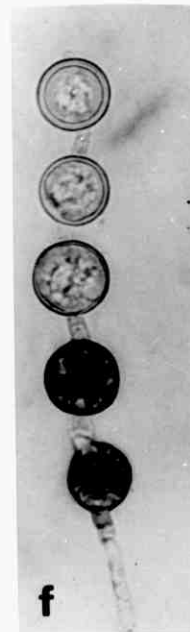
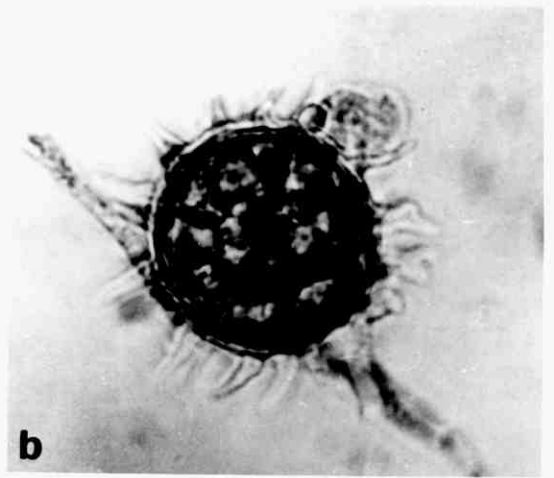
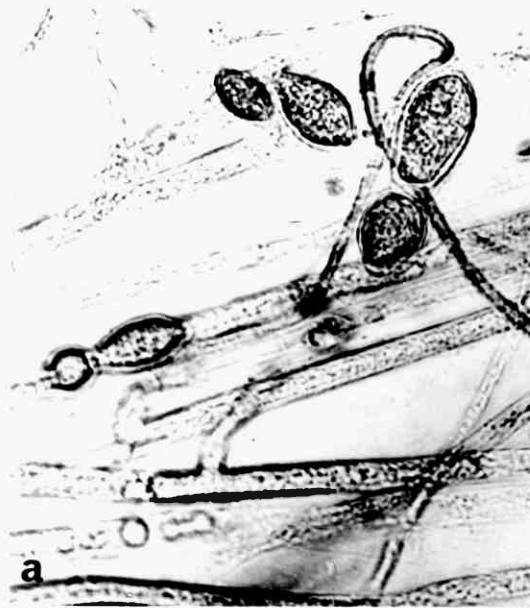


Plate 3.1

Plate 3.2 Reproductive stages of Pythium species.
(a) Oogonia and antheridia of P. heterothallicum
in a paired culture on carrot agar. (b) Large,
persistent multiple antheridia (often forming
dense 'knot' around oogonium) induced by a
contamination of Penicillium sp. in a paired
culture on CMA - P. heterothallicum.
(c) Intercalary and aplerotic oospore -
P. irregulare. (d) Multiple antheridial stalks
encircling oogonium - P. sylvaticum.
(e) Stalked antheridium and oogonium with
small protuberance - P. irregulare.
(f) Branched antheridial stalked and oogonium -
P. sp 998.

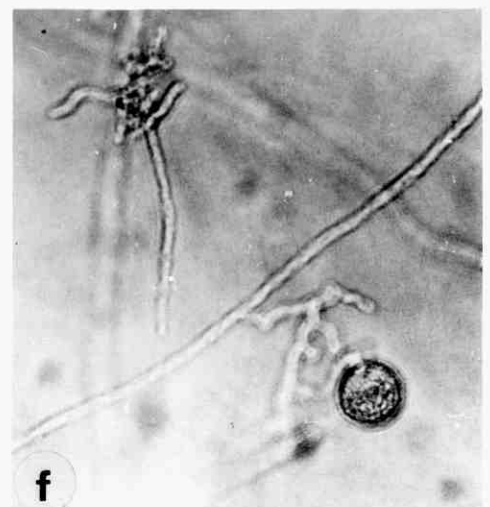
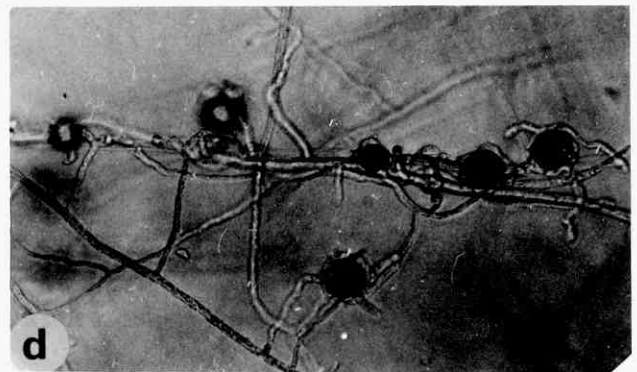
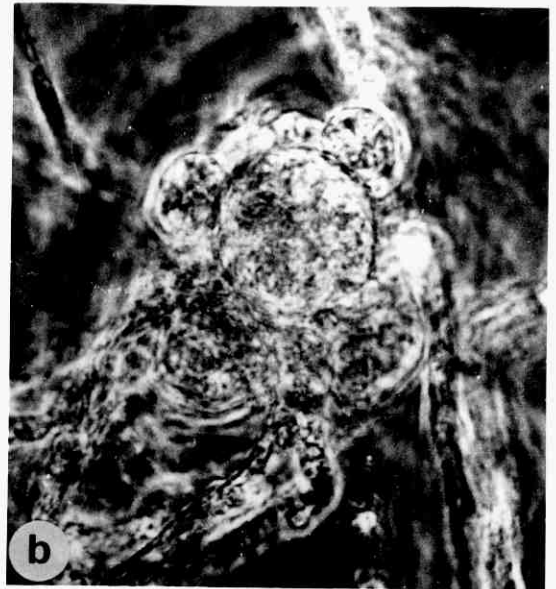
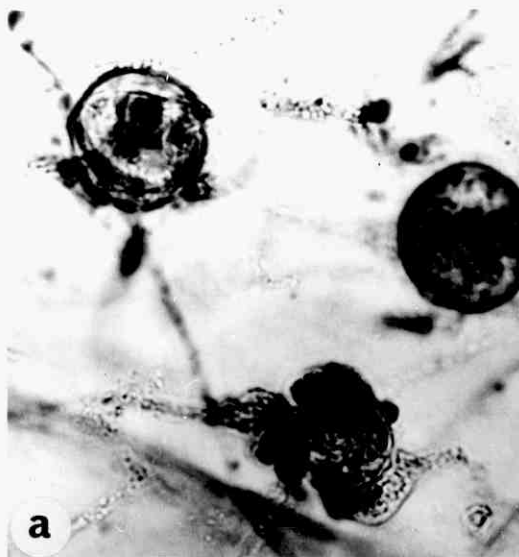


Plate 3.2

During these studies it was observed on carrot agar that paired cultures of P. sylvaticum (female) and P. intermedium (male), and vice versa, resulted respectively in the development of oogonia resembling those of P. sylvaticum and of P. intermedium. It was not possible to trace the hyphal origin of antheridia or oogonia. However, the observation indicates at least that under certain conditions intra-specific stimulation of sexual reproduction may occur.

Stimulation of sexual reproduction in P. heterothallicum on CMA was observed in one paired culture contaminated by a Penicillium sp. (Plate 3.3 b): such intra-generic stimulation might be of considerable importance in nature.

The reproductive stages of P. sylvaticum and P. heterothallicum are illustrated in Plate 3.2.

3.5. The composition of the English orchard soil flora

Heterothallic Pythium spp. were outstandingly the dominant fungi isolated from a wide range of apple orchard soils. This confirms the report of Sewell (1981) which contains the only record of a heterothallic species (P. sylvaticum) in England. P. heterothallicum is first reported in England in this investigation. (P. acanthophoron is also a first record if the identification is correct).

It is possible that the dominance of the heterothallic spp. was an artefact of the isolation method. The method was chosen because it favoured the isolation of P. sylvaticum which was the most pathogenic species to apple (Sewell, 1981). It has been reported that MPV is toxic to P. ultimum (Lumsden et al, 1975): but this sp. and P. intermedium and P. irregulare grew more rapidly on MPV than the far more frequently isolated P. heterothallicum and P.x.

A dominance of P. sylvaticum was also found (by other methods) in hardwood forest soils of the South-eastern U.S.A., but it was infrequent there in apple orchard soils where the 'P.irregulare-debaryanum complex' dominated (See Part I of this thesis). Clearly geographical differences in Pythium-apple associations must be anticipated.

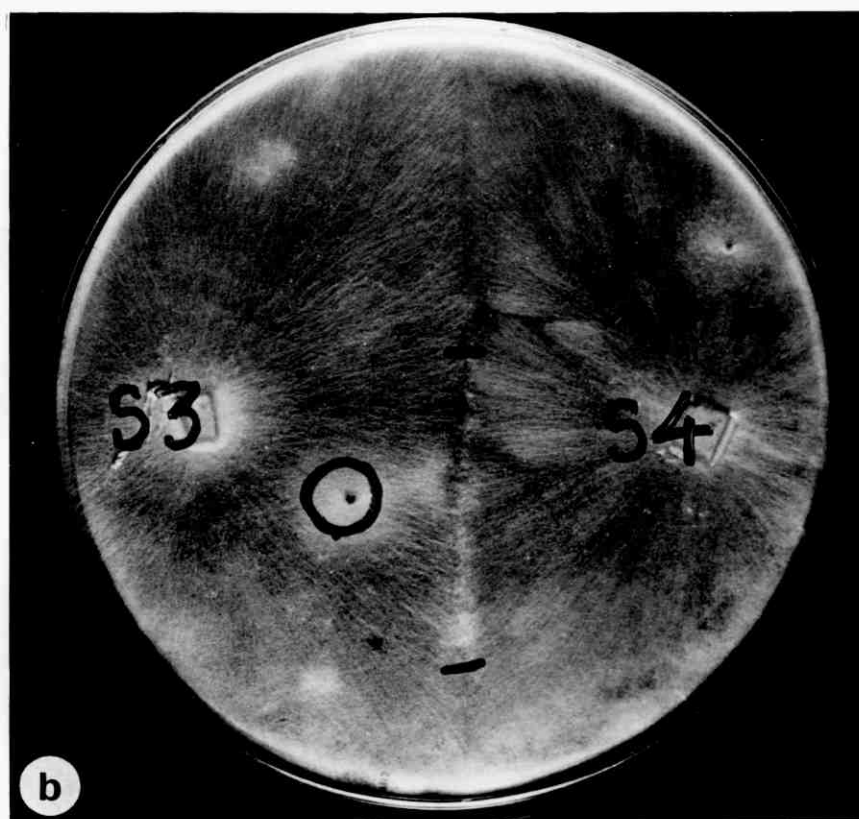
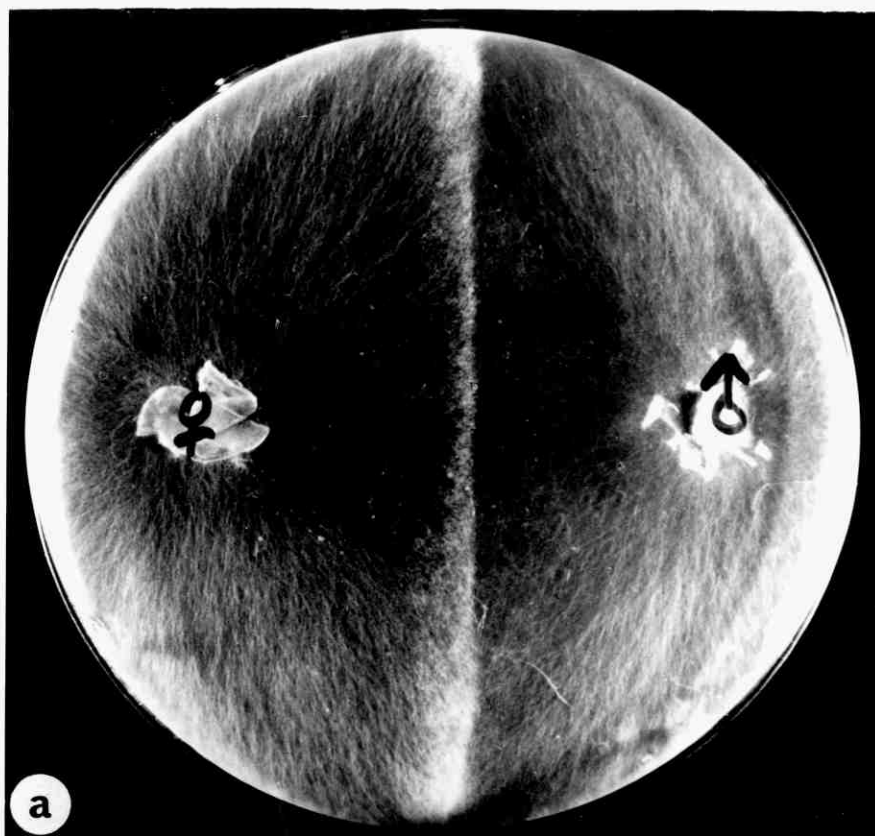


Plate 3.3.a Mating reaction on CMA between male and female isolates of P. sylvaticum.

Plate 3.3.b Male (S3) and female (S4) strains of P. heterothallicum on CMA. Mating reaction only induced by a contamination of Penicillium sp. (colony marked by a circle).

Table 3.1. Cultural characteristics of frequently isolated Pythium spp.
(sylvaticum, heterothallicum, intermedium, x).

	Pythium spp.				
	syl	het	int	X (A)	X (B)
Relative growth rate	+++	+	++	+	+
Colony zonate		+		+	+
Conidial diam (μ)	28-32	< 16	15-20	15-22	12-15
Conidia in MPV (7 d)	+	+	+	++	+
Conidia catenulate			+		
Appressoria in MPV	+		+++		+++
Intra-group mating CMA	+				
Do. carrot agar	+	+	+		

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4.1. Introduction

This investigation was undertaken with two objectives. Firstly, to examine the relative incidences of Pythium spp. and replant disease of apple. Secondly, to contribute to the sparse information available on the ecology of (especially heterothallic) Pythium spp. in soil.

Previous work (Sewell, 1981) showed that apple seedling growth was significantly reduced by eight Pythium spp., and that P. sylvaticum was the most pathogenic. This species was also the dominant fungus on MPV soil dilution plates prepared from ten different soils. Because of the possible particularly important role of P. sylvaticum in apple replant disease (ARD), the same isolation method was used in this study. Assessment of associations between Pythium and ARD in 135 apple orchard soils was possible only because of the existence of the ADAS soil fumigation test service for growers. These tests provide an essential background to the isolation studies and are therefore discussed separately, in detail, below (Ss 4.2.)

Ecological studies of Pythium spp. in soil are few and have mostly been made following the development of selective isolation methods in the 1960's (Campbell & Hendrix, 1973). Those made before 1967 are of diminished value because until then heterothallism in Pythium was unrecognised (Campbell & Hendrix, 1967a).

4.2. The ADAS apple seedling test for the incidence of replant disease in orchard soils.

Testing the benefits of chloropicrin fumigation of orchard soils was commenced by ADAS as a service for growers in 1965. The soil testing method was basically the bio-assay developed during 1962-63 in the Netherlands (Hoestra, 1968) and later modified to suit the facilities available at Wye (Upstone, 1975). The test consists of comparing the growth of apple seedlings in fumigated and untreated soil.

Soil samples are collected from orchards during Feb. - March, by spade to a depth of 25-30 cm, at 20 random points throughout areas up to 4 ha to give approximately 20 litres of soil. The soil is shredded mechanically, thoroughly mixed and divided into two portions, one to be fumigated and the other untreated. Fumigation is carried out by adding 2.5 ml chloropicrin to 10 litre soil in a

polyethylene bag which is then sealed and incubated in a metal box at ambient temperature for 7 days. The bags are opened to the atmosphere and the contents shaken by hand daily for up to 3 weeks to allow all fumes to escape.

A cress germination test is carried out on a representative number of sub-samples to confirm that no chloropicrin remains in the soil. (Cress seeds fail to germinate or seedlings grow poorly in the presence of traces of chloropicrin).

Fumigated and untreated soils are then each placed in 10 clay pots (600 ml capacity) without drainage holes. In early May, single seedlings of Malus pumila, selection 'Bittenfelder' (raised in sterilized compost) at the first true-leaf stage are planted into each pot. Pots are then plunged into a water saturated peat/sand medium in cold frames. Nutrient solution is applied to the pot soils every 10 days and foliar sprays (currently benomyl and demeton-methyl) are applied as necessary to control mildew and aphids.

After 12 weeks growth the height of each seedling is measured and the mean heights of plants in fumigated soil (F ht) and untreated soil (U ht) determined. Comparison of these mean heights indicates the increase (or decrease) in growth resulting from fumigation, and the result of the test is expressed as a percentage response (% R) to fumigation:

$$\% R = \left(\frac{\text{Mean F ht}}{\text{Mean U ht}} \times 100 \right) - 100$$

Growth increases of less than 50% are considered to indicate that fumigation would not be economically advisable. With responses of 50–100%, fumigation would be recommended if trees on dwarfing rootstocks (especially sensitive to ARD) are to be planted. Field fumigation is recommended before replanting all types of tree when the response is 100% or more.

Soil fumigation may also result in diminished seedling growth (a negative response.) These negative responses have been surprisingly frequent, and occurred in 11% of soils tested during 1965–74 (Upstone, 1975), and in 30% of the 135 soils included in this study. The cause of these negative responses was not understood until 1981 (Sect. 4.2.2.) but it was assumed by ADAS that soils giving negative responses did not have an ARD problem.

4.2.1. Classification of ADAS results: response groups and growth classes.

In this study the ADAS % responses to soil fumigation are given in five

'response groups' (often abbreviated RG), numbered 1-5, as follows:-

- (1) negative responses, (2) 0-49%, (3) 50-99%,
- (4) 100-149%, and (5) $\geq 150\%$.

Response groups 1 and 2 have been considered by ADAS to indicate little or no replant disease problem present. Soils in response group 3 are considered to have 'significant', and those in groups 4 and 5 to have 'very significant' replant disease problems.

In this laboratory attention has also been given to the relative growth of seedlings in unfumigated soils (U growth) which alone might reflect disease levels. Because growth varies considerably between years according to climatic conditions, an arbitrary classification is given for U growth which permits results from different years to be combined. The difference between the mean heights of the best and worst sets of replicate plants is divided equally into five: these five 'growth classes' are signified A,B,C,D and E. Class A represents the poorest, and class E the best growth in U soils. (Plant heights in growth classes A and E were generally in the order of 8 and 30 cm).

The response group (RG) and growth class (GC) for each of the 135 soils included in this study are given in Table 4.39. (Section 4.8.).

4.2.2. Soil phosphorus content, response group and growth class.

During 1981, an unusually large proportion (21%) of the soils tested by ADAS gave negative responses to fumigation and a highly significant association was found between these soils and low soil phosphorus content (G.W.F. Sewell, unpublished).

These analyses revealed that seedling growth in untreated soils was independent of P content (from 1-150 ppm), but that growth in fumigated soils increased linearly over the range of 1-40 ppm P: at very low P levels (< 20 ppm) growth in fumigated soils was generally less than that in untreated soils (resulting in negative responses). In fumigated soils with $P > 40$ ppm growth was virtually unaffected by P content. Sewell considered that these effects were related to the essential dependence of apple on vesicular-arbuscular mycorrhiza (VAM) when soil P content was low. His analyses (on all ADAS results for the period 1975-81) thus showed that 'growth class' was unaffected by P content, but that 'response

group' was very greatly influenced by P content. In low-P soils apple seedlings were unable to respond to the potential benefits of fumigation.

These findings were fully expressed within the 135 soils included in this study and are of great importance to the interpretations of the results of Pythium assays.

The effects of soil phosphorus on the interaction between growth classes and response groups are given in Tables 4.1. (a)–(b). In high-P soils (a) 'significant' responses ($\geq 50\%$ growth increases) occurred in 67% of soils: in low-P soils (b) they occurred in only 9%. In high-P soils, the mean growth class value was linearly, inversely related to response group, and the mean response group value was linearly, inversely related to growth class. In low-P soils there was no association between mean values of growth class and response group.

The significances of these associations are given in contingency tables (Tables 4.2. (a)–(c)). That growth class was not associated with phosphorus, and was very highly significantly related to response group, suggests that growth class alone might provide an indication of disease severity in all soils. The analysis (b) of response group and growth class was not possible in soils with $P < 40$ ppm, and was not significant when all soils together were included.

Table 4.1. Frequency of occurrence of soils in fumigation response groups and seedling growth classes in relation to soil phosphorus content.

(a) $P \geq 40$ ppm

Response group	Growth class					Sum	Mean value*
	A	B	C	D	E		
1	0	1	1	0	4	6	4.2
2	0	1	4	4	3	12	3.8
3	1	11	5	6	1	24	2.8
4	0	7	0	1	0	8	2.3
5	1	3	0	0	0	4	1.8
Sum	2	23	10	11	8	(54)	
Mean value*	4.0	3.4	2.4	2.7	1.6		

(b) $P < 40$ ppm

Response group	Growth class					Sum	Mean value
	A	B	C	D	E		
1	5	13	11	2	4	35	2.6
2	12	12	7	4	4	39	2.4
3	1	0	0	1	0	2	2.5
4	1	2	1	0	0	4	2.0
5	1	0	0	0	0	1	(1.0)
Sum	20	27	19	7	8	(81)	
Mean value	2.1	1.7	1.5	1.9	1.5		

* Mean value: Growth class A-E and response groups 1-5 respectively scored 1-5: $\sum(n \times \text{score}) / n = \text{mean value.}$

Table 4.2. Contingency tables showing observed and expected (in parenthesis) frequencies of occurrence of soils in relation to phosphorus content, growth class and response group.

P (ppm)	Response group		Sum
	1—2	3—5	
< 40	74 (55.2)	7 (25.8)	81
> 40	18 (36.8)	36 (17.2)	54
Sum	92	43	135

(a) Phosphorus x response group

$$X^2 = 50.26$$

$$(P < 0.001 \text{ at } 10.8)$$

Response group	Growth class		Sum
	AB	CDE	
1—2 (< 50%)	2 (8.3)	16 (9.7)	18
3—5 (≥ 50%)	23 (16.7)	13 (19.3)	36
Sum	25	29	54

(b) Response group x growth class

$$P \geq 40 \text{ ppm}$$

$$X^2 = 13.31$$

$$(P < 0.001 \text{ at } 10.8)$$

P (ppm)	Growth class		Sum
	AB	CDE	
< 40	47 (43.2)	34 (37.8)	81
≥ 40	25 (28.8)	29 (25.2)	54
Sum	72	63	135

(c) Phosphorus x growth class

$$X^2 = 1.79$$

$$(P < 0.05 \text{ at } 3.84)$$

4.3. Materials and Methods

4.3.1. The assay method

Pythium populations were assayed by the soil dilution plate method, using MPV medium (Sect. 1). In preliminary studies this medium was found to permit greater colony counts and to be more selective than a medium containing gallic acid (Flowers & Hendrix 1969). Mortierella spp. were virtually the only other fungi that developed on MPV within the assessment period (48 h).

Mortierella spp. were generally readily distinguished (from spreading, deeply-penetrating Pythium colonies) by their compact, superficial habit. The method, however, was clearly not only selective of Pythium, but of those Pythium spp. with the most rapid growth rates.

Few Pythium spp. sporulated on the selective isolation medium within the brief (48 h) assessment period and, therefore, quantitative and qualitative assessments required to be made separately (Ss 1.1). This is an important difference from assays of soil populations of other genera (which can often be identified directly on soil isolation plates), and means that quantitative assessments of individual species probably have a low level of accuracy. In this study most isolates were heterothallic species, requiring mating with authenticated male or female isolates for specific identification (Ss 2.3); this provided a further, laborious, complication. P. sylvaticum isolates mated readily on most media, but P. heterothallicum did not; a suitable medium for the latter species was only discovered half-way through this study, and no suitable medium was found for the (presumed heterothallic Pythium) Pythium x.

The results of Pythium isolations from the 157 soil assays in this study are given in detail in Table 4.40 (Section 4.8).

4.3.2. The soils assayed

All orchard soils assayed were supplied by the Agricultural Development and Advisory Service (ADAS) Sub-Centre, at Wye, Kent. ADAS provide a service to growers (Ss 4.2.) aimed at estimating the economic benefits of chloropicrin soil fumigation to the growth of replanted apple. Each year a varying number of orchard soils are collected for testing from apple-growing regions throughout England.

Selected, unfumigated, soil samples were collected from pot-plants for Pythium assays after the ADAS tests in 1979 (25 soils), 1980 (48 soils) and 1981 (22 soils). Samples of surplus soils were also collected before the ADAS tests in 1981 (62 soils).

Together with these soil samples ADAS also provided the test results (% growth response to fumigation), data on soil texture, pH and, recently, chemical soil analyses. These materials and data therefore, provided an invaluable foundation for studies of Pythium spp. in a large number of widely distributed English apple orchard soils, of the possible influences of soil texture and pH on occurrence of Pythium spp., and the severity of replant disease (as indicated by apple growth improvements after soil sterilisation).

ADAS data on the 135 soils included in this study are given in Tables 4.39 (a) - (c) (Section 4.8.)

4.4. Pythium populations in orchard soils before the ADAS tests (1981)

4.4.1. General observations on the incidence of Pythium spp.

Pythium spp. occurred in all (62) orchard soils with an overall mean population of 550 prop/g (Table 4.3.) The commonest species were, Pythium x, P. heterothallicum and P. sylvaticum, and, with these, frequency of occurrence was reflected by relative abundance. P. intermedium and P. irregulare were less frequent but, where they occurred were relatively abundant. 'Other spp.' isolated (from 35% soils) included: P. acanthophoron, P. coloratum, P. dissimile, P. paroecandrum, P. rostratum, P. salpingophorum, P. torulosum, P. ultimum, P. vanterpoolii, and two unidentified spp. (Isolate 491 and Isolate 1089).

Pythium x was of very frequent occurrence in all soils, irrespective of pH (Table 4.4.) and there was no obvious effect of pH on mean population size (Table 4.6.) The frequency of occurrence of P. heterothallicum, P. irregulare and 'other spp.' increased with increases in soil pH (Table 4.4.) and these associations between frequency and soil pH were highly or very highly significant (Table 4.5.) For P. heterothallicum and 'other spp.' increasing frequency was accompanied by increasing population sizes. P. sylvaticum showed a reverse effect with both frequency and abundance diminishing as soil pH increased (Table 4.4 - 4.6).

Analysis of Pythium populations in relation to soil texture was not possible because the bulk (57) of these soils were restricted to fine or very fine sandy loams.

Table 4.3. The incidence of Pythium spp. in 62 apple orchard soils assayed before the ADAS apple seedling tests (1981).

<u>Pythium</u> spp.	<u>Frequency</u>		<u>Abundance</u>
	No. Soils	%	(Mean p/g) *
<u>All spp.</u>	62	100	550
<u>P. x</u>	58	94	306
<u>P. heterothallicum</u>	34	55	199
<u>P. sylvaticum</u>	19	31	118
<u>P. intermedium</u>	11	18	156
<u>P. irregulare</u>	8	13	262
<u>Other spp.</u>	22	35	161

* Mean p/g = Mean propagules per gm dry wt. of soil.

Table 4.4. Frequency (%) of occurrence of Pythium spp. in relation to soil pH
(In 62 orchard soils before the ADAS apple seedling tests, 1981).

Soil pH	No. soils	Pythium spp.					
		x	het	syl	int	irr	others
< 5.4	5	100	0	80	20	0	20
5.4 - 5.9	4	100	50	0	25	0	0
6.0 - 6.4	8	88	0	38	0	13	50
6.5 - 6.9	15	93	47	53	20	13	20
7.0 - 7.4	18	89	72	17	11	17	50
≥ 7.5	12	100	100	8	33	17	50

Table 4.5. Contingency tables of observed and expected (in parenthesis)
occurrence of Pythium spp. in relation to soil pH (Table 4.4).

Soil pH	No. soils	<u>P. heterothallicum</u>		<u>P. sylvaticum</u>		<u>P.int., irr., others</u>	
		+	-	+	-	+	-
≤ 6.4	17	2 (9.3)	15 (7.7)	8 (5.5)	9 (11.5)	8 (11.5)	9 (5.5)
6.5 - 6.9	15	7 (8.2)	8 (6.8)	8 (4.8)	7 (10.2)	8 (10.2)	7 (4.8)
≥ 7.0	30	25 (16.5)	5 (13.5)	4 (9.7)	26 (20.3)	26 (20.3)	4 (9.7)
(Sum)	62	24	28	20	42	42	20
X ² (2 Df)		22.8		9.8		9.8	
P <		0.001		0.01		0.01	

Table 4.6. Abundance (Mean p/g) of Pythium spp. in relation to soil pH
(In orchard soils before the ADAS apple seedling tests, 1981.)

Soil pH	Pythium spp.					
	x	het	syl	int	irr	others
≤ 5.4	173	—	115	123	—	56
5.5 - 5.9	581	113	—	120	—	—
6.0 - 6.4	320	—	192	—	260	111
6.5 - 6.9	304	76	114	66	350	72
7.0 - 7.4	323	195	80	339	307	182
≥ 7.5	238	291	61	149	108	198

4.4.2. Pythium incidence in relation to the subsequent effects of soil fumigation on apple seedling growth.

The results of Pythium isolations from soils in the five fumigation response groups are given in Tables 4.7 - 4.9. There were no marked differences in Pythium populations between soils responding (groups 3-5) and not responding (groups 1-2) to fumigation. P. sylvaticum, P. heterothallicum and P. intermedium were slightly more frequent in response groups 3-5, but were not more abundant. Pythium x, P. irregulare and 'other spp.' were slightly more frequent and abundant in response groups 1-2.

Because responses to fumigation may be strongly influenced by soil phosphorus content (Ss 4.2.2.), and so not necessarily reflect disease levels, the results were analysed by two other approaches. Firstly, only high phosphorus soils ($P \geq 40$ ppm) were included. Secondly, seedling growth in unfumigated soil was considered to reflect disease levels (Ss 4.2.3.)

In high phosphorus soils (Table 4.9.), the most striking feature was that the proportion of soils in response groups 3-5 increased to 67% (cf 27% in all soils, Tables 4.7 - 4.8). The incidence of Pythium spp. (with the possible exception of P. sylvaticum), however, showed no relationship with the subsequent growth of apple seedlings: most species were slightly more frequent and abundant in low response soils.

Table 4.7. Frequency (%) of occurrence of Pythium spp. in relation to subsequent apple seedling growth responses to soil fumigation (ADAS tests 1981).

Pythium spp.	Response group (no. soils)				
	1 (21)	2 (24)	3 (10)	4 (5)	5 (2)
All spp.	100	100	100	100	100
P. x	95	96	90	80	100
P. heterothallicum	52	54	60	60	50
P. sylvaticum	33	25	30	40	50
P. intermedium	19	13	40	0	0
P. irregulare	14	17	0	20	0
Other spp.	38	42	10	60	50

Table 4.8. Abundance (Mean p/g) of Pythium spp. in relation to subsequent apple seedling growth responses to soil fumigation (ADAS tests 1981).

Pythium spp.	Response group				
	1	2	3	4	5
All spp.	623	550	411	601	345
P. x	346	297	233	376	182
P. heterothallicum	274	155	201	163	56
P. sylvaticum	112	114	145	58	226
P. intermedium	169	252	71	—	—
P. irregulare	155	343	—	260	—
Other spp.	152	156	87	211	45

Table 4.9. Incidence of *Pythium* spp. in relation to subsequent apple seedling growth response to fumigation, in soils with high phosphorus content ($P \geq 40$ ppm) (ADAS Tests, 1981).

	<u>Frequency (%)</u>		<u>Abundance (p/g)</u>	
	<u>Response group</u>		<u>Response group</u>	
<i>Pythium</i> spp.	1-2	3-5	1-2	3-5
All spp.	100	100	718	425
<i>P. x</i>	100	86	412	244
<i>P. heterothallicum</i>	63	57	147	172
<i>P. sylvaticum</i>	25	29	55	166
<i>P. intermedium</i>	13	4	(528)	71
<i>P. irregulare</i>	13	7	(630)	(260)
Other spp.	25	29	223	108
(No. soils)	8	14	8	4

4.4.3. Pythium incidence in relation to the subsequent growth of apple seedlings in unfumigated soils.

The results (Tables 4.10-11) showed that Pythium x occurred with similarly high frequency and abundance in good growth (GC C-E) and poor growth (GC A-B) soils. P. heterothallicum, P. sylvaticum and P. intermedium were slightly more frequent in poor than in good growth soils: the first two species also had larger populations in poor growth soils. P. heterothallicum occurred with significantly greater frequency ($P < 0.05$) than expected in poor growth soils ($X^2 = 4.73$, 1 Df) : the X^2 test for P. sylvaticum was not significant. P. irregulare and 'other spp.' were more frequent and abundant in good growth soils, but isolations of these spp. were too infrequent to permit analyses.

Table 4.10. Frequency (%) of occurrence of Pythium spp. in relation to subsequent growth of apple seedlings in unfumigated soils (ADAS Tests 1981).

Pythium spp.	Growth class (no. soils)						
	A	B	C	D	E	A-B	C-E
	(14)	(26)	(14)	(5)	(3)	(40)	(22)
All spp.	100	100	100	100	100	100	100
P. x	93	92	93	100	100	93	95
P. heterothallicum	86	54	43	20	33	65	36
P. sylvaticum	29	38	29	20	0	35	23
P. intermedium	29	15	21	0	0	20	14
P. irregulare	7	8	14	40	33	8	23
Other spp.	36	27	43	60	33	30	45

Table 4.11. Abundance (Mean p/g) of Pythium spp. in relation to subsequent growth of apple seedlings in unfumigated soils (ADAS Tests 1981).

Pythium spp.	Growth class						
	A	B	C	D	E	A-B	C-E
All spp.	518	550	461	684	895	538	571
P.x	230	324	221	435	637	291	331
P. heterothallicum	209	214	174	62	175	212	160
P. sylvaticum	92	154	72	48	—	136	67
P. intermedium	152	104	231	—	—	128	231
P. irregulare	40	218	360	324	250	158	324
Other spp.	146	162	140	162	350	155	168

4.4.4. Summary and discussion of Section 4.4.

Sixteen Pythium spp. were isolated from 62 orchard soils before the growth of apple seedlings. Populations, however, were strongly dominated by P. x, P. heterothallicum and P. sylvaticum. In general both frequency of occurrence and abundance of spp. increased with increase of pH: P. sylvaticum was an exception and its incidence was greatly diminished in soils of pH 7.0 or more.

There was no association between Pythium spp. populations and the subsequent growth responses to fumigation. In soils with high phosphorus content (where fumigation responses can be considered to be valid) P. sylvaticum was slightly more frequent and considerably more abundant in soils which subsequently gave 'significant' (> 50%) growth increases after fumigation. P. sylvaticum (and P. heterothallicum) was also more frequent and abundant in unfumigated soils which permitted only poor growth of apple seedlings. These latter observations therefore did not deny the possibility that P. sylvaticum might be implicated in the poor growth of apple: associations, however, were not clearly defined.

These studies of Pythium populations in soils before the apple seedling bio-assay offered little or no promise of providing a method for predicting the effects of chloropicrin fumigation on apple growth.

4.5. Pythium populations in orchard soils after the ADAS apple seedling tests (1979-81).

4.5.1. General observations on the incidence of Pythium spp.

The frequencies of occurrence of the common Pythium spp. differed little between years and these results are therefore bulked together in this section. Pythium spp. were isolated from all but two (93/95) orchard soils and their overall mean population was 869 prop/g (Table 4.12.). The most frequently occurring species were, in order, Pythium x, P. sylvaticum and P. heterothallicum, the most abundant sp. (1980 data, Table 4.13.) was P. sylvaticum. P. intermedium and P. irregulare were of infrequent occurrence but abundant when present (1980 data). Other species isolated (from 9% of soils) included: P. acanthophoron, P. coloratum, P. vanterpoolii and two unidentified species (Isolate - 491 and Isolate - 1089).

The incidence of Pythium spp. in soils from the five major apple-growing

regions of England is given in table 4.14. Numbers of samples were small except from Kent, but the data indicated certain differences in regional incidence for P. sylvaticum and P. heterothallicum. The X^2 test showed significantly different associations with Kent (Region I) and East Anglian (Regions II and III) soils for P. sylvaticum ($P < 0.05$) and for P. heterothallicum ($P < 0.01$). Inspection indicates that these species respectively were more frequent and more infrequent in Regions IV and V (Although there are no geographical or pedological reasons for combining these regions for analytical purposes).

The frequency of occurrence of P.x and P. heterothallicum increased with increasing soil pH and that of P. sylvaticum decreased with increasing pH (Table 4.15.). The reverse relationships between frequency and pH were very highly significant for P. sylvaticum and P. heterothallicum (Table 4.16.) : P. intermedium and 'other spp.' showed no relationship between frequency and soil pH. Where significant associations existed between frequency and soil pH, they were little reflected by differences in population sizes (Tables 4.17 - 18), except perhaps for P. heterothallicum: this lack of correlation may indicate that soil pH does not exert a direct influence on the incidence of certain Pythium spp. The overall mean Pythium population increased sharply in soils of pH ≥ 7.5 (Tables 4.16 - 17).

The association between soil texture and the frequency of occurrence of the three most common Pythium spp. is given in Tables 4.19 - 20. The significant associations for P. sylvaticum and P.x were largely due to the apparent absence of these species in a large proportion of the light-textured soils. (The only two soils which were apparently free of all Pythium spp. also belonged in this class, one being a sandy-loam and the other a fine sandy loam.) There was no indication of larger than expected Pythium frequencies of occurrence in heavy-textured soils.

Table 4.12. The incidence of Pythium spp. in 95 orchard soils assayed after the ADAS apple seedling tests (1979-81).

Pythium spp.	Frequency		Abundance *
	No. soils	%	(Mean p/g)
All spp.	93	98	869
P. x	69	73	
P. sylvaticum	68	72	443
P. heterothallicum	39	41	
P. intermedium	9	9	
Other spp.	20	21	

* Data incomplete for 1979, 1981 : See Table 4.13.

Table 4.13. The incidence of Pythium spp. in 48 orchard soils assayed after the ADAS apple seedling tests (1980).

Pythium spp.	Frequency		Abundance
	No. soils	%	(Mean p/g)
All spp.	47	98	892
P. x	33	69	411
P. sylvaticum	31	65	527
P. heterothallicum	18	38	490
P. intermedium	2	4	343
P. irregulare	4	8	396
Other spp.	6	13	152
(P. irreg + other spp.)	10	21	252)

Table 4.14. The frequency (%) of occurrence of *Pythium* spp. in soils from five regions in England (Soils assayed after the ADAS apple seedling tests, 1979-81).

	Regions* (no. soils)				
	I	II	III	IV	V
<i>Pythium</i> spp.	(59)	(14)	(8)	(8)	(6)
All spp.	98	93	100	100	100
<i>P. x</i>	80	57	63	75	50
<i>P. sylvaticum</i>	75	57	38	100	83
<i>P. heterothallicum</i>	36	64	75	13	33
<i>P. intermedium</i>	10	7	25	0	0
<i>P. irregulare</i>	7	7	13	0	17
Other spp.	17	7	13	25	17

* Regions: I Kent

IV Worc., Hereford, Glos., Oxon

II Norfolk, Suffolk, Camb.

V Surrey, Sussex, Hants

III Essex

Table 4.15. Frequency (%) of occurrence of Pythium spp. in relation to pH
(In 95 orchard soils after the ADAS apple seedling tests, 1979-81).

Soil pH	No. soils	Pythium spp.					
		All	x	syl	het	int	others
< 5.5	8	88	38	88	0	13	25
5.5 - 5.9	13	100	62	85	8	0	30
6.0 - 6.4	19	95	74	89	11	5	16
6.5 - 6.9	18	100	78	94	44	17	17
7.0 - 7.4	21	100	76	52	62	5	29
> 7.4	16	100	88	31	94	19	13

Table 4.16. Contingency table of observed and expected (in parenthesis)
occurrence of P. sylvaticum and P. heterothallicum in relation to
soil pH (Table 4.15).

Soil		<u>P. sylvaticum</u>		<u>P. heterothallicum</u>	
pH	No. soils	+	—	+	—
< 6.0	21	18 (15.0)	3 (6.0)	1 (8.6)	20 (12.4)
6.0 - 6.4	19	17 (13.6)	2 (5.4)	2 (7.8)	17 (11.2)
6.5 - 6.9	18	17 (12.9)	1 (5.1)	8 (7.4)	10 (10.6)
> 6.9	37	16 (26.5)	21 (10.5)	28 (15.2)	9 (21.8)
(Sum)	95	68	27	39	56
χ^2 (3 Df)		24.29		41.65	
P < 0.001 at 16.3					

Table 4.17. Abundance (Mean p/g) of Pythium in relation to soil pH. (In orchard soils after the ADAS apple seedling tests, 1979 - 81).

Pythium spp.	Soil pH					
	< 5.5	5.5 - 5.9	6.0 - 6.4	6.5 - 6.9	7.0 - 7.4	> 7.4
All spp.	763	831	849	791	825	1114
P. sylvaticum	563	489	455	433	327	424

Table 4.18. Abundance (Mean p/g $\pm$ S.E.) of Pythium spp. in relation to soil pH. (In orchard soils after the ADAS apple seedling test, 1980.)

Soil pH	Pythium spp.				
	All	x	syl	het	Others
< 5.5	730 $\pm$ 385	0	707 $\pm$ 398	0	(70)
5.5-5.9	714 $\pm$ 87	328 $\pm$ 118	490 $\pm$ 115	0	485
6.0-6.4	753 $\pm$ 124	442 $\pm$ 142	420 $\pm$ 51	(120)	0
6.5-6.9	901 $\pm$ 142	278 $\pm$ 48	550 $\pm$ 144	290	(150)
7.0-7.4	809 $\pm$ 135	338 $\pm$ 43	600 $\pm$ 43	529 $\pm$ 161	120
> 7.4	1206 $\pm$ 158	619 $\pm$ 119	575	547 $\pm$ 120	269

NB. Values in parenthesis were from single samples

Table 4.19. Frequency of occurrence (%) of P. sylvaticum, P. heterothallicum and P.x in relation to soil texture. (In 95 soils after the ADAS tests 1979-81).

Soil text.*	No soils	Pythium sp.		
		syl	het	x
LS	2	100	0	0
LFS	3	67	33	67
SL	2	0	0	0
FSL	17	47	41	53
VFSL	31	84	42	84
Zyl	16	75	44	88
L	5	20	60	80
SCL	5	80	40	60
CL	1	100	0	100
ZL	11	91	36	73
C	2	100	100	100

* See Ss 4.8.

Table 4.20. Contingency tables of observed and expected (in parenthesis)
incidence of P. sylvaticum and P.x in relation to soil texture.

Soil text.	No soils	<u>P. syl</u>		<u>P.x</u>	
		+	—	+	—
Light	24	12	12	11	13
(LS-FSL)		(17.2)	(6.8)	(17.4)	(6.6)
Medium	47	38	9	40	7
(VFSL-Zyl)		(33.6)	(13.4)	(34.1)	(12.9)
Heavy	24	18	6	18	6
(L-C)		(17.2)	(6.8)	(17.4)	(6.6)
(Sum)		68	27	69	26
χ^2 (2 Df)		7.6		12.4	
P <		0.05		0.01	

4.5.2. Pythium incidence in relation to the effects of soil fumigation on apple seedling growth.

The results of Pythium isolations from soils in the five fumigation response groups (Ss 4.2.2.) are given in Tables 4.21 -24. Pythium x, P. heterothallicum and P. irregulare tended to occur more frequently in soils not responding to fumigation (groups 1-2, growth increases < 50%); P. sylvaticum and 'other spp.' occurred more frequently in soils responding to fumigation (3-5, growth increases > 50%) (Table 4.21.) These associations were significant ($P < 0.05$) for P. sylvaticum and P. heterothallicum, but not for P.x (Table 4.22.)

The abundance of Pythium spp. (overall) diminished through the response groups 1-5 (Table 4.23.) and populations in response group 1 were significantly ($P < 0.01$) greater than populations in response groups 4-5 ($t = 2.88$, 36 Df). Differences in populations of P. sylvaticum were not significant (Table 4.23.)

Full data for specific abundance were available only for the 1980 ADAS tests (48 soils) and these are given in Table 4.24. Comparison of the abundance of all species and of P. sylvaticum in 1979-81 (Table 4.23.) and in 1980 (Table 4.24.) indicates that the 1980 data can be considered representative of those for all soils: this is further supported by the close similarity of specific frequency of occurrence in 1980 (Table 4.25.) and in 1979-81 (Table 4.2.) The 1980 data (Table 4.24.) show that 'all species' diminished in abundance through response groups 1-5, however, the results for P. sylvaticum populations in groups 1 + 2 (mean = 558 ± 129) and in groups 4 + 5 (mean = 380 ± 54) were not significantly different ($t = 1.14$, 21 Df).

These results, for both frequency and abundance, indicate that P. heterothallicum and P.x are not associated (in fact the opposite) with high responses to soil fumigation, and therefore, are probably not implicated in apple replant disease. A similar conclusion might also be reached for P. intermedium and P. irregulare, but soils from which these species were isolated were very few. The frequency of occurrence of P. sylvaticum was significantly associated with high response soils, but differences in population sizes in high and low response soils were not significant.

Of the 95 soils assayed after the ADAS tests in 1979-81, 40 had low phosphorus content (< 40 ppm) which might have invalidated the seedling

bio-assay results. Results of isolations from high phosphorus content soils ($P \geq 40$ ppm) therefore, were inspected separately. In high-P soils (Table 4.26.), the frequency of occurrence of P. heterothallicum did not greatly diminish through response groups 1-5, and P.x was of constant frequency. These results contrasted with those for all soils (cf Table 4.21.) The frequency of occurrence of P. sylvaticum was similar in both analyses, increasing continuously (and significantly) through the response groups 1-5. In high P-soils, as in all soils, the abundance of Pythium spp. overall and individually, diminished through response groups 1-5 (Table 4.26.) Certain differing results for frequency and abundance in high-P and in all soils (especially with Pythium x) indicated that phosphorus content of soils was directly or indirectly influencing the structure of Pythium populations (As distinct from influencing apple seedling responses to fumigation). The frequency of occurrence of Pythium x was found to be 60% greater in low P soils (Table 4.27.) and this association was significant in a 2 x 2 contingency table ($\chi^2 = 13.7$, $P < 0.001$). 'Other spp.' also occurred with greatly and significantly ($P < 0.05$) increased frequency in low P soils. All species, except P. heterothallicum, occurred more abundantly in low P soils with populations increased by 40 - 300%.

The frequency of P. sylvaticum in high fumigation response soils was consistent with a possibly causal role in replant disease. However, in certain 'valid' soils ($P > 40$ ppm) large populations were present in 11 low response soils (groups 1 and 2) (Table 4.27.) Seven of these soils contained possibly growth-limiting phosphorus levels (< 50 ppm), but others were (for English orchard soils) of high P content. Clearly therefore, large populations of P. sylvaticum were not invariably associated with severe disease, as reflected by response to fumigation.

Table 4.21. Frequency (%) of occurrence of Pythium spp. in relation to apple seedling growth responses to soil fumigation (ADAS tests, 1979-81).

	Response group (no. soils)				
	1	2	3	4	5
Pythium spp.	(23)	(32)	(25)	(10)	(5)
All spp.	100	100	92	100	100
P. x	91	69	68	70	40
P. sylvaticum	52	69	80	90	100
P. heterothallicum	57	47	28	30	20
P. intermedium	9	6	16	10	0
P. irregulare	13	6	8	0	0
Other spp.	13	13	12	30	20

Table 4.22. Contingency tables of observed and expected (in parenthesis) occurrence of Pythium spp. in relation to seedling growth responses to soil fumigation (Table 4.20.)

Growth responses	No. soils	P. x		P. syl		P. het	
		+	-	+	-	+	-
< 50%	55	43	12	34	21	28	27
(1 - 2)		(39.9)	(15.1)	(39.4)	(15.6)	(22.6)	(32.4)
≥ 50%	40	26	14	34	6	11	29
(3 - 5)		(29.1)	(10.9)	(28.6)	(11.4)	(16.4)	(23.6)
Sum	95	69	26	68	27	39	56
X ² (1 Df)		2.09		6.19		5.20	
		NS		(P < 0.05 at 3.84)			

Table 4.23. Abundance (Mean p/g) of Pythium spp. in relation to apple seedling growth responses to soil fumigation (ADAS tests, 1979-81).

Pythium spp.	Response group				
	1	2	3	4	5
All spp.	1025	878	857	636	614
<i>P. sylvaticum</i>	547	443	422	304	446

Table 4.24. Abundance (Mean p/g) of Pythium spp. in relation to apple seedling growth responses to soil fumigation (ADAS tests, 1980).

Pythium spp.	Response group				
	1	2	3	4	5
All spp.	1128	967	851	569	517
<i>P.x</i>	552	377	452	269	(80)
<i>P. sylvaticum</i>	725	483	661	333	490
<i>P. heterothallicum</i>	499	613	153	(120)	—
<i>P. intermedium</i>	(110)	(575)	—	—	—
<i>P. irregulare</i>	(190)	(660)	(75)	—	—
Other spp.	(200)	(180)	65	210	—

N.B. Values in parenthesis were from single samples.

Table 4.25. Frequency of occurrence of Pythium spp. in relation to apple seedling growth responses to soil fumigation (ADAS tests, 1980).

	Response group (no. soils)				
	1	2	3	4	5
Pythium spp.	(11)	(16)	(11)	(7)	(3)
All spp.	100	100	91	100	100
P. x	91	69	55	71	33
P. sylvaticum	36	56	73	100	100
P. heterothallicum	64	50	18	14	—
P. intermedium	9	6	—	—	—
P. irregulare	9	13	9	—	—
Others	9	6	18	29	—

Table 4.26. Frequency of occurrence of Pythium spp. in relation to apple seedling growth response to soil fumigation (ADAS tests, 1979-81, for soils with more than 40 ppm phosphorus.

	Response group (no. soils)		
	1/2	3	4/5
Pythium spp.	(19)	(24)	(12)
All spp.	100	92	100
P.x	58	58	58
P. sylvaticum	58	79	92
P. heterothallicum	47	25	33
P. intermedium	5	17	8
Other spp. + P. irregulare	5	13	25

Table 4.27. Abundance (Mean p/g $\pm$ SE) of Pythium spp. in relation to apple seedling growth responses to soil fumigation (ADAS tests, 1979-81, for soils with more than 40 ppm phosphorus).

Year	group	Pythium spp.				
		All spp.	syl	x	het	others + irr
1979-81	1/2	924 $\pm$ 123	545 $\pm$ 100			
	(19)	(19)	(11)			
	3	823 $\pm$ 78	390 $\pm$ 54			
	(22)	(22)	(19)			
	4/5	578 $\pm$ 92	301 $\pm$ 42			
	(12)	(12)	(11)			
1980	1/2	997 $\pm$ 202	488	223	795	180
	(6)	(6)	(4)	(3)	(4)	(1)
	3	766 $\pm$ 101	549 $\pm$ 86	153	153	68
	(10)	(10)	(7)	(2)	(2)	(3)
	4/5	433 $\pm$ 54	306 $\pm$ 52	120	120	150
	(7)	(7)	(7)	(1)	(1)	(1)

Nos. in parenthesis = no. / sample.

P. intermedium was not isolated from 1980 high P soils.

Table 4.28. Incidence of Pythium spp. in relation to soil phosphorus content.

			Pythium spp.					
	P ppm	No. Soils	All	x	syl	het	int	Others
Frequency (%)	< 40	55	100	93	68	50	8	33
(1979-81)	≥ 40	40	96	58	75	35	11	13
Abundance	< 40	22	1041	487	647	474	343	360
(1980)	≥ 40	25	732	295	441	515	—	89

4.5.3. Pythium incidence in relation to the growth of apple seedlings in unfumigated soils.

The relative sizes of apple seedlings in unfumigated soils was not related to the frequency of occurrence of Pythium spp. (Table 4.29.): the frequencies of P.x and P. sylvaticum were very high in soils permitting all classes of seedling growth. The frequency of P. sylvaticum was higher (80%) in CD than in AB (69%) soils. Soil populations (Table 4.30.) of P. x, P. sylvaticum and P. heterothallicum (averaging 480 prop/g) were also very high, but, except for P. sylvaticum, showed little association with relative seedling growth. With P. sylvaticum, the combined results for 1979-81 and (especially) those for 1980 alone, showed a curvi-linear population increase as plant growth increased through classes A-D and a dramatic population diminution in the best growth (Class E) soils. (This result was also reflected to some extent in the frequency of occurrence of P. sylvaticum). Differences in population sizes with growth classes D and E were significant ($t = 2.18$, 12 Df, $P < 0.05$) for 1980 alone but not for 1979-81. The result, for P. sylvaticum, might indicate that class E soils, permitting best apple growth, were inhibitory to the development of P. sylvaticum populations. If this was a real effect, the inhibitory soil factor(s) clearly did not affect P. heterothallicum, whose populations in class E soils greatly exceeded those in all other soils.

Table 4.29. Frequency of occurrence of *Pythium* spp. in relation to the growth of apple seedlings in unfumigated soils (ADAS tests, 1979-81).

	Growth class (no. soils)						
	A	B	C	D	E	A-B	C-E
<i>Pythium</i> spp.	(11)	(34)	(20)	(15)	(15)	(45)	(50)
All spp.	91	97	100	100	100	96	100
<i>P. x</i>	73	68	75	67	87	69	76
<i>P. sylvaticum</i>	55	74	80	80	60	69	74
<i>P. heterothallicum</i>	45	50	35	20	47	49	34
<i>P. intermedium</i>	0	12	15	0	13	9	10
<i>P. irregulare</i>	18	6	15	0	0	9	6
Other spp.	27	6	20	13	20	11	18

Table 4.30. Abundance (Mean p/g) of *Pythium* spp. in relation to the relative growth of apple seedlings in unfumigated soils (ADAS tests, 1979-81 and 1980).

	Growth class						
	A	B	C	D	E	A-B	C-E
<u>1979-81</u>							
All spp.	757	872	818	929	944	846	889
<i>P. sylvaticum</i>	338	371	449	645	432	365	509
<u>1980</u>							
All spp.	834	893	930	924	882	914	909
<i>P. x</i>	438	460	373	373	373	452	373
<i>P. sylvaticum</i>	440	477	630	768	290	469	575
<i>P. heterothallicum</i>	310	473	(330)	215	835	429	586
<i>P. intermedium</i>	—	(575)	(110)	—	—	—	—
Other spp.	320	325	(270)	(60)	125	323	145

4.5.4. Summary and discussion of Section 4.5.

Ten Pythium spp. were isolated from 95 orchard soils after the growth of apple seedlings. The Pythium spp. most frequently occurring (on dilution plates) were P.x, P. heterothallicum and P. sylvaticum. The most abundant species was P. sylvaticum (1980 data). P. intermedium and P. irregulare were of infrequent occurrence, but relatively abundant where present. P. sylvaticum occurred least frequently and P. heterothallicum most frequently, in soils from East Anglia.

Increases in soil pH were associated with increasing frequencies of P.x and P. heterothallicum, and decreasing frequency of P. sylvaticum. Pythium incidence did not (as might have been anticipated) increase with heaviness of soil texture, but P. sylvaticum and P. x were of less frequent occurrence in light-textured soils.

P. sylvaticum, therefore, was of less frequent occurrence in E. Anglian and high pH and light-textured soils. Information of this type is of interest but gives no indication of interaction effects between these (and other) features and impracticably large samples would be required to develop an understanding of such effects. The limited available statistics, however, do indicate 'background' factors that are likely to affect interpretations of associations between Pythium spp. and the growth of apple.

Associations overall between the incidence of Pythium spp. and apple growth responses to soil fumigation indicated that P. heterothallicum, P. x, P. intermedium and P. irregulare were probably not causally involved in ARD. The evidence against P. heterothallicum, however, was partly removed when only 'valid' fumigation responses were considered (i.e. for soils with $P \geq 40$ ppm). The evidence against P. intermedium and P. irregulare was based on small samples and not strong: both species have been shown to cause greatly diminished growth of apple seedlings in infested compost (Sewell, 1981).

The frequencies of occurrence of P. sylvaticum and 'other spp.' were positively associated with responses to fumigation in both 'all soils' and 'high-P soils' and these therefore may have a causal role in ARD. There was, however, no association between P. sylvaticum population size and fumigation response, and P. sylvaticum was abundant in a few soils with high-P contents giving low response to fumigation. The results suggest that if P. sylvaticum is a causal

factor, its direct or indirect effects on apple growth may be considerably influenced by soil environmental (perhaps especially microbial) factors. The role of 'other spp.' is of great interest, but in these studies (which were deliberately largely directed at P. sylvaticum) isolations were too few to be properly assessed.

The growth of apple in unfumigated soils (which, at least statistically, reflect disease levels) revealed little other than a direct relationship between seedling size and P. sylvaticum population size. However, in 'best growth' soils (Class E) an inhibitory environmental factor affecting P. sylvaticum was again indicated.

Phosphate content of soil clearly provided a further complicating 'background' factor (to be added to location, pH and texture) because most Pythium spp. were more frequent and abundant in low-P soils. Low-P soils were often deficient or poor in other nutrients also and the result, therefore, conflicts with some earlier views that Pythium spp. were more prevalent in agricultural soils (Remy 1949; Warcup 1952; Barton 1958).

4.6. Effects of apple seedling growth on the Pythium floras of orchard soils.

This section is concerned with comparisons of the results obtained from soils assayed before (Sect 4.4.) and after (Sect 4.5.) the ADAS apple seedling tests. This comparison is of value in studying the effects of apple seedling root growth concentrated in small (600 ml) volumes of orchard soils. The distribution of disease is known to be very uneven in orchards (Ross & Crowe, 1973) and soil collections for the ADAS tests are made by a random sampling system that is unrelated to the past or present distribution of trees. Variation in apple root density within these small soil samples, therefore, is likely to be even greater than that indicated by the effects of replant disease within orchards.

The Pythium assays of (62) pre-plant soils were made during the latter part of this study, primarily with the hope that they might provide a method for disease prediction. This did not materialise, but it became apparent that populations, in size and structure, differed from those of earlier post-plant soil assays. The importance of re-assaying the same soils after the ADAS tests (after November 1981) was realised but time restricted the number of same-soil assays that could be made: for this reason the assays were restricted to those soils

likely to give 'valid' fumigation responses ($P \geq 40$ ppm), and were only 22 in number.

The abundance of Pythium spp. overall was 58% greater in post - than pre - test soils (Table 4.31.): the greatest population increases occurred with P. sylvaticum (x 4.5), P. heterothallicum (x 2.5), and P. intermedium (x 2.2). Increased abundances of species were not (except for P. sylvaticum) reflected by increased frequencies of occurrence. P. sylvaticum frequency more than doubled in post - test soils and the diminished frequencies of other species may have been associated with increased competition from P. sylvaticum on dilution plates.

For P. sylvaticum the results from all soils (Table 4.31.) were similar to the results from the 22 soils sampled both before and after apple growth (Table 4.32.) The larger samples presented in Table 4.31. are therefore probably valid despite the largely differing soils and seasons. The associations between apple seedling growth and P. sylvaticum frequencies of occurrence were very highly or highly significant respectively in analyses of 'all soils' and of 'same soils' assays (Table 4.33.)

Detailed results of the 'same soil' assays for all Pythium spp. and for P. sylvaticum are given in Table 4.34. In each soil but two (nos 39, 54) total Pythium populations increased in abundance after apple growth, on overall average by 79%: differences between populations in pre - and post - tests soils were highly (nearly very highly) significant ($t = 3.34$ with 42 Df). P. sylvaticum increased in frequency from 27 to 68%, and mean populations increased significantly by 147% ($P < 0.05$; $t = 2.16$ with 19 Df) (Table 4.34.)

P. sylvaticum was not detected in seven soils (32%) after apple growth: these soils tended to be more frequently than expected of high pH (> 7.0) and to give fewer than expected 'significant' responses ($\geq 50\%$) to fumigation (Table 4.35.)

'Significant' responses to fumigation occurred in 80% of soils with P. sylvaticum (Table 4.35.), but there was no association between degree of response and population size (Table 4.34.)

Table 4.31. Incidence of Pythium spp. in orchard soils assayed before and after the ADAS apple seedling growth tests.

Pythium spp.	% frequency (years)			Abundance (years)		
	Before (1981)	After (1980)	After (1979-81)	Before (1981)	After (1980)	After (1979-81)
All spp.	100	98	98	550	892	869
P. x	94	69	73	306	411	
P. heterothallicum	55	38	41	199	490	
P. sylvaticum	31	65	72	118	527	443
P. intermedium	18	4	9	156	343	
P. irregulare	13	8	7	262	396	
Other spp.	35	13	21	161	155	
(No. soils)	(62)	(48)	(95)	-	-	-

Mean no. p/g Data incomplete for 1979 and 1981.

Table 4.32. Incidence of P. sylvaticum in orchard soils assayed before and after the ADAS apple seedling growth tests, in different and common soils.

Incidence	Before (no. soils)		After (no. soils)		
	1981 (62)	1981 (22)*	1979 (25)	1980 (48)	1981 (22)*
No. soils	19	6	22	31	15
% soils	31	27	88	65	68
Mean p/g	118	131	362	527	352

* Soils common to before and after apple growth.

Table 4.33. Contingency tables showing observed and expected (in parenthesis) incidence of *P. sylvaticum* in soils before and after the ADAS apple seedling growth tests.

P. sylvaticum	All soils			Common soils		
	Before	After	Sum	Before	After	Sum
+	19 (34.4)	68 (52.6)	87	6 (10.5)	15 (10.5)	21
—	43 (27.6)	27 (42.4)	70	16 (11.5)	7 (11.5)	23
Sum	62	95	157	22	22	44
χ^2	25.59			7.38		
	P < 0.001 at 10.83			P < 0.01 at 6.63		

Table 4.34. Response to fumigation and abundance (Mean p/g) of all *Pythium* spp. and *P. sylvaticum* before and after the growth of apple seedlings. (ADAS tests, 1981: $P > 40$ ppm in all soils).

Soil No	<u>All <i>Pythium</i> spp.</u>		<u><i>P. sylvaticum</i></u>		Response Group
	Before	After	Before	After	
1	500	850	275	425	3
62	565	740	226	370	5
58	605	630	121	250	3
60	610	1500	61	150	1
26	950	1370	48	550	2
47	165	540	41	50	4
35	875	960	—	600	3
39	790	600	—	600	2
43	125	780	—	390	5
54	520	400	—	360	4
59	400	1760	—	350	3
33	175	830	—	330	3
41	665	1350	—	140	3
42	360	710	—	140	3
57	205	750	—	80	3
9	200	200	—	—	2
11	1750	1930	—	—	1
27	125	1350	—	—	2
38	435	440	—	—	1
40	580	1440	—	—	4
48	205	800	—	—	3
55	880	1040	—	—	2
No soils					
(%)	22 (100)	22 (100)	6 (27)	15 (68)	
Mean p/g	531 $\pm$ 97	953 $\pm$ 98	129 $\pm$ 41	319 $\pm$ 47	

Table 4.35. Incidence of *P. sylvaticum* in 22 orchard soils after the growth of apple seedlings in relation to soil pH and seedlings responses to fumigation (ADAS tests, 1981).

P. sylvaticum	Soil pH		% response	
	> 7.0	≤ 7.0	≥ 50	< 50
Present (15)	5	10	12	3
Absent (7)	5	2	2	5
Sum	10	12	14	8

4.7. Discussion of Section 4 with special reference to associations between *Pythium* spp. and apple replant disease.

The growth of apple seedlings in small pot volumes of orchard soils was associated with significant increases in both *Pythium* populations and the frequency of occurrence of *P. sylvaticum*. It has been suggested, for soil-borne *Phytophthora* spp., that such population increases indicate the plant grown to be a host (Orlikowski & Hendrix, 1977). That suggestion is debatable, but stability or reduction of a fungal population in the presence of a particular plant might more strongly indicate the plant to be a non-host. The evidence for effects of apple seedling growth on *Pythium* populations does not deny that *Pythium* spp. may be parasitic on apple roots (and result in poor growth), even in the absence of observations of soils growing other plant species, or unplanted.

The *Pythium* populations were found (by the methods used) to be strongly dominated by *P. sylvaticum*, *P. heterothallicum* and a fungus presumed to be a *Pythium* and presumed also to be a heterothallic sp. (*Pythium* x). Apart from one report (Sewell, 1981) heterothallic *Pythiums* have not previously been recorded in English soils. *P. sylvaticum* is an extremely rapidly growing sp. (Ss 2.4.) and undoubtedly largely precluded the isolations of other spp: there was a general inverse relationship between the dominance of *P. sylvaticum* and *P. heterothallicum* and a wider range of *Pythium* spp. was detected in orchard soils before apple seedling growth, when these two spp. were less abundant.

Seedling growth responses to chloropicrin fumigation of soils were very strongly associated with soil phosphorus content (Table 4.37.), and this effect required always to be taken into account when relating fumigation responses to the presence or absence of apple replant disease (ARD). Growth responses to fumigation were also significantly associated with soil pH: high responses (growth increase $\geq 50\%$) occurred less frequently than expected in soils of pH ≥ 7.0 (Table 4.38 (c)).

Incidence of *P. heterothallicum* was inversely related to increasing growth responses in 'all soils' and in 'high phosphorus' soils. Incidence of *P. x* was inversely related to increasing growth responses in 'all soils' and unrelated in 'high phosphorus' soils. *P. sylvaticum* incidence was directly and significantly related to increasing growth responses in all analyses (Tables 4.21, 22, 25, 26).

These results indicated that P. sylvaticum (but not P. heterothallicum or P. x) might be causally implicated in ARD, and subsequent discussion is largely confined to this sp.

P. sylvaticum was present in 68 of the 95 orchard soils assayed after apple seedling growth, and occurred with greater frequency (85%) in high than in low (62%) response soils (Table 4.36.) In soils with P. sylvaticum the incidence of high responses increased, and the incidence of low responses diminished, with increasing levels of soil phosphorus (Table 4.36.)

Of the 40 high response soils, P. sylvaticum was present in 34 (85%) and not detected in six. High responses to fumigation only occurred in soils with more than 20 ppm P: in these (80) soils response to fumigation was very highly significantly associated with the incidence of P. sylvaticum (Table 4.38. (b)). Both the incidence of high responses (Table 4.38. (c)) and of P. sylvaticum (Table 4.16.) were significantly less in soils of pH ≥ 7.0 .

Of the 55 low response soils, P. sylvaticum was present in 34 (62%). Responses to fumigation in 30 of these soils could have been totally, largely or partly inhibited by soil phosphorus levels (< 20 , 20-39, 40-50 ppm respectively). There was no obvious reason for the low response in the remaining four P. sylvaticum-containing soils (Soil nos 79/8, 79/41, 80/36, 81/60).

The incidence of ARD (reflected by fumigation response) therefore could be associated with the combined effects of P. sylvaticum and soil P content in 85 of 89 soils. In six of the 95 soils, poor growth must be attributed to other causes.

Consideration of the ADAS results in relation to high and low P content soils (Ss 4.2.2.) suggested that seedling growth in unfumigated soil (growth classes A-E) might reflect disease levels in soils, irrespective of P. content, because in high-P soils (fumigation responses valid) the association between growth class and response was very highly significant (Table 4.2. (b)). The data for high-P soils (Table 4.1. (a)) indicated that ARD was present in 92% soils in growth class AB, 57% soils in growth class CD, and 42% soils in growth class E. The Pythium assay results (data derived from Table 4.29.) showed P. sylvaticum to be present in 69% AB soils, 80% CD soils, and 60% E soils. There was therefore no agreement between these data: growth class, however, might be expected to be influenced considerably by nutritional and physical soil factors,

as well as by disease.

Results so far discussed have been concerned only qualitatively with the incidence in soils of P. sylvaticum. The abundance of P. sylvaticum increased as plant size increased through growth classes A-D, but diminished sharply in 'best growth' (growth class E) soils (Table 4.30.) The abundances of P. sylvaticum (and of total Pythium populations) diminished as growth responses to fumigation increased (Tables 4.24, 4.27). Reference to Table 4.1. shows that mean plant sizes also diminished as growth responses to fumigation increased. (The mean growth values for all soils together for RG 1-5 respectively were 2.9, 2.7, 2.8, 2.2, 1.6). Both sets of data thus showed that abundance of P. sylvaticum was directly related to plant size (except in 'best growth' soils). P. sylvaticum populations, therefore, increased when apple per se was grown (Table 4.34.) and also in direct relationship to the density of root growth.

These quantitative observations are consistent with the concept of replant diseases in general (i.e. that growth of a particular plant species encourages the establishment and accumulation in soil of pathogens to that plant). It is also consistent with observations on the replant disease of cherry (Sewell, 1975), that the pathogen population requires to be uniformly dispersed (ahead of growing roots) in order to cause diminished growth. Pathogen populations developing with the growth of roots may have negligible effects on plant growth.

With cherry replant disease, however, disease levels in soils could confidently be predicted by assays of Thielaviopsis soil populations (Pepin et al , 1975). No such prediction was possible from assays of P. sylvaticum populations in soils before the seedling growth test (Ss 4.4.). If, therefore, P. sylvaticum is to be considered a major cause of ARD, other factors influencing the host-pathogen relationship must be importantly involved.

Some aspects of these studies did indicate other interacting factors. In soils with pH > 7.0, P. sylvaticum occurred with greatly diminished frequency but, where present, populations were comparable with those in soils of other pH levels (Table 4.18.). These differing results for frequency and abundance suggest that the pH influences on P. sylvaticum populations were indirect. Again, P. sylvaticum frequency increased as plant growth increased (through growth classes A-E), but abundances increased only through growth classes A-D. In growth class E soils P. sylvaticum populations diminished and, in 1980 soils, were

less than in all other growth classes (Table 4.30.) That a direct association between population size and plant size was abruptly reversed in soils giving best apple growth strongly indicated that these soils were inhibitory to the development of P. sylvaticum populations.

P. sylvaticum appears to be implicated in ARD but the host - parasite relationship is not simple, and this particular species was not associated with poor apple growth in all orchard soils. Other Pythium spp. (mostly isolated by other methods) are known to be strongly pathogenic to apple seedlings (Sewell, 1981). Some of these known pathogenic spp., and other spp., were isolated in this study, but too infrequently to permit any estimations of their possible significance in apple replant disease.

Table 4.36. Distribution of soils in relation to response to fumigation, incidence of *P. sylvaticum*, and phosphorus content.

Phosphorus (ppm)	Growth increase after fumigation				Sum
	Low (< 50%)		High (≥ 50%)		
	+ P. syl	– P. syl	+ P. syl	– P. syl	
< 20	14	1	0	0	15
20 — 39	9	12	4	0	25
40 — 50	7	3	11	2	23
> 50	4	5	19	4	32
Sum	34	21	34	6	95

Table 4.37. Contingency table showing observed and expected (in parenthesis) frequencies of occurrence of soils in relation to soil phosphorus and fumigation response.

Fumigation response	Phosphorus (ppm)				Sum
	< 20	20 — 39	40 — 50	> 50	
Low	15	21	10	9	55
(< 50%)	(8.7)	(14.5)	(13.3)	(18.5)	
High	0	4	13	23	40
(≥ 50%)	(6.3)	(10.5)	(9.7)	(13.5)	
Sum	15	25	23	32	95

$$\chi^2 = 31.30 \quad (3 \text{ Df})$$

$$P < 0.001 \text{ at } 16.27$$

Table 4.38. Contingency tables showing observed and expected (in parenthesis) frequencies of occurrence of soils in relation to associations between some factors studied.

(a) Soil phosphorus x incidence of *P. sylvaticum*

P. syl	Phosphorus (ppm)			Sum
	0 – 39	40 – 49	≥ 50	
+	27 (28.6)	17 (15.0)	24 (24.3)	68
–	13 (11.4)	4 (6.0)	10 (9.7)	27
Sum	40	21	34	95
$\chi^2 = 1.26$ (2 D f) $P < 0.05$ at 5.99				

(b) Fumigation response x incidence of *P. sylvaticum* (Soil P ≥ 20 ppm)

P. syl	Fumigation response		Sum
	Low	High	
+	20 (27)	34 (27)	54
–	20 (13)	6 (13)	26
Sum	40	40	80
$\chi^2 = 11.17$ (1 D f) $P < 0.001$ at 10.83.			

Table 4.38. Continued

(c) Fumigation response x soil pH			
Soil pH	Fumigation response		Sum
	Low	High	
< 7.0	26 (33.6)	32 (24.4)	58
≥ 7.0	29 (21.4)	8 (15.6)	37
Sum	55	40	95
$\chi^2 = 10.50$			
$P < 0.01$ at 6.6			

4.8. Tabulations of ADAS soil data and Pythium isolations

In table 4.39. the following abbreviations are used for column headings:

No	The ADAS soil number for each year
GC	Growth class of apple seedlings in unfumigated soils (See Ss 4.2.1.)
RG	Response group. The classified growth response of apple seedlings in fumigated soils (See Ss 4.2.1.)
P	Soil phosphorus content (ppm)
pH	Soil reaction
T	Soil texture: C = clay, F = fine, L = loam(y), S = sand(y), V = very, Z = silt, Zy = silty (In table 4.19. the textural types included in this study are listed in order of increasing 'heaviness').

In table 4.40. the following abbreviations are used for column headings:

No	The ADAS soil number for each year
Total	The estimated mean abundance of <u>Pythium</u> spp. in the soil expressed as propagules/g (O.D. wt) (p/g)
syl	<u>P. sylvaticum</u>
int	<u>P. intermedium</u>
het	<u>P. heterothallicum</u>
x	<u>Pythium x</u>
irr	<u>P. irregulare</u>
others	<u>Pythium</u> spp. other than listed and, individually, of infrequent occurrence.

Table 4.39. ADAS data on the soils assayed

(a) Soils tested in 1979 (25)

No	GC	RG	P	pH	T	No	GC	RG	P	pH	T
8	E	2	73	5.4	VFSL	30	C	1	19	7.4	IZyl
10	B	3	49	6.3	VFSL	40	B	3	43	6.3	SCL
14	B	2	20	7.7	C	41	C	2	84	5.5	SCL
15	D	1	4	6.2	Zyl	43	D	3	77	6.8	SCL
16	E	2	36	6.8	Zyl	46	A	1	8	7.3	ZL
17	C	3	48	7.4	ZL	47	A	2	15	7.3	ZL
18	C	3	46	7.4	ZL	48	C	2	35	6.8	ZL
20	C	1	15	6.0	ZL	49	E	1	68	6.2	SCL
21	C	2	15	5.8	ZL	50	C	1	16	7.1	ZL
24	C	1	19	5.2	ZL	55	C	2	40	5.4	ZL
25	B	2	1	5.9	ZL	56	E	1	10	5.8	Zyl
26	B	2	32	7.4	C	104	D	2	43	6.0	VFSL
27	C	1	14	6.1	SCL						

(b) Soils tested in 1980 (48)

1	D	4	68	6.1	VFSL	9	B	1	9	5.8	Zyl
2	B	2	12	6.9	VFSL	10	B	5	83	6.1	VFSL
3	D	3	133	6.3	VFSL	11	B	3	47	7.4	LFS
4	D	3	95	6.6	VFSL	12	B	2	32	7.8	FSL
5	B	4	42	6.5	LFS	13	D	3	29	5.4	FSL
6	A	2	22	7.3	FSL	14	C	4	39	5.5	FSL
7	A	2	33	7.2	SL	15	E	2	53	7.4	Zyl
8	B	4	48	5.7	Zyl	16	E	2	49	6.5	Zyl

continued over

Table 4.39 (b) 1980 continued

No	GC	RG	P	pH	T	No	GC	RG	P	pH	T
17	E	2	34	6.2	Zyl	33	A	2	30	7.7	VFSL
18	D	1	30	7.7	Zyl	34	D	3	80	6.0	LS
19	A	1	28	7.9	Zyl	35	D	3	66	7.3	VFSL
20	B	1	26	8.0	L	36	D	2	96	6.5	VFSL
21	E	1	50	8.0	Zyl	37	B	2	22	5.7	FSL
22	E	1	32	6.5	Zyl	38	D	3	54	5.5	FSL
23	E	2	28	5.7	Zyl	39	C	1	23	7.6	VFSL
24	E	2	33	7.1	Zyl	40	B	2	41	7.6	VFSL
25	E	1	30	7.7	L	41	A	6	28	5.7	VFSL
26	D	2	18	6.6	FSL	42	B	4	41	6.4	VFSL
27	A	3	86	4.6	FSL	43	B	3	83	6.5	CL
28	B	1	45	7.6	FSL	44	B	5	80	5.4	LFS
29	A	4	134	6.6	VFSL	45	E	3	94	5.2	LS
30	A	4	28	6.6	Zyl	46	C	1	9	5.6	L
31	C	3	42	6.3	VFSL	47	B	2	32	7.8	FSL
32	B	2	33	7.3	VFSL	48	B	1	29	7.4	FSL

(c) Soils tested in 1981 (62)

1	B	3	43	6.4	VFSL	7	B	1	9	5.5	FSL
2	A	2	24	7.3	FSL	8	C	1	17	4.4	FSL
3	C	1	8	5.5	VFSL	9	B	2	45	7.8	FSL
4	B	1	9	5.6	VFSL	10	A	3	14	6.8	FSL
5	C	1	13	6.8	FSL	11	E	1	156	7.3	L
6	C	2	14	7.3	FSL	12	A	2	9	6.8	VFSL

continued over

Table 4.39. (c) 1981 continued

No	GC	RG	P	pH	T	No	GC	RG	P	pH	T
13	A	2	15	7.0	VFSL	38	E	1	43	6.7	VFSL
14	A	1	21	7.6	VFSL	39	C	2	43	7.2	VFSL
15	A	2	27	7.5	VFSL	40	B	4	72	5.9	VFSL
16	A	4	25	7.5	VFSL	41	B	3	100	7.1	VFSL
17	E	1	21	6.5	VFSL	42	B	3	100	6.6	VFSL
18	C	1	15	7.2	VFSL	43	A	6	64	6.0	VFSL
19	B	1	6	7.7	VFSL	44	B	1	32	7.2	FSL
20	D	2	39	6.7	VFSL	45	A	2	30	7.3	VFSL
21	C	2	31	7.1	VFSL	46	A	1	20	7.5	VFSL
22	C	2	14	7.4	VFSL	47	B	4	79	7.3	VFSL
23	D	2	21	6.5	VFSL	48	C	3	79	7.5	VFSL
24	D	2	23	7.1	VFSL	49	B	4	28	6.5	VFSL
25	C	2	11	7.1	VFSL	50	B	1	8	4.5	VFSL
26	D	2	50	6.2	VFSL	51	B	1	5	5.2	VFSL
27	D	2	69	7.2	VFSL	52	B	2	20	5.0	VFSL
28	B	2	17	6.1	VFSL	53	B	1	6	6.2	VFSL
29	B	2	11	6.1	VFSL	54	B	4	49	6.0	FSL
30	A	2	10	7.5	VFSL	55	C	2	115	7.1	L
31	A	1	27	7.1	VFSL	56	B	1	15	7.1	FSL
32	B	1	19	7.9	VFSL	57	C	3	117	4.1	FSL
33	B	3	45	6.9	VFSL	58	B	3	90	6.5	VFSL
34	B	2	38	6.5	VFSL	59	B	3	82	6.0	VFSL
35	B	3	48	7.5	VFSL	60	C	1	59	7.5	FSL
36	B	1	9	6.8	L	61	A	2	31	7.8	SL
37	A	2	9	6.6	L	62	B	5	89	6.5	FSL

Table 4.40. Abundance (p/g) or incidence (+) of Pythium spp.

(a) Soils assayed after the ADAS tests, 1979 (25)

No	Total	syl	int	het	x	irr	others
8	1200	960			+		
10	0	0					
14	1100	220		+	+		
15	1000	300			+		
16	1100	440	+	+	+		
17	300	150			+		
18	800	460			+	+	
20	950	270			+	+	
21	750	180			+		+
24	900	600				+	+
25	500	120			+		
26	400	80		+	+		
27	950	850			+		
30	1100	600		+	+		
40	1200	600			+		
41	900	900					
43	270	50		+			+
46	350	140		+	+		
47	600	180		+			+
48	500	280		+			+
49	50	0	+	+	+		
50	500	0		+	+		
55	300	180			+		
56	1500	750			+		+
104	700	700					

continued over

Table 4.40. continued.

(b) Soils assayed after the ADAS tests, 1980 (48)

No	Total	syl	int	het	x	irr	others
1	450	360	0	0	90	0	0
2	1050	350	0	350	350	0	0
3	1300	520	0	0	780	0	0
4	920	320	0	230	370	0	0
5	190	80	0	0	110	0	0
6	500	0	0	300	200	0	0
7	620	0	0	0	0	620	0
8	400	400	0	0	0	0	0
9	800	160	0	0	640	0	0
10	450	450	0	0	0	0	0
11	600	0	0	75	450	75	0
12	1100	0	0	360	740	0	0
13	1500	1500	0	0	0	0	0
14	760	490	0	0	0	0	270
15	900	0	0	720	0	0	180
16	400	200	0	0	200	0	0
17	750	190	0	0	560	0	0
18	900	0	0	200	700	0	0
19	1500	0	0	190	1120	190	0
20	1400	0	0	0	1200	0	200
21	1600	0	0	1280	320	0	0
22	1300	780	0	0	520	0	0
23	440	90	0	0	350	0	0
24	1600	200	0	1000	400	0	0

continued over

Table 4.40. (b) continued

No	Total	syl	int	het	x	irr	others
25	600	0	0	340	260	0	0
26	1750	1570	0	0	180	0	0
27	0	0	0	0	0	0	0
28	1600	1000	0	600	0	0	0
29	600	150	0	0	300	0	150
30	980	490	0	0	490	0	0
31	1100	440	0	0	660	0	0
32	1000	1000	0	0	0	0	0
33	880	0	0	440	440	0	0
34	620	620	0	0	0	0	0
35	400	0	0	0	340	0	60
36	750	600	0	0	150	0	0
37	700	0	0	0	0	700	0
38	650	650	0	0	0	0	0
39	660	0	110	330	220	0	0
40	730	150	0	580	0	0	0
41	760	680	0	0	80	0	0
42	600	360	0	120	120	0	0
43	1070	960	0	0	110	0	0
44	340	340	0	0	0	0	0
45	350	280	0	0	0	0	70
46	1200	960	0	0	240	0	0
47	2300	0	575	1150	575	0	0
48	850	0	0	550	300	0	0

continued over

Table 4.40. continued

(c) Soils assayed after the ADAS tests, 1981 (22)

No	Total	syl	int	het	x	irr	others
1	850	425			+		
9	200	0		+	+		
11	1930	0		+	+		
26	1370	550			+		
27	1350	0			+		
33	830	330		+			
35	960	600		+	+		
38	440	0			+		
39	600	600					
40	1440	0		+	+		
41	1350	140			+		
42	710	140	+	+	+		
43	780	390			+		+
47	540	50	+	+	+		
48	800	0	+	+	+		
54	400	360					+
55	1040	0		+	+		
57	750	80	+		+		
58	630	250	+		+		
59	1760	350			+		
60	1500	150		+	+		
62	740	370		+			

continued over

Table 4.40. continued.

(d) Soils assayed before the ADAS tests, 1981 (62)

No	Total	syl	int	het	x	irr	others
1	500	275	0	0	225	0	0
2	315	0	0	65	250	0	0
3	485	0	0	0	485	0	0
4	1120	0	0	110	1010	0	0
5	260	105	0	0	130	0	25
6	260	80	0	0	155	0	25
7	485	0	120	0	365	0	0
8	120	40	0	0	80	0	0
9	200	0	0	120	80	0	0
10	120	40	0	40	40	0	0
11	1750	0	0	175	1225	0	350
12	235	39	78	39	78	0	0
13	700	120	0	60	520	0	0
14	820	0	330	410	40	40	0
15	680	0	0	136	408	0	136
16	1000	0	0	333	333	0	333
17	500	0	0	0	250	250	0
18	515	0	0	412	0	0	103
19	1040	0	0	375	250	0	415
20	520	0	0	62	333	0	125

continued over

Table 4.40. (d) continued

No	Total	syl	int	het	x	irr	others
21	390	0	0	0	78	0	312
22	1000	0	0	0	690	90	220
23	900	0	0	0	450	450	0
24	925	0	0	0	460	200	265
25	350	0	0	70	123	0	157
26	950	48	0	0	807	0	95
27	125	0	0	0	125	0	0
28	390	0	0	0	390	0	0
29	215	0	0	0	215	0	0
30	725	0	0	507	218	0	0
31	120	0	0	40	80	0	0
32	1170	0	175	350	470	175	0
33	175	0	0	175	0	0	0
34	165	0	0	40	125	0	0
35	875	0	0	613	175	0	87
36	665	133	0	0	466	0	66
37	570	170	0	0	400	0	0
38	435	0	0	0	435	0	0
39	790	0	0	80	80	630	0
40	580	0	0	116	464	0	0
41	665	0	0	133	522	0	0

continued over

Table 4.40. (d) continued

No	Total	syl	int	het	x	irr	others
42	360	0	60	120	180	0	0
43	125	0	0	0	80	0	45
44	750	0	0	85	500	0	165
45	745	0	150	595	0	0	0
46	470	0	50	160	210	0	50
47	165	41	0	41	41	0	41
48	205	0	41	123	41	0	0
49	740	74	0	0	666	0	0
50	210	105	0	0	105	0	0
51	410	90	0	0	320	0	0
52	560	224	0	0	280	0	56
53	420	252	0	0	126	0	42
54	520	0	0	0	0	260	260
55	880	0	528	116	236	0	0
56	730	0	0	657	73	0	0
57	205	0	123	0	82	0	0
58	605	121	60	0	424	0	0
59	400	0	0	0	400	0	0
60	610	61	0	244	305	0	0
61	620	0	0	124	330	0	166
62	565	226	0	56	283	0	0

5.	<u>Effects of apple orchard soils on the growth (in vitro) of <i>P. sylvaticum</i> and other species.</u>	
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5.1. Introduction

Fumigation responses in apple orchard soils showed that not all soils were affected by apple replant disease and widely varying results were obtained after chloropicrin fumigation of soils with a long history of apple growth (Hoestra, 1968; Upstone, 1974). Some of this variation was associated with an artifact of the apple seedling test (soil phosphorus and absence of VA mycorrhiza) and some was associated with soil pH (Section 4). Results from the soil population studies indicated that Pythium sylvaticum was implicated in the disease but that the host-pathogen relationship was not a simple one, and was probably affected by unknown (possibly microbial) soil factors. The studies in this section therefore were undertaken to determine whether soils differed in their fungistatic effects on the mycelial growth of Pythium spp.

5.2. Materials and methods

Soils from the ADAS tests in 1980 (42) and 1981 (12 high phosphorus soils) were selected for study of their fungistatic effects on the growth of P. sylvaticum and other Pythium species. All 54 soils were collected after the ADAS apple seedling tests.

Strips (10 mm wide) of glass-fibre filter paper (Whatman GF/D) were placed across the bases of plastic petri-dishes (9 cm diam), with 3 cm protruding.

Sieved (1 mm) soil samples (40 g/dish) were spread evenly in the dishes, over the filter paper strips, and uniformly compacted with a 500 g balance weight.

The soil plates were placed on small plastic dishes (5 cm diam, 2 cm deep) above bench level. The protruding filter paper strips were then directed into petri-dishes at bench level containing distilled water. During 24 H the soils wetted-up, approximately to field capacity (1 cm suction). Boiled cellophane discs (85 mm diam) were then placed over the wet soil surfaces.

Two sterile, rectangular, glass plates (8 x 12 cm), separated by two glass microscopic slides (1 mm thick) were placed horizontally. The cavity between the plates was filled (by pipette) with molten plain agar (2% PWA). When the agar had solidified, the glass plates were separated leaving the uniform, 1 mm thick, agar layer on one of the plates. The agar was then cut into rectangular strips (60 x 7 mm). Five agar strips were placed separately on the cellophane, covering each soil plate. For controls, soil was replaced by

2% PWA (30 ml/plate). The soil plates were then incubated overnight at approximately 22° C.

After incubation, each agar strip was inoculated at one end, with a disc (4 mm diam) from a 7-day old, PWA plate culture of Pythium. The soil plates were then incubated, at approximately 22° C for 48 h or more when mycelial growth on the agar strips was measured under the microscope.

5.3. Results

Detailed records of mycelial growth are tabulated in Ss 5.5., where soils are separated into the sets in which they were studied. Fungistasis is expressed as the percentage inhibition of mycelial growth in agar strips on soils compared with growth in agar strips on PWA. The summarised results presented in this Section were derived from the detailed data presented in Tables 4.39., 4.40., 5.17., 5.18.

5.3.1. General observations on soil fungistatic effects on the growth and incidence of *P. sylvaticum*.

The mycelial growth of *P. sylvaticum* on PWA slabs on orchard soils was inhibited by differing amounts from 0–100%. Half of the soils tested caused more than 40% growth inhibition (Table 5.1.) *P. sylvaticum* was present in 35 of the 54 soils tested. Strong fungistasis (> 40% growth inhibition) was found in a greater proportion of soils without than with *P. sylvaticum*.

The incidence in orchard soils of *P. sylvaticum* was very highly significantly associated with levels of soil fungistasis (Table 5.2.): this fungus was present in 93% of soils with low inhibitory properties ($\leq$ 40% inhibition), but in only 37% of soils with high inhibitory properties (> 40%).

When *P. sylvaticum* was present in orchard soils the strength of fungistatic effects was not significantly associated with mean population sizes: *Pythium* spp., other than *P. sylvaticum*, however, occurred in far greater abundance in soils strongly inhibitory to *P. sylvaticum* (Table 5.3.)

5.3.2. Fungistatic effects on *P. sylvaticum* in relation to soil fumigation response groups.

Soils that had given small growth responses to fumigation were mostly (86%) highly inhibitory to the growth of *P. sylvaticum*. Soils that had given large growth responses to fumigation were mostly (92%) not strongly inhibitory to the

growth of P. sylvaticum. This inverse association was very highly significant (Table 5.4.)

The quantitative data on the incidence of fungistasis was supported by qualitative data on the strength of fungistasis. Low response soils (RG 1-2) resulted, on average, in 52% inhibition of mycelial growth. High response soils (RG 3-5) resulted, on average, in 21% inhibition of mycelial growth (Table 5.5.)

5.3.3. Fungistatic effects on P. sylvaticum in relation to growth classes.

Poor apple seedling growth (ABC) soils tended to be less inhibitory to the growth of P. sylvaticum than good growth (DE) soils, but the association was not significant in a 2 x 2 contingency test (Table 5.6.) Qualitatively the mean strength of fungistasis was significantly greater in good growth soils (Table 5.7.)

Earlier results (Ss 4.5.3.) had shown a drastic diminution of P. sylvaticum populations in best growth (E) soils that could not be associated with soil features. Within the smaller number of soils included in these fungistasis tests this population effect was also present (Table 5.8.) The fungistasis results did not show a dramatic increase in the strength of fungistasis (although it was greatest) in class E soils (Table 5.7.), but they did show a sharp increase in the frequency of occurrence of highly inhibitory soils (Table 5.6.)

5.3.4. Fungistasis and soil phosphorus content.

Strongly inhibitory soils (to P. sylvaticum) were associated more frequently than expected with low soil phosphorus content. The association just failed to reach significance when soils were divided at the 40 ppm P level, but was highly significant at the 60 ppm P level (Table 5.9.)

In soils containing 0-59 ppm P the mean strength of fungistasis was almost double that in soils containing greater amounts of phosphorus (Table 5.10.)

5.3.5. Fungistasis and soil pH.

The strength of fungistasis (to P. sylvaticum) did not vary significantly in soils over the range pH 4.1 - 7.0. In alkaline soils (pH > 7.0) the inhibition of mycelial growth increased sharply in both mean strength and incidence. Differences between alkaline and acid soils were very highly significant (Tables 5.11., 5.12.)

5.3.6. Fungistasis and soil texture.

The strength of soil fungistasis was not associated with any particular texture classes, despite a tendency for increasing 'heaviness' of texture to be associated with increasing soil pH: the maximum mean pH of any texture class, however, was only 7.1 (Table 5.13.) A 3 x 2 contingency test of the association between the incidence of weakly and strongly fungistatic soils and light (LS-FSL), medium (VFSL) and heavy (Zyl-CL) soil was not significant.

5.3.7. Comparison of soil fungistatic effects on *P. sylvaticum*, *P. intermedium*, *P. heterothallicum* and *P.x*.

On overall average the growth of *P. sylvaticum* was less inhibited than that of *P. intermedium*, *P. heterothallicum* and two isolates of *P.x* (types A and B). On overall average, the 11 soils tested occurred in approximately the same order of inhibitory strength, as they did to *P. sylvaticum* alone. One soil (no.34), however, had very marked differential effects on the isolates (Table 5.14.)

In general, *P. sylvaticum*, *P. intermedium* and the two isolates of *P.x* were most strongly inhibited by a similar range of soils (Table 5.15.) *P. heterothallicum* differed markedly from these species and this result indicated qualitative differences in soil fungistatic factors which may differentially influence the activities of *Pythium* spp.

Comparison of the results for *P. sylvaticum* from this set of experiments (Table 5.18.) with those from experiments in table 5.17. demonstrated the reproducibility of fungistasis tests: ten of the soils remained in the same inhibition classes; one soil (no.34) moved from the low to the high class by virtue of a change from 37 to 43% inhibition.

Table 5.1. The distribution of fungistatic effects (% inhibition) on P. sylvaticum and the incidence of P. sylvaticum in 54 orchard soils.

Inhibition (%)	No Soils	<u>P. sylvaticum</u>	
		+	—
0 — 20	13	12	1
21 — 40	14	13	1
41 — 60	20	6	14
61 — 80	4	2	2
81 — 100	3	2	1
Sum	54	35	19

Table 5.2. Contingency table of observed and expected (in parenthesis) incidence of P. sylvaticum in 54 soils in relation to soil fungistasis.

P. sylvaticum	<u>Growth Inhibition (%)*</u>		Sum
	0 — 40	41 — 100	
+	25 (17.5)	10 (17.5)	35
—	2 (9.5)	17 (9.5)	19
Sum	27	27	54
$\chi^2 = 18.27.$ $P < 0.001$ at 10.83.			

* P. sylvaticum

Table 5.3. Abundance (Mean p/g $\pm$ S.E.) of Pythium spp. in orchard soils in relation to soil fungistasis.

Inhibition (%)	<u>P. sylvaticum</u>		<u>Other Pythium spp. *</u>	
	No Soils	p/g	No Soils	p/g
0 — 40	25	438 $\pm$ 66	27	373 $\pm$ 66
41 — 100	10	545 $\pm$ 154	27	897 $\pm$ 107

* t = 4.17 (52 Df)

P < 0.001 at 3.5.

Table 5.4. Contingency table of observed and expected (in parenthesis) growth inhibition of P. sylvaticum in 54 soils in relation to apple seedling growth responses to soil fumigation.

Response Group	Inhibition (%) of mycelial growth		
	0 – 40	41 – 100	Sum
1 – 2 (< 50%)	4 (14.5)	25 (14.5)	29
3 – 5 (≥ 50%)	23 (12.5)	2 (12.5)	25
	27	27	54
$\chi^2 = 32.85$	$P < 0.001$ at 10.83.		

Table 5.5. Mean inhibition (% $\pm$ S.E.) of mycelial growth of P. sylvaticum on 54 soils in relation to fumigation response groups.

	Response group*				
	1	2	3	4	5
No Soils	12	17	12	8	5
Inhibition (%)	51.2	53.3	28.1	24.9	6.6
S.E.	5.7	3.9	4.4	2.8	3.2

* RG 1 – 2 vs 3 – 5 : $t = 6.76$ (52 Df) $P < 0.001$ at 3.5.

Table 5.6. The incidence of soils with low and high fungistatic properties (% inhibition) in five growth classes (A - E).

Inhibition (%)	Growth class				
	A	B	C	D	E
(a) 0 — 40	3	12	5	6	1
(b) 41 — 100	4	9	2	4	8
Sum	7	21	7	10	9
% (b) in GC	57	43	29	40	89
	43			63	

Table 5.7. Mean inhibition (% $\pm$ S.E.) of mycelial growth of P. sylvaticum in 54 soils in relation to growth class (A - E).

	Growth class*				
	A	B	C	D	E
No Soils	7	21	7	10	9
Inhibition (%)	37.0	33.5	31.9	43.4	52.2
S.E.	8.6	4.2	11.2	7.4	4.8

* ABC vs DE : $t = 2.29$ (52 D f) $P < 0.05$ at 2.0

Table 5.8. Abundance (mean p/g) of P. sylvaticum in relation to growth classes of soils included in fungistasis tests.

	Growth class				
	A	B	C	D	E
No Soils	4	15	4	8	4
Mean p/g	428	404	420	761	218
S.E.		84		174	

Table 5.9. Contingency tables of the observed and expected (in parenthesis) growth inhibition of P. sylvaticum on 54 soils in relation to soil phosphorus content.

Growth inhib (%)	No Soils	P (ppm)		P (ppm)	
		0 – 39	≥ 4.0	0 – 59	≥ 60
0 – 40	27	8 (11.5)	19 (15.5)	12 (17.5)	15 (9.5)
41 – 100	27	15 (11.5)	12 (15.5)	23 (17.5)	4 (9.5)
Sum		23	31	35	19
χ^2		3.71 NS		9.83	P < 0.001

Table 5.10. Mean inhibition (% $\pm$ S.E.) of mycelial growth of P. sylvaticum on 54 soils in relation to soil phosphorus content.

	P (ppm)*		
	0 – 39	40 – 59	60
No Soils	23	12	19
Inhibition (%)	43.3	49.6	25.1
S.E.	4.2	6.9	3.9

* 0 – 59 vs ≥ 60 ppm : t = 3.58 (52 Df) P < 0.001 at 3.50

Table 5.11. Mean inhibition (% $\pm$ S.E.) of mycelial growth of P. sylvaticum on 54 soils in relation to soil pH.

pH	No soils	Inhibition (%)	
		Mean	S.E.
4.1 — 5.5	6	27.5	6.4
5.6 — 6.0	7	26.3	7.5
6.1 — 6.5	11	30.3	8.0
6.6 — 7.0	5	33.8	7.8
7.1 — 7.5	14	47.4	5.3
7.5	11	52.3	4.6

pH 4.1 — 7.0 vs > 7.0 : $t = 3.80$ (52 Df) $P < 0.001$

Table 5.12. Contingency tables of observed and expected (in parenthesis) growth inhibition of P. sylvaticum in relation to soil pH.

Soil pH	Inhibition		
	0 — 40	41 — 100	Sum
≤ 7.0	23 (14.5)	6 (14.5)	29
> 7.0	4 (12.5)	21 (12.5)	25
(Sum)	27	27	54
$\chi^2 = 21.53$ $P < 0.001$ at 10.8			

Table 5.13. Mean inhibition (% $\pm$ S.E.) of mycelial growth of P. sylvaticum on 54 soils in relation to soil Texture.

Soil Texture*	No soils	Inhibition (%)		Mean pH
		Mean	S.E.	
LS	2	45.0		5.6
LFS	3	27.3		6.4
SL	1	(43.0)		(7.2)
FSL	13	42.3	6.7	6.5
VFSL	19	36.2	6.1	6.8
Zyl	10	40.1	5.0	6.9
L	5	44.4	4.9	7.1
CL	1	(14.0)		(6.5)

* Soils arranged in order of increasing 'heaviness' (See Ss 4.8. for explanation of symbols).

Table 5.14. The inhibition (%) of mycelial growth of P. sylvaticum,
P. intermedium, P. heterothallicum and two isolates of P.x by eleven
orchard soils.

Soil No*	Pythium spp.					Mean
	syl	int	het	xA	xB	
9	12	33	53	44	46	37.6
30	14	37	67	37	28	36.6
5	16	29	20	26	33	24.8
10	20	37	70	33	20	36.0
13	28	28	53	32	38	35.8
41	29	51	54	50	31	43.0
34	43	53	100	91	74	72.2
45	45	56	28	55	42	45.2
15	52	57	24	80	67	56.0
24	55	56	47	80	46	56.8
37	64	83	90	100	100	87.4
Mean	34.4	47.3	55.1	57.1	47.7	

* ADAS tests, 1980. Soils arranged in order of increasing inhibition of
P. sylvaticum.

Table 5.15. As Table 5.14., with weak (unmarked), intermediate (+), and strong (++) inhibition indicated for 12-42, 43-71, and 72-100% inhibition respectively.

Soil no	Pythium spp.				
	syl	int	het	X 1	X 2
9			+	+	+
30			+		
5					
10			+		
13			+		
41		+	+	+	
34	+	+	++	++	++
45	+	+		+	
15	+	+		++	+
24	+	+	+	++	+
37	+	++	++	++	++

5.4. Discussion of Section 5

Strongly fungistatic soils were significantly associated with a greatly diminished incidence of P. sylvaticum, small responses to fumigation, and good growth of apple seedlings in unfumigated soils. The incidence of strong fungistasis was greatest in 'best apple growth' soils, and in soils of pH > 7.0, in which ARD occurred infrequently (as reflected by fumigation responses). These associations all add considerably to the evidence for a causal role of P. sylvaticum in ARD in England.

Although a smaller number of soils (54) were included in the fungistasis tests they appeared to be representative of the larger sample included in Pythium population studies (Sect 4). Within these 54 soils (as in those in Sect 4), P. sylvaticum populations were greatly diminished in growth class E soils (Table 5.8.), and similar, very highly significant associations existed between fumigation response groups and soil pH and phosphorus content (Table 5.16.) There were therefore several interacting associations (if not links) and these are summarised diagrammatically in Fig. 5. 1. The relative strengths of the associations shown are indicated by X^2 values all derived similarly from 2 x 2 contingency tables of results from the 54 soils. In order, they indicate response group (and presumably ARD) to be strongly associated with soil fungistasis, soil pH and soil phosphorus. Soil phosphorus may be unimportant in practice because all trees planted in fumigated soils are infected by VA mycorrhiza in the nursery. However, the association between weak fungistasis (large responses) and high levels of soil P may be an influential factor in the field. The association between soil pH and fungistasis was very strong and it is possible that this is reflected by the association between pH and response group.

These studies also indicated that fungistatic factors may differentially affect the activity of Pythium spp. Soil fungistasis is generally considered to be of biological origin (Watson & Ford, 1972). While Pythium spp. have been reported to be particularly sensitive to microbial antagonism (Barton, 1958, Mitchell & Hurwitz, 1965, Vaartaja & Agnihotri, 1969), differing levels of tolerance to a range of antibiotics by Pythium spp. was demonstrated by Vaartaja & Agnihotri (1969). The present results overall suggest that P. heterothallicum is not implicated in ARD, but the differing fungistatic reactions shown by this species point to the possibility that P. spp. other than P. sylvaticum may be implicated in ARD

Table 5.16. Contingency tables showing observed and expected (in parenthesis) incidence of 54 soils included in fungistasis tests in relation to response group, soil pH, and soil phosphorus content.

Response group	Sum	pH		P (ppm)	
		≤ 7.0	> 7.0	0 - 39	≥ 40
1 — 2	29	8 (15.6)	21 (13.4)	19 (12.4)	10 (16.6)
3 — 5	25	21 (13.4)	4 (11.6)	4 (10.6)	21 (14.4)
Sum	54	29	25	23	31
χ^2 (P < 0.001 at 10.8)		17.3		13.3	

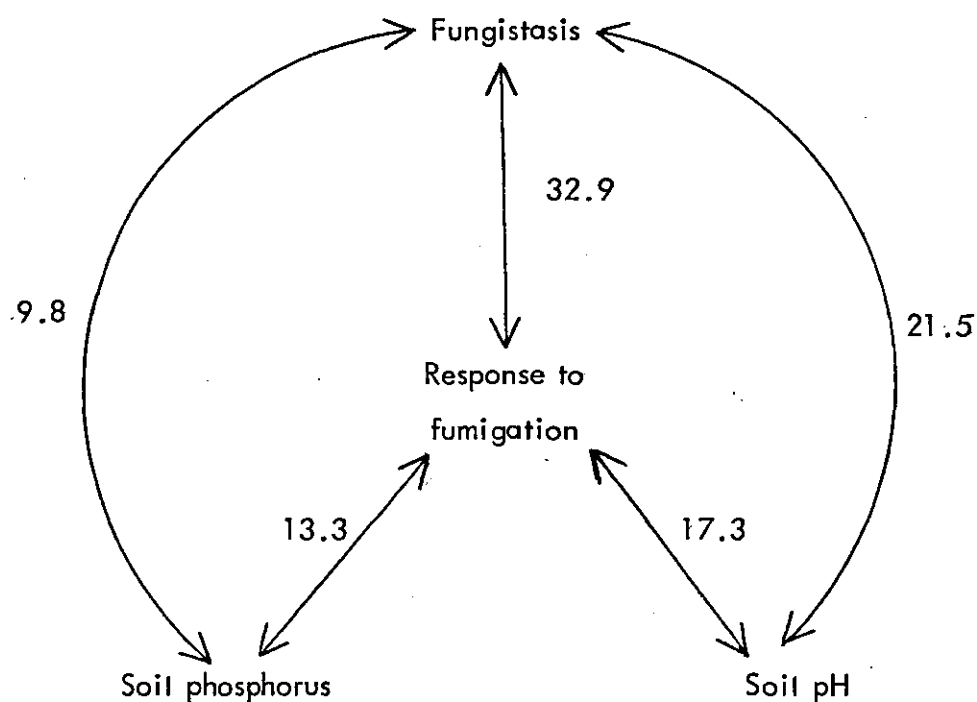


Fig 5.1 Significant associations between apple replant disease (response to fumigation) and soil factors (numbers are χ^2 values).

in certain ecological situations.

While the presence or absence of P. sylvaticum in orchard soils was strongly associated with fungistasis, population sizes of this species were not. For Pythium spp., other than P. sylvaticum, populations were significantly larger in strongly fungistatic soils. This result is of great interest and worth further investigation. Colonies on soil dilution plates mostly originate from spores (pers obsn), and it is possible that in inhibitory soils sporulation is induced (at the cost of mycelial activity) and that spores, inhibited from germinating, are more persistent. These effects would result in higher spore counts. Alternatively, populations and activities of antagonistic micro-organisms may be directly related to the size of the Pythium population.

A further unresolved feature of the results was that while population size of P. sylvaticum was not associated with strength of fungistasis, it was greatly diminished in growth class E (best growth) soils. This may indicate inhibitory factors in certain soils that were not demonstrated by the method used : alternatively the slightly greater fungistasis levels in E than in D soils (Table 5.7.) may be critical.

5.5. Tabulations of experimental results

Table 5.17. Mycelial growth (mm) of P. sylvaticum (no 483) in PWA strips placed on orchard soils or on PWA (controls).

(Soils in Sets 1-5 and 6-8 respectively were from ADAS tests in 1980 and 1981. Incubation period 48-52 H).

Set	Soil no	Mycelial growth (mm)			S.E.	Inhibition %
		Min	Max	Mean		
1	7	12	25	17.8	2.4	43
	8	21	28	24.0	1.2	23
	10	25	33	29.6	1.3	5
	12	18	24	22.0	1.1	30
	37	7	15	11.6	1.4	63
	40	15	23	18.2	1.5	42
	41	32	35	33.6	0.6	0
	43	26	29	27.0	0.6	14
	44	23	29	26.2	1.4	16
	47	10	22	17.2	2.1	45
	PWA	29	33	31.2	0.7	
2	13	25	29	27.4	0.8	24
	14	28	32	30.0	0.7	17
	15	16	22	19.0	1.1	48
	16	14	29	20.6	3.3	43
	18	18	23	20.4	0.9	44
	19	14	24	19.2	1.6	47
	20	15	22	17.6	1.5	51
	21	11	22	17.2	2.1	53
	24	7	16	11.4	1.8	69
	25	16	25	19.0	1.6	48
	PWA	35	37	36.2	0.4	

Continued.

Table 5.17 Continued

Set	Soil no	Mycelial growth (mm)				Inhibition %
		Min	Max	Mean	S.E.	
3	1	20	27	22.6	1.2	27
	2	15	20	17.2	0.9	44
	3	20	21	20.2	0.2	34
	4	24	26	25.0	0.4	19
	6	9	14	11.2	0.8	64
	17	18	22	20.4	0.8	34
	33	11	15	13.0	0.6	58
	38	18	20	18.4	0.4	40
	39	0	8	3.6	1.5	88
	PWA	30	31	30.8	0.2	
4	9	25	29	26.5	0.9	22
	26	12	15	14.2	0.6	59
	29	23	25	24.2	0.8	29
	30	26	29	27.8	0.6	18
	32	12	15	14.0	0.6	59
	34	19	23	21.6	0.8	37
	45	15	18	16.0	0.6	53
	48	18	21	19.5	0.6	43
	PWA	30	38	34.0	1.3	
5	5	28	29	28.4	0.2	16
	11	15	19	17.0	0.6	50
	28	8	15	10.4	1.2	69
	42	21	25	23.8	0.7	30
	46	24	25	24.8	0.2	27
	PWA	33	35	33.8	0.4	

Continued.

Table 5.17 Continued

Set	Soil no	Mycelial growth (mm)				Inhibition %
		Min	Max	Mean	S.E.	
6	11	23	30	26.2	1.2	41
	26	0	0	0	0	100
	55	15	27	20.0	2.3	55
	58	30	34	30.8	0.8	30
	60	5	14	8.2	2.0	81
	PWA	42	45	44.0	0.6	
7	27	20	28	23.4	1.4	50
	43	40	43	40.6	0.6	12
	47	28	29	28.2	0.2	39
	57	35	42	39.6	1.2	15
	PWA	45	48	46.4	0.5	
8	41	26	34	29.4	1.6	19
	48	35	36	35.6	0.2	2
	62	31	45	37.8	2.4	0
	PWA	35	37	36.2	0.4	

Table 5.18. Mycelial growth (mm) of *P. sylvaticum*, *P. intermedium*, *P. heterothallicum* and two isolates of *P.x* in PWA strips placed on orchard soils or on PWA (controls).

(All soils were from ADAS 1980 tests. Differing periods of incubation were used between experiment sets.

Incub period	Soil no	<i>P. sylvaticum</i>			<i>P. intermedium</i>		
		Mean	S.E	Inhib (%)	Mean	S.E	Inhib (%)
48 H	5	26.6	0.8	16	22.6	1.6	29
	15	16.0	1.5	52	13.8	1.2	57
	45	18.2	0.7	45	14.0	0.6	56
	PWA	33.0	0.0		31.8	0.8	
48 H	10	24.0	0.8	20	19.8	0.4	37
	24	13.4	0.5	55	14.0	0.7	56
	34	17.2	0.5	43	14.8	0.9	53
	PWA	30.0	0.3		31.6	0.5	
56 H	37	15.8	1.0	64	6.6	1.7	83
	41	30.6	0.7	29	19.0	0.3	51
	PWA	43.2	0.6		38.4	1.0	
60 H	9	51.0	0.3	12	37.0	0.9	33
	13	42.0	1.0	28	39.8	3.4	28
	30	49.8	0.2	14	34.6	0.9	37
	PWA	58.0	0.0		55.0	0.3	

Continued

Table 5.18. Continued

Incub period	Soil no	P.x (type A)			P.x (type B)		
		Mean	S.E	Inhib (%)	Mean	S.E	Inhib (%)
48 H	5	16.2	0.6	26	16.8	1.0	33
	15	4.4	0.4	80	8.4	0.5	67
	45	9.8	0.6	55	14.6	0.8	42
	PWA	21.8	0.2		25.2	0.5	
48 H	10	14.4	0.2	33	21.0	1.0	20
	24	4.2	0.5	80	14.2	1.7	46
	34	2.0	1.2	91	6.8	0.7	74
	PWA	21.6	0.4		26.2	0.2	
60 H	9	22.8	1.6	44	23.2	1.7	46
	13	27.4	1.6	32	26.4	1.2	38
	30	25.8	1.4	37	30.8	1.2	28
	PWA	40.8	0.7		42.6	0.4	
4d	37	0.0	0.0	100	0.0	0.0	100
	41	26.0	0.4	50	38.2	3.0	31
	PWA	52.0	0.3		55.6	0.4	

Continued

Table 5.18. Continued

Incub period	Soil no	<i>P. heterothallicum</i>		
		Mean	S.E	Inhib (%)
48 H	5	11.4	0.6	20
	15	10.8	0.2	24
	45	10.2	0.2	28
	PWA	14.2	0.2	
48 H	10	6.4	0.4	70
	24	11.4	0.7	47
	34	0.0	0.0	100
	PWA	21.4	0.4	
60 H	9	13.4	0.9	53
	13	13.4	0.5	53
	30	9.2	0.4	67
	PWA	28.2	0.6	
4 d	37	3.6	2.2	90
	41	16.0	1.6	54
	PWA	34.4	0.7	

6.	<u>Seasonal variations in the isolation of <i>Pythium</i> spp. from an apple orchard soil.</u>	
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6.1. Introduction

Few data are available on the seasonal activity in soil of Pythium spp. (Literature review). These experiments, therefore, were designed to study possible changes in the abundance of Pythium propagules and the activity of Pythium species in situ in a restricted area of orchard soil.

6.2. Materials and methods

The soil area used for these studies was confined to one row of apple trees (Cox's Orange Pippin on M9 root-stocks), severely dwarfed by replant disease, on a Research Station plot (PR 117). The soil was sampled every month from May 1980 until April 1982 inclusive, by two assay methods.

Pythium spp., were assayed by the soil dilution plate method using MPV medium as described in Section 1. Soil samples were collected from 0 - 6 cm depth at the mid-point between the apple trees (spacing in row 3 m), and assayed within one day of collection.

The slide-trap method employed in this study was basically the same as that described by Sewell (1956) for the isolation of actively growing soil fungi. However, the selective medium MPV was introduced into the traps to restrict isolations to Pythium species.

The slide-trap consisted of a shallow agar-filled chamber covered by a glass microscope slide. A 'Perspex' strip (76 x 25 mm, 2.4 mm thick) formed the base of the chamber. Narrow lengths (3.2 x 2.4 mm cross-section) of the same material formed the four side walls and the chamber was divided into two unequal parts (one third and two thirds) by a cross piece. Chloroform was used to fuse the 'Perspex' surfaces.

The chambers were stored in alcohol and sterilized by flaming when required. Melted MPV agar was introduced into the larger section of the chamber by means of a sterilized pipette. When the agar had solidified, the chamber was covered by a sterilized glass microscope slide, which was firmly fixed in position by rubber bands.

Not less than twelve slide-traps were partially buried in soil at the selected site on about the 15th day of every month. Slot-shaped holes to receive the slide-traps were prepared with a square-ended chisel blade. Slide-traps were pressed vertically into the prepared holes to such a depth that the larger

agar-containing portion of the chamber was completely buried. The traps were buried each month in a group (30 x 10 cm) and protected from rain by an inverted plastic tray. Sample traps were examined after two or more days for fungal colonisation. When fungal growth was detected, all slide-traps were returned to the laboratory, examined under the microscope, and colonies marked. The individual colonies were transferred aseptically to fresh MPV agar strips for cleaning and then subcultured for identification.

6.3. Results

Monthly rainfall and soil temperature data (collected at the EMRS meteorological plot) for the period of these studies are given in Table 6.1. Detailed results of Pythium spp. isolated in each month by the soil dilution plate and slide-trap methods respectively are given in Tables 6.2. and 6.3. : these results are summarised in Tables 6.4 - 6.7.

6.3.1. Pythium spp. isolated by the soil dilution plate method

Pythium spp. were isolated from each of the 24 monthly samples. Total populations in these samples varied from 270 to 1100 p/g and averaged 644 p/g (Table 6.2). Outstandingly, the dominant species in this soil were Pythium x and P. ultimum. P. heterothallicum was moderately abundant, and P. sylvaticum was sparsely represented. Among 'other spp.' isolated were P. acanthophoron, P. vanterpoolii (isolated only from October samples), P. rostratum and two unidentified species (nos 1, 442).

Pythium populations overall tended to increase with soil temperature and with rainfall but these increases were statistically non-significant (Table 6.4.) and remained so when both high soil temperature and high rainfall jointly were examined. Examination of periodic results (as opposed to monthly results) indicated that populations tended to diminish during periods of diminishing rainfall (e.g. Oct 1980 - Feb 1981, Sep - Nov 1981, Dec 1981 - Feb 1982. Tables 6.1., 6.2.) Populations were very highly significantly greater during the months Aug - Jan than during the months Feb - July (Table 6.4); for these two periods overall mean rainfall and soil temperature were virtually identical, and population differences therefore may have been associated with increased plant debris during the latter part of the year.

P. sylvaticum was isolated only from soils sampled in the period Feb - May ;

P. x, P. ultimum and P. heterothallicum were detected virtually throughout the year (Table 6.5). Populations of P. ultimum appeared to increase progressively from March to December and then to sharply decline (Table 6.5). The incidence of P. acanthophoron and P. vanterpoolii only in October samples suggests vigorous activity either over very limited periods or within very localised soil areas.

6.3.2. Pythium spp. isolated by the slide-trap method.

Pythium spp. were isolated from soils in 20/24 samplings: none was isolated in certain winter months (Feb 1981 and 1982, Dec 1981 and Jan 1982) (Table 6.3). From March to November inclusive there was little variation in the total numbers of monthly isolations; the highest total number of isolations occurred in September.

The most frequently isolated species, in order, were P. x, P. ultimum and P. heterothallicum. P. sylvaticum and P. irregulare were isolated infrequently. Included in 'other species' (Table 6.3.) were P. intermedium (March only), P. acanthophoron and an unidentified species (no. 614).

P. x was isolated in all samplings except those when soil temperatures were less than 5° C. On overall average (Table 6.6.) this species was isolated with high frequency in all months except February and most frequently during September - November. P. ultimum was isolated only when soil temperatures exceeded 8° C; the frequency of isolation increased progressively, from March to September and then sharply declined. This latter pattern of isolation results was somewhat similar with P. heterothallicum (Table 6.6). P. sylvaticum was isolated infrequently during March - May, and from single slide-traps in July and November. Pythium spp. showed differing minimum temperature restrictions on their isolation, and there was no obvious effect of rainfall on the frequencies of isolation (Table 6.4).

6.3.3. Discussion of Section 6

The soil dilution plate and slide-trap methods placed P. x, P. ultimum, P. heterothallicum and P. sylvaticum in the same order of dominance. P. acanthophoron was also detected by both methods. P. intermedium was detected only by the slide-trap method, and P. rostratum and P. vanterpoolii only by the dilution plate method.

Of the species identified in this soil, P. ultimum, P. sylvaticum, P. intermedium, P. irregulare, P. heterothallicum and P.x are known to include strains pathogenic to apple (Sewell, 1981; Sect 8 and 9, this Thesis).

It was perhaps unfortunate that this severe replant disease site was unusual in having a low soil population of P. sylvaticum (and a high population of P. ultimum). However, the results, from both isolation methods, indicated differing periodicities for these two spp. jointly extending over the whole apple growing season.

Most colonies on soil dilution plates originate from spores (pers obsn) and it has been questioned (Agnihotri & Vaartaja, 1967) whether frequency of isolation or abundance are related to mycelial activity in soil. In this study the relative abundance of colonies on dilution plates varied from 0 to 100% for all spp. except P. heterothallicum (11-100%) (Table 6.7).

The fluctuations in colony numbers of P.x and P. heterothallicum were erratic and not related to obvious seasonal factors; however, that the normally abundant spp. P.x and P. ultimum were both not isolated on dilution plates in February 1981 and 1982 suggests that their populations in surface soils were severely diminished, if not eliminated, during the winter cold. It also appeared probable that P. sylvaticum was partly, if not completely, eradicated from surface soils during the warmest summer months after May.

Most colonies in slide-traps probably originated from mycelia which had grown over glass or 'Perspex' surfaces into the agar. Condensed water on these surfaces may have reduced fungistatic effects and permitted germination of spores. The slide-trap therefore may permit isolations when (or where) spores are abundant and when soil temperature permits mycelial growth. If this is true a correlation would be expected between results from slide-traps and dilution plates, when soil temperatures are not limiting. Such a correlation occurred with P.x, P. heterothallicum and P. sylvaticum (Table 6.7). With P. ultimum abundance on dilution plates and frequency in slide-traps both increased more or less progressively from March to September. During October - December, slide-trap isolations of P. ultimum were severely diminished, but dilution plate abundancies continued to increase. In October - November, when soil temperatures (at 10 cm depth) were 5-9°C (Table 6.1.), slide-trap isolates were dominated by P.x (Table 6.3.) Laboratory observations of the growth rates of Pythium spp. isolated

from this soil showed that at 8-10°C Pythium x was faster growing than other abundant species including P. ultimum (Table 6.8). It is probable therefore, that after September, P.x was competitively superior to P. ultimum and precluded its isolation in slide-traps. Competitive effects, based simply on growth rates, do not explain the general failure to isolate P. sylvaticum during summer months after May : the results suggest that P. sylvaticum activity was suppressed during periods of high temperature which favoured its growth in vitro (Table 6.8).

In summary: the results indicated a relatively brief period of activity for P. sylvaticum, and increasing activity of P. ultimum as soil temperatures increased; for other spp. the results indicated localised activity (when soil temperature permitted) which resulted in great variation between samples; the competitive balance between species varied with soil temperature. If propagule abundance in soil is not a valid measure of past or present mycelial activity, the latter is probably largely not reflected by slide-trap isolations either.

Table 6.1. Total monthly rainfall (mm) and mean monthly soil temperature ($^{\circ}\text{C}$ at 10 cm depth) during the period of Pythium isolations from PR117 soil. (EMRS data).

Month	Rainfall			Soil temp.		
	1980	1981	1982	1980	1981	1982
Jan		25	56		3	3
Feb		13	32		2	4
Mar		86	57		7	5
Apr		27	13		8	9
May	18	91		13	12	
Jun	80	36		16	16	
Jul	89	59		16	17	
Aug	61	26		18	18	
Sep	28	125		15	15	
Oct	85	86		9	9	
Nov	41	40		5	7	
Dec	37	80		4	2	

Table 6.2. Abundance (mean p/g) of *Pythium* spp. on soil dilution plates prepared from PR 117 soils sampled at monthly intervals.

Year	Month	Pythium sp.					Others
		All	P.x	P.ult	P.het	P.syl	
1980	May	300	66	66	66	66	33
	Jun	430	108	54	160	0	108
	Jul	335	201	67	0	0	67
	Aug	1100	110	0	220	0	880
	Sep	720	72	216	360	0	0
	Oct	1100	220	110	440	0	330
	Nov	850	680	0	170	0	0
	Dec	715	572	0	143	0	0
1981	Jan	720	144	144	432	0	0
	Feb	300	0	150	0	150	0
	Mar	300	0	200	100	0	0
	Apr	700	140	140	350	0	70
	May	480	60	120	300	0	0
	Jun	365	0	182	110	0	73
	Jul	775	194	0	281	0	300
	Aug	880	196	98	196	0	390
	Sep	1050	630	0	420	0	0
	Oct	1080	360	90	540	0	90
	Nov	190	38	76	38	0	38
	Dec	720	540	36	144	0	0
1982	Jan	735	0	0	735	0	0
	Feb	270	0	90	0	180	0
	Mar	640	64	128	0	256	192
	Apr	700	70	70	210	140	210
(Mean)		644	186	85	226	33	116

Table 6.3. Number of slide-traps (max 10) colonised by Pythium spp. after burial in PR 117 at monthly intervals.

Year	Month	P.x	P.ult	P.het	P.syl	P.irr	Others
1980	May	3	1	2	2	1	4
	June	4	4	2	0	2	0
	Jul	6	2	2	0	0	1
	Aug	4	3	1	0	0	3
	Sep	8	7	1	0	4	1
	Oct	9	2	0	0	0	1
	Nov	8	2	0	0	1	0
	Dec	10	0	0	0	0	0
1981	Jan	10	0	0	0	0	0
	Feb	0	0	0	0	0	0
	Mar	8	0	3	1	0	1
	Apr	7	2	1	0	0	0
	May	5	7	1	0	0	1
	Jun	3	7	2	0	0	0
	Jul	4	7	7	1	0	1
	Aug	3	4	2	0	0	2
	Sep	7	6	5	0	0	1
	Oct	8	0	1	0	0	1
	Nov	5	0	1	1	0	6
	Dec	0	0	0	0	0	0
1982	Jan	0	0	0	0	0	0
	Feb	0	0	0	0	0	0
	Mar	2	2	4	4	0	0
	Apr	4	3	3	4	0	0
(Mean)		4.9	2.5	1.6	0.5	0.3	1.1

Table 6.4. Mean monthly occurrence of *Pythium* spp. on soil dilution plates (DP; p/g) and in slide-traps (ST; no. isolation records) in relation to soil temperature, rainfall and periods of the year.
(PR 117 soil isolations May 1980-Apr 1982).

Factor		DP	ST
Temp (°C) < 6	8	619 $\pm$ 76	5.4 $\pm$ 2.0
	8	606 $\pm$ 12	12.8 $\pm$ 0.8
	8	707 $\pm$ 107	14.9 $\pm$ 1.6
Rf (mm) < 35	8	574 $\pm$ 86	10.3 $\pm$ 2.6
	8	671 $\pm$ 100	11.1 $\pm$ 1.9
	8	687 $\pm$ 122	11.4 $\pm$ 1.9
Period Feb-Jul	12	466 $\pm$ 54	10.7 $\pm$ 1.8
	12	822 $\pm$ 74	11.2 $\pm$ 1.7

ANOVA: $F_{2,22} = 3.88$ (22 Df) with $P < 0.001$ at 3.79.

Table 6.5. Mean abundance (p/g) of Pythium spp. on soil dilution plates prepared from PR 117 soils sampled at monthly intervals, May 1980-Apr 1982.

Month	Pythium sp.				
	P.x	P.ult	P.het	P.syl	other spp.
Jan	584	72	72		
Feb			120	165	
Mar	50	32	164	128	96
Apr	280	105	105	70	140
May	183	63	93	30	17
Jun	135	54	118		91
Jul	141	198	34		184
Aug	208	153	49		635
Sep	390	351	108		
Oct	490	325	100		210
Nov	104	359	38		19
Dec	144	556	18		
(Mean)	214	189	85	33	116

Table 6.6. Mean frequency (%) of isolation of Pythium spp. from slide-traps buried in PR 117 soil at monthly intervals (May 1980 - Apr 1982).

Month	Pythium sp.				
	P.x	P.ult	P.het	P.syl	others
Jan	50	0	0	0	0
Feb	0	0	0	0	0
Mar	50	10	35	25	5
Apr	55	25	20	20	15
May	40	40	15	10	30
Jun	35	55	20	0	10
Jul	50	45	45	5	10
Aug	35	35	15	0	25
Sep	75	65	30	0	30
Oct	85	10	5	0	10
Nov	65	10	5	5	35
Dec	50	0	0	0	0
Mean :	49.2	24.6	15.8	5.4	14.2

Table 6.7. Relative monthly frequency of isolations by slide-traps (ST) and relative monthly abundance of colonies on soil dilution plates (DP).
(PR 117 soil isolations May 1980-Apr 1982).

Month	P.x		P.ult		P.het		P.syl	
	ST	DP	ST	DP	ST	DP	ST	DP
Jan	59	100		13		44		
Feb						73		100
Mar	59	9	15	6	78	100	100	78
Apr	65	48	38	19	44	64	80	42
May	47	31	62	11	33	57	40	20
Jun	41	23	85	10	44	72		
Jul	59	24	69	36	100	21	20	
Aug	41	36	54	28	33	30		
Sep	88	67	100	63	66	66		
Oct	100	84	15	58	11	61		
Nov	76	18	15	65	11	23	20	
Dec	59	25		100		11		

Isolation results for each species and method expressed as a percentage of the maximum record.

Table 6.8. Effect of temperature on the colony diameter (mm) of *Pythium* spp. isolated from PR 117 soil (CMA, 48 h)

Pythium sp.	Temp (C)						
	8	10	13	20	25	30	33
<i>P. ultimum</i>	7	22	42	87+	87+	87+	78
<i>P. x</i>	16	28	34	61	68	43	0
<i>P. sylvaticum</i>	14	36	42	87+	87+	87+	11
<i>P. heterothallicum</i>	8	25	27	55	64	53	0
<i>P. intermedium</i>	17	28	50	87+	76	36	0
<i>P. irregulare</i>	7	16	34	83	87+	84	0

7. Recolonisation by Pythium spp. of a chloropicrin-fumigated
apple orchard soil

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7.1. Introduction

Apple 'replant' disease, as a decline phenomenon in layer-beds, has frequently been observed in nurseries (Savory, 1967 b). This indicates the possibility of the transfer of causal organisms from nursery to orchard soils. Also, in orchard soils, fumigated or not, the growth of trees in fumigated soil is often better only during the first two years after planting (Savory, 1966 ; Jackson, 1973). Observations were therefore made of the recolonisation by Pythium spp. of a chloropicrin-fumigated apple orchard soil.

7.2. Materials and methods

The site selected for this study was a research station plot (CW 106). Strips of land (1.5 m wide) were fumigated with chloropicrin (280 ℓ /ha) on 24th August 1979 and the strips were covered with polyethylene film. The fumigated strips were separated by grassed alleys (5 m wide). Apple trees (Cox's Orange Pippin and Discovery on MM 106 rootstocks) were planted into the fumigated strips (4.25 m spacing) on 6th and 7th December 1979, 15 wk after fumigation.

Pythium populations in these soils were assayed by the soil dilution plate method using MPV medium (Sect 1). In initial assays 1/10 dilution was used. Assays were first made 21 weeks after planting (36 wk after fumigation) and subsequently, at 10-19 wk intervals, for a period of 118 wk after planting. These studies were primarily concerned with the recolonisation of sterilised soil by Pythium per se, and specific identifications were made only of isolates obtained from the second assay (31 wk after planting).

In routine assays (eight assays between 6.5.80 and 17.3.82 soil samples were taken at 10-15 cm distance from the bases of 12 trees, and from 12 mid-points between trees (c 2 m distant from trees). In the second routine assay, when Pythium spp. were identified, and at other times soil samples were also collected from the edges of the (non-fumigated) grassed alleys.

More detailed surveys of the distribution of Pythium were made 61 and 64 wk after planting. Soil samples were collected at 16 cm intervals between tree or mid-tree points and the grassed alley, and at 16 cm intervals between tree and mid-tree sites.

7.3. Results

7.3.1. Routine assays

Pythium spp. were not detected (using 1/10 dilution) in soils 36 wk after fumigation (21 wk after planting apple trees) (Table 7.1). At this time, May 1980, the Pythium population in the grassed, non-fumigated, alley was 900 p/g. Pythium spp. were detected near tree sites and mid-tree sites 31 wk after planting, in July 1980: populations tended to remain stable and low through winter 1980/81 and then progressively increased in subsequent samplings at both tree and mid-tree sites. The frequency of occurrence of Pythium spp. was considerably less at mid-tree than at tree sites during 70 wk after planting (Table 7.2). During 31–60 wk after planting populations at tree sites were 9–23 times greater than at mid-tree sites. In subsequent samplings (70–118 wk after planting) the difference between populations at tree sites and mid-tree sites diminished from $\times 5$ to $\times 2$ (Table 7.1). Within each assay there was very great variation between replicate samples, indicating localised differences in Pythium activity.

In the final assay, 133 wk after fumigation, Pythium populations at tree and mid-tree sites respectively were only 29% and 13% of that in non-fumigated soil from the grassed alley.

7.3.2. Pythium spp. isolated

Pythium spp. were identified only from isolations made in July 1980. In the grassed alley soil P.x was the most abundant species, constituting 60% of the population. P. intermedium constituted 30% of this population, and the rest was contributed by P. heterothallicum and a species tentatively identified as P. rostratum: one isolation was made of P. sylvaticum (male strain).

In fumigated soil P. intermedium (both mating strains) was the dominant species followed by P.x and P. heterothallicum. P. sylvaticum (female strain) and P. irregulare were each isolated from a single tree (but no mid-tree) site. P. irregulare was the only species not isolated from the non-fumigated alley.

7.3.3. Detailed assays

In February 1981, Pythium populations were assayed in soil samples taken at 16 cm intervals from a tree to a mid-tree site (X1, Table 7.3), and from tree to

alley and from mid-tree to alley (X2, Table 7.4).

Along the centre-line of the fumigated zone, 76 wk after treatment, Pythium spp. were not detected in 7/15 samples. Appreciable populations, of 120 and 400 p/g respectively were detected at the 16 and 64 cm distances from the tree. On average, populations were ten times greater near (0-64 cm) to the tree than distant from the tree (Table 7.3).

Pythium populations increased progressively from the fumigated centre-line towards the grassed alley (Table 7.4, 11.2.81) and were generally greater for transects at tree sites than at mid-tree sites.

Transects from tree site to alley were studied in greater detail three weeks later (Table 7.4, 3.3.81), when Pythium populations had increased considerably. Pythium spp. were detected in 39/40 samples from the fumigated strip. Populations, however, showed an uneven gradation from the grassed alley towards the fumigated centre-line tree site. Pythium populations in the fumigated zones 0-35 cm and 35-75 cm from the grassed alley were, on average, 66% and 17% of the population in the alley. Maximum populations recorded under grass, and at 0-35 and 35-75 cm from the grass, were 1200, 900 and 325 p/g respectively.

7.4. Discussion of Section 7

Pythium spp. were detected at the centre-line of the fumigated strip 46 (but not 36) weeks after fumigation. Two and a half years after fumigation Pythium populations at the centre-line were only 13-29% as large as that in the non-fumigated soil. These results indicated a strong and persistent inhibitory effect of fumigation, on the recolonisation of this soil by Pythium spp.

The relationship between Pythium populations and apple root density could not be determined because the planting was designed for other experiments. However, it is probable that apple roots had extended to the edge of the fumigated zone (75 cm) by the end of the first growing season (1980). Soil populations were consistently larger near to than distant from the trees along the centre-line of the fumigated strip. Differences in frequencies of occurrence were also very great: 31 wk after planting Pythium spp. were present in 83% of samples from tree sites but in only 17% of samples from mid-tree sites. (Tables 7.1 - 3). This may indicate that Pythium spp. were introduced with the trees, and this is supported by the occurrence of P. irregulare at one tree site but not in the grassed alley.

On the other hand, the presence of apple roots may simply have stimulated the activity of the recolonising flora. Much larger fumigated areas would be required to resolve this question.

The evidence for lateral recolonisation from the non-fumigated alley was much stronger: populations at the centre-line and from the grassed alley to the centre-line progressively increased. There was no evidence to suggest that vertical recolonisation (from non-fumigated soil depths of c 30 cm) was important.

The results do show that, even with a strong, persistent inhibitory effect, considerable Pythium recolonisation of fumigated soil occurred by the third year. The apparent 'improvement' of growth of trees in non-fumigated soil therefore, may merely be due to diminishing growth of control trees in fumigated soil.

Table 7.1. Mean abundance (p/g $\pm$ S.E.) of *Pythium* spp. along the centre-line of the fumigated strip at differing times after planting apple.
(Minimum and maximum records in parenthesis Replication x 12).

Date		Wk after Planting	Distance from tree base (cm)	
			10 - 15	210
6.5.1980	(a)	21	0	0
15.7.1980		31	47 $\pm$ 18 (0 - 200)	5 $\pm$ 5 (0 - 55)
24.9.1980		41	53 $\pm$ 12 (10 - 120)	3 $\pm$ 1 (0 - 10)
5.2.1981	(b)	60	45 $\pm$ 11 (10 - 130)	2 $\pm$ 1 (0 - 5)
15.4.1981		70	82 $\pm$ 22 (10 - 260)	17 $\pm$ 5 (0 - 60)
25.8.1981		89	118 $\pm$ 17 (30 - 240)	30 $\pm$ 6 (5 - 70)
23.11.1981		102	165 $\pm$ 27 (65 - 350)	68 $\pm$ 14 (10 - 180)
17.3.1982	(c)	118	308 $\pm$ 29 (200 - 450)	140 $\pm$ 33 (35 - 400)

Pythium populations in grassed alley (a) 900 (b) 300 (c) 1050 p/g.

Table 7.2. Frequency of occurrence (%) of Pythium spp. along centre-line of the fumigated strip at differing times after planting apple.

Wk after planting	Distance from tree (cm)	
	10 - 15	210
21	0	0
31	83	17
41	100	58
60	100	50
70	100	83
89 - 118	100	100

Table 7.3. Abundance (p/g $\pm$ S.E) of Pythium spp. at differing distances from tree to mid-tree sites (11.2.81).

	Distance from tree site (cm)	
	0 — 64	80 — 224
No. samples	5	10
Mean p/g	105 $\pm$ 77	11 $\pm$ 5
Range p/g	0 — 400	0 — 50

Table 7.4. Abundance (p/g) of Pythium spp. at differing distances from tree or mid-tree sites towards grassed alley (GA).

Distance (cm)	Site (11.2.81) *		Tree site (3.3.81)
	Tree	Mid-tree	
0	50	0	91 $\pm$ 38
16	75	15	147 $\pm$ 47
32	145	85	113 $\pm$ 31
48	225	160	438 $\pm$ 100
64	180	285	469 $\pm$ 81
GA	325	258	685 $\pm$ 50

* Replication x 2, x 4 for GA.

Replication x 8, x 16 for GA.

8.	<u>Laboratory studies with apple seedlings on the pathogenicity and parasitism of Pythium spp.</u>	
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8.1. Effects of PWA inoculum slabs on apple seedling roots

Methods of seedling inoculation and root-clearing, developed and in routine use at EMRS, were used in these studies of the pathogenicity and parasitism of Pythium spp.

8.1.1. Materials and methods

The test plants were Bittenfelder or (occasionally) Worcester Pearmain seedlings. Seeds were sown in moist sand in seed trays and subjected to cold treatment (10 wk at 4 ° C). Seedlings were used at the early cotyledon stage (6–8 days after removal from the cold store) when the unbranched radicle was 3–4 cm long.

Pythium isolates were cultured for 7–14 days in plastic petri-dishes (9 cm) containing measured volumes (30 ml) of 2% PWA.

A strip (5 cm) of wall of each petri-dish base was removed and seven seedlings were placed in the dish with cotyledons and part of the hypocotyls protruding. The distal portions of the seedling roots were then covered with a semi-circular PWA slab removed from a culture (or uninoculated) plate. Proximal root portions and hypocotyl parts within the petri-dish were covered with moistened semi-circles of filter paper. This system was held gently in position by synthetic foam strips just touching the lid which was strapped in position with adhesive tape.

The positions of root tips were marked with waterproof ink on the petri-dish bases and the plates were incubated in a vertical position in a growth-room.

Root growth was recorded after 24, 48 and 72 h incubation in most experimental sets but only after 72 h in some. After 72 h the extent of root necrosis was assessed and roots were preserved in alcohol for subsequent microscope examination.

Root tissues were cleared by simmering for 30 min in sodium hydroxide (0.08 N), rinsing in fresh NaOH, and then submerging in hydrogen peroxide until just bleached. The roots were then washed repeatedly in distilled water, and submerged in hydrochloric acid (0.2 N) for 15 min before staining. Roots were stained by simmering in alcoholic-lactophenol-trypan blue (ALT) for 20 min and then mounted on slides in 70% glycerol.

(ALT: glycerol 20 ml, lactic acid 20 ml, DW 20 ml, phenol 20 g, trypan blue 40 mg; thoroughly mix and add to twice the volume of alcohol).

8.1.2. Results

The root extension growth results for each experiment were subjected to analyses of variance by Mr. G.P. Barlow. Most experiments included isolates of several Pythium species. To simplify presentation, the results for each Pythium species (from several experiments) are grouped together in Tables 8.1 – 8.3. The results are given as the percentages of growth inhibition under inoculated, compared with uninoculated, PWA slabs: significance values refer to comparisons of actual root extensions beneath uninoculated and inoculated slabs. In these experiments mean root growth under uninoculated slabs during 72 h varied from 11 to 20 mm (mean 16.3 mm). Replication was x 7. Root necrosis was assessed as the proportion (%) of total root length that was necrotic or discoloured.

All (25) isolates of P. sylvaticum caused significant reductions in the growth of apple seedling radicles during the 72 h observation period, and most (68%) isolates caused immediate and complete cessation of growth (Table 8.1). Discoloration usually first appeared on roots immediately above the agar slab. With most isolates reddish-brown root discoloration was present after 48 h and extensive discoloration and decay occurred within 72 h: eight isolates caused complete root discoloration (Table 8.4). Two isolates (nos. 19 and 22, Table 8.1) were not associated with root discoloration although causing appreciable (but diminishing) levels of growth inhibition. Microscope examination of roots inoculated with P. sylvaticum showed that infections mostly occurred within 24 h. After 72 h only hyphal swellings were visible in the invaded tissues and virtually all invading mycelium was lysed (Plate 8.1).

All but one isolate of P. heterothallicum caused significant reductions in root growth ranging from 54 to 93% after 24 h, but the inhibitory effects progressively diminished with time. (Table 8.1). Five of these isolates caused slight root discoloration particularly near to root tips (Table 8.4), but no mycelial penetration of roots was observed with P. heterothallicum, except in association with physical injury.

Types A and B of Pythium x differed considerably in the strengths of their inhibitory effects on root growth (Table 8.2): significant reductions of root growth occurred with four of eleven isolates of type A and with all (6) isolates of type B. Inhibitory effects of type A tended to diminish, and those of type B to be

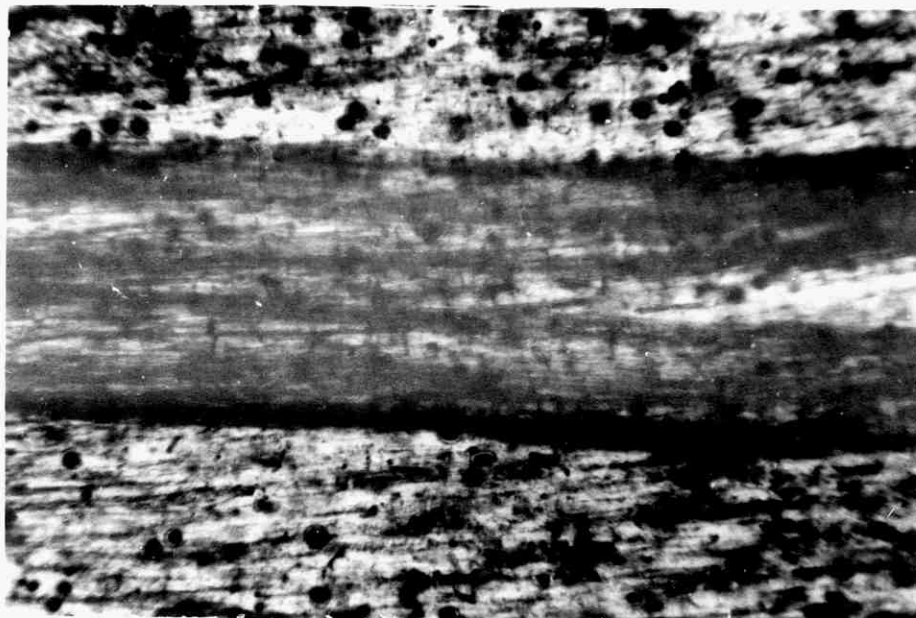


Plate 8.1 Hyphal swellings of *P. sylvaticum* (upper) and oospores of *P. irregulare* (lower) in apple seedling roots 72 h after application of PWA inoculum slabs.

maintained, with time. Type A isolates caused no root discoloration and type B only traces of discoloration. Root invasion was observed with one type B isolate and the colonisation was mainly restricted to epidermal and superficial tissues.

Of other Pythium isolates tested, P. intermedium, P. ultimum, P. paroecandrum, P. coloratum and P. irregulare (3/4 isolates) caused complete inhibition of root growth and extensive root necrosis. Oospores of the latter four species were produced in abundance in invaded tissues (Plate 8.1). Less, but significant root growth inhibition, without tissue invasion, occurred with single isolates of P. acanthophoron and P. vanterpoolii (Tables 8.3, 8.4).

Table 8.1. Inhibition (%) of apple seedling root growth 24, 48 and 72 hours after applying PWA slabs inoculated with P. sylvaticum (isolates 1-25) or with P. heterothallicum (isolates 26-32).

Isolate No	Hours		
	24	48	72
1 - 17	100	100	100
18	100	86	86
19	91	81	81
20	77	77	80
21	96	83	80
22	91	79	65
23	28	36 *	55 **
24	40 *	31 *	54 ***
25	17	37 *	38 *
26	93	86	68
27	81	88	59
28	65	61	49
29	54	54	46
30	74 ***	95 ***	38 **
31	89 ***	75 ***	26 *
32	—	—	21

Significant differences from uninoculated treatments:

* $P < 0.05$

** $P < 0.01$

*** $P < 0.001$

For isolates 1-22 and 26-29 all results were very highly significant ($P < 0.001$).

Table 8.2. Inhibition (%) of apple seedling root growth 24, 48 or 72 hours after applying PWA slabs inoculated with P.x type A (isolates 33-43) or with P.x type B (isolates 44-49).

Isolate No	Hours		
	24	48	72
33	83	74	59
34	63	54	31 **
35	39	58	56
36	9	13	0
37	0	2	0
38	—	—	36 *
39	—	—	20
40	—	—	20
41	—	—	17
42	—	—	8
43	—	—	3
44	100	100	100
45	93	88	91
46	86	88	91
47	79	63	62
48	—	—	83
49	—	—	70

Significant differences from uninoculated treatments :

* $P < 0.05$ ** $P < 0.01$

All records greater than 36 were very highly significant ($P < 0.001$).

Table 8.3. Inhibition (%) of apple seedling root growth 24, 48 and 72 hours after applying PWA slabs inoculated with isolates of seven Pythium spp.

Pythium sp.	No. isolates	Hours		
		24	48	72
<i>P. intermedium</i>	6	100	100	100
<i>P. ultimum</i>	4	100	100	100
<i>P. irregulare</i>	3	100	100	100
<i>P. irregulare</i>	1	54 *	47 *	31
<i>P. paroecandrum</i>	1	100	100	100
<i>P. coloratum</i>	1	100	100	100
<i>P. acanthophoron</i>	1	60 ***	46 ***	33 ***
<i>P. vanterpoolii</i>	1	26 **	16	11

Significant differences from uninoculated treatments:

* $P < 0.05$

** $P < 0.01$

*** $P < 0.001$

Table 8.4. Mean root necrosis (%) after 72 h, and mean inhibition (%) of root growth after 24 h, induced in apple seedlings by Pythium spp.

Pythium sp.	No. isolates	Root necrosis		Growth inhibn.
		mean	range	
<i>P. intermedium</i>	6	100	—	100
<i>P. ultimum</i>	4	83	83	100
<i>P. sylvaticum</i>	25	76	0 - 100	90
<i>P. paroecandrum</i>	1	—	67	(100)
<i>P. irregulare</i>	4	54	0 - 83	89
<i>P. coloratum</i>	1	—	50	(100)
<i>P. heterothallicum</i>	7	12	0 - 17	76
<i>P. x</i> type B	4	3	0 - 17	90
<i>P. acanthophoron</i>	1	—	0	(60)
<i>P. x</i> type A	6	0	—	37
<i>P. vanterpoolii</i>	1	—	0	(26)

8.2. Tests for the production of diffusible toxins by Pythium spp.

The immediate arrest of root growth when certain inoculum slabs were placed on roots indicated that some cultures contained growth inhibiting substances. Experiments therefore were made to test this possibility.

8.2.1. Materials and methods

The methods used were similar to those described in Subsection 8.1.1. with two exceptions. Firstly, in some treatments seedlings in petri-dishes were covered with boiled 'Cellophane' discs (8 cm diam) before placing inoculum slabs. Secondly, smaller inoculum sectors (taken from standardised plate-cultures) were used to permit exposed cellophane edges to dry and diminish risks of mycelial growth from inocula.

8.2.2. Results

In Exp 8.1 (Table 8.5) an isolate of P. sylvaticum completely inhibited root growth and caused severe necrosis when placed directly onto roots. This isolate caused 78 and 68% growth inhibition respectively after 48 and 72 h when separated from roots by cellophane without necrosis: no mycelial penetration was observed. In Exp 8.2 (Table 8.6) an isolate of P. intermedium completely inhibited root growth when in direct contact with roots, but had no effect when separated by cellophane. In these two experiments cellophane had negligible (slightly retarding) effects on root growth beneath uninoculated PWA slabs. Uninoculated control treatments made significantly greater growth under cellophane in Exp 8.3 (Table 8.7). After 24 h incubation (Exp 8.3) isolates of P. sylvaticum, P. heterothallicum and P.x B respectively caused 100, 84 and 43% growth inhibition ($P < 0.001$) when placed directly onto roots; when separated by cellophane growth inhibitions were 82, 52 ($P < 0.001$) and 6% (NS). After 48 and 72 h incubation growth continued to be retarded under cellophane with P. sylvaticum and P. heterothallicum but relatively poor and good growth of control sets diminished the value of these later records. However, with P. sylvaticum root growth was clearly diminished by a diffusible toxin(s) which also caused considerable swelling of the root tip tissues (Plate 8.2). Similar but less marked effects were caused by P. heterothallicum. No diffusible toxin was demonstrated with P. intermedium (Exp 8.1) or P.x type B (Exp 8.3).

Table 8.5. (Exp 8.1) Mean root growth (mm) of apple seedlings under PWA slabs inoculated with P. sylvaticum or uninoculated, and with (+) or without (—) cellophane between roots and slabs.

Incubation (hours)	Cellophane	PWA slab inoculum	
		Nil	P. syl
48	—	10.4	0.0
	+	10.1	2.2
72	—	19.2	0.0
	+	16.7	5.3

Table 8.6. (Exp 8.2) As Table 8.5., but with P. intermedium.

Incubation (hours)	Cellophane	PWA slab inoculum	
		Nil	P. int
24	—	5.6	0.0
	+	5.6	6.1
48	—	10.6	0.0
	+	9.0	10.1
72	—	14.6	0.0
	+	13.1	11.6

Table 8.7. (Exp 8.3). As Table 8.5., but with P. sylvaticum, P. heterothallicum and P. x type B.

PWA slab inoculum	24 h		48 h		72 h	
	—	+	—	+	—	+
Nil	6.7	6.8	10.3	13.8	14.0	20.8
P. syl	0.0	1.2	0.0	5.3	0.0	10.5
P. het	1.1	3.2	3.7	7.8	7.8	11.2
P. x	2.5	6.3	7.2	11.0	11.8	16.3
LSD: P = 5%		1.8		3.2		6.0
P = 1%		2.4		4.3		8.1
P = 0.1%		3.1		5.6		—

8.3. Discussion of Section 8

The isolates tested in these laboratory studies fell into two clearly defined groups, either strongly parasitic on apple roots or with negligible parasitic ability. The former included P. coloratum, P. intermedium, P. irregulare, P. paroecandrum, P. sylvaticum and P. ultimum: other isolates of all these species were found significantly to reduce growth of apple seedlings in pot-plant tests (Sewell, 1981). The non - or sparingly parasitic species included P. acanthophoron, P. heterothallicum, P. vanterpoolii and P.x types A and B: of these only P. vanterpoolii was tested by Sewell (1981) who found two isolates to have no effect on apple seedling growth.

All strongly parasitic species had very strong inhibitory effects on root growth. These effects were immediate and therefore appeared to be of different cause from that resulting from parasitism. Strong or moderately strong growth inhibitory effects were also shown by species with little or no parasitic ability (P. heterothallicum, P.x type B and P. acanthophoron). The production of diffusible toxin(s) was demonstrated for P. sylvaticum and P. heterothallicum but not for P. intermedium and P.x. Of these four species P. sylvaticum and P. intermedium were the strongest inhibitors of root growth and it is therefore unlikely that differences in test results (Exp 8.1 - 3) were associated merely with quantities of toxin produced.

Effects of P. sylvaticum toxin on root morphology (Plate 8.2) strongly indicated, in agreement with other work, that the toxin was hormonal. Thus inhibition of flax root growth was directly related to the concentrations of autoclaved P. sylvaticum culture filtrate applied (Blok, 1970, 1973), and indole-acetic acid was identified in the culture filtrates by Posthumus (1973). Killed mycelium of P. sylvaticum strongly inhibited apple seedling root growth (Sewell, unpublished) and IAA has been identified with certainty as a major metabolite of P. sylvaticum by other EMRS workers (unpublished).

These present results suggest the possibility that certain Pythium species may be pathogenic either with or without accompanying parasitism. A similar conclusion has been reached, involving species of both Pythium and Phytophthora, by other workers (Wilhelm 1965, Csinos & Hendrix, J.W. 1977., Kanlong & Hendrix, J.W. 1977., Csinos 1979).

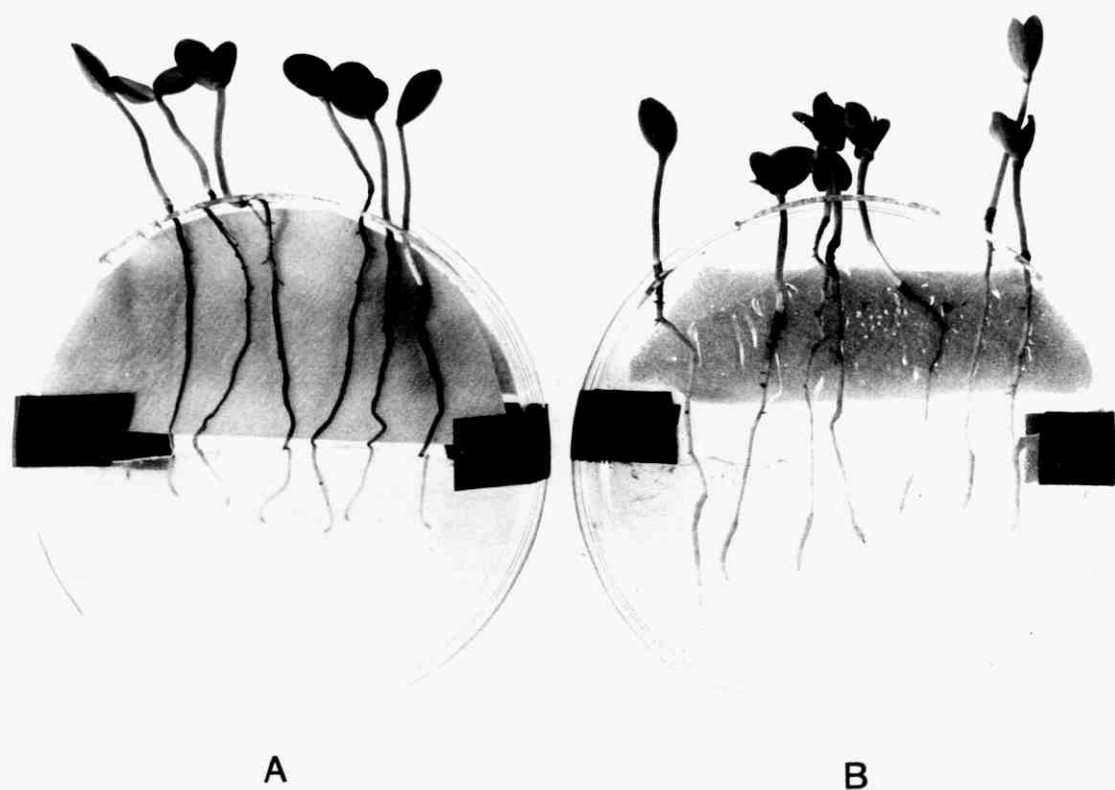


Plate 8.2 Upper - Apple seedling plates used for observing effects of PWA/P. sylvaticum inoculum slabs on root growth and infection.
 A. roots and inoculum in contact (NB. root necrosis).
 B. inoculum and roots separated by cellophane (NB. swollen root tips).
Lower - Effects of P. sylvaticum toxin, after diffusion through cellophane, on apple seedling root morphology.

9. Pot-plant studies of the effects of Pythium isolates on the growth of apple seedlings.

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9.1. Introduction

The effects of compost infestation with Pythium isolates on the growth of apple seedlings were studied in four glasshouse experiments during 1981 and 1982. In these experiments a total of 24 isolates of 11 Pythium species were tested. Five isolates originated from the EMRS plot PR 117, and the remainder from ADAS test soils (1979 - 81).

9.2. Materials and methods

The materials and methods used were similar to those described by Sewell (1981). Isolates were cultured for 3-5 wk on a sterilised medium (CMSS) comprising equal volumes of (1.7 mm sieved) loam and washed sand amended with commeal (3% v/v). The CMSS (75 ml) was spread evenly in 300 ml capacity medicine flats and wetted with distilled water (27 ml) prior to autoclaving and inoculating with CMA culture pieces. On this medium certain isolates (P. sylvaticum, P. coloratum, P. intermedium and P. ultimum) formed dense floccose mycelium; others (P. acanthophoron, P. heterothallicum, P. rostratum and P.x (B)) formed only sparse superficial mycelium; growth of P.x (A) was visible only by microscope examination.

Compost (5 parts sterilised loam, 2 parts sand and John Innes No.1 base fertiliser) was spread evenly and thinly on clean brown paper, and finely chopped inoculum was sprinkled over the surface: uniform inoculum dispersal was obtained by repeatedly spreading and 'rolling' the mixture. The rate of inoculum amendment was 3% v/v.

Composts (infested or not) were distributed into black plastic pots (500 ml capacity) and planted with single apple seedlings at the first leaf-pair stage: the seedlings were either open-pollinated Worcester Pearmain or the M. pumila selection 'Bittenfelder'.

Treatment replication was x 6 or x 7 with or without double replication of the uninfested control treatment. Experiment designs were fully randomised blocks or Latin squares. The experiments were made during the months May - September in a shaded glasshouse. Seedling heights were recorded at two-week intervals for a period of 11-12 wk. Analyses of variance of the results were kindly made by Mr. G.P. Barlow.

9.3. Results

Details and results of the four experiments are given in Tables 9.1 - 4. Compost infestation with 16 of the 24 isolates was associated with diminished apple growth but growth reductions were statistically significant (after 12 wk) with only five isolates of three species, P. heterothallicum (Expt 2), P. irregulare and P. sylvaticum (Exp 3). There was a tendency for effects on growth to be most marked during the first six weeks and then to diminish. However isolates of P. sylvaticum and P. heterothallicum caused 68 and 48% growth reductions respectively (Tables 9.2, 9.3); such growth reductions are equivalent to 213 and 92% growth increases in terms of fumigation responses. Growth reductions after 12 wk ranged from 15 to 68% with five isolates of P. sylvaticum, and from 16 to 48% with three isolates of P. heterothallicum.

In soils infested with certain isolates of P. sylvaticum and P. heterothallicum the seedling cotyledons rapidly became chlorotic and developed peach-like colouration before early necrosis and abscission. Later, the only symptoms were stunting and rosetting. One isolate tested of P. ultimum (Table 9.1) caused significant growth reduction only in the early stages of growth. Single isolates of P. coloratum, P. rostratum and P. vanterpoolii did not significantly reduce seedling growth.

Isolates of P.x (Tables 9.3, 9.4) appeared at times to be associated with improved rather than diminished growth. In compost infested with P.x 811 (Table 9.3) growth was significantly better than the control after 6 wk but the effect was lost after 12 wk. P.x 1060 and P.x 1080 were associated with improved growth after 12 wk which just failed to be significant at the 5% level.

9.4. Discussion

The results showed that some isolates of certain Pythium species can induce poor growth of apple seedlings in compost. Within species there were considerable variations between the pathogenicity of isolates.

The pathogenicity of P. sylvaticum, reported earlier by Mulder (1969) and Sewell (1981) was confirmed in these experiments. Very few reports are available on the pathogenicity of P. heterothallicum. Watanabe et al (1977) reported isolation of heterothallic species from strawberry roots, but the identification of P. heterothallicum was not confirmed by mating. Stephens & Powell (1982)

reported that P. heterothallicum was not pathogenic to a range of plants including Ageratum, Celosia, Salvia, Vinca, cabbage, egg-plant, pepper and tomato. In this study two of three isolates were strongly pathogenic to apple, but could not be readily reisolated from apple roots after vigorous washing. (Re-isolations of P. heterothallicum were obtained with 20-30% frequency compared with 80-90% for P. sylvaticum.)

Some Pythium spp. (P. coloratum, P. intermedium, P. ultimum) that caused severe root necrosis in in-vitro studies (Sect 8) did not cause significant growth reductions in pot-plant studies. Mulder (1969) made similar observations with P. ultimum.

The tendency of many isolates to appear decreasingly harmful over the course of these experiments could be associated with many factors, including, possibly, that steam-sterilised loam may provide a variably hostile environment for Pythium spp. (V.J. Freeman, pers comm and pers obsn).

Table 9.1. (Exp 1) Mean ht (cm for control, % control for treatments) of Worcester Pearmain seedlings in uninfested or Pythium-infested composts, (Repln x 6/x 12. Randomised blocks. Planted 15.5.81.)

Pythium		Soil origin	Wk after planting	
Sp.	No.		6	11
Nil (control)	—	—	<u>15.4</u>	<u>34.1</u>
P. intermedium	725	1980/39	81	75
P. sylvaticum	84	1979/18	88	85
P. sylvaticum	97	1979/18	60 *	75
P. sylvaticum	486	1980/56	79	74
P. ultimum	148	1979/24	65 *	74
P. vanterpoolii	17	PR 117	84	89

* $P < 0.05$

Table 9.2. (Exp 2) Mean ht (cm for control, % control for treatments) of Worcester Pearmain seedlings in uninfested or Pythium-infested composts. (Repln x 6. Latin square. Planted 18.6.81).

Pythium		Soil origin	Wk after planting	
Sp.	No.		6	12
Nil (control)	—	—	<u>15.6</u>	<u>36.8</u>
P. acanthophoron	297	PR 117	85	78
P. heterothallicum	623	1980/6	81	84
P. heterothallicum	730	1980/21	44 **	52 **
P. heterothallicum	798	1980/24	54 **	56 **
P. sp	491	PR 117	77	78

** $P < 0.01$

Table 9.3. (Exp 3) Mean ht (cm for control, % control for treatments) of Worcester Pearmain seedlings in uninfested or Pythium-infested composts. (Rep1n x 6/x 12. Randomised blocks. Planted 29.6.81).

Pythium		Soil origin	Wk after planting	
Sp.	No.		6	12
Nil (control)	—	—	<u>8.6</u>	<u>22.7</u>
P. irregulare	47	1979/16	64 *	74 *
P. irregulare	83	1979/18	90	100
P. sylvaticum	483	1980/56	47 **	32 ***
P. sylvaticum	765	1980/23	52 **	76 *
P. x (type A)	52	1979/17	131	121
P. x (type A)	768	1980/23	131	101
P. x (type B)	811	1980/30	137 *	87
P. sp	491	PR 117	95	82

* $P < 0.05$

** $P < 0.01$

*** $P < 0.001$

Table 9.4. (Exp 4) Mean ht (cm for control, % control for treatments) of Bittenfelder seedlings in uninfested and Pythium-infested composts. (Repln x 7. Latin square. Planted 4.5.82).

<u>Pythium</u>		Soil origin	<u>Wk after planting</u>	
Sp.	No.		6	12
Nil (control)	—	—	<u>17.1</u>	<u>28.0</u>
P. coloratum	996	1981/19	86	111
P. rostratum	1140	PR 117	90	121
P. ultimum	505	PR 117	77	94
P. x (type A)	1089	1981/38	84	106
P. x (type B)	1060	1981/28	99	128
P. x (type B)	1090	1981/40	92	126

GENERAL DISCUSSION

In assays of 137 orchard soils from 12 counties in the South-East, the West-Midlands and eastern England the most frequently occurring and abundant Pythium species were P. heterothallicum and P. sylvaticum (heterothallic), and a presumed heterothallic species (or species-complex) P.x. P. intermedium (heterothallic) and P. irregulare were less frequent but abundant where present. Other species isolated included: P. acanthophoron, P. coloratum, P. dissimile, P. paroecandrum, P. rostratum, P. salpingophorum, P. torulosum, P. ultimum and P. vanterpoolii. P. ultimum was a co-dominant member of the flora of one orchard soil. Among these identified species only three (P. coloratum, P. salpingophorum, P. vanterpoolii) were observed to be zoosporic. The dominance of heterothallic species in orchard soils confirmed Sewell's (1981) less extensive observations; however a similar isolation method (the MPV soil dilution plate) was used in both studies. Pythium floras dominated by heterothallic species were reported from hardwood and mixed hardwood forest soils (but not apple orchard soils) in the South-East United States (Campbell & F.F. Hendrix 1967b; F.F. Hendrix & Campbell 1968a, 1970; F.F. Hendrix et al 1971).

P. sylvaticum occurred with significantly lower frequency in soils from the eastern counties (Cambridge, Essex, Norfolk, Suffolk) than from Kent. P. sylvaticum and P.x occurred less frequently in light-textured (loamy sands to fine sandy loams) than in heavier-textured soils. P. heterothallicum and P. irregulare increased in frequency of occurrence with increasing soil pH; P. sylvaticum occurred with greatly diminished frequency in soils of pH greater than 7.0 (in high pH soils, however, P. sylvaticum was not less abundant when present). All species (except P. heterothallicum) were more abundant in soils of low fertility (as reflected by phosphorus content).

Of the soils assayed, 135 were tested by ADAS at Wye, Kent, for effects on apple-seedling growth of chloropicrin soil fumigation. This enabled studies to be made on effects on the Pythium populations of concentrated apple root growth in small (unsterilised) soil volumes. It also permitted studies of associations between effects of soil sterilisation on apple growth and the Pythium floras.

The growth of apple seedlings in small pot volumes (600 ml) of soil was associated with a mean increase in total Pythium abundance of 58%. The greater increases in abundances occurred with P. sylvaticum (x 4.4), P. heterothallicum (x 2.5) and P. intermedium (x 2.2). Only P. sylvaticum, however, also increased in frequency of occurrence (from 27 to 68%) with the growth of apple seedlings. Within a group of 22 soils commonly assayed before and after seedling growth P. sylvaticum was detected after but not before in 9/16 soils: in six of these nine soils final populations were greater than those in the P. sylvaticum-containing soils assayed before the seedling test. The abundance of P. sylvaticum (and other spp.) was also directly related to amount of seedling growth, except in a class of 'best apple growth' soils where populations of P. sylvaticum were very severely diminished.

Both apple growth per se and amount of apple growth therefore were associated with considerable increases in populations of Pythium species in general, and P. sylvaticum in particular. P. sylvaticum clearly was initially present in many orchard soils (randomly sampled without regard to past or present tree positions) at low, and undetectable, population levels. Such small populations were also clearly capable of rapid or 'explosive' increase in the presence of apple roots. This feature, together with highly variable suppressive effects of soils on Pythium growth (discussed below), may explain why estimates of Pythium populations before the ADAS tests failed to provide a basis for prediction of subsequent effects of soil sterilisation on apple growth. (P. sylvaticum and P. heterothallicum, however, were of slightly more frequent occurrence in soils subsequently giving 'significant' responses to fumigation, and were both more frequent and abundant in poor apple growth soils).

The great differences in Pythium populations in field and post pot-test soils indicated that the incidence of Pythium spp. in soil was strongly influenced by the incidence of roots, and therefore was likely to be of markedly discontinuous and non-uniform distribution in orchard soils. This feature was also indicated during attempts to study seasonal variations in Pythium populations in a restricted area of field soil.

Associations between Pythium populations in soils after the ADAS tests and results of the tests provided further evidence for the involvement of P. sylvaticum in the 'poor-growth disease' of apple. The frequency of occurrence of

P. sylvaticum increased progressively with increasing magnitude of apple growth responses to soil fumigation (i.e. with increasing disease severity). In soils of pH more than 7.0 both incidence of disease and P. sylvaticum were greatly diminished. P. sylvaticum was present in 34/40 soils giving 'significant' fumigation responses; its absence from six soils showed that this species was not solely or universally associated with poor apple growth. P. sylvaticum was also present in 34/55 soils giving insignificant fumigation responses, but in most (30) of these soils responses to fumigation would probably have been partly or wholly prohibited by soil phosphorus deficiency.

The production of diffusible toxin(s), parasitism and pathogenicity of certain isolates of P. sylvaticum were demonstrated in laboratory and glasshouse studies, and the association between incidence of disease and P. sylvaticum in ADAS test soils was very highly significant. The results, however, also showed that P. sylvaticum was not solely causal of poor growth, and that large populations of P. sylvaticum were not invariably associated with severe disease. The latter finding especially indicated that the P. sylvaticum/apple relationship was not a simple one.

The most frequently occurring and abundant Pythium in orchard soils, designated Pythium x, was not greatly increased in abundance by the growth of apple seedlings and its frequency of occurrence was inversely associated with increasing fumigation responses. In laboratory studies, isolates of this species complex caused some inhibition of root growth but were capable of little or no parasitism. In pot-plant studies, isolates were apparently beneficial rather than harmful to the growth of apple seedlings. Members of the P.x complex therefore were considered unlikely to be implicated in ARD.

The role of P. heterothallicum was more equivocal. This species increased in abundance more than two-fold with growth of apple seedlings, occurred with slightly greater frequency in soils giving 'significant' fumigation responses, and was more frequent and abundant in poor apple growth soils. In laboratory studies, P. heterothallicum produced a diffusible toxin significantly inhibiting root growth. The inhibition diminished in strength with time, but this might perhaps be attributable to effects of growth room illumination on a light-sensitive hormone (as IAA). Isolates of P. heterothallicum that were very sparingly (no. 730) or non-parasitic (no. 798) on apple roots were associated with highly significant

seedling growth reductions of 48 and 44% respectively in pot-plant tests. These results indicated that certain Pythium isolates might be associated with pathogenic effects in the absence (or virtual absence) of parasitic ability.

P. heterothallicum was also of particular interest in revealing differential fungistatic effects of soils on the growth of Pythium isolates, and therefore perhaps that the relative pathogenic importance of species might differ in different environments.

The dominance of rapidly growing heterothallic species on MPV soil dilution plates possibly resulted in the relatively few isolations of other (mainly homothallic) spp. P. intermedium (heterothallic) increased in abundance with growth of apple seedlings. This species, together with P. coloratum, P. irregulare, P. paroecandrum and P. ultimum, caused very strong or complete inhibition of root growth, and extensive necrosis, through parasitism, in laboratory studies. P. acanthophoron and P. vanterpoolii were inhibitory but non-parasitic. In pot-plant tests with P. coloratum, P. irregulare, P. rostratum, P. ultimum and P. vanterpoolii, only P. ultimum (during early growth) and P. irregulare (throughout) significantly reduced seedling growth. In other, parallel, pathogenicity tests during 1981 and 1982 isolates of known high virulence caused smaller than expected growth reductions: it is possible therefore that glasshouse conditions or compost batches (completely inhibitory to P. sylvaticum in unreported fungistasis tests) used were inimical to the full expression of pathogenicity. Isolates of several species identified in this study (P. coloratum, P. intermedium, P. irregulare, P. sylvaticum and P. ultimum) were shown by Sewell (1981) to be highly pathogenic to apple seedlings.

The fact that ARD only occurs, and is highly variable, in a proportion of soils with long histories of apple culture (the reason for the ADAS soil testing service) strongly suggests the existence of disease conducive/suppressive soil factors. In these studies certain aspects indicated, for P. sylvaticum, that the host/pathogen relationship was not simple and was subject to strong environmental influence, viz: the absence of growth responses after sterilisation of some soils containing large populations of P. sylvaticum and high levels of phosphorus, indicated disease suppressive soil factors; the greatly diminished frequency of occurrence, but not abundance, of P. sylvaticum in alkaline soils indicated that pH effects were not directly influential: that P. sylvaticum population size

progressively increased with increase of apple growth but dramatically diminished in a group of 'best apple growth' soils indicated the possible presence in those soils of a strongly inhibitory soil factor.

The circumstantial evidence of conducive/suppressive soil effect on ARD and Pythium spp. was supported by the results of soil fungistasis tests. Tests of the effects of diffusible soil factors on the growth of P. sylvaticum, and other species, showed that soils varied from 0-100% in their growth-inhibiting effects. Half of the (54) soils tested caused inhibition of mycelial growth of P. sylvaticum by more than 40%. The incidence of strong fungistasis closely matched several features of the incidence of both P. sylvaticum and ARD. Thus, strong fungistasis occurred with greatly increased frequency in alkaline soils and in 'best apple growth' soils, and was associated with greatly diminished frequencies of occurrence of both P. sylvaticum and large apple growth responses to soil fumigation. This set of associations provided strong supportive evidence for an important causal role of P. sylvaticum in apple replant disease in English orchard soils. It also indicated the importance of (probably microbial) soil factors interacting with and influencing the host/parasitic relationship.

The relative fungistatic properties of (11) soils affected P. sylvaticum, P. intermedium and P.x similarly, but affected P. heterothallicum differently. P. sylvaticum and P. heterothallicum also differed markedly in soil incidence and pathology. These aspects emphasised the importance of recognising specific identity and the inappropriateness of grouping isolates (perhaps particularly the heterothallic spp.) into 'species complexes' as some workers have done (e.g. F.F. Hendrix & Papa, 1974; Watanabe et al 1977).

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