

**STUDIES ON SEED-BORNE PATHOGENS
OF GRAIN LEGUMES.**

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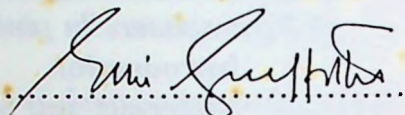
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DECLARATION

I hereby declare that the whole of the work now submitted in this thesis is the result of my own investigations except where reference is made to published literature and where assistance is acknowledged.

Candidate.....

Director of Studies.....

CERTIFICATE

I hereby certify that the work embodied in this thesis has not already been submitted in substance for any degree and is not being concurrently submitted in candidature for any degree.

Candidate.....Quir.....

Date.....28/9/1993.....

DEDICATION

To my family for their patience during my absence from home.

1. GENERAL INTRODUCTION

Species of *Ascochyta* infecting peas

Three species of *Ascochyta* infect peas: *Ascochyta pisi* Lib. causing leaf and pod spot; *Ascochyta pinodes* Jones, causing blight; *Ascochyta pinodella* Jones, causing foot rot.

Ascochyta pisi, which has no known teliomorph, attacks only aerial tissues (leaves, stems, flowers, pods and seeds). *Ascochyta pinodes* (teliomorph *Mycosphaerella pinodes* (Berk. & Blox.) Vestergr.; synon. *Didymella pinodes* (Berk. & Blox.) Petrak) attacks all aerial parts and also causes a foot rot. *Ascochyta pinodella* (now usually referred to as *Phoma medicaginis* var *pinodella* (Jones) Boerema) causes a foot rot but is less conspicuous on aerial parts; like *Ascochyta pisi* no teliomorph is known (Dixon, 1981; Lawyer, 1984).

Distinguishing between the three species in the field is not easy since the disease syndrome incited by *M. pinodes* embraces the symptoms induced by *A. pisi* and *P. medicaginis* var *pinodella* (Dixon, 1981). In culture, however, they are more easily differentiated. *P. medicaginis* var *pinodella*, unlike the other two species, produces pycnidiospores which are predominantly non-sepate; the bi-celled pycnidiospores of *A. pisi* are slightly larger than those of *A. pinodes* ($14 \times 4.2 \mu\text{m}$ compared to $12.3 \times 4.5 \mu\text{m}$) and the spore masses (cirrhi) are carrot red rather than buff. Both *M. pinodes* and *Phoma medicaginis* var *pinodella* produce chlamydospores in the hyphae while *A. pisi* does not. (Noble 1971). *A. pinodes* produces perithecia (pseudothecia) readily in culture and provides a clear distinction from the other species (Lawyer, 1984).

Of the three species, *M. pinodes* is certainly the most important. It was noted as causing severe damage to the pea crop in New York in 1925 (Jones, 1927) and was considered the most important pathogen in the pea crop in Wisconsin (Hare and Walker, 1944). Wallen (1964) regarded it as the most important pathogen of peas in the USA and also reported that it was the

principal pathogen found on pea seed in Canada (Wallen, 1967). In Britain, *M. pinodes* and *A. pisi* were reported as the most important whilst *P. medicaginis* var *pinodella* was unimportant (Maude, 1965). *M. pinodes* is potentially a very destructive pathogen capable of reducing yields by 50-75% (Lawyer, 1984). It is widely distributed throughout the world (Fig.1.1) and is particularly important in temperate areas of N.America, Europe, Australia and New Zealand where peas are grown extensively (Carter, 1959; Wallen, Wong and Jeun, 1967; Lawyer, 1984), but it has also been reported from sub-tropical areas of Africa, Central and South America (Lawyer, 1984).

Mycosphaerella Pinodes

M. pinodes, a homothallic fungus with a chromosome number of 15 (Warmelo, Van 1966), has been described in detail (Fig.1.2) and on the characteristics of its perithecia (pseudothecia) and asci is, apparently, better placed in the genus *Didymella* as *Didymella pinodes* (Berk. & Blox.) Petrak (Sivanesan, 1984; Jellis & Punithalingam, 1991).

The bi-celled pycnidiospores ($8-16\ \mu\text{m} \times 3-6.6\ \mu\text{m}$; average $12.3\ \mu\text{m} \times 4.5\ \mu\text{m}$) are produced in globose, light brown or black, ostiolate pycnidia ($100-200\ \mu\text{m}$ in diam.) (Fig.1.2). They are discharged from the pycnidium in a gelatinous cirrhus when the pycnidium is moistened. Pycnidiospores, which have a thin gelatinous coating that enables them to adhere to surfaces on which they are placed, are dispersed by rain splash.

The perithecia are, like the pycnidia, which they superficially resemble, globose, dark brown, with papillate ostioles and measure $90-180\ \mu\text{m}$ in diam. The bitunicate asci ($50-80\ \mu\text{m} \times 10-15\ \mu\text{m}$) contain eight 2-celled ascospores, which measure, on average, $7.9-17.2\ \mu\text{m}$. When perithecia are moistened the outer membrane of the ascus ruptures at the apex, the inner membrane stretches, and the ascospores move towards the apex. Finally, the inner membrane bursts and the ascospores are forcibly ejected through the ostiole for a distance of 2-3 mm (Hare & Walker, 1944), or up to 5 mm (Carter, 1963). Thereafter they may be wind dispersed over long distances.

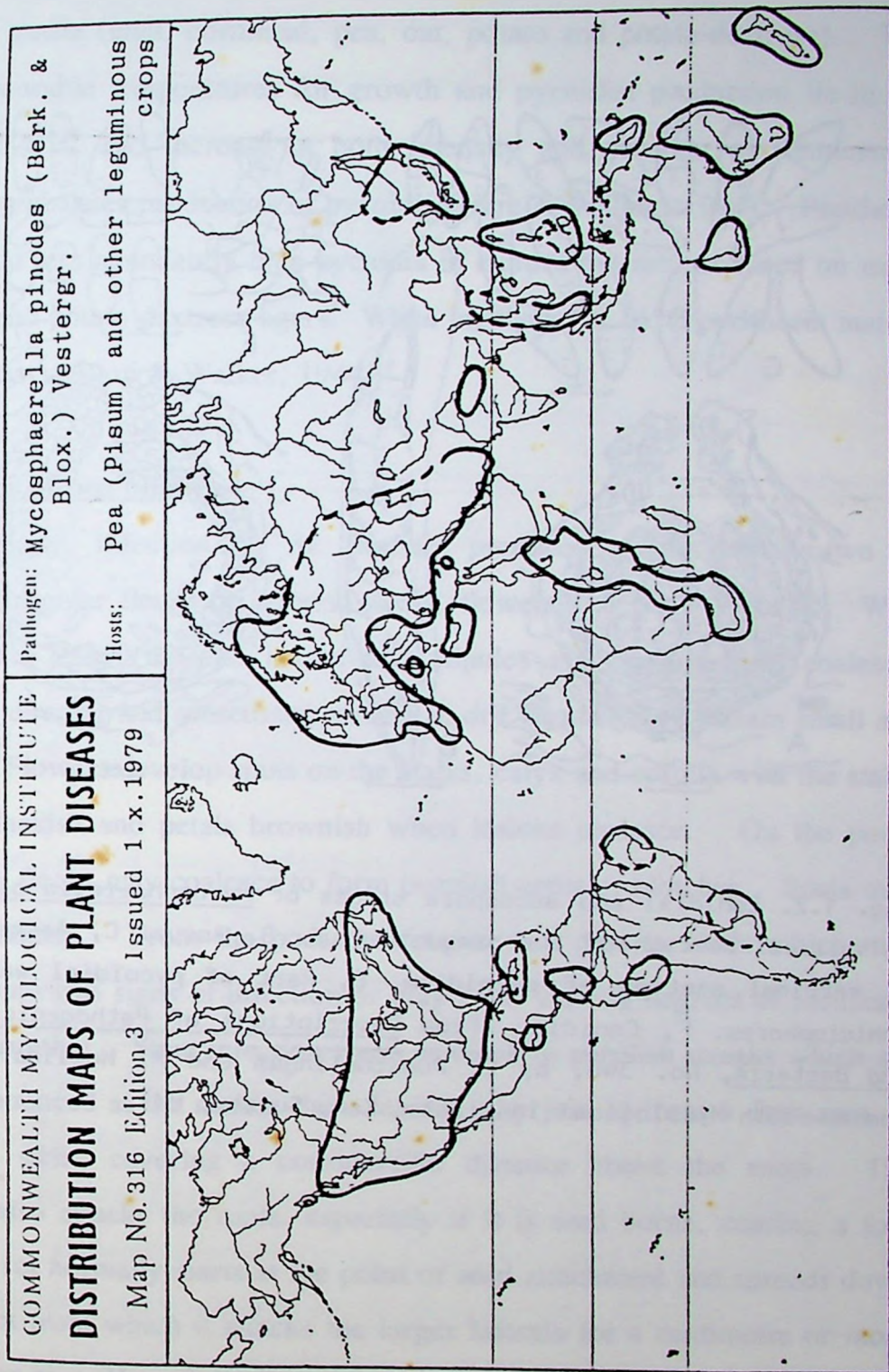


Fig. 1.1. Distribution of *Mycosphaerella pinodes*.

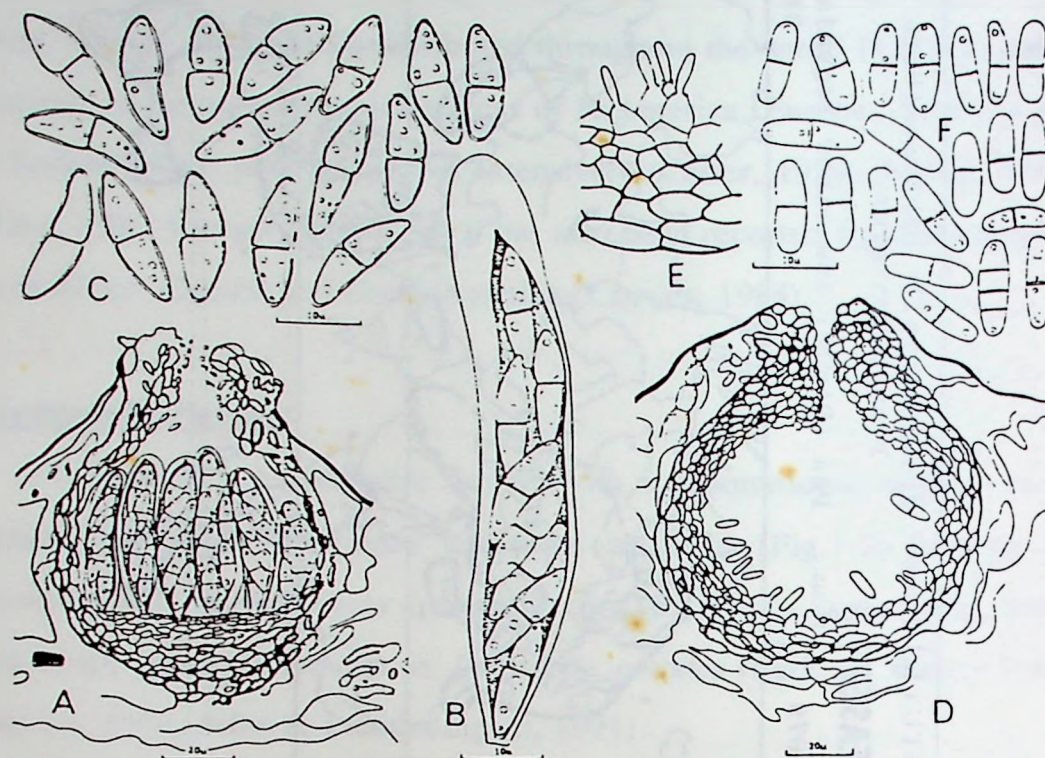


Fig. 1.2. Conidial and ascospore stages of Mycosphaerella pinodes. A, Vertical section of the pseudothecium. B, Ascus. C, Ascospores. D, Vertical section of pycnidium. E, Part of pycnidial wall and conidiophores. F, Conidia. (From Descriptions of Pathogenic Fungi and Bacteria, No. 340, by E. Punithalingam and P. Holliday, 1972 Commonwealth Mycological Institute, Kew, Surrey, UK).

In culture, *M. pinodes* forms light to dark grey colonies which are often zonate in appearance. Pycnidia are rapidly and abundantly produced on a wide range of media (malt, cornmeal, pea, oat, potato and potato-dextrose). The most favourable temperatures for growth and pycnidial production lie in the range 20-28°C and increase in both intensity and duration of illumination markedly enhances production of pycnidia (Hare & Walker, 1944). Perithecia are formed less abundantly than pycnidia in culture but are produced on malt, oat, pea and potato-dextrose agars. When incubated at 16°C perithecia mature in 25-30 days (Hare & Walker, 1944).

Symptoms of pea blight

Initially, infection by *M. pinodes* produces small, dark brown or purplish irregular flecks on leaves, stems, flowers and pods (Plate 1). With heavy rain, lesions on the leaves and stipules may enlarge and coalesce, becoming circular and sometimes zonate; in dry weather they remain small and distinct. Flowers develop spots on the stalks, calyx and corolla with the stalks turning blackish and petals brownish when lesions coalesce. On the pods, very many flecks may coalesce to form purplish areas or blotches. Spots may become sunken. From the pod, the fungus may pass to seeds which may show no outwards signs of infection or may show varying degrees of shrinkage and discoloration. Stem infection starts as black to purplish streaks which are more pronounced at the nodes; these lesions may join to form a dark area on the entire stem, covering a considerable distance above the roots. The pathogen also attacks the roots, especially if it is seed borne, causing a foot rot. The rot normally starts at the point of seed attachment and spreads down the tap root from which it attacks the larger laterals for a centimetre or more from the tap root (Hare & Walker, 1944).

Epidemiology

There are four means of transmission of *M. pinodes*: water splash of conidia, carriage of ascospores in air currents, survival between pea crops in

soil and plant debris (as chlamydospores, sclerotia, mycelium and spores), and via seed (Dixon, 1981). As a result of diversified ways of survival the disease has become endemic in pea growing areas (Lawyer, 1984).

On pea debris, the pathogen survives well as a saprophyte and, even if the debris is buried, it competes well with other soil saprophytes. The pathogen can survive for a long time in the soil in the absence of its host, retaining its pathogenicity, as evidenced by the isolation of a virulent isolate from soil in which peas had not been grown for 20 years (Wallen, Wong & Jeun, 1967).

On land completely free from the pathogen, infection of the crop may arise from two sources; infected seed and wind blown ascospores.

As infected seed germinates, lesions develop at the point of cotyledonary attachment, causing a foot-rotting symptom along the length of the developing plumule and radicle. Diseased seedlings may die before emergence, but if emergence is achieved, lesions become evident on the above-ground portions. Pycnidia developing on these lesions provide inoculum for infection of adjacent plants.

Infection by pycnidiospores on healthy plants is by direct penetration of germ tubes through the cuticle into the cell walls (Lawyer, 1984; Clulow, Lewis & Matthews, 1991; Nasir, Hoppe & Ebrahim-Nesbat, 1992) but information on the methods of penetration by ascospores is not available. However, whether infection is by pycnidiospores or ascospores, symptoms appear in 2-4 days. Pycnidia develop rapidly on lesions but spread of the disease by rain splashed pycnidiospores is slow and localised. The subsequent appearance of perithecia on senescent tissues, however, usually brings a marked change in the progress of the disease since production of airborne ascospores permits widespread distribution of the disease. Indeed, the development of an epidemic is dependent upon seasonal conditions favouring maturation of perithecia and the release, transportation and infection of ascospores deposited on plant surfaces. Prolonged dry weather retards perithecial maturation whilst it is favoured by intermittent dry and wet periods.



Plate 1.1. Symptoms of pea blight (*Mycosphaerella pinodes*). A. Early symptoms: spotting on leaves, stipules, stems and pods. B. Late symptoms: necrosis of stipules, tendrils and prominent stem lesions. C. lesions on pods. Basal stem lesions.

Ascospore release requires moisture and Carter & Moller (1961) found that 0.2 mm of rain was the minimum amount required to initiate ascospore discharge. Many experiments have shown that if perithecia are kept dry, no ascospores are discharged.

Wind is important in ascospore dispersal. Hare & Walker (1944) caught 8 ascospores per cm² at a distance of 300 m from infected pea plants. It has been suggested (Lawyer, 1984) that spores may be carried for 1.6 km or even more. Curiously, temperature variation does not seem to have a marked effect on the epidemiology of pea blight. Temperature had little effect on perithecial production nor on discharge of ascospores which occurred between 0° and 37°C (Hare & Walker, 1944). Equally temperature did not influence the time required for ascospore discharge which occurred immediately at all temperatures from 0° and 37°C once perithecia were moistened.

Control of Pea Blight

(i) Chemical Control

Since all three *Ascochyta* species can be seed borne it is not surprising that chemical control has focused on seed treatment. Good control of seed infection of all three pathogens has been achieved, initially with formulations of thiram and captan (Maude, 1965; Wallen *et al.*, 1967) and more recently with the systemic fungicide, thiabendazole (Bretag, 1985).

The impact of seed treatment on the diseases caused by the three *Ascochyta* species is not, however, equally good. It has been most effective against *A. pisi*, which, surviving poorly on debris in or on the soil, is very dependent on seed transmission. As a consequence of moving seed production to drier climates and the universal adoption of seed treatment "the importance of *A. pisi* in the major pea producing areas has become largely historical" (Lawyer, 1984). Seed treatment also gives good protection against soil borne infection by chlamydospores of *P. medicaginis* var *pinodella* which is enhanced when rotations are employed.

M. pinodes is more recalcitrant. Effective seed treatment removes only one source of infection; airborne infection by ascospores derived from plant debris or from nearby infected crops poses a hazard which is little affected by seed treatment. Control using five foliar sprays of thiabendazole has been reported by Bretag (1985) but was considered to be uneconomic.

(ii) Cultural Practices

Rotations, in areas where peas are a major crop every year, do not significantly reduce infection caused by *M. pinodes* (Lawyer, 1984) but may, of course, help in controlling other diseases. Deep ploughing, as soon after harvest as possible, has been strongly advocated to diminish survival of pathogens on debris (Carter & Moller, 1961; Sheridan, 1973).

(iii) Resistant Cultivars

In view of the difficulties of controlling *M. pinodes* by chemical and cultural practices, the possibility of exploiting host resistance has received considerable attention, although, to date, with little success.

Screening of commercial cultivars has regularly failed to reveal a significant level of resistance (Jones, 1927; Hare & Walker, 1944; Ali *et al.*, 1978; Lawyer, 1984; Zimmer & Sabourin, 1986). Not uncommonly seedling resistance has been observed but this declines with maturity. More encouraging results have been obtained recently. Clulow (1989) observed resistance in both stems and leaves in some pea lines obtained from primary (Central Asia) and secondary (East Mediterranean) centres of origin.

Evaluating resistance is complicated by the existence of pathotypes of *M. pinodes*. Clulow, Lewis and Matthews (1991) examined 45 isolates from widely varying geographic locations. Based on nine differential host lines, these could be classified, by stem symptoms, into 9 groups or, by leaf symptoms, into 16 groups. The existence of variation of this kind is likely to make breeding resistant cultivars difficult in view of the possibility (? probability) of the emergence of resistance-breaking pathotypes. The

alternative approach of seeking more broad ranging resistance ('horizontal resistance', *sensu* Vanderplank, 1963) may offer the prospect of durable resistance.

Scope and Aims of the present study

1. To provide a better understanding of the biology of *M. pinodes* by examining some aspects of the environmental component of its epidemiology; in particular leaf wetness, temperature and humidity.
2. To evaluate partial resistance in present-day commercial cultivars as a possible basis for future breeding work.
3. To investigate the possible use of free-radical scavengers, some of which are natural compounds, in the control of pea blight.
4. To study the mode of infection of both pycnidiospores and ascospores (using Scanning Electron Microscopy) in order to improve knowledge about the infection processes in *M. pinodes*.

2. GENERAL MATERIALS AND METHODS

(i) Isolation and culture of *M. pinodes*

The isolate of *M. pinodes* used in all experiments reported in this thesis was derived from infected peas (cultivar unknown) provided by the National Institute of Agricultural Botany (NIAB), Cambridge, UK.

Seeds were soaked in 10% Chlorox solution for 10 mins, washed in two changes of sterile water and briefly dried in filter paper before transferring them to agar plates. Two media, Malt Extract Agar and Potato Dextrose Agar (both Lab M) with and without streptomycin sulphate (100 ppm), were used initially and plates were incubated in the laboratory (20 - 22°C) under fluorescent lights. *M. pinodes* colonies grew readily on both media and, since bacterial contamination was not a problem there was no benefit from use of streptomycin. Other fungi (*Botrytis*, *Penicillium*, *Fusarium*) occurred occasionally but there was no difficulty in sub-culturing to fresh plates and obtaining cultures of *M. pinodes*, free from contamination.

Several media were subsequently tested in order to select one which optimised production of both pycnidia and perithecia. These included three proprietary media (Czapek-Dox Agar (Oxoid); Potato Dextrose Agar (Lab M); Malt Extract Agar (Lab M) and two 'home made' media (Potato Dextrose Agar prepared according to the formula of Hare & Walker (1944) and Oat Agar (3g oatmeal powder, 15g agar, 1l water)).

Plates were inoculated with mycelial plugs (7.6mm diameter) cut from colonies of *M. pinodes* grown on PDA (Lab M) and incubated under three different regimes: 16°C dark, 24°C dark and 24°C in a NUV (near ultra violet light) cabinet with a 16h 'day'. Good growth was obtained on all media but was faster at 16°C than 24°C. No pycnidia were formed on colonies grown without illumination but there was profuse pycnidial development in illuminated cultures. Perithecia and ascospores were only found on 'home made' PDA in cultures incubated in darkness at 16°C. However, PDA (Lab M) was used routinely because of convenience in preparation.

Cultures of *M. pinodes* were regularly passed through pea leaves to prevent loss of virulence. Following inoculation with pycnidiospores pea leaves rapidly develop lesions from which the fungus can be re-isolated using the same procedures used for isolations from seed except that sterilisation with 10% Chlorox is for 5 rather than 10 min.

(ii) Preparation of pycnidiospore suspensions

Large quantities of pycnidiospore suspensions were needed for inoculation of field plots. Cultures on PDA were grown under illumination (usually on the laboratory bench under a single bank of fluorescent tubes) for 10-14 days by which time they were about 60mm in diameter and sporulating profusely. Plates were flooded with distilled water and the colony surface scraped gently with a scalpel. The suspension produced was filtered twice through four layers of muslin to remove mycelial fragments and then centrifuged twice at 3000 rpm for 5 min, each time decanting the supernatant and resuspending the spores in distilled water. Spore concentrations were determined with a haemocytometer and adjusted to the required level. For inoculation of plants, 0.025% 'Tween 20' was added to facilitate wetting of leaves.

(iii) Collection of ascospores

Although ascospores occupy a central role in the epidemiology of *M. pinodes*, there is little specific information about them. This seems to be due to the technical problems of capturing sufficient numbers to carry out experiments. Perithecia and ascospores are produced in culture but somewhat erratically and not in great numbers. Potentially the best source of ascospores is infected pea haulm from which, following wetting, large numbers of ascospores are freely discharged. However, they still have to be captured. Use of an Andersen sampler (Plate 2.1) (kindly loaned by Dr. John Hedger, Department of Life Sciences, UCW Aberystwyth), enabled this to be achieved

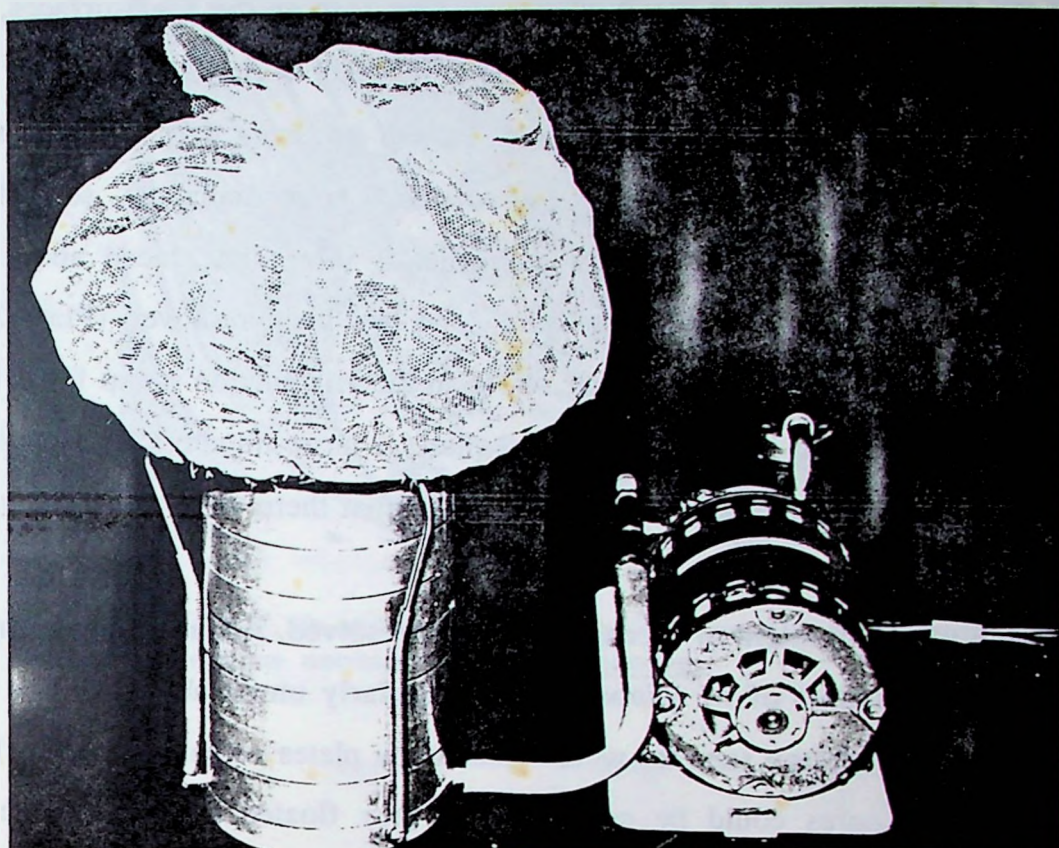


Plate 2.1. Andersen sampler and pump (right) with moistened pea debris in nylon net in position used for capturing ascospores of M. pinodes.

The Andersen sampler allows air to be drawn through a stack of six circular plates, perforated with holes of progressively smaller size and below which are placed petri dishes. Particles are impacted on the agar surfaces, the largest particles at the top and the smallest at the bottom of the stack. Dry pea haulm, infected with *M. pinodes* and known to have large numbers of perithecia, was tied in a nylon net bag (holes 0.1mm diameter), soaked for about 5 min, allowed to drain for a further 5 min. and placed above the Andersen sampler from which the nose cone had been removed (Plate 2.1). Air was drawn through the sampler using an electric suction pump for 2 min. Using PDA plates it was quickly found that (a) large numbers of ascospores were collected in the upper four dishes and (b) that there were remarkably few contaminating organisms.

With the problem of ascospore capture resolved, it was disappointing to find that ascospores of *M. pinodes* were singularly unwettable. When agar plates in the Andersen sampler were replaced by plates containing water, large numbers of spores could be collected but they floated on the surface and aqueous suspensions of ascospores could not be prepared using non-lethal concentrations of surfactant ('Tween 20'). Consequently, for plant inoculation, a different approach had to be devised (see below). However, the Andersen sampler proved invaluable for studies on effects of temperature on ascospore germination (Section 3.3.1), on effects of humidity on ascospore survival (Section 3.3.2) and in capture of ascospores for scanning electron microscopy (Section 6).

(iv) Use of a dust-bin as a sedimentation tower

Because of the difficulty of making aqueous suspensions of ascospores for plant inoculation an alternative approach was used in which a galvanised steel dust-bin served as a sedimentation tower. The dust-bin was 550mm high and 450mm in diameter at the top, tapering to 400mm at the base. Potted plants to be inoculated, or petri dishes containing detached leaves or agar

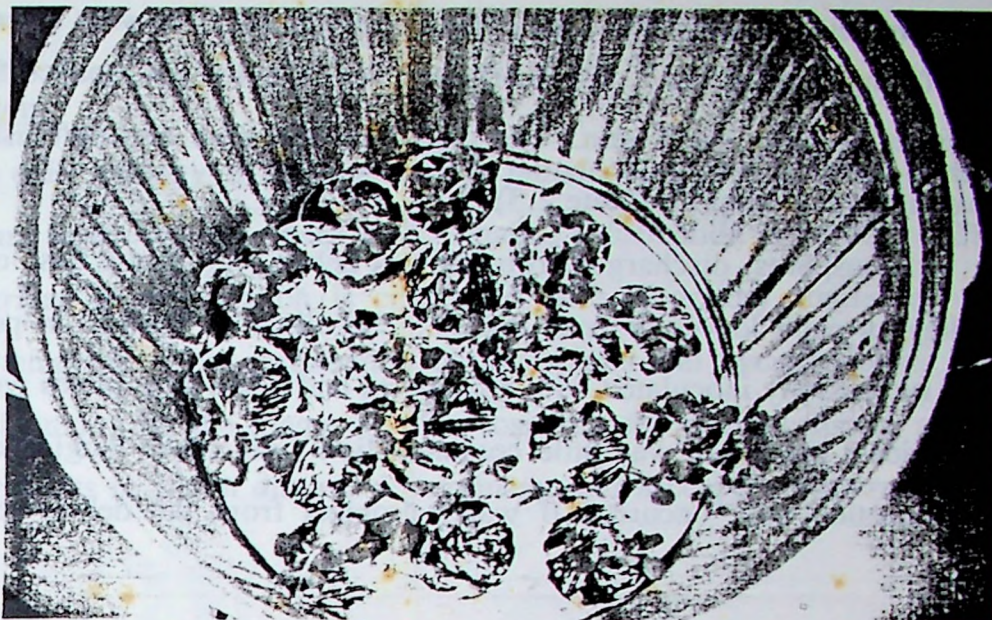


Plate 2.2. Dust bin with potted pea seedlings in position to receive ascospores of M. pinodes.



Plate 2.3. Dust bin with moistened pea debris in position above nylon netting for discharge of ascospores of M. pinodes.

media were placed at the bottom of the bin (Plate 2.2). The top was covered with wire netting over which was a layer of nylon net to prevent large particles from falling into the bin. Pea debris was tied in nylon net, soaked for 5 min. and after draining for 5 min. was placed on the wire netting/muslin before covering with the bin lid (Plate 2.3).

Ascospores, discharged from the pea debris, fell to the base of the bin, landing on plant leaves or petri dishes. Seedlings or leaflets in petri dishes were successfully inoculated with ascospores and when agar plates were used it was evident that there was little contamination from other fungi and bacterial contamination only occurred if water droplets from the debris reached the plates.

Sadly, the experiments for which this equipment was developed failed for other reasons and have not been reported in the thesis although they are briefly referred to in the discussion at the end of Section 3.3.3.

3. STUDIES ON ASPECTS OF THE EPIDEMIOLOGY OF *MYCOSPHAERELLA PINODES*

Introduction

The epidemiology of *Mycosphaerella pinodes* is known in broad outline but there are many aspects where more detailed information is needed. The work described in this section is concerned with laboratory studies on (a) effects of temperature on germination of ascospores and pycnidiospores, (b) effects of humidity on the longevity of ascospores and pycnidiospores and (c) field experiments in which disease development and ascospore dispersal were monitored.

3.1 EFFECT OF TEMPERATURE ON GERMINATION OF PYCNIDIOSPORES AND ASCOSPORES OF *MYCOSPHAERELLA PINODES*

Introduction

Effects of temperature on mycelial growth, pycnidial and perithecial development in culture have been reported by Hare & Walker (1944). They found that mycelial growth and pycnidial production was greatest at c. 24°C, whilst perithecial production was greatest at c. 16°C. Sorgel (1956) found that the optimum temperature for germination of pycnidiospores was c. 24°C but no detailed studies have been reported on effects of temperature on germination of ascospores, despite their relevance to the epidemiology of pea blight.

Materials and Methods

Spore suspensions (10^5 spores ml⁻¹) were prepared from 14 day old cultures. Droplets (20μl) were placed on the surface of 1% water agar (Oxoid Agar No.2) contained in small (50mm diam.) Petri dishes. The droplets were spread over the agar surface so that individual spores were well separated.

After inoculation plates were placed on a thermogradient bar assembly (Plate 3.1). This consisted of four metal bars (64cm long x 5cm wide) the ends of which passed into thermostatically controlled water baths, one maintained at 35°C and the other at 3°C. Each bar could accommodate six 50mm Petri dishes which were placed such that temperatures across the diameter of the dish were 4°, 10°, 16°, 22°, 28° and 34°C. The four bars served as replicates.

In a series of experiments, germination was recorded after, 3, 6, 9, 12 and 15h. Fresh samples of spores were used on each occasion. At the end of each time interval spores were killed by introducing a small amount of formalin into each dish.

Germination was assessed on spores across the diameter of a dish corresponding to the temperature mark on the bar when it was subdivided.

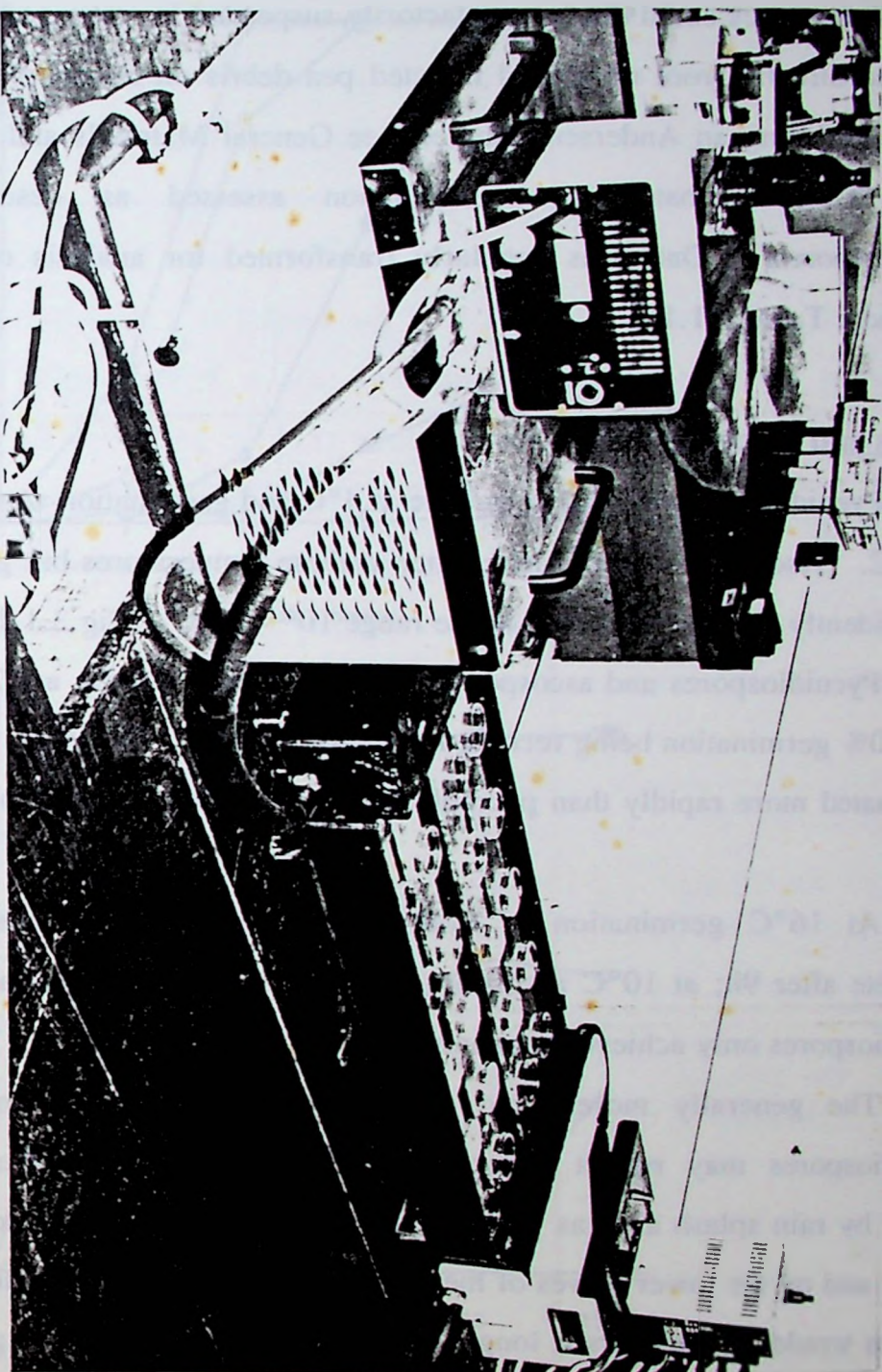


Plate 3.1.1. Thermogradient bar assembly; cooling unit on left and heating unit on right. Petri dishes are shown spaced on each of the four thermogradient bars

This minimised effects of temperature variation within the dish. Spores were considered germinated if their germ tubes were equal to or exceeded the diameter of the cells from which they developed. A total of 200 spores were counted per dish.

Ascospores could not be satisfactorily suspended in water and they were collected directly from moistened infected pea debris on 50mm plates of 1% water agar using an Andersen sampler (see General Materials and Methods). Plates were incubated and germination assessed as described for pycnidiospores. Data was angularly transformed for analysis of variance (Appendix Table 3.1.1).

Results and Discussion

Pycnidiospores did not germinate at 4°C and germination was very poor at 34°C. Ascospores germinated better at these temperatures but germination was evidently much poorer than in the range 10° - 28°C. (Fig.3.1.1).

Pycnidiospores and ascospores germinated most rapidly at 22° and 28° C, 100% germination being recorded for both after 6h. However, ascospores germinated more rapidly than pycnidiospores, c. 90% compared to 30% after 3h.

At 16°C germination of both pycnidiospores and ascospores was complete after 9h; at 10°C ascospore germination was complete after 9h but pycnidiospores only achieved maximum germination after 12h.

The generally more rapid germination of ascospores compared to pycnidiospores may reflect differences in dispersal. Pycnidiospores are spread by rain splash and, as pointed out by Lawyer (1984) tend to be spread locally and on the lower leaves of the pea vine canopy. In these situations, leaf wetness would tend to persist longer and the need for rapid spore germination would be less acute. By contrast, ascospores, being wind dispersed, tend to be deposited on upper leaves; these dry out more quickly and hence speedier germination would facilitate infection.

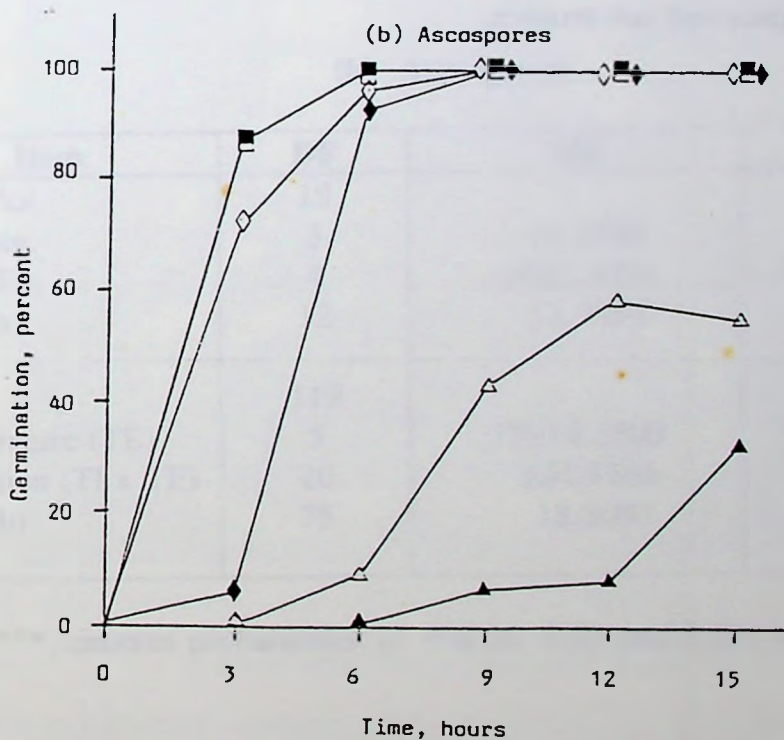
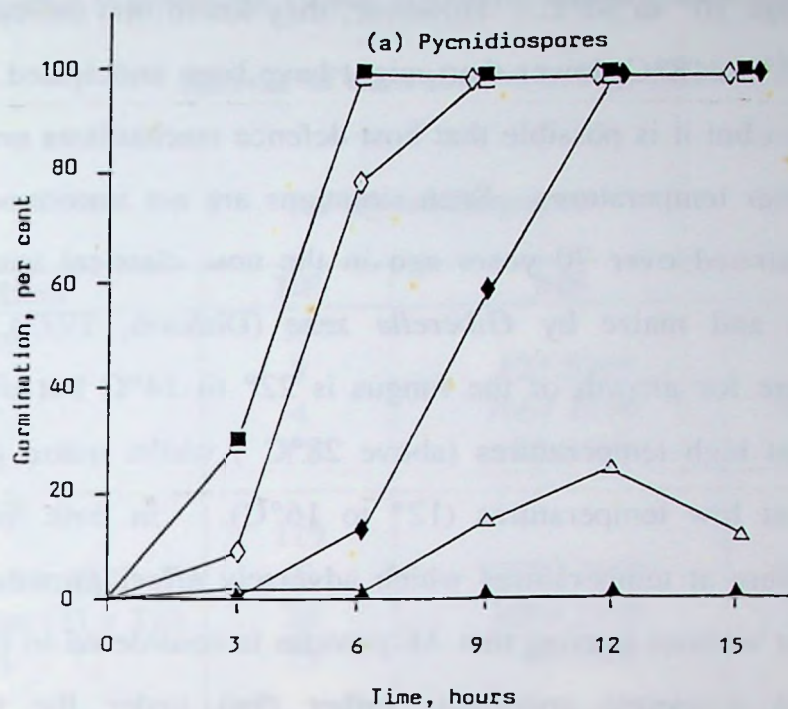


Fig. 3.1.1. Effect of temperature on germination (% untransformed) of (a) pycnidiospores and (b) ascospores of *M. oinodes*.
 (▲, 4°C; ◆, 10°C; ◇, 16°C; ■, 22°C; ◻, 28°C; △, 34°C)

These observations would seem to indicate that temperatures within the range 10° and 28°C would be favourable for infection and, indeed, Wallen *et al.*, (1967) reported that infection of pea seedlings occurred over a similar temperature range viz. 10° to 30°C. However, they found that the optimum for infection was 15° to 18°C, lower than might have been anticipated. The reasons are unknown but it is possible that host defence mechanisms are more effective at the higher temperatures. Such situations are not uncommon and was clearly demonstrated over 70 years ago in the now classical studies of infection of wheat and maize by *Giberella zeae* (Dickson, 1923). The optimum temperature for growth of the fungus is 22° to 24°C but wheat is severely damaged at high temperatures (above 28°C) whilst maize is most severely damaged at low temperatures (12° to 16°C). In both instances damage is most severe at temperatures which adversely affect growth of the host. It is also not without interest that *M. pinodes* is considered to be most serious under cool temperate conditions rather than under the warmer conditions of the tropics and sub-tropics.

Appendix Table 3.1.1

Effect of temperature on germination of pycnidiospores and ascospores of *M. pinodes*. (Percentage values angularly transformed before analysis).

Anovar of data shown in Fig. 3.1.1

(a) Pycnidiospores

Item	DF	MS	VR
Main Plot	19		
Replicate	3	127.5365	5.0727 *
Time (TI)	4	7667.2190	304.9575 ***
Error (a)	12	25.1419	
Total	119		
Temperature (TE)	5	19509.3600	485.8766 ***
Interaction (TI x TE)	20	1009.5330	25.1422 ***
Error (b)	75	40.1529	

(b) Ascospores

Item	DF	MS	VR
Main Plot	19		
Replicate	3	16.2396	1.1680 NS
Time (TI)	4	5842.3050	420.1995 ***
Error (a)	12	13.9036	
Total	119		
Temperature (TE)	5	19910.1800	1058.5360 ***
Interaction (TI x TE)	20	524.9266	27.9080 ***
Error (b)	75	18.8092	

*, **, ***, denotes probabilities of < 0.05, 0.01 and 0.001 respectively.

3.2 EFFECT OF HUMIDITY ON SURVIVAL OF PYCNIDIOSPORES AND ASCOSPORES OF *MYCOSPHAERELLA PINODES*

Introduction

The effect of humidity on the survival of pycnidiospores and ascospores of *M. pinodes* after their dispersal, but before the onset of favourable conditions for their germination, are unknown. However, previous studies on other fungi (Page, Sherf & Morgan, 1947; Teitell, 1958 and Griffiths & Peverett, 1980) have shown that survival of spores is much influenced by relative humidity. In the three experiments described here, both pycnidiospores and ascospores of *M. pinodes* were exposed to a range of relative humidities, and for varying periods of time, before providing them with favourable conditions for germination.

Materials and Methods

Aqueous suspensions of pycnidiospores (5×10^5 spores, ml^{-1}), prepared from 14 day old cultures, were sprayed on to cellophane sheets using an atomiser (Humbrol Ltd., U.K.). The sheets were allowed to air dry before transfer to humidity chambers (below). For ascospores, an Andersen sampler was set up in the normal way except that cellophane circles replaced agar in the petri dishes. Ascospores, discharged from infected pea stubble, were collected on the cellophane circles (see General Materials and Methods).

Humidity chambers were made from large (1.5l) glass jars with tightly fitting lids ('Kilner' preserving jars). Each jar contained 200ml of a water/glycerol mixture, different proportions of glycerol and water providing different known relative humidities (Carson, 1931; see Appendix Table 3.2.2) at the temperature of 25°C which was used in all experiments.

Strips of cellophane (c. 2.5 x 1cm) bearing pycnidiospores or ascospores were cut from the cellophane sheets or circles and fixed to long strips of paper which were suspended in the humidity chambers such that the bottom edge of a paper strip was 2cm above the level of the glycerol/water

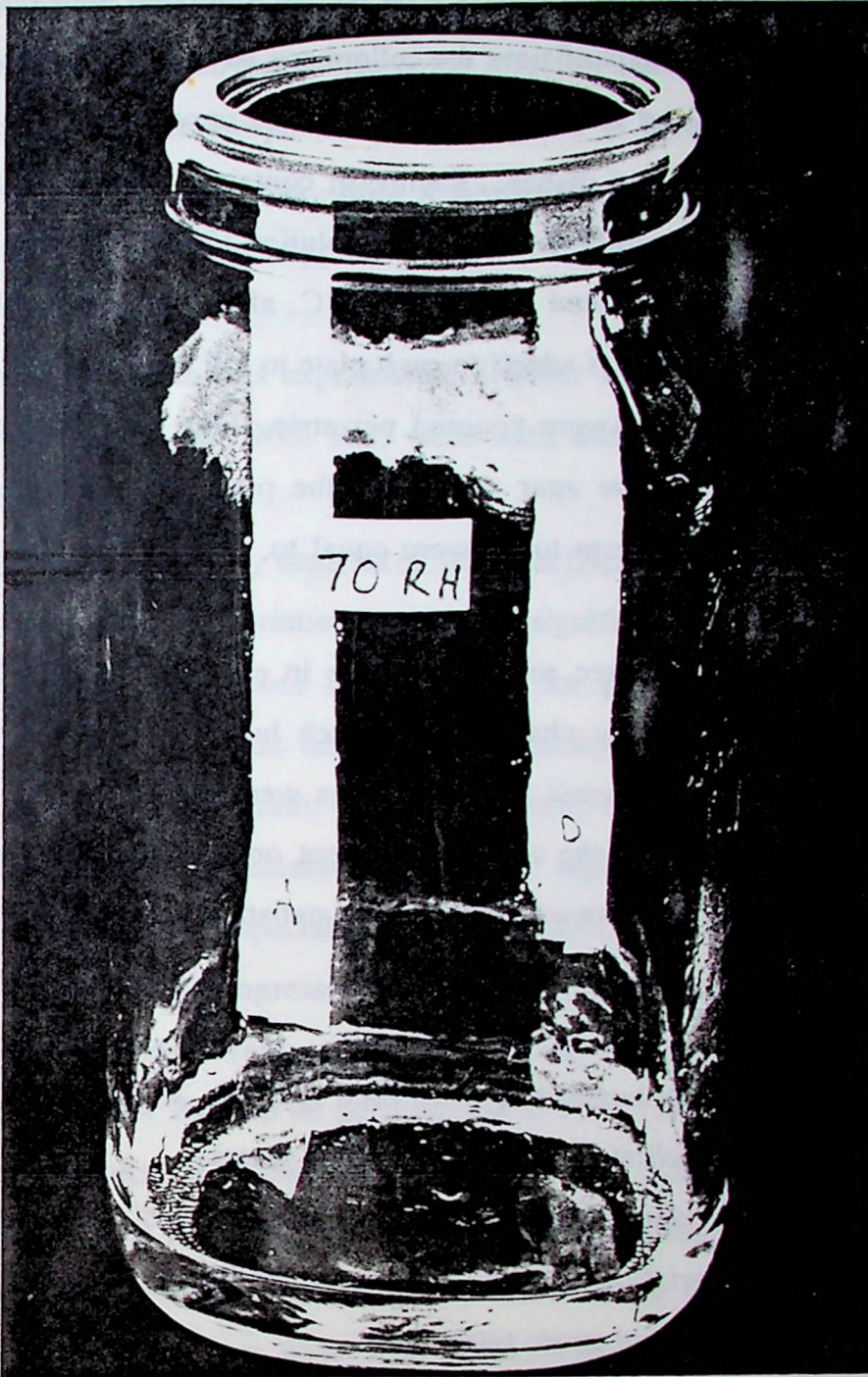


Fig. 3.2.1. Humidity jar with paper strips (A,P) and cellophane strips with spores attached at the bottom ends. Bottom edges of the cellophane strips are 2 cm above the glycerol-water mixture.

mixture. After inserting the paper strips, the humidity chambers were sealed with parafilm and put in an incubator at 25°C (Plate 3.2.1).

After different periods of time the cellophane strips were removed from the humidity chambers and placed (spore bearing side uppermost) on 1% water agar in 90mm diameter petri dishes. Each dish contained 10ml agar to which had been added 5 μ l of streptomycin sulphate solution (50mg ml⁻¹) to suppress bacteria. Plates were incubated for 12h at 25°C, after which a small amount of formalin (37-41% w/v) was added to each plate to kill the spores.

Two hundred spores were counted per strip along their long axes and whilst they were still on the agar surface in the petri dishes. Spores were considered germinated if germ tubes were equal to, or exceeded the diameter of the spore.

Experiment 1 had three replicates while in experiments 2 and 3 there were four replicate humidity chambers for each humidity. In the first two experiments both pycnidiospores and ascospores were placed in each chamber (i.e. a split-plot design); in the third experiment only ascospores were used. Percentages were angularly transformed before statistical analysis of the data.

Results

Experiment 1. Four humidities were used (30, 50, 70 and 90% RH) and germination was recorded after 12, 24, 48 and 72 hours.

Analysis of the data (Appendix Table 3.2.1) showed that there was no significant replicate variation but that there were highly significant differences attributable to time, RH and spore type.

Up to 12h RH had little effect on pycnidiospores; at this time ascospore germination was best at 30% RH but there was little difference at the higher humidities (Fig. 3.2.1).

At 24, 48 and 72h pycnidiospore germination declined progressively and the decline was hastened by increasing RH so that after 48h no germination was found at 90% RH. Ascospore germination showed a similar pattern

though the detrimental effect of high humidity was apparent much earlier; after 24h germination was nil at 70 and 90% RH (Fig. 3.2.1).

In view of the obvious differential effect of humidity and its duration on the viability of pycnidiospores and ascospores it is not surprising that the interactions, time x humidity, time x spore type and humidity x spore type, were all highly significant (Appendix Table 3.2.1).

Experiment 2. In this experiment, a wider range of humidities (10, 30, 50, 60, 65, 70, 75, 80, 90% RH) were used and an additional time interval (36h) was incorporated.

As in experiment 1, analysis of the data (Appendix Table 3.2.1) showed no significant replicate variation but highly significant differences attributable to time, humidity and spore type. Data for pycnidiospores (Fig. 3.2.2.) showed a somewhat erratic response to humidity. Up to 24h, whilst the highest rate of germination (c. 80%) occurred at the lowest humidity (10% RH) germination remained reasonably high (50-70%) up to 80% RH before falling steeply (especially at 24h) at 90% RH. For the longer time intervals there was a progressive decline with both increasing humidity and increase in the time interval. After 36, 48 and 72h there was less than 20% germination at 80% RH and no germination at 90% RH.

The response of ascospores (Fig. 3.2.3) was more clearly defined. At low humidities (10 and 30% RH) there were high rates of germination at all time intervals. Thereafter, except for the 12h interval, germination fell steeply and was virtually zero at 65% RH. Germination then increased so that between 65 and 90% RH (when germination was again zero except for the 12h interval) there was a peak at 80% RH for the 12, 24 and 36h time intervals. This was such a curious result that a further experiment was carried out with ascospores, to examine again the behaviour of these spores at the higher range of humidities.

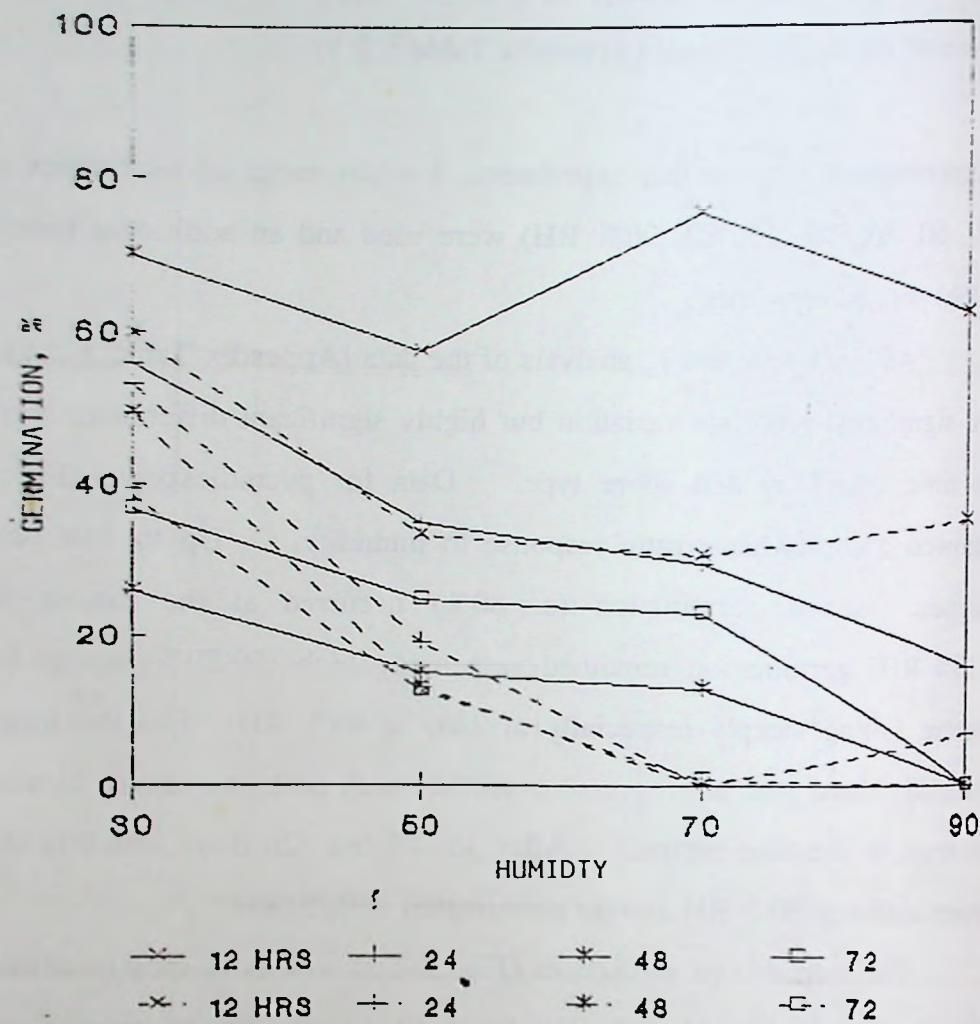


Fig. 3.2.1. Effect of relative humidity (%) on survival of pycnidiospores (solid lines) and ascospores (broken lines) after 12, 24, 48 and 72 h at 25°C.

Experiment 3. Seven humidities (50, 60, 65, 70, 75, 80, 90% RH) and four time intervals (24, 36, 48, 72 hours) were examined. Analysis of the data (Appendix Table 3.2.1) revealed highly significant effects of time and humidity but replicate variation was small and not significant. In this experiment (Fig. 3.2.4) there was again, following a decline between 50 and 60% RH (for all time intervals) an increase with a peak at 80% RH for the 24h but not for the 36h time interval.

Discussion

The longevity of both pycnidiospores and ascospores of *M. pinodes*, after dispersal is evidently much affected by humidity. Both survived longest at low humidity (<30% RH); at high humidity (90% RH) there was virtually complete loss of viability after 24h. In general pycnidiospores were less sensitive than ascospores to the deleterious effects of increasing humidity, particularly in the range 30 - 70% RH.

These results are similar to those obtained with pycnidiospores of *Septoria nodorum* (Griffiths & Peverett, 1980). They pointed out that, not only are low humidities rare in the UK, where average values are rarely below 60% RH (Anon, 1952), but that higher values would tend to occur within crop canopies. As a consequence they concluded that few spores of *S. nodorum* would remain viable after 24h. The results obtained here point to a similar conclusion for both pycnidiospores and ascospores of *M. pinodes*. It may be further argued that, since dispersal of both types of spore require rainfall, high humidities would tend to occur post dispersal, even if leaf wetness was of short duration.

An explanation for the damaging effects of high humidity has been put forward by Tervi & Mochizuki (1947; cit. Sussman & Halvorson, 1966). At high humidity respiration of spores (*Aspergillus spp.*) as measured by oxygen uptake, was found to be much enhanced and it was argued that reserves are more rapidly exhausted. This explanation would explain the positive

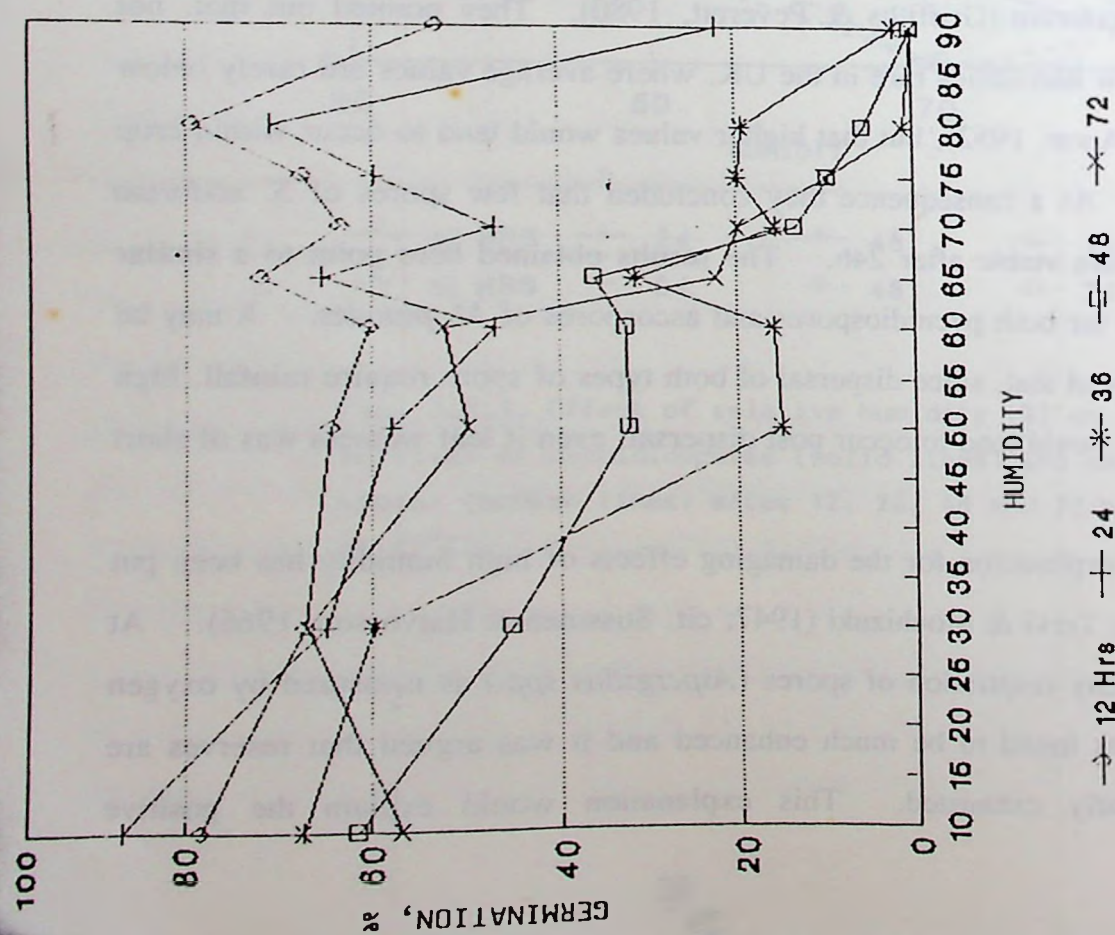


Fig. 3.2.2. Effect of relative humidity (%) on survival of pycnidiospores (Experiment 2)

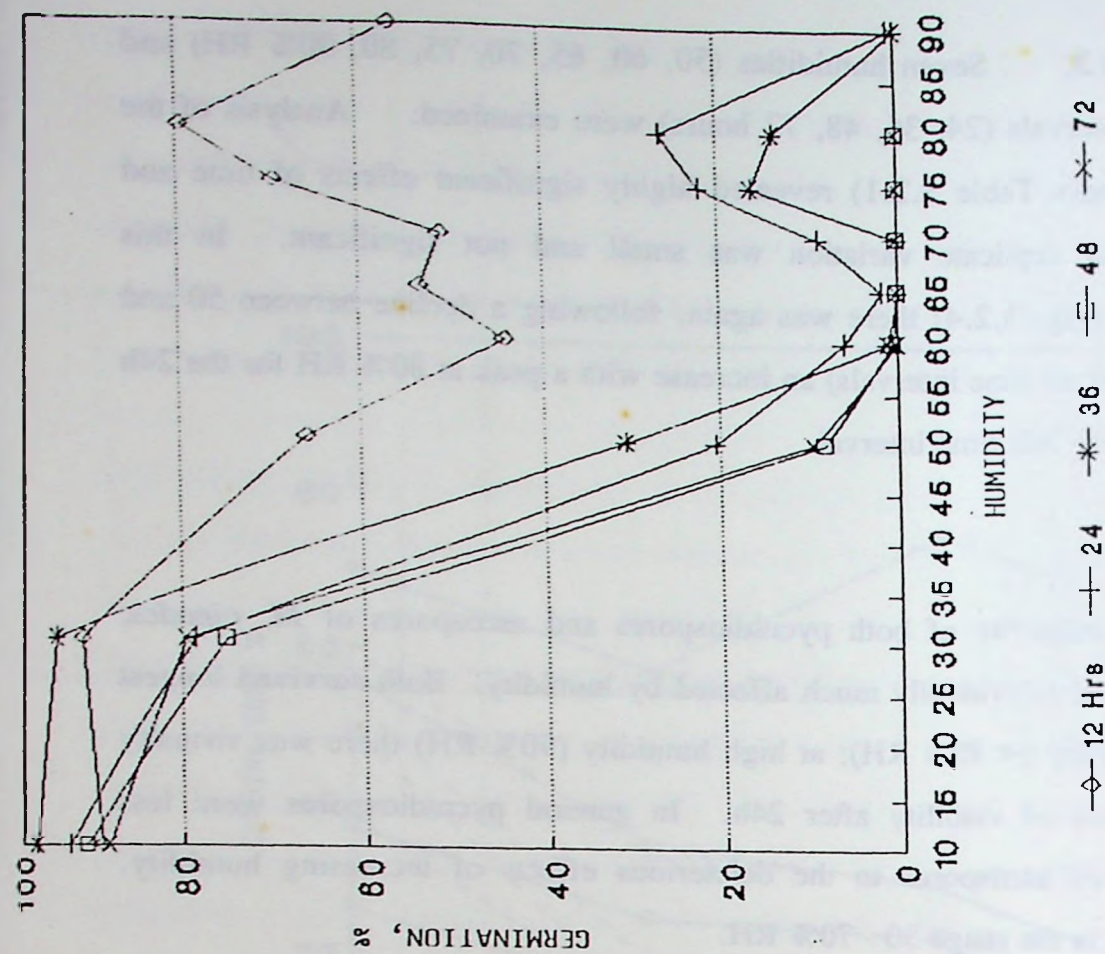


Fig. 3.2.3. Effect of relative humidity (%) on survival of ascospores (Experiment 2)

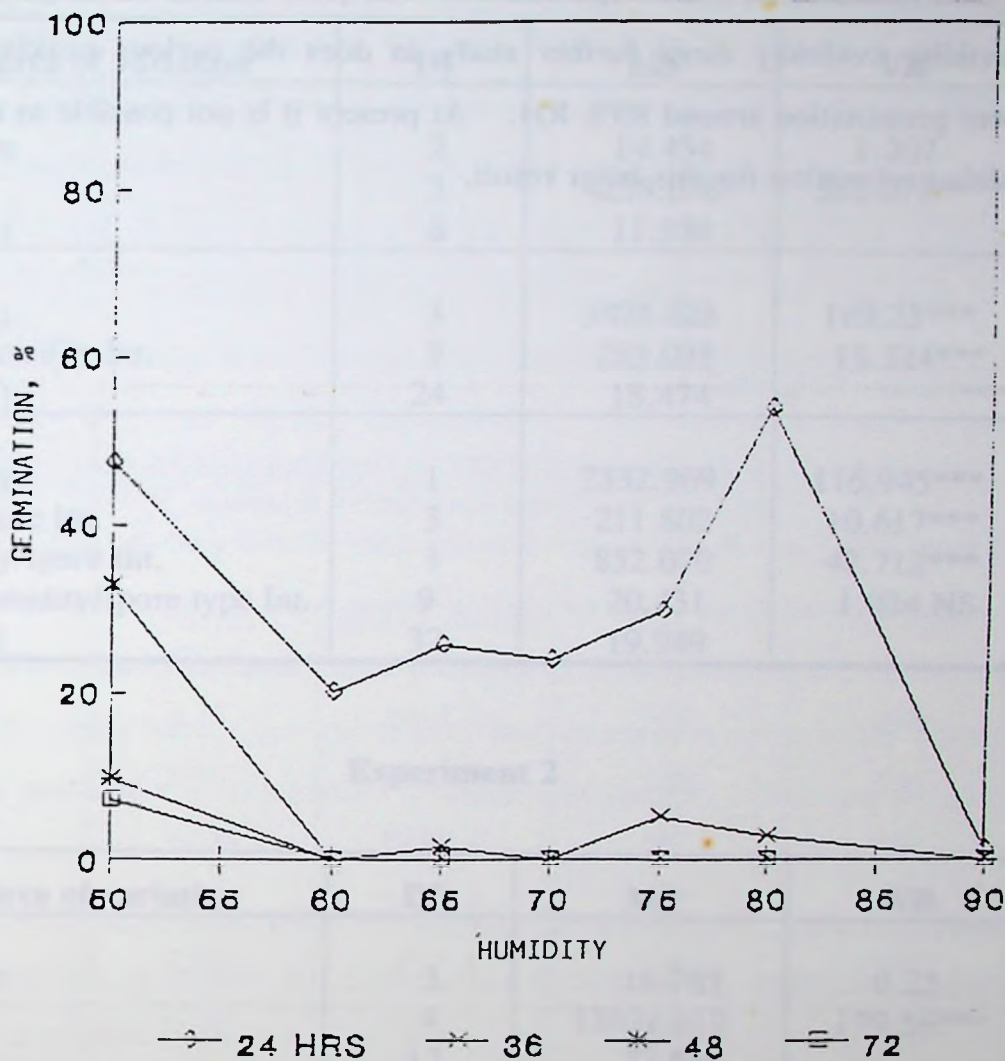


Fig. 3.2.4. Effect of high relative humidity (%) on survival of ascospores (Experiment 3)

interaction of humidity with temperature noted by Page *et al.*, (1947) who examined longevity of spores of *Helminthosporium oryzae*.

Only one temperature (25°C) was used in the present study and at normal field temperatures spores of *M. pinodes* would probably survive for longer than indicated in these experiments. The joint effects of temperature and humidity evidently merit further study as does the curious peaking of ascospore germination around 80% RH. At present it is not possible to offer a plausible explanation for this latter result.

Appendix Table 3.2.1.

Analyses of germination (% angles) of pycnidiospores and ascospores of *M. pinodes* in three experiments.

Experiment 1.

Source of variation	Df	MS	VR
Replicate	2	14.434	1.207
Time	3	4258.078	356.076***
Error (a)	6	11.958	
Humidity	3	3495.828	189.23***
Time/humidity Int.	9	283.095	15.324***
Error (b)	24	18.474	
Spore type	1	2332.969	116.945***
Time/spore Int.	3	211.802	10.617***
Humidity/spore Int.	3	852.070	42.712***
Time/humidity/spore type Int.	9	20.431	1.024 NS
Error (c)	32	19.949	

Experiment 2

Source of variation	Df	MS	VR
Replicate	3	16.765	0.22
Time	4	13931.619	179.56***
Error (a)	12	77.586	
Humidity	8	11803.314	249.81***
Time/humidity Int.	32	654.062	13.84***
Error (b)	120	47.250	
Spore type	1	8772.637	221.15***
Time/spore type Int.	4	1529.563	38.56***
Humidity/spore type Int.	8	2743.618	69.16***
Time/humidity/spore type Int.	32	138.968	3.50***
Error (c)	135	39.668	

Appendix Table 3.2.1 continued.

Experiment 3.

Source of variation	Df	MS	VR
Replicate	3	5.662	0.261
Time	3	4936.585	227.162***
Error (a)	9	21.732	
Humidity	6	1081.788	64.708***
Time/humidity Int.	18	170.269	10.185***
Error (b)	72	16.718	

Appendix Table 3.2.2.

Combinations of glycerol and water to produce specific relative humidities at 25°C in closed containers (Carson, 1931).

Humidity % RH	Glycerol % Wt	Specific Gravity (SG)	Glycerol % Vol	Water % Vol
10*	95*	1.245*	75.6	5.0
30*	89*	1.229*	70.8	11.0
50*	79*	1.203*	62.8	21.0
60*	72*	1.184*	57.3	28.0
65*	68.5	1.176	54.5	31.5
70*	64*	1.162*	50.9	36.0
75	58.5	1.150	46.5	41.5
80*	51*	1.127*	40.6	49.0
90*	33*	1.079*	26.3	67.0

SG of Glycerol = 1.257

* Figures by Carson (1931); others by calculation.

3.3 FIELD EXPERIMENTS, 1991

Introduction

Two field experiments, examining aspects of the epidemiology of *Mycosphaerella pinodes*, were carried out in 1991.

The first experiment was designed to examine the effects of additional irrigation periods on disease development. The epidemic was initiated by artificial inoculation with pycnidiospores (the only source of inoculum in this experiment) but disease progress after inoculation was so slow that it seemed possible that the experiment might have to be abandoned. For this reason the decision was taken to sow a second experiment of identical design and to re-inoculate the plants in the first experiment. This second inoculation led to successful establishment of the disease which subsequently developed rapidly.

By the time the plants in experiment 2 were ready for inoculation, it was apparent that, because of the frequency of rainy days, the planned programme of irrigation treatments could not be satisfactorily implemented in experiment 1. Also, there were clear indications that ascospores were being liberated from the pea haulm in experiment 1 which was nearby. The impact of this external source of wind-blown inoculum on disease development in experiment 2 was of obvious interest and it was decided to abandon the planned irrigation programme and to exploit the opportunity of monitoring ascospore dispersal using simple spore traps.

Materials and Methods

Experimental design

Both experiments were located in a field at Frongoch Experimental Farm near Aberystwyth. The experimental areas were ploughed during the winter and rotavated prior to marking out the plots and sowing. Experiment 1 was sown on 25 April and experiment 2 on 16 June. In each experiment there were 16 plots, 2m x 2m, separated by 0.5m paths. Each plot was sub-divided to accommodate two cutlivars, Orb and Conquest, semi-leafless and standard.

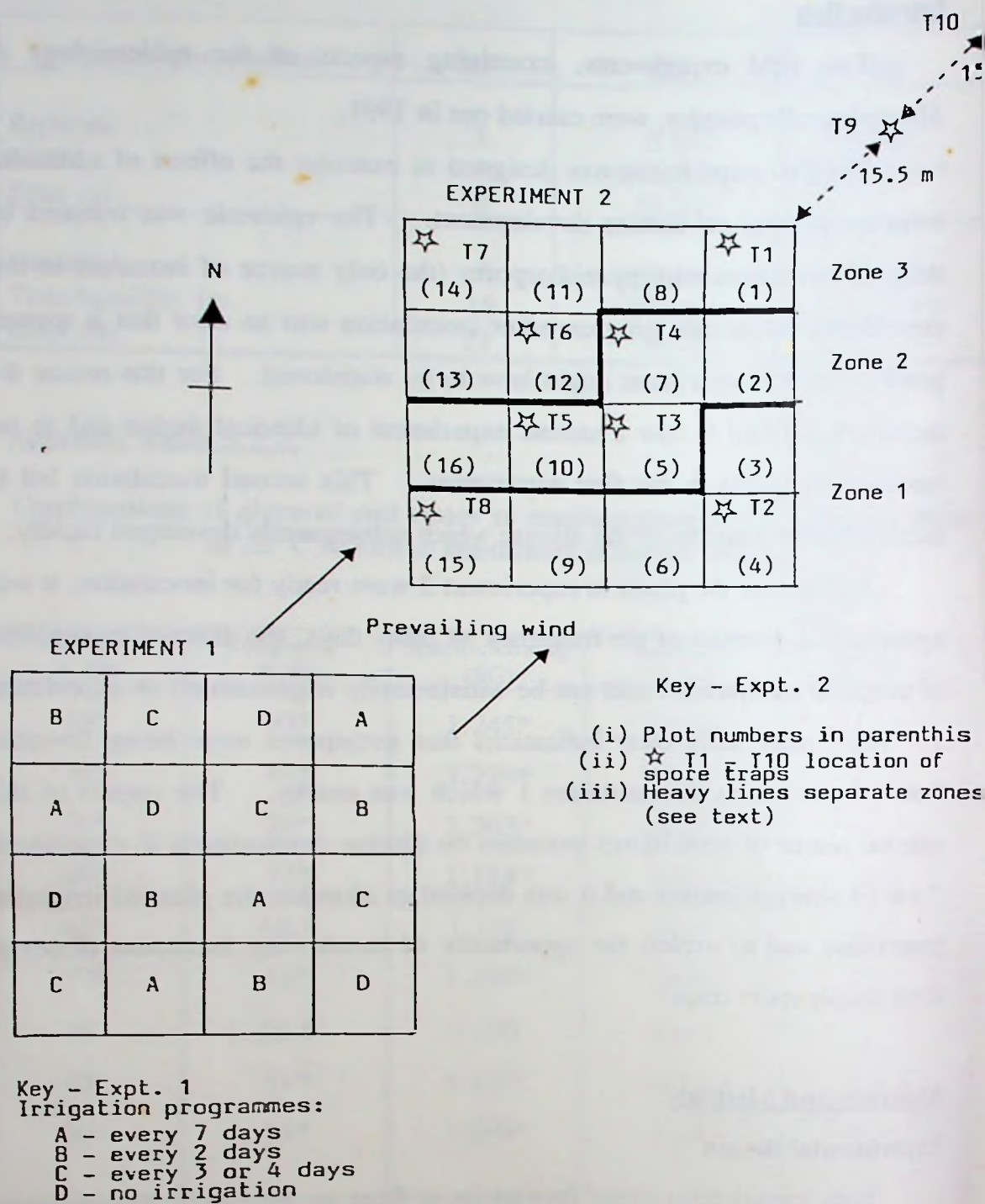


Fig. 3.3.1. Arrangement and orientation of field experiments at Frongoch Experimental Farm, 1991.



Plate 3.3.1. General view of field plots at Frongoch, 1991.
Dead haulm of plots of Expt. 1 in foreground: Expt. 2 with
spore traps beyond.



Plate 3.3.2. View of plots of Expt. 2, Frongoch, 1991.

respectively (Plate 3.3.3). Seeds were sown 5cm apart in row 20cm apart so that in each sub-plot there were 5 rows, each with 40 seeds. The layout of the plots in both experiments together with their orientation is shown in Fig. 3.3.1 (Plate 3.3.1, & 3.3.2)

Inoculation

Suspension of pycnidiospores (5×10^5 spores ml⁻¹) were prepared as described earlier (General Materials and Methods) and Tween 20 added at a rate of 250µl per litre. Plants were inoculated at growth stage 104 (Biddle, Knott and Gent, 1988) with 250ml spore suspension per plot using a pneumatic knapsack sprayer. In experiment 1 a second inoculation was carried out 3 weeks later. Prior to inoculation plots were irrigated for about 1 hour using reciprocating sprinklers. Inoculation was always done in the evening to maximise the duration of wetness.

Disease recording

In experiment 1, three assessments were made (6 July, 24 July & 6 August) when plants were at growth stages 111, 118 and 120 respectively (Biddle *et al.*, 1988). In experiment 2, five assessments were made (2 August, 16 August, 27 August, 8 September and 15 September).

One plant was removed for assessment from each of the three centre rows of each sub-plot but excluding the five plants at the end of each row. There were approximately 30 plants available for assessment in each row and to avoid bias, plants to be assessed were identified using random number tables and tagged at an early stage of the experiment.

For each plant, assessments of necrosis were made on the leaves, stipules, stems and pods. The proportion of necrotic tissue was assessed visually on leaves (tendrils of the semi-leafless cv. Orb), stipules and pods (when present) at each node. On stems, necrotic areas were assessed on individual internodes. The assessments were expressed as disease

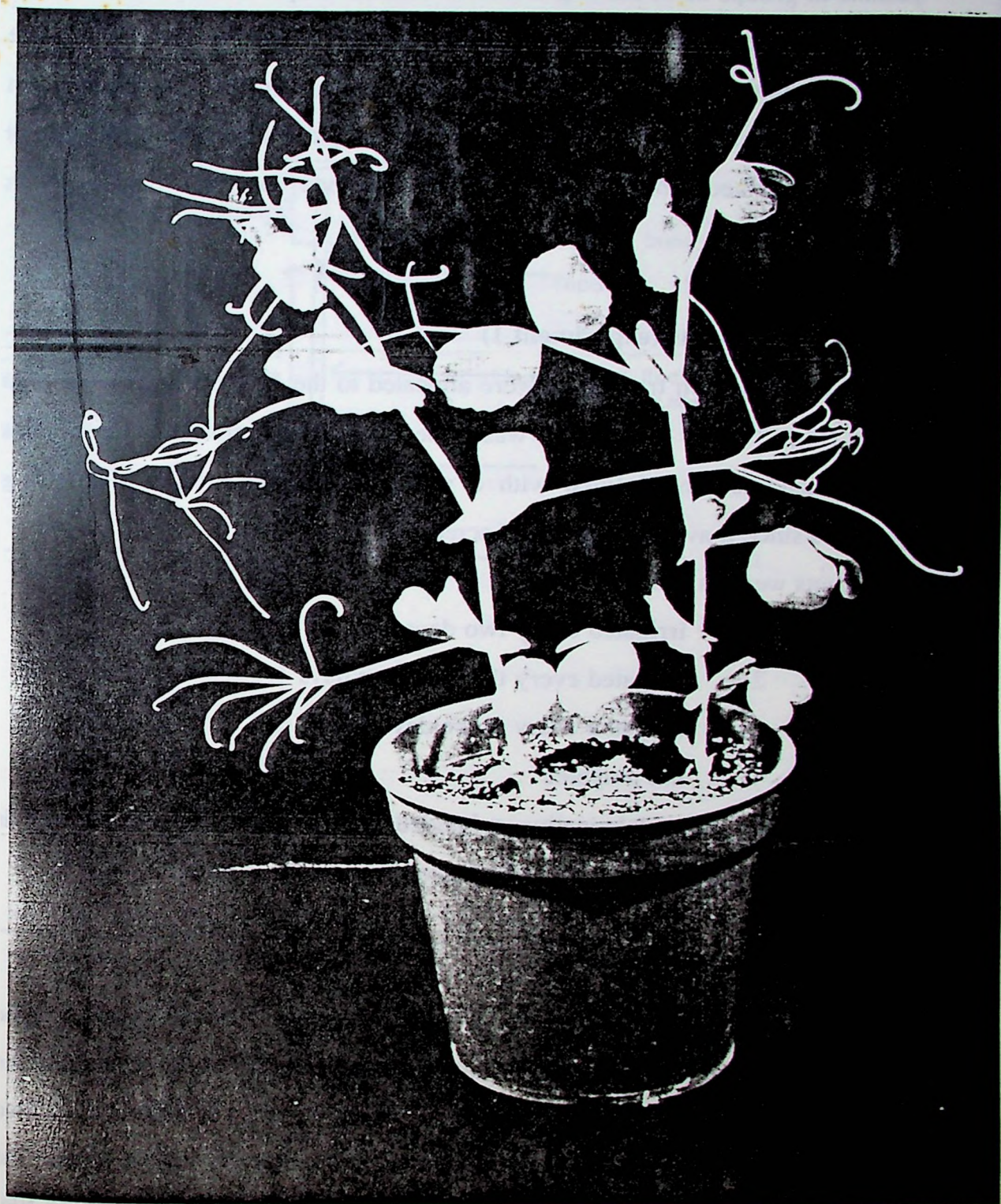


Plate 3.3.3 Seedlings of semi-leafless cultivar Orb (left) and standard cultivar Conquest (right).

scores, 0 - 4 (0 = 0%; 1 = 1 - 24%; 2 = 25 - 49%; 3 = 50 - 74%; 4 = 75 - 100%).

In experiment 1 individual nodes were arranged in groups (as far as possible in groups of 5) starting from the base of the plant. Mean scores for 'node groups' enabled a comparison between cultivars to be made of disease progress from the base to the top of the plant. In experiment 2, a simple mean per plant was calculated from all the scores at individual nodes; a sub-plot mean was derived from the mean score per node for each of the plants sampled.

Irrigation treatments (experiment 1)

Four irrigation treatments were allocated to the 16 plots as a 4 x 4 latin square (Fig. 3.3.1). Irrigation was done by watering plots for 15 minutes using a hand-held hose fitted with a rose. This was simpler than using sprinklers since it avoided the use of screens.

Treatments were as follows:

1. irrigated every two days
2. irrigated every three or four days
3. irrigated every seven days
4. not irrigated

Where a planned irrigation coincided with a day on which rain fell it was omitted. The irrigation programme commenced on 3 June.

Spore traps

The spore traps used (Fig. 3.3.2) were the vertical cylinder type (Gregory, 1973) made from a cylindrical wooden rod, 27mm in circumference and 500mm long. On the rod, double-sided 'Selotape' was used to hold two pieces of clear Polythene, each 16 x 27mm, which were wrapped around the rod, 150 and 450mm from its base. A 200 x 200mm polystyrene ceiling tile, which acted as a rain guard, was fixed to the top of the rod using a rubber bung.

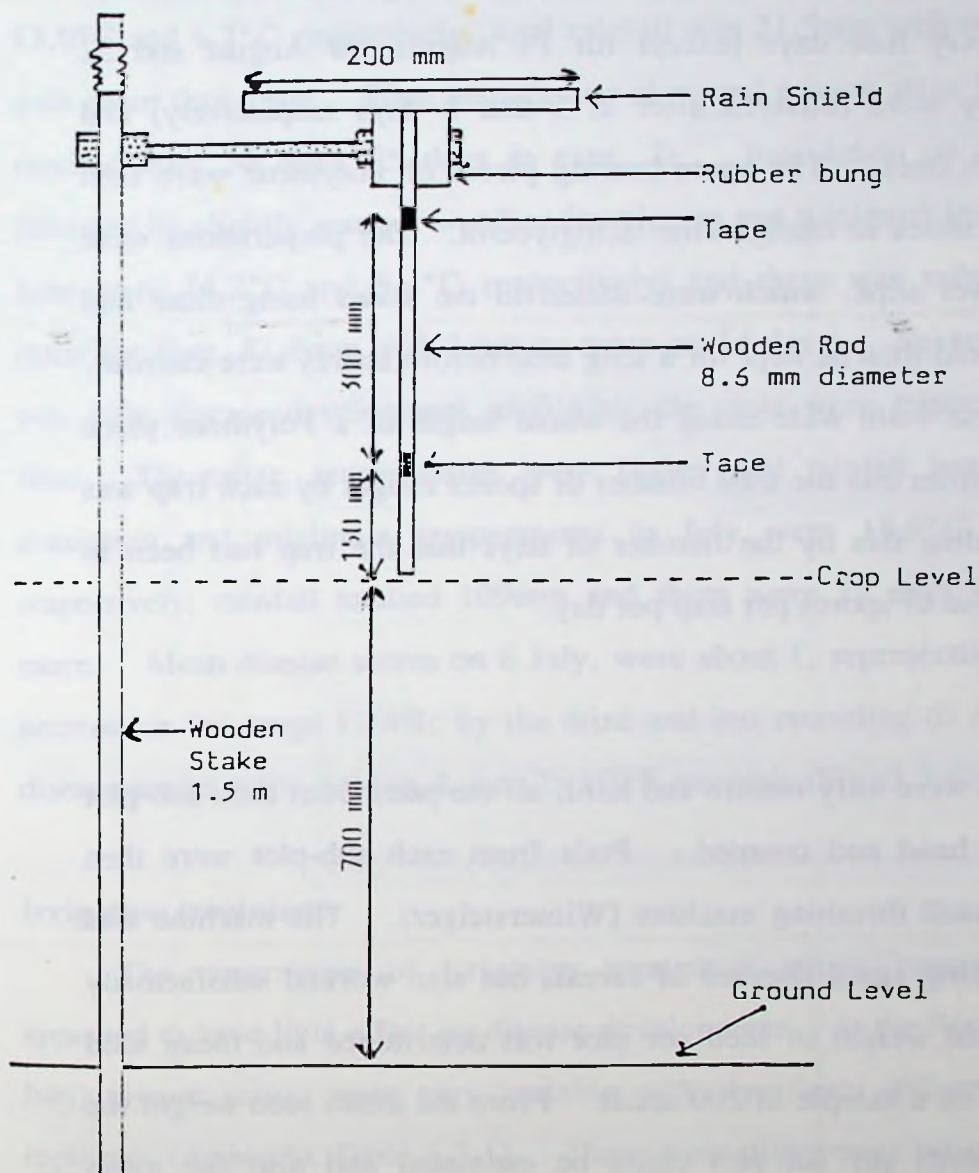


Fig. 3.3.2. Design of spore traps used in experiment 2 (Frangoch, 1991)

Individual traps were fixed to wooden stakes, 1.5m long, using retort stand clamps. The stakes were inserted into the ground so that traps were vertical and the lowest point of the trap rod just touched the crop canopy which was about 700mm above ground level.

The two pieces of Polythene were coated with a thin layer of petroleum jelly to facilitate the trapping of spores. The Polythene pieces were removed from the traps every four days (except for 14 August, 19 August and 22 August, when they were removed after 2, 5 and 3 days respectively) and replaced with fresh ones. The spore-bearing pieces of Polythene were then mounted on glass slides in methyl blue/lactoglycerol. The preparations were protected with cover slips, which were sealed to the slides using clear nail varnish. Slides could then be kept for a long time before spores were counted. Spores in a traverse 4mm wide along the whole length of a Polythene piece were counted and from this the total number of spores caught by each trap was calculated. Dividing this by the number of days that the trap had been in position gave a value of spores per trap per day.

Harvesting

When seeds were fully mature and hard, all the pods from each sub-plot were removed by hand and counted. Pods from each sub-plot were then threshed using a small threshing machine (Wintersteiger). The machine was designed for threshing small sheaves of cereals but also worked satisfactorily for peas. The total weight of seed per plot was determined and mean seed weight determined on a sample of 200 seeds. From the mean seed weight the total number of seeds per sub-plot could be estimated and also the mean number of seeds per pod.

Results

(i) Experiment 1

Weather conditions and disease development

Following sowing on 25 April, the weather was cool and dry (Fig.3.3.3). Mean maximum and minimum temperatures for May were 13.0°C and 6.7°C respectively; total rainfall was 21.5mm with only five days with more than 1mm. Plant growth was slow and growth stage 104 was only reached after 36 days (25 days in expt. 2). Inoculation on 31 May was followed by slightly warmer weather (maximum and minimum temperatures in June were 14.2°C and 8.4°C respectively) and there was rather more rain (total for June, 81.6mm with 1mm or more on 14 days). Nevertheless, there was little disease development until after the plots were reinoculated on 24 June. Thereafter, temperatures were higher and rainfall heavier. Mean maximum and minimum temperatures in July were 18.9°C and 12.9°C respectively; rainfall totalled 109mm and there were 12 days with 1mm or more. Mean disease scores on 6 July, were about 1, representing percentage necrosis in the range 1-24%; by the third and last recording (6 August) mean disease scores were close to 4, i.e. 75-100% necrosis (Fig. 3.3.4).

Irrigation treatments

The programme of irrigation treatments which began on 3 June appeared to have little effect on disease development. At the first recording (6 July) disease scores were very variable with significant differences between replicates (Appendix Table 3.3.1). There were differences between irrigation treatments ($P < 0.05$) for leaves and stipules (but not stems) but it is difficult to attach much importance to this result since the highest disease score was on the non-irrigated control. At the two later recordings there was no indication of differences between either irrigation treatments or replicates (Appendix Table 3.3.1).

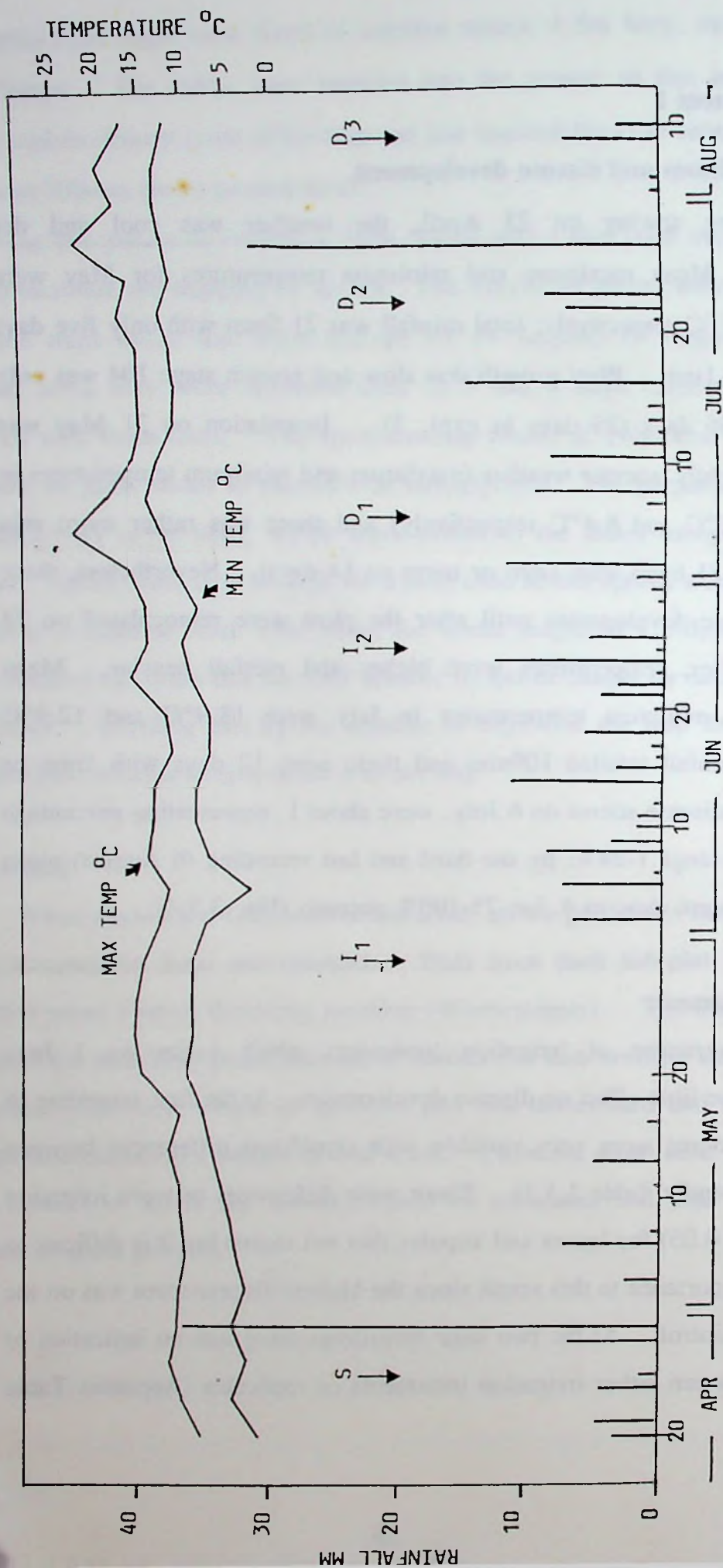


Fig. 3.3.3. Maximum and minimum temperatures (3-day means) and daily rainfall (mm) during Experiment 1 (Frongoch, 1991). (S = sowing; I₁ and I₂ inoculation dates; D₁, D₂, D₃, recording dates).

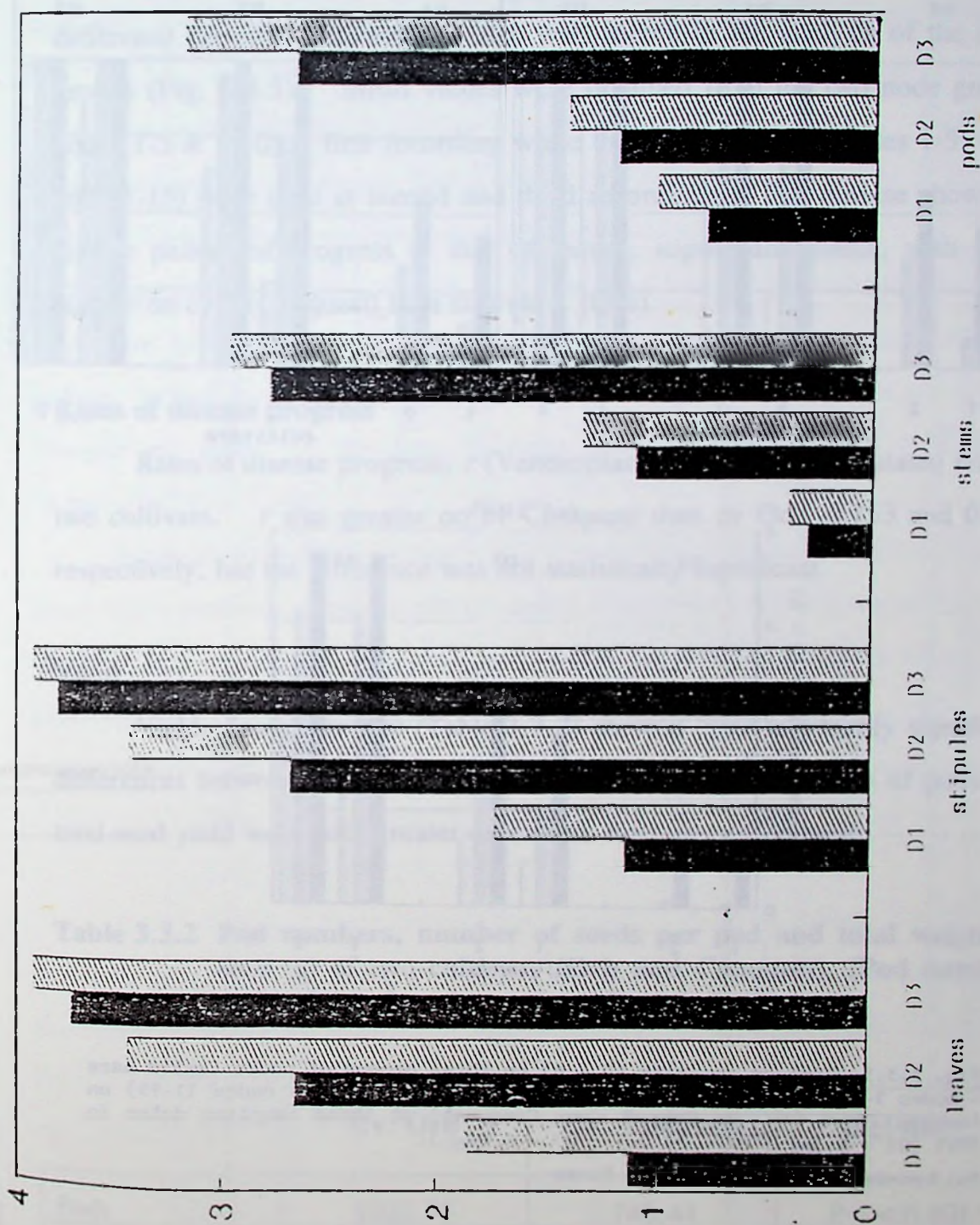


Fig. 3.3.4 Disease severity (0 - 4) on leaves, stipules, stems and pods on two cultivars (Orb - solid bars; Conquest - hatched bars) at three sampling dates in 1991 (D1 - 6 Jul.; D2 - 24 Jul.; D3 - 6 Aug.).

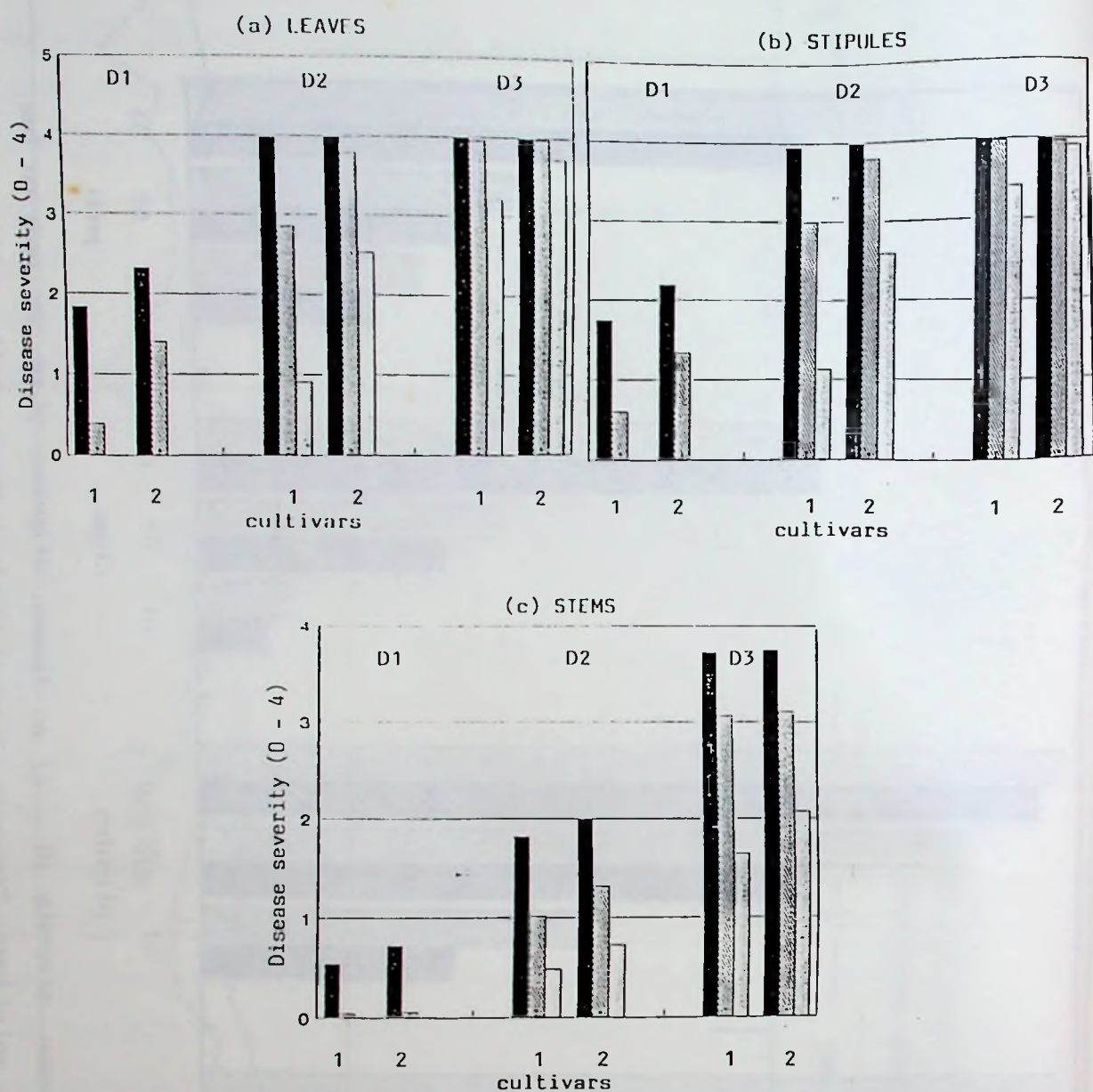


Fig. 3.3.5. Disease severity (0 - 4) on three groups of nodes (solid bars - nodes 1-5; hatched bars - nodes 6-10; stippled bars - nodes 11-15) on two cultivars (1 - cv Orb; 2 - cv Conquest) at three sampling dates in 1991 (D1 - 6 Jul.; D2 - 24 Jul.; D3 - 6 Aug.).

(a) Leaves: (b) Stipules: (c) Stems

Cultivars

Despite much variation between replicates and hence high error variances at the first recording, differences between cultivars emerged clearly except at the last recording when disease scores were very high. Cv Orb, the semi-leafless cultivar, was the more resistant (Fig.3.3.4, Table 3.3.1) and this difference between cultivars was also well shown in the analysis of the node groups (Fig. 3.3.5). Mean values were obtained from the two node groups nodes 1-5 & 6-10) at first recording while three node groups (nodes 1-5, 6-10 and 11-15) were used at second and third recordings. Pod disease showed a similar pattern of progress to that of leaves, stipule and stems, with more disease on cv 2 (Conquest) than cultivar 1 (Orb).

Rates of disease progress

Rates of disease progress, r (Vanderplank, 1963) were calculated for the two cultivars. r was greater on cv Conquest than cv Orb, 0.153 and 0.138 respectively, but the difference was not statistically significant.

Yield

Yield of seed per plot (Table 3.3.2) showed large and highly significant differences between cultivars (Appendix Table 3.3.2). Numbers of pods and total seed yield were both greater on cv Orb than on cv Conquest.

Table 3.3.2 Pod numbers, number of seeds per pod and total weight of seed (g) of two cultivars (Orb and Conquest). (Pod numbers and weight of seeds are for sub-plots 2m²).

	Cv. Orb	Cv. Conquest	Sig. diff.
Pods	1226.75	740.44	P = < 0.001
Weight Seeds (g)	699.4	315.1	P = < 0.001
Seeds/Pod	4.82	4.08	P = < 0.01

Table 3.3.1.1 *M. pinodes* infection (0 - 4) on leaves, stipules and stems of two cultivars of *Pisum sativum* (Cv.1 = Orb; Cv.2 = Conquest) at three sampling dates (July 6, July 27, August 6) in 1991 (Experiment D).

Sampling Date	Leaves			Stipules			Stems		
	Cv.1	Cv.2	Mean	Cv.1	Cv.2	Mean	Cv.1	Cv.2	Mean
July 6	1.11	1.86	1.49	1.14	1.74	1.44	0.29	0.38	0.34
SED for Cv means	(0.115)			(0.117)			(0.051)		
Error Df 12									
July 27	2.66	3.44	3.05	2.69	3.45	3.07	1.11	1.35	1.23
SED for Cv means	(0.099)			(0.066)			(0.096)		
Error Df 12									
August 6	3.723	3.906	3.81	3.80	3.91	3.85	2.81	2.98	2.90
SED for Cv means	(0.083)			(0.081)			(0.161)		
Error Df 12									

(ii) Experiment 2

Disease development

The conditions for both plant growth and disease development were much more favourable in expt. 2 (Fig. 3.3.6). From sowing (16 June) to the end of September, daily maximum temperatures were generally between 15 and 20°C whilst daily minimum temperatures were generally between 10 and 15°C. July was wet (109mm rain) and although August was drier (50mm rain) there were still 9 days with 1mm rain (12 days in July). Disease progressed rapidly and by the first recording (2 August) mean scores of about 2 (25-49%) were found on leaves and stipules.

Analysis of the data (Fig. 3.3.7; Appendix Table 3.3.3) showed highly significant differences between recording dates reflecting the progress of the disease, which, as in expt.1, was more severe on cv. Conquest than cv. Orb. Disease on stems of the two cultivars was not significantly different. (Appendix Table 3.3.3).

Replicate variation was highly significant and observation indicated that there was a gradient across the plots roughly from SW to NE with most disease in plots near to expt.1. This was examined in more detail by dividing the plots into three groups or zones:

Zone 1 - plots 3, 4, 6, 9, 15

Zone 2 - plots 2, 5, 7, 10, 6

Zone 3 - plots 1, 8, 11, 12, 13, 14

Mean disease scores (leaves) for zones 1 - 3 were 2.766, 2.535 and 2.374 respectively and using T - tests for unpaired values it could be shown that there were highly significant differences ($p < 0.001$) between all three. Analysis variance on this data is difficult because the zones do not contain equal numbers of plots (six in zone 3 compared to five in zones 1 and 2). However, by omitting plot 12 from zone 3 produced negligible effects on means and with fully orthogonal sets of data it was possible to make a more detailed study. Analysis (Appendix Table 3.3.4) showed clear and highly significant differences between zones for leaves, stipules and stems

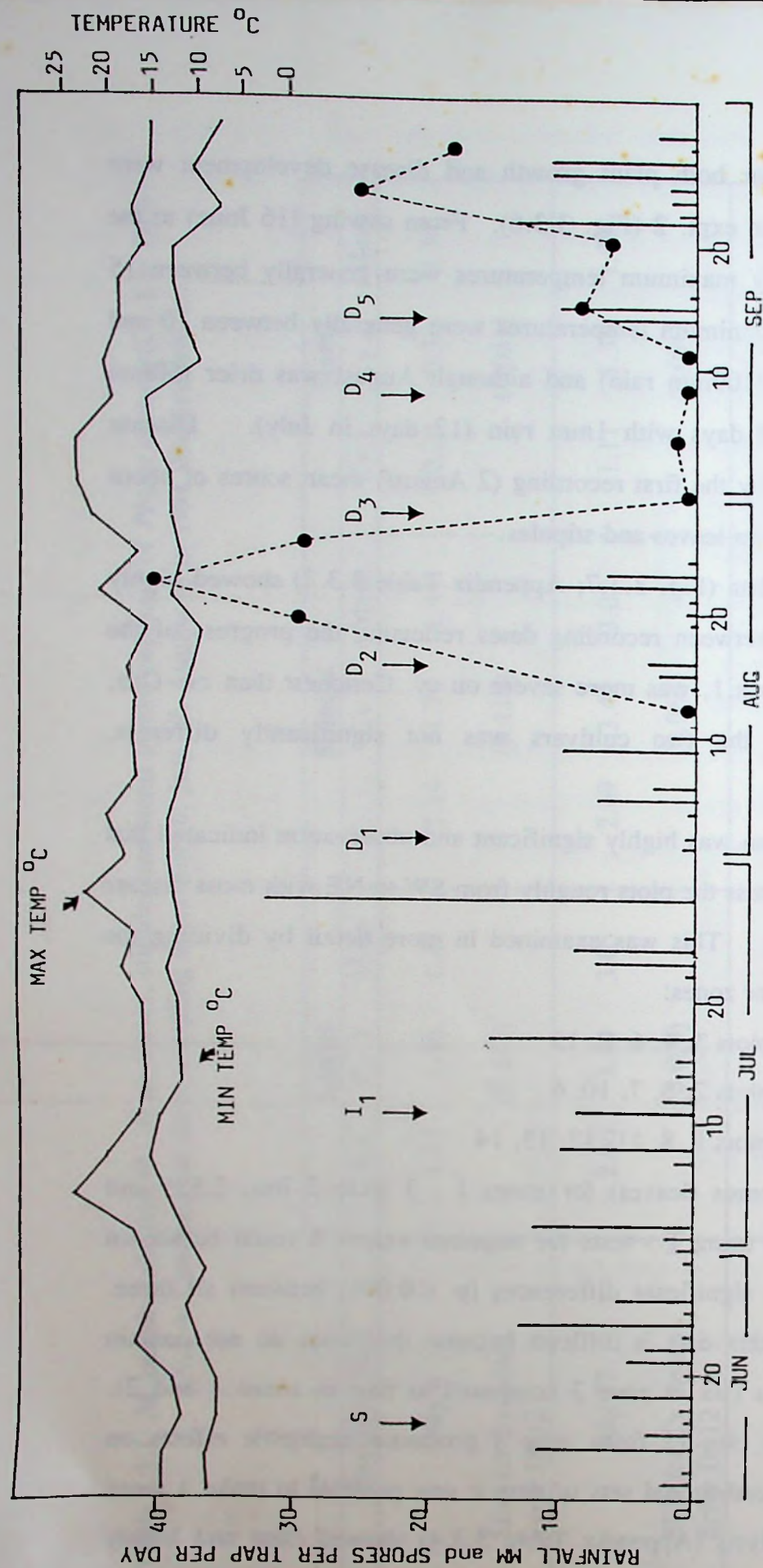


Fig. 3.3.6. Maximum and minimum temperatures (3-day means) and daily rainfall (mm) during Experiment 2 (Frangoch, 1991). (S = sowing; I₁ inoculation; D₁ - D₅, recording dates; ---●---, mean number of spores caught per trap per day within the experimental area).

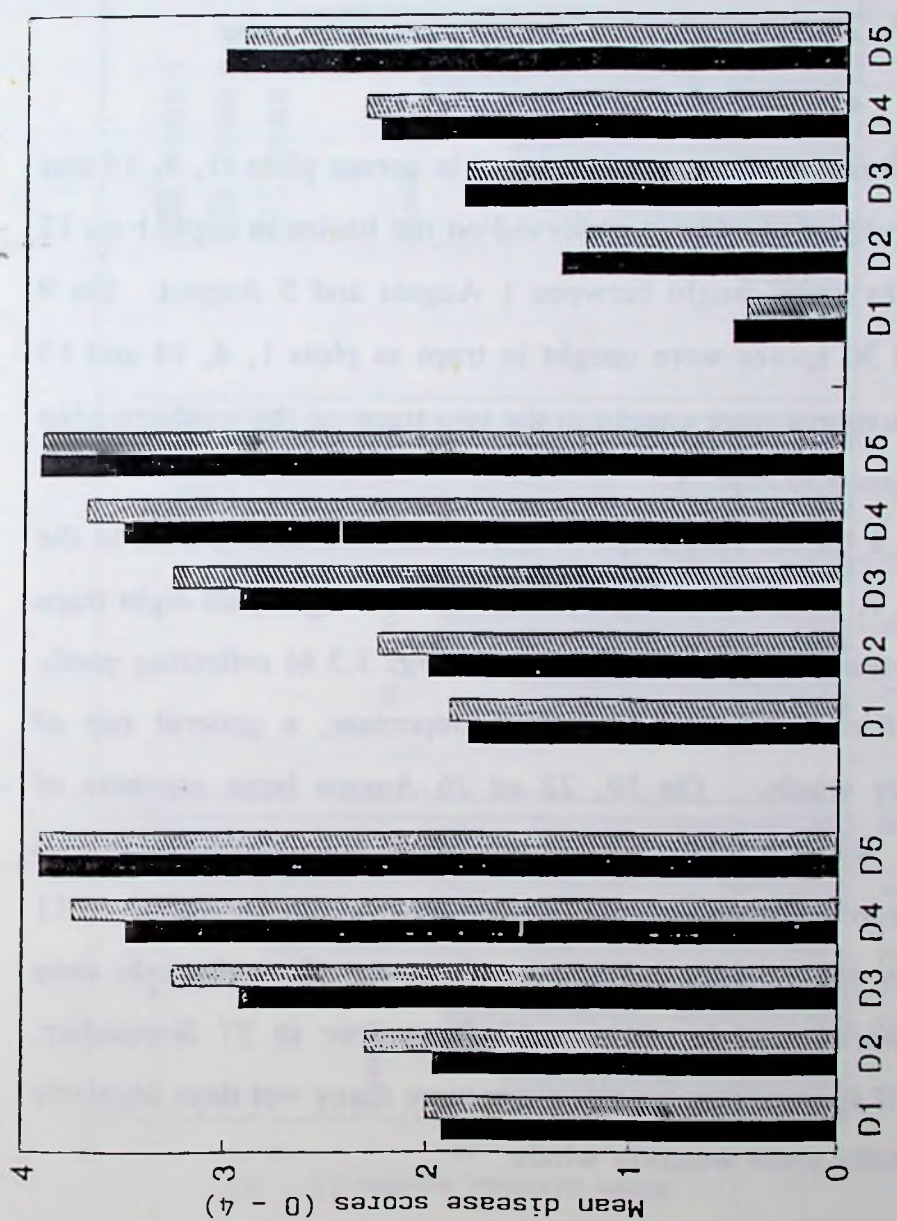


Fig. 3.3.7. Mean disease scores (0 - 4) on two cultivars (cv 0rb, solid bars; cv Conquest, hatched bars) at five sampling dates (D1 - D5) in 1991. (Experiment 2).

(Fig. 3.3.8). It is also evident that differences between zones account for most of the replicate variation noted earlier (Appendix Table 3.3.3) since the residual replicate variation i.e. within zones was very small and not significant (Appendix Table 3.3.4). Interactions (dates x zones, dates x cultivars, zones x cultivars and dates x zones x cultivars were at times highly significant (Appendix Table 3.3.4) but their magnitude was very small compared to the main effects and they were not examined further.

Spore trapping

On 1 August, four spore traps were placed in corner plots (1, 4, 14 and 15 in Fig. 3.3.1). *Perithecia* had been observed on the haulm in expt. 1 on 12 July but no ascospores were caught between 1 August and 5 August. On 9 August, 1, 50, 0 and 30 spores were caught in traps in plots 1, 4, 14 and 15 respectively i.e. most spores were caught in the two traps on the southern edge of expt. 2 i.e. that nearest to expt. 1.

On 12 August a further four traps (T3, T4, T5, T6, were placed in the four central plots (5, 7, 10 and 12; Fig. 3.3.1). On 14 August, all eight traps were checked but virtually no spores were found (Fig. 3.3.6) reflecting partly the small amount of rain but probably more important, a general run of northerly and easterly winds. On 19, 22 and 26 August large numbers of spores were caught. Rainfall was evidently not heavy but there were several showers and winds were mainly southerly to westerly. From 26 August to 11 September hardly any spores were caught; there was no rain and winds were mainly easterly or north easterly. From 15 September to 27 September, significant numbers of spores were caught; there were many wet days regularly associated with generally south westerly winds.

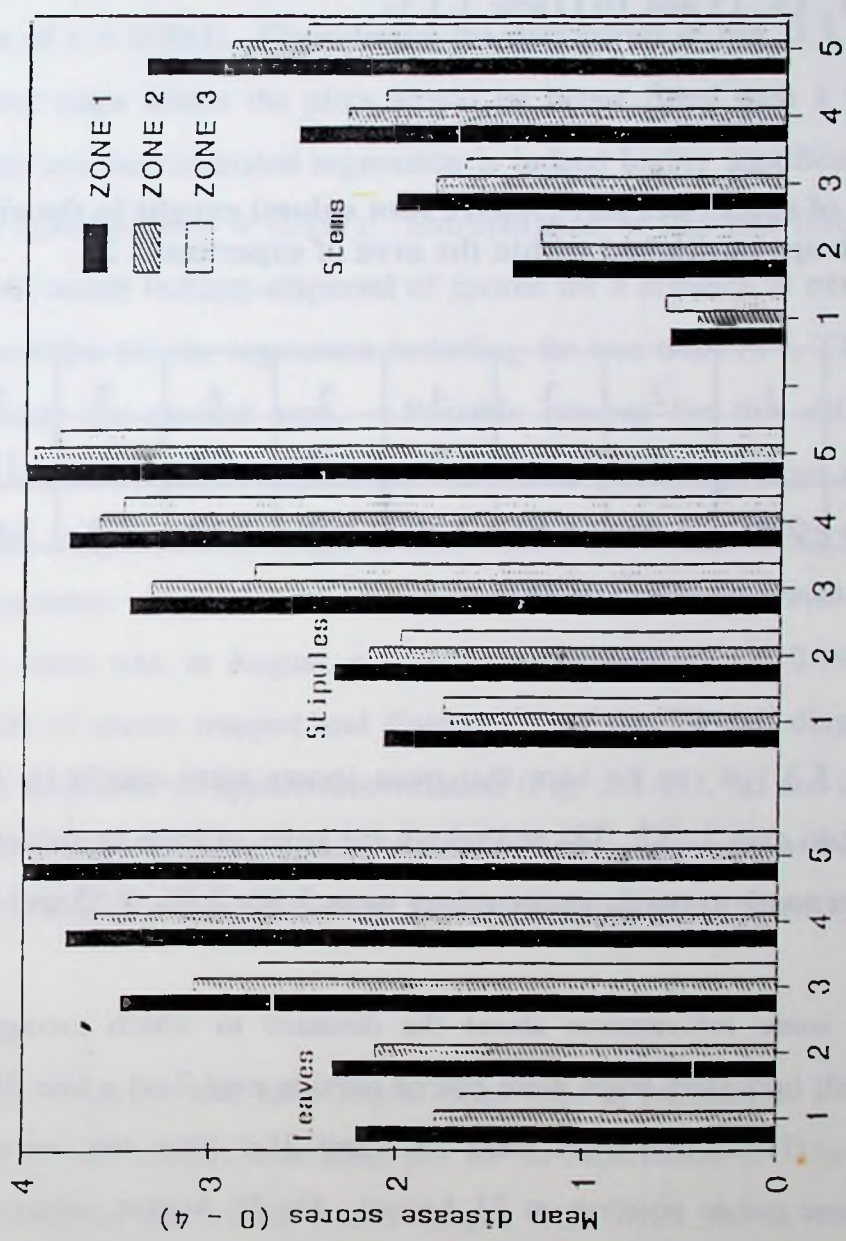


Fig. 3.3.8. Mean disease scores (0 - 4) on leaves, stipules and stems in three zones (see Fig. 3.3.1) at five sampling dates (1 - 5). Experiment 2, 1991)

Mean values of spores caught by the eight traps within the experimental area were analysed after transformation to square roots and assigning a value of 0.5 in place of zero values (Appendix Table 3.3.5). There was large and highly significant differences between dates as might be expected but there was also highly significant differences between traps. Most spores were caught in traps T2, T3, T8 and least in T7; intermediate and rather similar numbers were caught in traps T1, T4, T5 and T6 (Table 3.3.3).

Table 3.3.3

Mean numbers of spores per day (square root values) caught in the eight spore traps positioned within the area of experiment 2.

Trap Number	1	2	3	4	5	6	7	8
Plot Number	1	4	5	7	10	12	14	15
Mean No. spores	2.85	3.45	3.40	2.50	2.50	2.60	1.5	3.60

SED for mean numbers of spores 0.257

Error DF, 84

From Fig. 3.3.1 it can be seen that most spores were caught by traps positioned nearest to expt.1 (T2, T8) and taking the pairs of traps in successive rows of plots from south to north, mean values were 3.50, 2.95, 2.55 and 2.00 respectively.

To obtain some information about the distance to which ascospores might be dispersed, two extra traps were placed outside expt.2 on a line SW to NE (Fig. 3.3.1). These two traps were 15.5 and 31m from the corner of plot 1 and they were put in position on 22 August. On 26 August, when most spores were caught within the experimental area, 5 spores were caught in each trap. Thereafter, few or no spores were caught until 23 September when spore catches within the experimental area were also relatively high. On this occasion 2 and 4 spores were caught in the traps outside the experiment.

If traps T2 (SE corner) and T7 (NW corner) are discounted, the remainder fall in a line SW-NE, the track of the prevailing wind. To plot spore catches along this line, mean values were used for traps in the central plots of expt. 2, i.e. means of T5/T3 and T6/T4. A simple plot of the data (Fig. 3.3.9) produces a typical curve for spore dispersal from a single source. Linear regression for $\log_{10}$ data (Fig. 3.3.10) is highly significant with a correlation of $r = 0.864$). Considering the data points in Fig. 3.3.10 it seems that the four traps within the plots would be better fitted with a less steeply sloping line and the calculated regression is indeed highly significant and with a stronger correlation ($r = 0.897$). Extrapolation of this line (broken line in Fig. 3.3.10) would indicate dispersal of spores for a distance in excess of 1km rather than 100m for the regression including the two traps (T9, T10: see Fig. 3.3.1) outside the planted area. Possible reasons for this difference are discussed later. There are two periods when relatively large numbers of spores were caught within the planted area of expt. 2 viz. 19-26 August and 15-27 September (Fig. 3.3.6). When these periods were considered separately, there was, in August, a very close correlation ($r = 0.996$) between the numbers of spores trapped and distance along the SW-NE diagonal but in September there was no apparent correlation (Fig. 3.3.11., (a) and (c)).

The disease gradient across expt. 2, clearly apparent in August (Fig. 3.3.11., (b)) had also disappeared by September (Fig. 3.3.11., (d)).

Seed Yield

Seed yields per plot were much lower than in experiment 1, mean yield being 64g per m^2 compared to 254g per m^2 in experiment 1. There was also much variation in plot yields which ranged from over 500 to 100g per plot (125 to 25g per m^2). Seed yield was closely correlated with pod numbers ($r = 0.965$; Fig. 3.3.12). The yield of the two cultivars again differed, the mean yields (g per m^2) being 72 (cv. Orb) and 56 (cv. Conquest).

In experiment 1, differences in plot yields were not correlated with mean disease scores but in experiment 2 there was a strong correlation.

$r = 0.879$ for mean yields and $r = 0.892$ for cv. Orb and $r = 0.786$ for cv. Conquest. (The correlation for cv. Conquest, however, becomes $r = 0.813$ if data for an exceptionally high yielding plot (circled in Fig. 3.3.13., (b)) is excluded.

As might be anticipated, differences between pod numbers and seed yield declined from zone 3 to zone 1 (Fig. 3.3.1) whilst disease, as noted earlier, increased (Fig. 3.3.14).

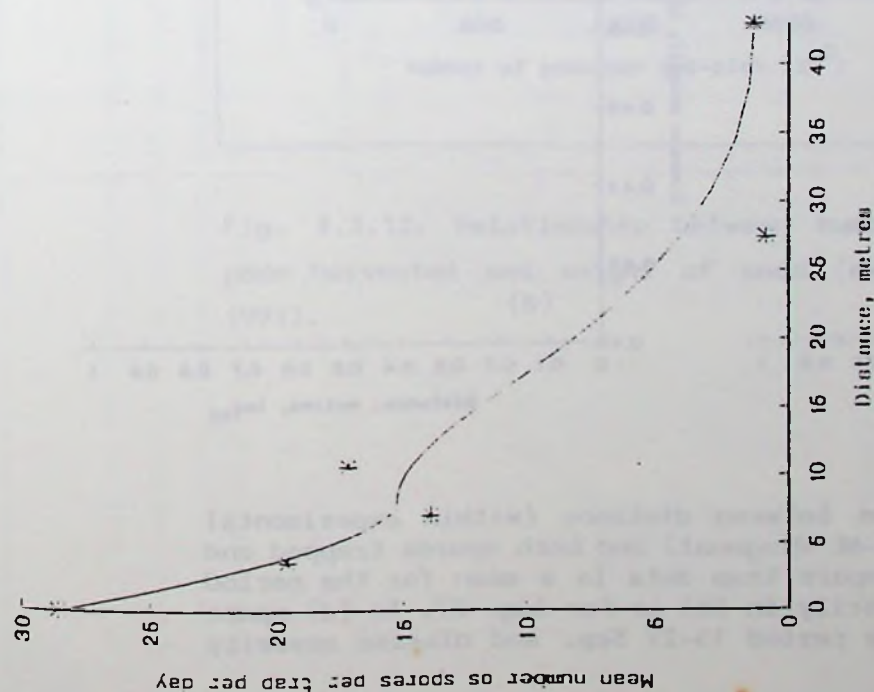


Fig. 3.3.9. Relationship between spores trapped and distance along a SW to NE line from the corner of expt. 2 (Fig. 3.3.1).

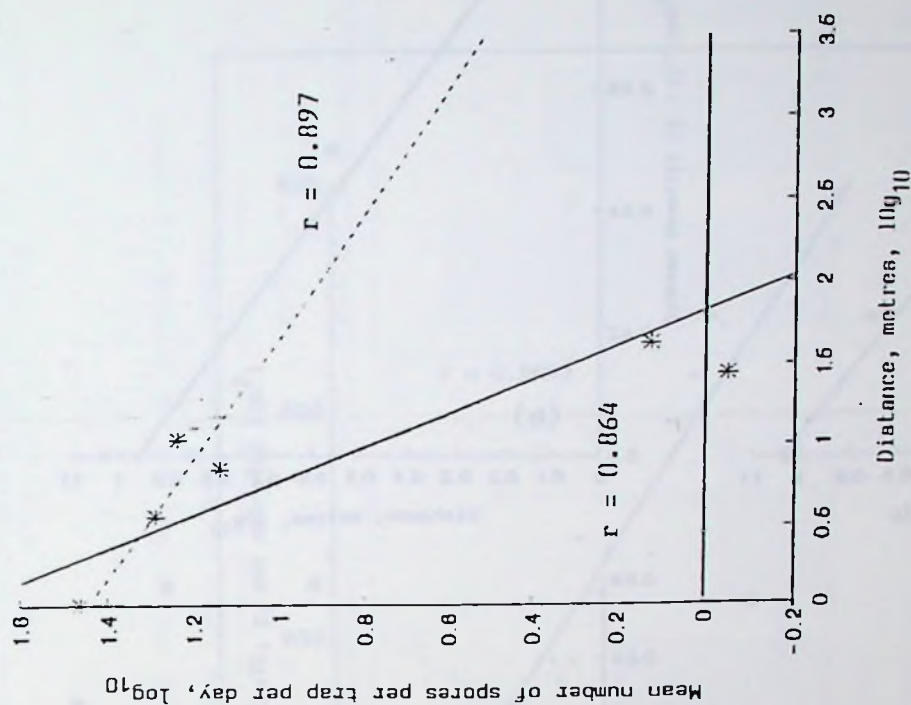


Fig. 3.3.10. As Fig. 3.3.9 but data transformed to logs. The linear regression for all six points (solid line) is significant as also is the regression for the first four points i.e. traps within plots in expt. 2 (broken line). Extrapolation of the broken line would indicate deposition of spores at distances in excess of 1 km from source.

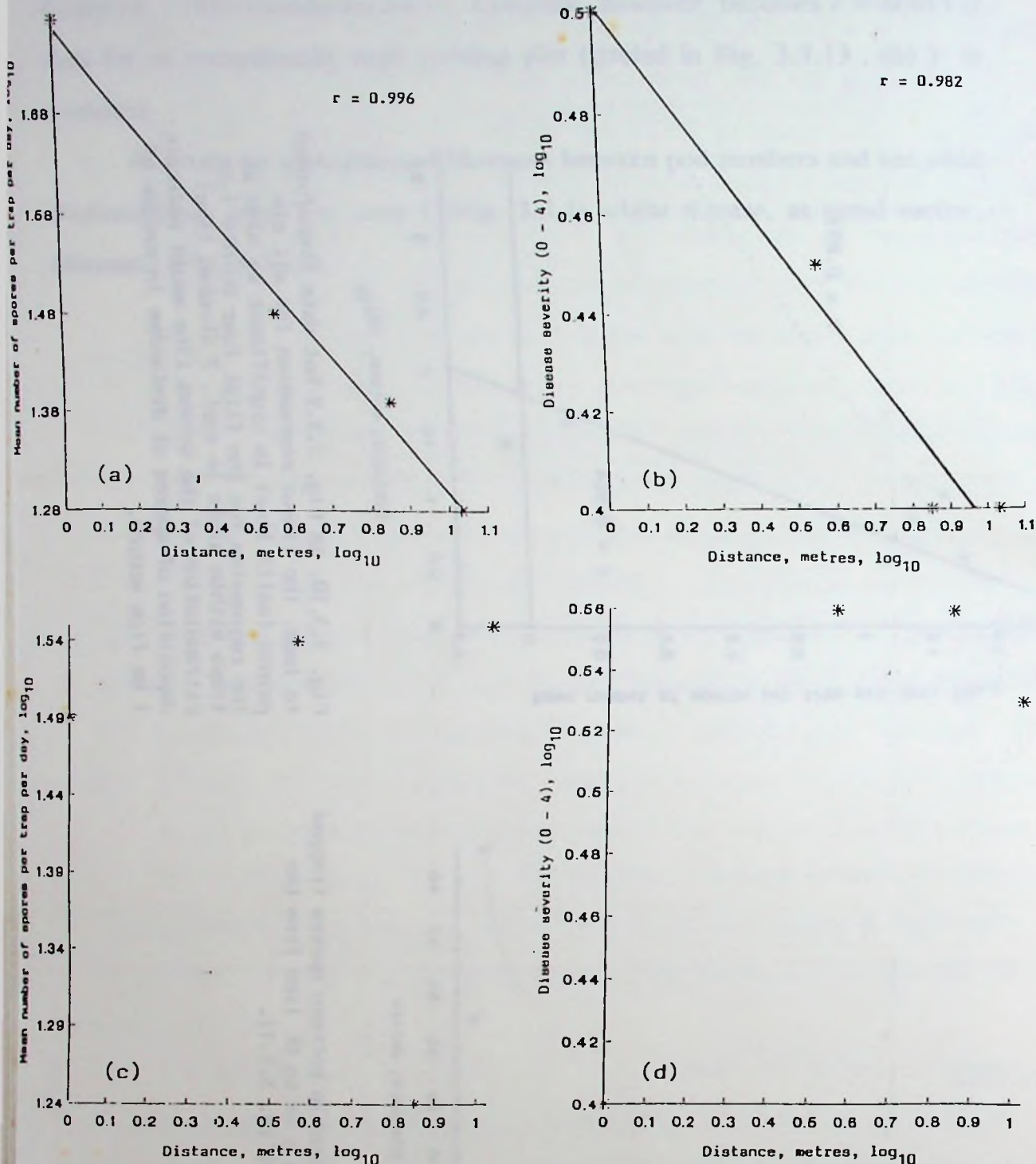


Fig. 3.3.11. Relationships between distance (within experimental plots of expt. 2 on the Sw-NE diagonal) and both spores trapped and disease severity. In (a) spore trap data is a mean for the period 19-26 Aug. and disease severity in (b) is for Aug. 27. In (c) spore trap data is mean for the period 15-27 Sep. and disease severity (d) for 15 Sep.

In August there was a close correlation between distance and both spores trapped and disease severity; in September there was no apparent relationship.

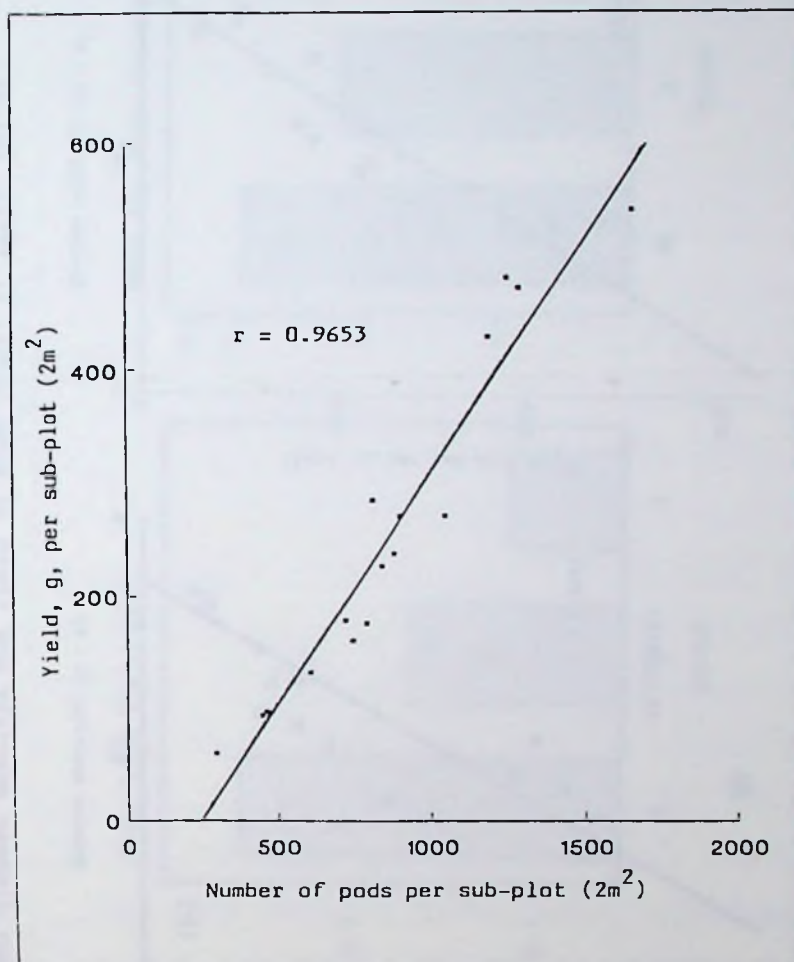


Fig. 3.3.12. Relationship between numbers of pods harvested and weight of seed (expt. 2, 1991).

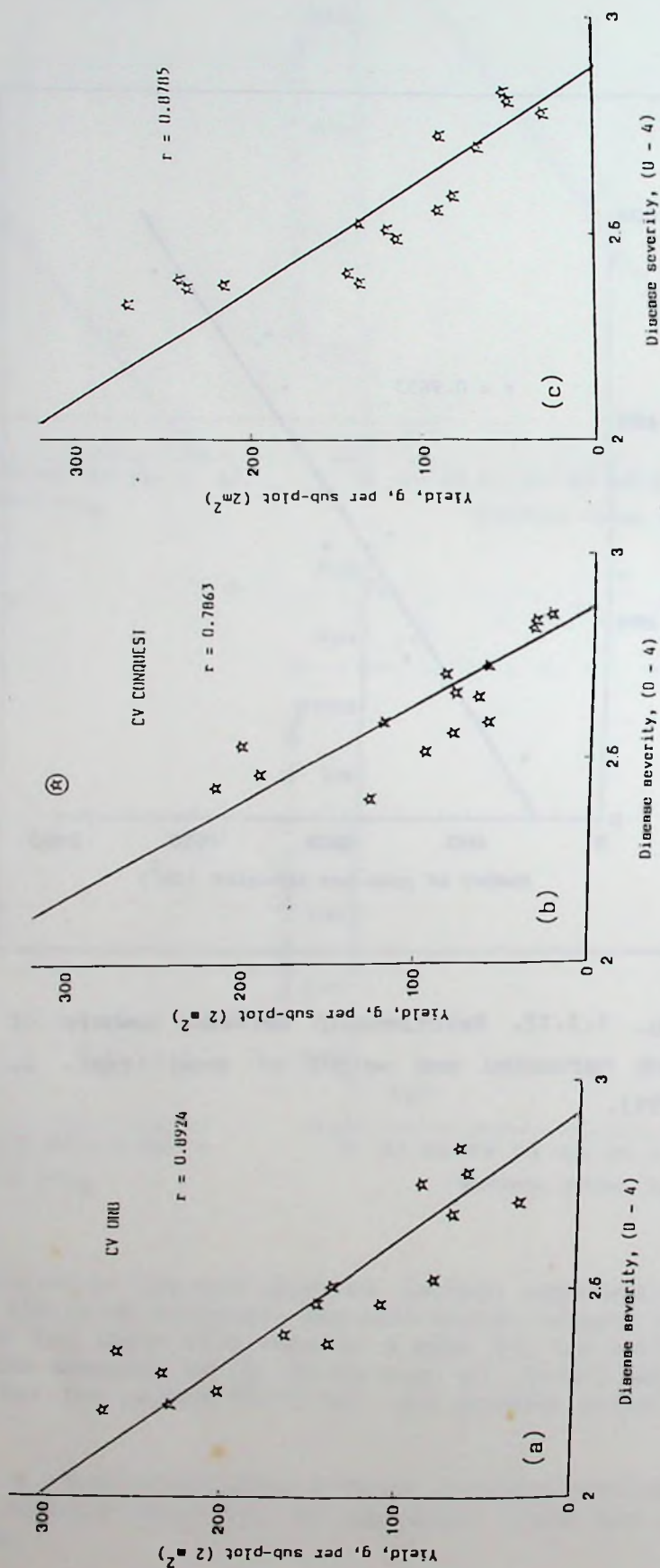


Fig. 3.3.13. Relationship between mean disease severity and yield of seed (expt. 2, 1991). (a) cv Orb; (b) cv Conquest; (c) mean of two cvs. (The correlation coefficient for cv Conquest increases to $r = 0.813$ if one point (circled) is omitted.)

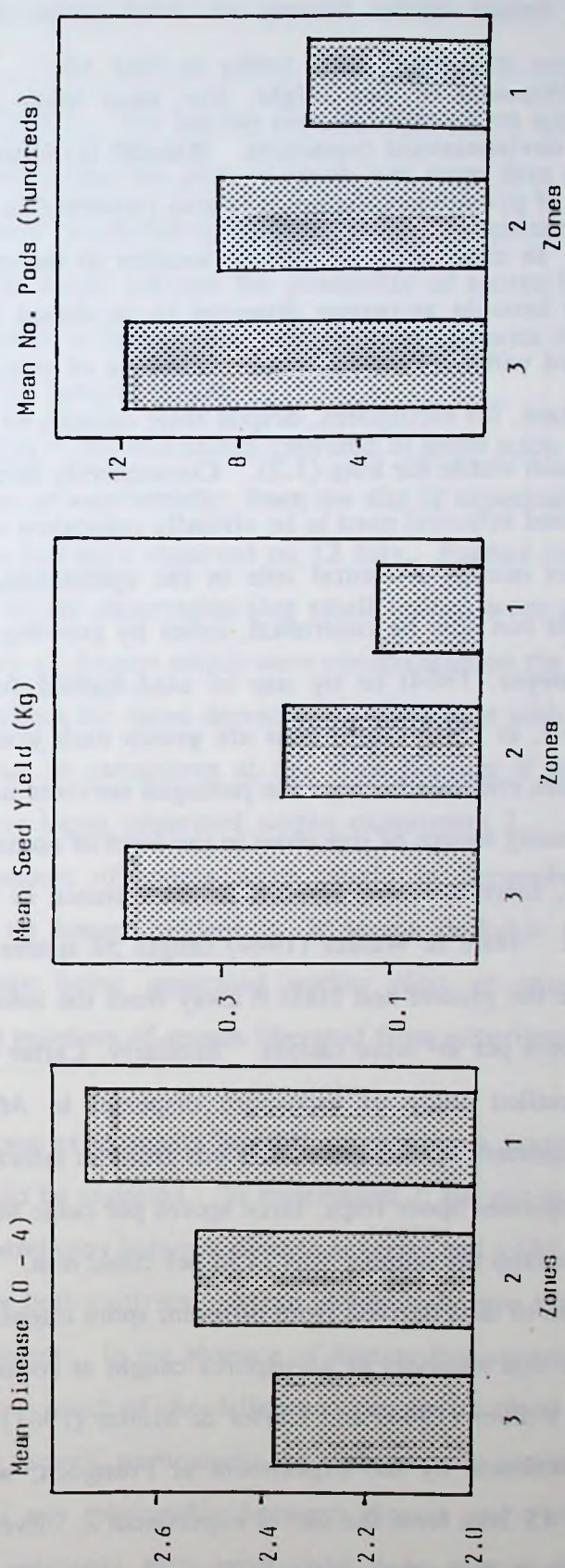


Fig. 3.3.14. Mean disease, seed yield and pod numbers in three zones (Fig. 3.3.1) in experiment 2, 1991. All differences are highly significant ($P < 0.001$).

Discussion

The development of pea blight, like most other diseases, is very dependent upon environmental conditions. Rainfall is obviously important for splash dispersal of pycnidiospores but it is also important in triggering release of ascospores. In experiment 2, the dry weather at the end of August and early September brought ascospore dispersal to an abrupt halt and dispersal was only resumed with the onset of rain. Linkage of ascospore discharge to rainfall is important, for ascospores, despite their capacity to germinate rapidly (3.1) do not remain viable for long (3.2). Consequently favourable conditions for germination and infection need to be virtually coincident with dispersal.

Ascospores occupy a central role in the epidemiology of pea blight. Seed transmission can now be controlled, either by growing crops in areas of low rainfall (Lawyer, 1984) or by use of seed-applied fungicides (Bretag, 1985). However, in areas where peas are grown each year on a large scale the disease remains endemic because the pathogen survives in the haulm which provides a continuing source of inoculum in the form of ascospores.

Inevitably, there has been interest in the distance to which ascospores can be dispersed. Hare & Walker (1944) caught 52 spores per in² on slides placed 3 ft above the ground and 1000 ft away from the infected plots; within the plots 833 spores per in² were caught. Similarly, Carter & Moller (1961), who made a detailed study of ascospore dispersal in *M. pinodes*, found relatively large numbers of ascospores 425 yds from an infected crop. At this distance, using aspirated spore traps, three spores per cubic mm were caught in 30min; within the crop the number was 1150 per cubic mm.

Comparison of data derived from different spore trapping methods is not easy but it seems that numbers of ascospores caught at considerable distances by both Hare & Walker (1944) and Carter & Moller (1961) were far greater than would be indicated by the experiment at Frongoch, where few spores were caught even 15-30m from the site of experiment 2. Even if it is assumed that ascospores were derived exclusively from the site of experiment 1, the distance would not be greater than 75m. The topography of the area may

offer a possible explanation. The two outlying traps, although on the track of the prevailing wind, were on ground which sloped gently away from experiment 2. They were in effect over a hill with respect to the sites of experiments 1 and 2. The log-log plot for distance on spore number indicates that the gradient across the plots is much less steep than the gradient derived from all the plots i.e. including the outliers. Extrapolating from the gradient within the plots would indicate the possibility of spores being deposited at a distance of 1000m, a figure much more comparable with those reported earlier and suggested by Lawyer (1984).

The origin of the ascospores captured in spore traps in experiment 2 was presumed to be, at least initially, from the site of experiment 1 where perithecia and ascospores had been observed on 12 July. Further support for this belief was provided by the observation that small lesions were present on plants on experiment 2 on 12 August which were conspicuous on the SW side of plants.

The gradient for spore deposition is consistent with experiment 1 being the main source of ascospores at this time because it is most unlikely that ascospores were being generated within experiment 2. Later in the season substantial numbers of spores were caught in September but the SW-NE gradient was no longer evident. It seems probable that by September, ascospores were being generated within plots of experiment 2 and the possibility that numbers of spores liberated from experiment 1 might also have diminished.

In the two experiments in 1991, disease was severe and reductions in seed yield would be expected. In experiment 2, but not in experiment 1, there was a close correlation between disease severity and yield, similar correlations being found for both cultivars. Mean yields in the two experiments, however, were very different. In the absence of disease-free controls there is no means of knowing how much of the difference can be ascribed to disease and how much to other factors, particularly the difference in sowing date. However, the absence of any relationship between disease and yield in experiment 1, coupled with relatively high seed yield does suggest that seed yield was

relatively unaffected by disease in experiment 1. Features of the experiment which make this a possibility are, first, that the initial inoculation failed and it was only after the second inoculation that the disease really developed. By this time flowering had started and much, possibly most, of the crop had presumably set. By contrast, in experiment 2, inoculation at growth stage 104 was successful and it is not unreasonable to presume that it was augmented by ascospores derived from the site of experiment 1. Infection was, consequently, much more severe at an earlier stage of crop development than in experiment 1, reducing pod numbers (and hence seed yield) in proportion to its severity across the experiment.

The interaction between epidemics of pea blight and stage of crop growth merits further study as also would be evaluation of the relative resistance of different plant organs. In *Vicia faba*, for example whilst leaves became more susceptible to *Botrytis fabae* with age, the pods became more resistant (Griffiths & Amin, 1977). Yield of seed is much reduced when infection occurs at flowering but is unaffected by later attacks.

Appendix Table 3.3.1

Anovar: mean scores (0.4) for leaves, stipules and stems at three dates (6 July, 7 August, 16 August) in 1991 (Field Experiment 1).

(i) Date 1, 6 July

Item	DF	Leaves		Stipules		Stems	
		MS	VR	MS	VR	MS	VR
Main plots	15						
Replicates	3	1.498	12.85***	1.176	11.09***	0.181	5.31*
Irrigations (I)	3	0.578	4.96*	0.471	4.45*	0.026	0.77
Error (a)	9	0.117		0.106		0.034	
Total	31						
Cultivar (C)	1	4.556	42.87***	2.886	26.18***	0.067	3.21
Interaction (C x I)	3	0.084	0.79	0.044	0.40	0.011	0.52
Error (b)	12	0.106		0.110		0.021	

(ii) Date 2, 7 August

Item	DF	Leaves		Stipules		Stems	
		MS	VR	MS	VR	MS	VR
Main plots	15						
Replicates	3	0.109	1.23	0.032	0.34	0.104	0.81
Irrigations (I)	3	0.079	0.89	0.008	0.09	0.033	0.26
Error (a)	9	0.089		0.093		0.125	
Total	31						
Cultivar (C)	1	4.914	62.83***	4.636	133.10***	0.483	6.48*
Interaction (C x I)	3	0.137	1.75	0.057	1.66	0.047	0.63
Error (b)	12	0.078		0.034		0.075	

(iii) Date 3, 16 August

Item	DF	Leaves		Stipules		Stems	
		MS	VR	MS	VR	MS	VR
Main plots	15						
Replicates	3	0.052	1.62	0.045	1.18	0.272	3.44
Irrigations (I)	3	0.004	0.12	0.002	0.05	0.072	0.91
Error (a)	9	0.032		0.038			
Total	31						
Cultivar (C)	1	0.270	4.92	0.107	2.05	0.263	1.25
Interaction (C x I)	3	0.077	1.40	0.063	1.20	0.085	0.41
Error (b)	12	0.055		0.052			

Appendix Table 3.3.2

Anovar: Seed yeild of two cultivars from *Pisum sativum* (see Table 3.3.3).

Item	DF	Pods/Plot		Wt. Seeds/Plot		No. of seeds per Pod	
		MS	VR	MS	VR	MS	VR
Main Plot	15						
Rows	3	7,557.7	0.196	8,855.7	1.144	2.8539	1.1360
Columns	3	12,782.9	0.332	8,341.9	1.077	2.1944	0.8735
Treatment (T)	3	2,224.4	0.058	906.3	0.117	2.4689	0.9828
Error (a)	6	38,552.3		7,744.0		2.5122	
Total	31						
Cultivar (C)	1	1,891,998.0	62.81***	1,181,569.0	120.75***	4.3633	17.7875**
C x T	3	25,496.0	0.85	2,108.7	0.22	0.2063	0.8412
Error (b)	12	30,126.0		9,785.3		0.2453	

Appendix Table 3.3.3

Anovar: Mean disease scores (0-4) on leaves, stipules and stems of two cultivars at five dates (Experiment 2, 1991)

Item	DF	Leaves		Stipules		Stems	
		MS	VR	MS	VR	MS	VR
Main Plot	79						
Replicate	15	0.333	4.95***	0.302	5.10***	0.304	5.02***
Dates (D)	4	24.487	363.39***	25.834	436.87***	27.787	458.35***
Error (a)	60	0.674		0.059		0.061	
Total	159						
Cultivar (1)	1	1.903	45.06***	1.502	48.12***	0.074	1.45
D x C	4	0.158	3.74***	0.119	3.82***	0.089	1.75
Error (b)	75	0.422		0.312		0.051	

Appendix Table 3.3.4

Anovar: Mean disease scores (0.4) on leaves, stipules and stems of two pea cultivars at five dates in three zones (Fig.3.3.1 : Experiment 2, 1991)

Item	DF	Leaves		Stipules		Stems	
		MS	VR	MS	VR	MS	VR
Main Plots	24						
Replicates	4	0.031	0.74	0.036	1.49	0.005	0.12
Dates (D)	4	22.942	553.37***	24.068	1007.21***	26.227	648.36***
Error (a)	16	0.042		0.024			
Sub-plots	74						
Zones (Z)	2	2.158	40.05***	1.853	43.32***	1.964	45.48***
D x Z	8	0.141	2.61**	0.130	3.04**	0.226	5.23***
Error (b)	40	0.054		0.043		0.043	
Sub-sub-plots	149						
Cultivar (C)	1	1.782	49.64***	1.520	52.27***	0.043	0.98
D x C	4	1.161	4.49***	0.137	4.72***	0.073	1.66
Z x C	2	0.118	3.28*	0.035	1.22	0.181	4.16*
D x C	8	0.083	2.30*	0.041	1.40	0.089	2.03
Error (c)	60	0.036		0.029		0.044	

Appendix 3.3.5 Pea Growth Stages (Biddle *et al.*, 1988)

Definitions and Codes for Stages of Development of the Pea

Code	Definition	Description
Germination and Emergence		
000	Dry seed	
001	Imbibed seed	
002	Radicle apparent	
003	Plumule and radicle apparent	
004	Emergence	
Vegetative Stage refers to main stem and recorded node. Two small scale leaves appear first and the nodes where these occur are not recorded: only nodes where a stipule and leaf stalk develop are counted. Definitions and codes for nodal development refer to all cultivars, descriptions in brackets refer to conventional leaved cultivars only.		
101	First node	First leaf fully unfolded, with one pair leaflets, no tendril present
102	Second node	Second leaf fully unfolded, with one pair leaflets, simple tendril
103	Third node	Third leaf fully unfolded, with one pair leaflets, complex tendril
:	:	
10x	x node	x leaf fully unfolded, with more than one pair of leaflets, complex tendril
:	:	
:	:	
1n	n, last recorded node	n - any number of nodes on the main stem with fully unfolded leaves according to cultivar
Reproductive Stage refers to main stem, and first flower or pod apparent		
201	Enclosed buds	small flower buds enclosed in terminal shoot
202	Visible buds	flower buds visible outside terminal shoot
203	First open flower	
204	Pod set	a small immature pod
205	Flat pod	
206	Pod swell	Pods swollen, but still with small immature seeds
207	Pod fill	green seeds fill the pod cavity
208	Green wrinkled pod	
209	Yellow wrinkled pod	seed 'rubbery'
210	Dry seed	Pods dry and brown, seed dry and hard
Senescence Stage refers to lower, middle and upper pods on whole plant		
301	Lower pods dry and brown, seed dry; middle pods yellow and wrinkled, seed 'rubbery'; upper pods green and wrinkled. Desiccant application stage.	
302	Lower and middle pods dry and brown, seed dry; upper pods yellow and wrinkled, seed 'rubbery'. Pre-harvest stage.	
303	All pods dry and brown, seed dry. Dry harvest stage.	

4. EVALUATION OF PARTIAL RESISTANCE TO *MYCOSPHAERELLA PINODES* IN CULTIVARS OF *PISUM SATIVUM*

Introduction

M. pinodes, unlike *Ascochyta pisi* and *Phoma medicaginis* var *pinodella*, cannot be adequately controlled by cultural practices (see General Introduction). For this reason, there has been a continuing and indeed a growing interest in attempts to find sources of host resistance (Jones, 1927; Hare & Walker, 1944; Ali *et al.*, 1978; Zimmer & Sabourin, 1986).

Screening of cultivars has often revealed variation in resistance (Ali *et al.*, 1978; Zimmer & Sabourin, 1986; Clulow *et al.*, 1990; Nasir & Hoppe, 1990; Clulow *et al.*, 1992) but, to date, commercial cultivars with a high level of resistance have not been developed. Recent studies (Clulow *et al.*, 1990; Nasir *et al.*, 1992; Clulow *et al.*, 1992) have provided evidence of distinct pathotypes within *M. pinodes* and this may well make the task of breeding resistant cultivars more difficult. It is too early to assess whether resistance to specific pathogen virulences can be successfully exploited in *Pisum sativum* to provide durable resistance to *M. pinodes*. Experience with similar host-pathogen systems does not offer much encouragement however, and for this reason it seems desirable to attempt an evaluation of partial resistance which tends to operate against all pathotypes.

Partial resistance operates by reducing the rate of epidemic development and is essentially a function of three components *viz.* infection frequency (IF), latent period (LP) and sporulation (SP). Quantitative measurement of these components can often provide good prediction of a cultivar's field performance. For example, Kari (1991) showed that field resistance of barley cultivars to *Rhynchosporium secalis* was highly correlated with total spore production on seedling leaves. For *M. pinodes* assessment of IF does not offer significant problems but assessment of LP and SP pose substantial difficulties. Two spore forms, pycnidiospores and ascospores, are involved

and methods for determining both the intervals from infection to their production (LP) and the quantity of spores generated have yet to be devised.

In the preliminary studies described here disease development was examined in small field-grown plots of 25 cultivars of *P. sativum* inoculated with a single isolate of *M. pinodes*. The aim was to determine the extent of variation to provide a basis for examining the relationship of field performance with IF which was measured on glasshouse-grown seedlings. In addition to measurement of IF assessments of lesion size (LS) were made on 12 cultivars. LS is not normally considered as a component of partial resistance but Parlevleit (1979) has observed that LS is often highly correlated with sporulation (SP). In the absence of any measure of SP for *M. pinodes* it was thought that it might prove a useful measurement to make.

4.1 FIELD EXPERIMENT AT FRONGOCH, 1992

Materials and Methods

The field experiment was sited on the University's Field Station at Frongoch (near Aberystwyth) in a field which had been ploughed during the winter and rotavated prior to planting. The experimental area was divided into four blocks each of which was further subdivided to accommodate 25 plots (1 x 1m). Plots were separated by 0.5m paths and blocks by 1m paths.

Fungicide-treated seeds of 25 commercial cultivars of *Pisum sativum* (Appendix Table 4.1) were kindly provided by the Processors and Growers Research Organisation (PGRO) at Peterborough, UK. Cultivars were randomly allocated to the 25 plots in each block. Seeds were sown in 5 rows (20cm apart) with 5cm between seeds within each row (100 seeds per plot). Planting took place over a 3-day period and was completed on May 23.

Plants were inoculated with a pycnidiospore suspension (7.5×10^5 spores, ml⁻¹) on July 3, when they were at growth stage 107 (7 fully expanded leaves). Immediately before inoculation, plants were wetted for 30 min using sprinkler irrigators. The pycnidiospore suspension was then applied as uniformly as possible using a 5 litre knapsack sprayer. Inoculation was carried out in late evening to enhance the possibility of maintaining leaf wetness sufficiently long for infections to be established.

Plants were sampled for disease assessment on July 17, 31, August 7, 14. On each occasion, two plants were removed from each plot at random and transferred to the laboratory where assessments were made on stipules, leaves, stems and pods on three groups of nodes, 5 & 6, 10 & 11, and 15 & 16. A simple mean of the six values from the two plants provided a plot mean for statistical analysis. Pods were assessed for disease at three dates only (July 31, August 7, 14).

Disease on stipules and leaves of standard cultivars was assessed using the key for common leaf spot of alfalfa (*Pseudopeziza trifolii*) as a guide (James, 1971). On semi-leafless cultivars, where only stipules were present, a

visual assessment was made of the proportion of the area of tendrils affected and these values were used for comparison with leaf assessments on standard cultivars. Assessments of disease on stems and pods were visual assessments of percentage area affected.

Rates of disease progress per day (r) were calculated using the formula of Vanderplank (1963):

$$r = \frac{1}{t_2 - t_1} \left\{ \log e^{\frac{x_2}{1 - x_2}} - \log e^{\frac{x_1}{1 - x_1}} \right\}$$

where x_1 and x_2 are the proportions of the plant part affected and at times t_1 and t_2 respectively.

Pods were harvested from the plots but conditions at harvest were very unfavourable and much seed was lost through shedding. Further damage took place when pods were being stored prior to assessing seed weights and numbers. Any yield data collected in these circumstances would have been unreliable and hence has not been presented.

Observations and Experimental Results

(i) Weather conditions and development of pea blight in 1992

From the beginning of July, when plots were inoculated, until mid-August, when the last disease assessment was made, temperatures showed little variation from day to day, with maximum temperatures in the range 17-20°C and minimum temperatures around 10°C (Fig. 4.1). Rain fell on many days (17 rain days in July and 18 in August); total rainfall for July was 87.5mm and for August 146.2mm (Fig. 4.1). It was unfortunately, not possible to maintain records of duration of leaf wetness, but, in view of the number of rainy days and the small numbers of sunshine hours (0 - 13.2h, mean 4.75, in July; 0 - 11.8, mean 4.9, in August) it is not unreasonable to assume that prolonged periods of leaf wetness were not uncommon.

It would seem from the available meteorological data that conditions for development of pea blight were generally favourable. Although temperatures

were rather below the optimum for spores germination and infection, they were very favourable for perithecial formation and ascospore production.

Following inoculation, a brief spell of dry weather appeared to have retarded disease development for an inspection of the plants on July 10 showed only small and sporadic amounts of infection. However, by July 17 substantial infection was present and thereafter the disease progressed rapidly.

(ii) **Disease severity on leaves, stipules, stems and pods.**

None of the 25 cultivars showed marked resistance to disease and at the last sampling date percentage infection on leaves ranged from 50 per cent (cv 22) to 85 per cent (cv 13). However, analysis of the data (Table 4.1; Appendix Table 4.2), showed that there were highly significant differences between cultivars.

Disease scores on leaves and stipules were similar and mean scores were strongly correlated (Fig. 4.2) with $r = 0.903$. It might have been expected that assessments on true leaves of standard cultivars would have correlated better with those on stipules than assessments made on tendrils of semi-leafless cultivars and this was found to be the case. However, the difference was not great, the correlation coefficients for standard and semi-leafless cultivars being $r = 0.893$ and $r = 0.814$ respectively.

Lower disease scores were recorded on stems and although the correlation with leaf scores (Fig. 4.3) was still significant ($r = 0.681$; $P < 0.001$) it was much weaker than the correlation between leaf and stipule scores. On pods, disease scores were low until the last recording (Table 4.1); mean values were correlated with both leaves ($r = 0.609$) and stems ($r = 0.623$).

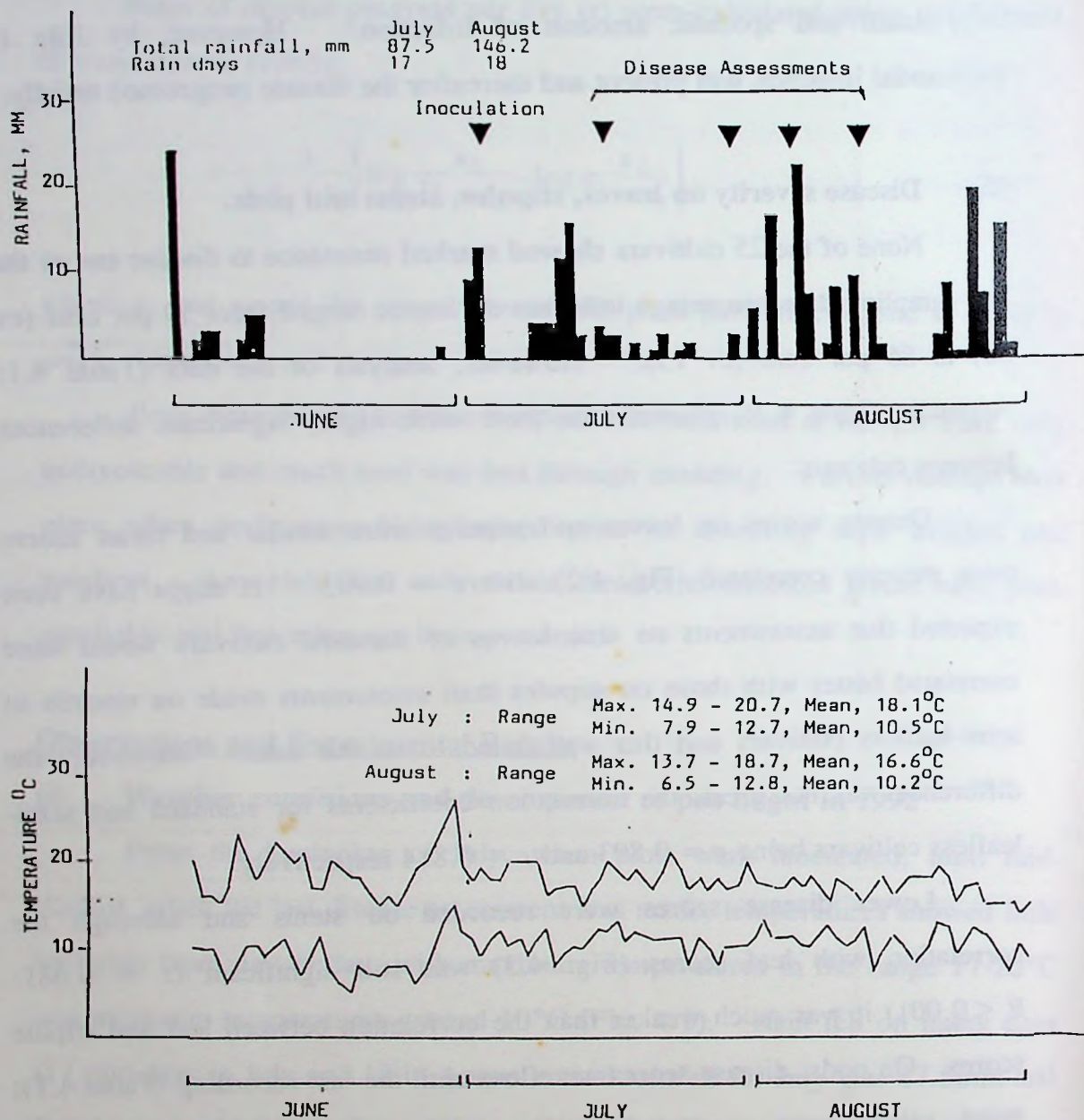


Fig. 4.1. Daily rainfall and temperatures recorded at Frongoch in June, July and August, 1992.

Table 4.1 Disease % (Angles) on Leaves, Stipules, Stems and Pods of 25 Cultivars of Peas at four dates (D1 - D4), Frongoch, 1992

	LEAVES				STIPULES				STEMS				PODS			
	D1	D2	D3	D4	D1	D2	D3	D4	D1	D2	D3	D4	D2	D3	D4	
1	32.32	35.428	46.434	52.635	30.326	38.533	47.740	54.820	7.6530	15.3665	20.4290	28.9713	6.9848	7.9934	25.8244	
2	16.484	33.532	40.146	56.424	12.574	35.318	44.982	57.981	4.6410	11.2722	20.5842	29.6653	5.0680	5.5465	30.1941	
3	21.029	34.919	39.346	48.740	23.004	33.301	34.218	46.109	8.1285	14.4359	20.5742	31.1416	7.9219	6.9347	24.2777	
4	32.767	45.182	56.616	62.542	31.459	47.340	56.632	62.040	8.0110	16.8394	25.3339	44.2360	6.5510	12.0296	32.8518	
5	25.918	42.128	50.894	63.678	23.916	39.688	48.486	64.021	8.4373	11.6118	15.4340	41.5753	5.8772	11.9579	25.4601	
6	22.991	37.312	37.420	55.901	23.297	39.875	39.759	54.431	7.1697	11.1813	16.1018	32.9502	6.3541	7.3957	21.4666	
7	13.053	33.097	41.238	52.486	17.832	35.024	43.881	57.355	6.2453	11.8021	17.1039	31.0896	5.0169	7.9640	20.4066	
8	23.721	40.597	47.763	57.737	17.960	39.647	46.844	55.045	7.5916	16.3568	26.0385	34.8825	5.1043	9.7591	28.8642	
9	31.404	45.889	50.029	54.667	26.240	47.480	50.646	55.339	8.9891	20.0875	23.6356	39.9819	8.0208	10.1250	28.0287	
10	25.192	44.425	49.125	54.912	16.322	42.686	46.458	54.147	6.7937	13.8939	20.1969	37.2329	5.9426	11.4350	24.6644	
11	28.944	42.523	47.578	63.926	24.862	41.729	46.019	63.813	7.0640	15.0921	21.1610	37.3662	11.0544	12.0527	35.8577	
12	25.458	42.954	49.859	56.552	24.827	41.762	50.760	52.287	5.9748	11.7543	22.0871	35.4871	6.9235	12.1185	24.6304	
13	26.906	45.300	57.707	66.886	16.611	40.688	56.596	65.974	8.0296	15.7170	33.8692	47.6802	10.1542	16.5393	43.1233	
14	17.084	32.948	37.643	51.589	24.765	37.159	41.160	53.355	6.6703	11.8819	15.3852	25.9350	5.7709	6.3369	22.3317	
15	27.606	43.734	45.807	57.285	23.384	37.355	42.333	56.171	4.8670	10.4518	12.1071	27.8825	8.6524	9.2645	24.7264	
16	19.553	34.127	40.594	54.366	19.101	34.756	46.542	54.355	6.6969	13.3008	19.1144	30.9519	5.2127	4.3044	26.4441	
17	19.702	34.964	39.318	51.944	20.328	35.266	38.453	50.677	6.4585	18.0952	21.6188	33.7722	6.2856	9.9616	30.9783	
18	8.879	31.416	37.566	45.774	13.113	31.346	36.056	43.681	5.3381	8.2026	14.0402	26.8463	4.3834	11.0753	29.9854	
19	17.942	33.441	40.005	52.864	26.627	36.924	46.870	56.150	7.3632	11.1518	19.5291	36.6203	5.1660	7.6154	22.0989	
20	7.802	33.638	37.517	50.672	16.583	35.226	38.636	52.124	6.3767	12.4251	15.4676	26.6169	6.2440	7.5324	27.7764	
21	20.775	33.287	34.913	48.311	23.968	33.701	35.768	48.797	9.4543	15.3725	21.1589	29.5857	8.0813	6.7980	27.0322	
22	12.816	29.966	37.612	44.680	16.645	30.169	38.882	44.974	6.2525	13.0115	20.2756	28.2044	5.6463	9.1637	24.3447	
23	32.319	45.386	50.289	56.997	29.928	44.246	49.446	57.381	6.8512	12.4878	22.0661	35.2257	9.7541	13.0363	31.2513	
24	26.124	41.378	47.328	59.934	22.858	38.146	46.593	56.624	7.0342	15.5698	18.1155	34.9962	5.3554	6.7980	23.9430	
25	15.065	31.768	37.600	48.415	16.004	33.637	40.473	49.993	9.0930	12.8401	19.2973	33.4496	6.6236	10.1323	22.9646	
SED	5.551	3.0440	3.4850	4.5540	5.5755	3.7240	3.7277	5.1788	1.7670	2.750	3.3940	5.0973	1.5763	2.7131	6.0760	
Error df	72	72	72	72	72	72	72	72	72	72	72	72	72	72	72	

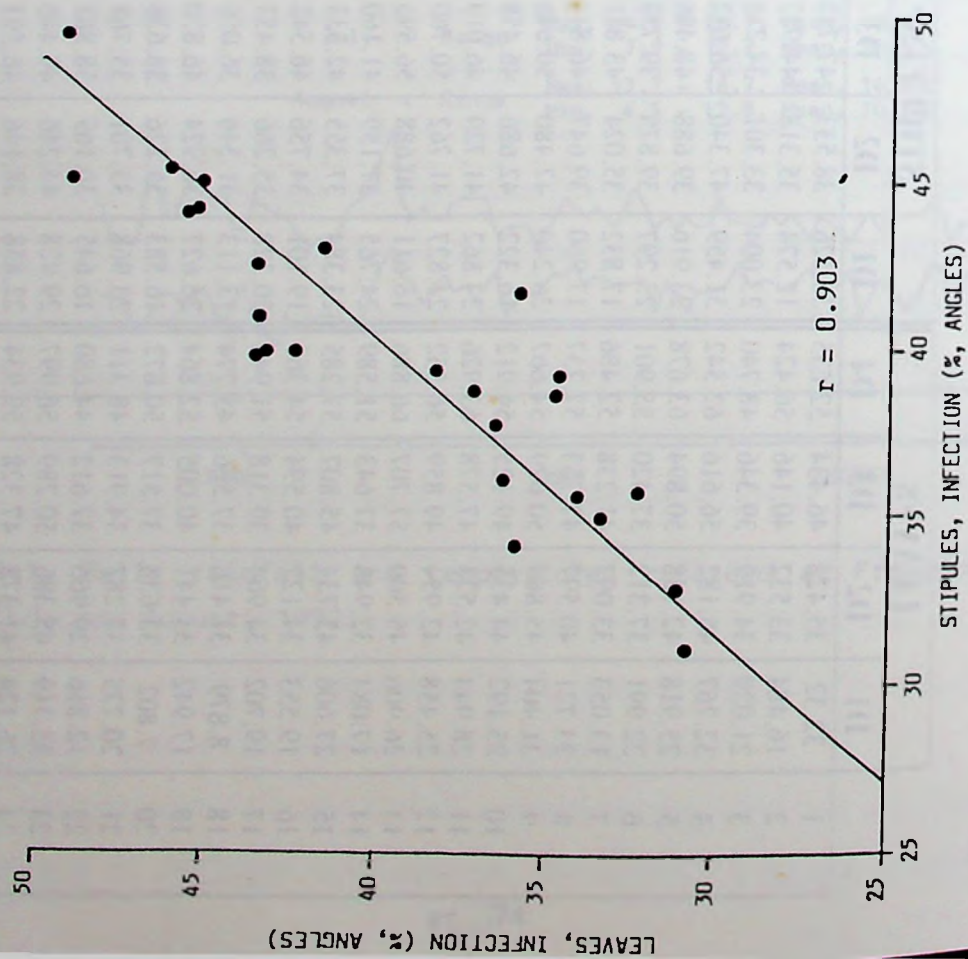


Fig.4.2. Correlation between % infection on stipules and leaves (Frongoch Field Experiment, 1992).

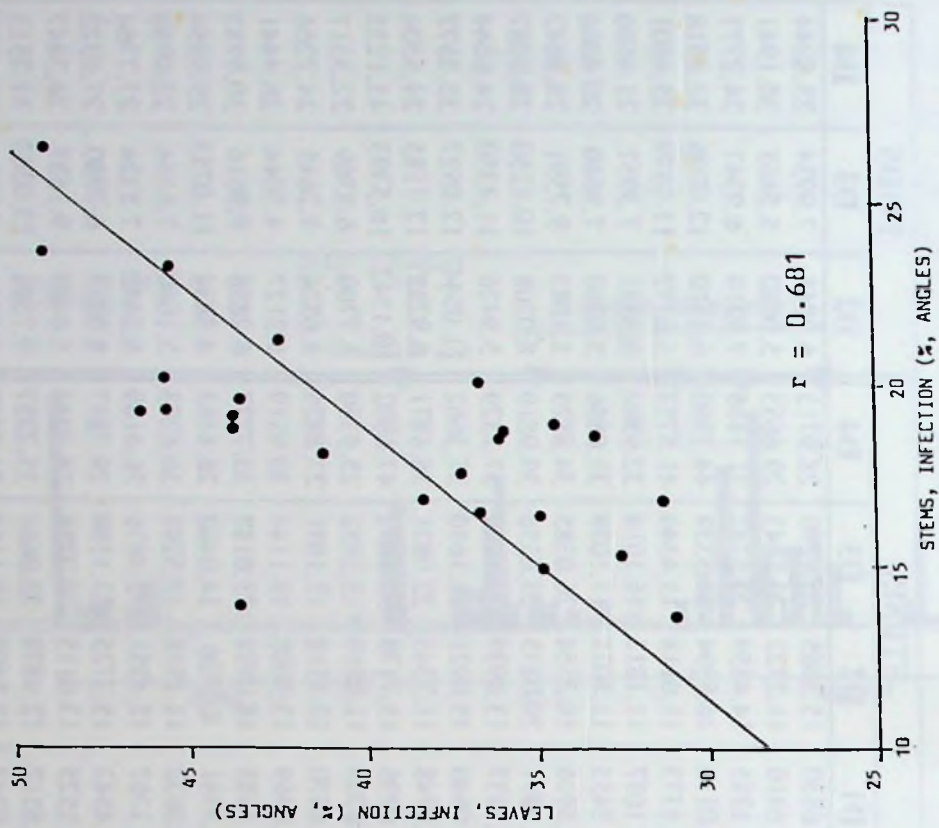


Fig. 4.3 Correlation between % infection on stems and leaves (Frongoch Field Experiment,

(iii) Ranking of cultivars

In Fig. 4.4 cultivars are shown ranked from most to least severely affected at the last recording. Ranking at other times is evidently not the same but correlation analysis, comparing ranking at different times for leaves reveals that a measure of consistency in rank order was maintained (Table 4.2).

Table 4.2 Correlation (r) between disease scores on leaves of 25 pea cultivars at four sampling dates (D_1 - D_4)

	D_2	D_3	D_4
D_1	0.83	0.78	0.67
D_2		0.90	0.79
D_3			0.82

Ranking was examined in more detail by dividing the 25 cultivars into 3 groups (8, 8 and 9 cultivars) as most, least and moderately affected at each sampling date. From these groupings, four sets of cultivars could be defined:

- (a) Amongst the most affected cultivars, three (4, 13, 23) appeared in the same group at all sampling dates and a further three (9, 11, 15) at three sampling dates.
- (b) Amongst the least affected cultivars, five (14, 18, 20, 22, 25) appeared in the same group at all sampling dates and a further two at (7, 21) at three sampling dates.
- (c) Amongst the moderately affected cultivars none appeared in the same group at all sampling dates but three (1, 6, 16) appeared in the same group at three sampling dates.
- (d) The remaining cultivars (2, 3, 5, 8, 10, 12, 17, 19, 24) appeared only once or twice in any one group.

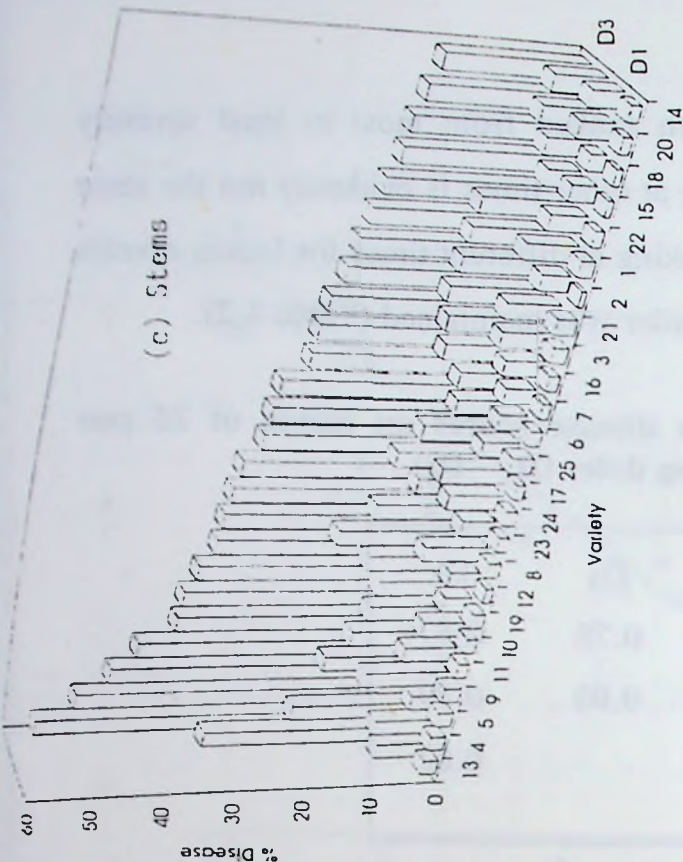
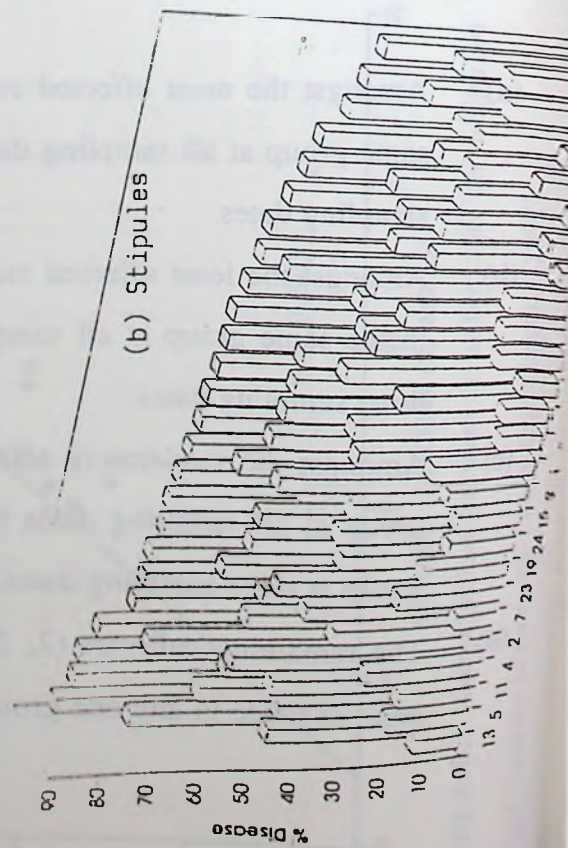
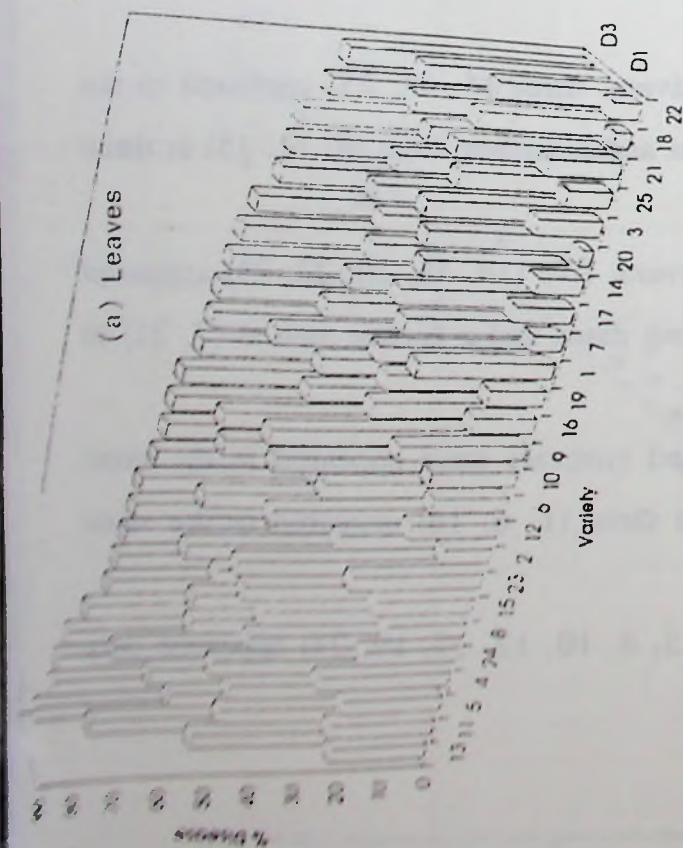


Fig. 4.4. Disease severity on (a) leaves, (b) stipules and (c) stems of 25 pea cultivars at four sampling dates (D1 - D4). Cultivars are ranked from most to least affected at D4. (Frongoch Field Experiment, 1992).

From this analysis it appears that rather more than half the cultivars (16/25) behaved fairly consistently, greatest consistency being shown by the most and least severely affected cultivars.

A similar analysis, based on disease scores on stems, showed an almost similar degree of consistency, 13/25 cultivars occurring 3/4 times in the same category. Three cultivars (4, 9, 13) fell regularly into the most severely affected category. Three cultivars (14, 15, 18) appeared regularly in the least affected category and three others (2, 7, 20) occurred 3/4 times in the same category. Four cultivars (2, 10, 23, 25) occurred 3/4 times in the moderately affected category.

(iv) Disease progress

With four successive disease recordings it was possible to make some assessment of disease progress on leaves and stems for individual cultivars and for three periods of the epidemic. Values of r (Vanderplank, 1963) are shown in Table 4.3 and analysis of the data in Appendix Table 4.3).

Mean rates of disease progress differed significantly ($P < 0.001$) on leaves and stems, 0.096 and 0.131 respectively. Mean rates also differed significantly ($P < 0.001$) between the three time intervals, 0.105, 0.091 and 0.145 respectively. There was, however a highly significant interaction between leaves and stems and intervals. From Table 4.4 it can be seen that whilst the rate on leaves was similar during intervals 1 and 3 it was much lower during interval 2. By contrast, on stems, rates increased progressively over the three intervals with the largest increase in interval 3. There was less rain in interval 2 than in intervals 1 and 3 (Fig. 4.1). This would seem to have had a greater effect on disease progress on leaves than on stems.

Table 4.3

Disease Progress ($\bar{r}$) on Leaves and Stems on 25 cultivars of Peas at three date Intervals (I), Frongoch, 1992.

Cultivar	Leaves			Stems		
	I 1	I 2	I 3	I 1	I 2	I 3
1	0.017	0.112	0.066	0.111	0.083	0.113
2	0.138	0.068	0.168	0.131	0.176	0.120
3	0.087	0.046	0.095	0.078	0.124	0.136
4	0.064	0.122	0.068	0.125	0.126	0.214
5	0.118	0.089	0.146	0.046	0.093	0.333
6	0.090	0.001	0.197	0.067	0.108	0.227
7	0.164	0.086	0.115	0.094	0.111	0.188
8	0.103	0.073	0.109	0.102	0.167	0.095
9	0.075	0.042	0.045	0.117	0.055	0.191
10	0.128	0.048	0.063	0.108	0.117	0.207
11	0.072	0.051	0.181	0.106	0.111	0.202
12	0.101	0.070	0.073	0.100	0.190	0.161
13	0.104	0.129	0.111	0.109	0.250	0.143
14	0.112	0.050	0.143	0.092	0.077	0.153
15	0.087	0.021	0.122	0.114	0.049	0.260
16	0.110	0.067	0.141	0.108	0.096	0.170
17	0.099	0.044	0.128	0.153	0.046	0.159
18	0.266	0.068	0.083	0.065	0.166	0.187
19	0.128	0.068	0.129	0.066	0.165	0.219
20	0.238	0.041	0.133	0.097	0.059	0.173
21	0.100	0.017	0.137	0.070	0.103	0.105
22	0.147	0.084	0.072	0.110	0.139	0.098
23	0.067	0.049	0.074	0.090	0.157	0.173
24	0.095	0.060	0.135	0.118	0.050	0.225
25	0.143	0.062	0.111	0.068	0.123	0.188
Mean	0.113	0.063	0.114	0.098	0.119	0.176
SED	0.053	0.036	0.054	0.043	0.075	0.076
Error DF	72	72	72	72	72	72

Table 4.4.

Mean rates of disease progress (r , Vanderplank, 1963) of *M. pinodes* on leaves and stems of peas during three intervals
(Frongoch Field Experiment, 1992)

	I ₁	I ₂	I ₃	Mean
Leaves	0.113	0.063	0.114	0.096
Stems	0.098	0.119	0.176	0.131
Mean	0.105	0.091	0.145	

(v) Replicate variation

In the analysis (Appendix Table 4.2) carried out on the data from this field experiment, replicate variation was found to be statistically significant. On closer examination it was found that this variation showed a similar pattern irrespective of the disease variable being scored (Table 4.5). The highest scores were on replicate 4 and the lowest (with only one exception) on replicate 1.

Table 4.5

Replicate Means for Disease (% Angles) on Leaves, Stipules, Stems and Pods
(Frongoch Field Experiment 1992)

Replicate	Leaves	Stipules	Stems	Pods
1	38.48	38.70	17.86	12.69
2	39.17	38.93	17.82	14.91
3	40.32	40.05	19.07	13.81
4	40.88	41.33	19.65	16.27
SED	0.860	0.925	0.703	0.914
Error DF	297	297	297	297

In field experiments comparing disease resistance of cultivars grown in contiguous small plots, there is always the risk that disease development will be influenced by inoculum transfer between plots. With *M. pinodes* on pea there is the further complication of two sorts of inoculum viz. splash borne pycnidiospores and airborne ascospores. The design of the experiment (below) was such that ascospores generated in replicate 1 would, on average, move toward replicate 4 possibly accounting for the observed differences in disease severity between replicates.

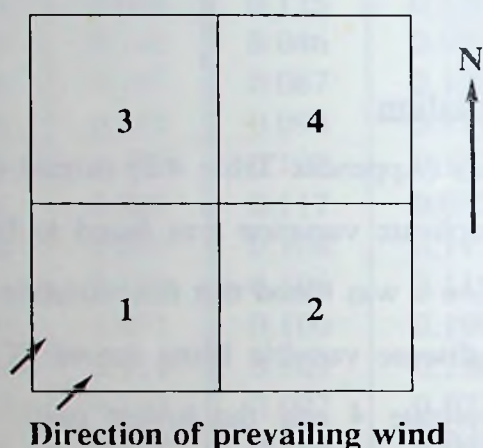


Figure 4.5

**Arrangement and orientation of replicate blocks
(Frongoch Field Experiment, 1992)**

This hypothesis would, however, only be sustainable if replicate differences increased with progress of the epidemic. There was, however, little evidence for this. At the first recording (Table 4.6) differences were not significant but at subsequent recordings, although infection was always significantly greater in replicate 4 than in replicate 1, the magnitude of the difference did not change.

Table 4.6**Replicate Means for Disease (% Angles) on Leaves at Four Sampling Dates
(D1 - D4) Frongoch Field Experiment 1992**

Replicate	D ₁	D ₂	D ₃	D ₄
1	23.45	36.24	42.37	51.87
2	20.03	37.56	44.29	54.80
3	23.38	37.90	44.14	55.87
4	21.44	40.19	45.25	56.64
SED		1.22	1.39	1.82
Error DF		72	72	72

4.2 ASSESSMENTS OF INFECTION FREQUENCY AND LESION SIZE.

Materials and Methods

(i) Infection frequency

Seeds of 25 pea cultivars (Appendix Table 4.1) were sown singly in 7.5cm diameter pots of John Innes No. 2 compost. There were 8 pots of each cultivar and seedlings were grown in the glasshouse (c. 20°C) until they were at growth stage 103. Pots were then transferred to the laboratory and each sprayed with 5ml pycnidiospore suspension (5×10^4 spores, ml⁻¹) using a Precision Sprayer (Burkhard Manufacturing Co., Rickmansworth, UK). After spraying, pots were enclosed in plastic bags for 4 days to ensure high humidity for spore germination and infection. The plastic bags were then removed and the pots returned to the glasshouse where they were arranged as 8 completely randomised blocks.

Eleven days after inoculation, when lesions were clearly visible, plants were returned to the laboratory. Leaves (each with two leaflets) or, in semi-leafless cultivars, stipules, were removed from nodes 2 and 3 and discs cut from the centre of each leaflet or stipule using a 10mm diam. cork borer. The total number of lesions on each disc was counted under a binocular microscope and a mean value for each plant obtained from the four discs scored. Mean values for individual cultivars varied widely, from over 12 to less than 1. A square root transformation was, therefore, used before statistical analysis of the data.

(ii) Lesion size

Twelve of the 25 cultivars tested in the field were selected to represent cultivars with high, low and intermediate disease scores in the field.

High	4, 11, 13, 23 (all standard)
Intermediate	3, 5, 10 (semi-leafless), 24 (standard)
Low	14, 18, 20, 21 (all semi-leafless)

Seeds of each cultivar were sown singly in 7.5cm diam pots of John Innes No. 2 compost. There were 12 pots of each cultivar and seedlings were grown in a greenhouse (c. 20°C) until they were at growth stage 103. All plants of each cultivar were inoculated by placing a single 5 μ l droplet of spore suspension (2×10^4 spores, ml⁻¹) on each member of a pair of leaflets (or pair of stipules for semi-leafless cultivars) at nodes 1 and 2. Following inoculation pots were enclosed in plastic bags for 4 days to ensure high humidity for spore germination and infection. Pots were then arranged as 4 completely randomised blocks.

Data was collected on four plant of four randomly selected pots for each cultivar. Lesion diameters were recorded (using a pair of dividers) after 17 days and a mean value for each plant obtained from measurements on the 4 leaflets or stipules. Data was collected on four plants randomly selected from the three in each replicate. Lesion diameters were recorded (using a pair of dividers) after 17 days and a mean value for each plant obtained from measurements on the four leaflets or stipules. Data was transformed to square roots before analysis.

Results

(i) Infection frequency (IF)

IF varied greatly between cultivars (Fig. 4.6) and analysis of the data (Appendix Table 4.4) showed that differences were highly significant ($P < 0.001$). Comparing values of IF with mean values for infection (leaves) in the field (Fig. 4.7) showed that they were significantly, if not strongly correlated ($r = 0.616$).

(ii) Lesion size (LS)

The twelve cultivars tested showed significant differences ($P < 0.001$) in the size of the lesions which developed on their leaves or stipules and these differences were correlated ($r = 0.600$) with mean values for infection of

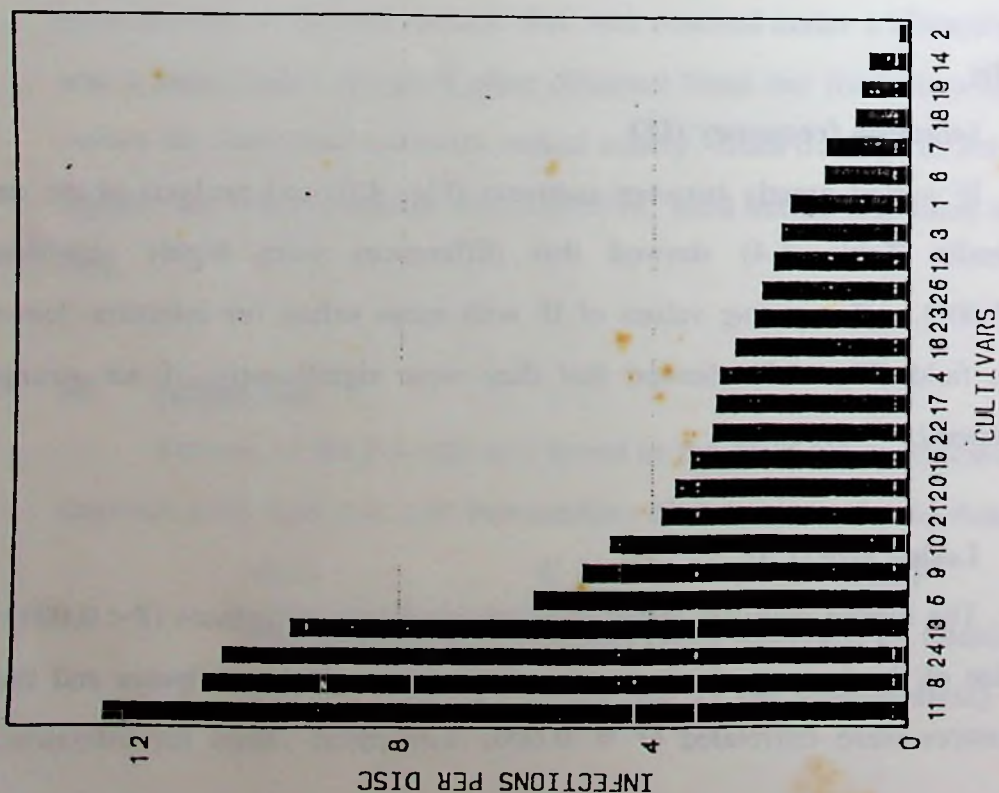


Fig. 4.6. Numbers of infections on 10 mm leaf on stipule discs of 25 pea cultivars after inoculation with a standard dose of

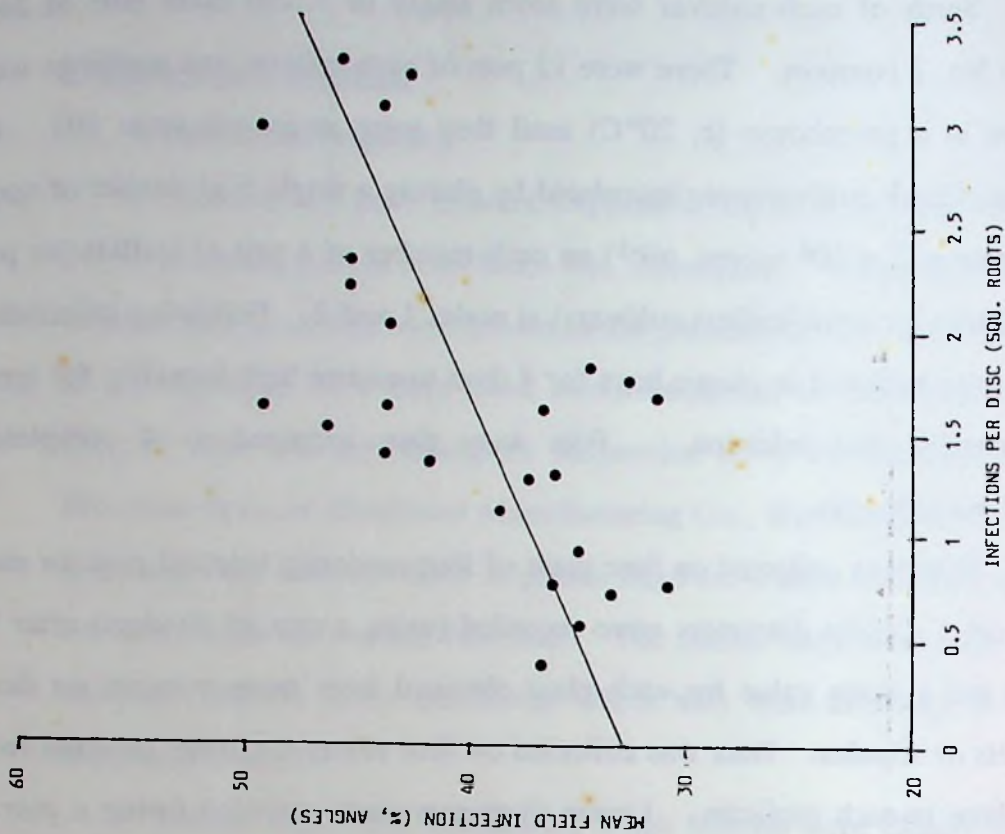


Fig. 4.7. Regression of numbers of infections on leaf discs (IF) on disease (%) in the field for 25 pea cultivars

leaves in the field (Fig. 4.8). For this group of cultivars the correlation of IF x field infection was $r = 0.621$ (Fig. 4.9).

(iii) Relationship between IF, LS and field scores

Plotting IF against LS showed that, for the twelve cultivars tested, there was no evidence of correlation, the calculated value of r being 0.307. However, combining values of IF and LS (viz. IF x LS) produced values which correlated significantly ($P < 0.01$) and relatively strongly ($r = 0.681$) with mean disease scores in the field (Fig. 4.10). This was a stronger correlation than that found for either IF or LS alone for the same 12 cultivars.

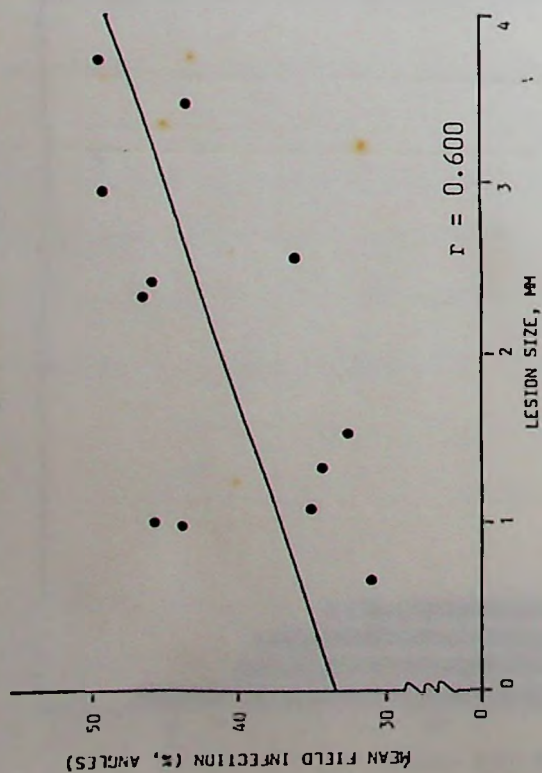


Fig. 4.8. Correlation between lesion size and infection in the field.

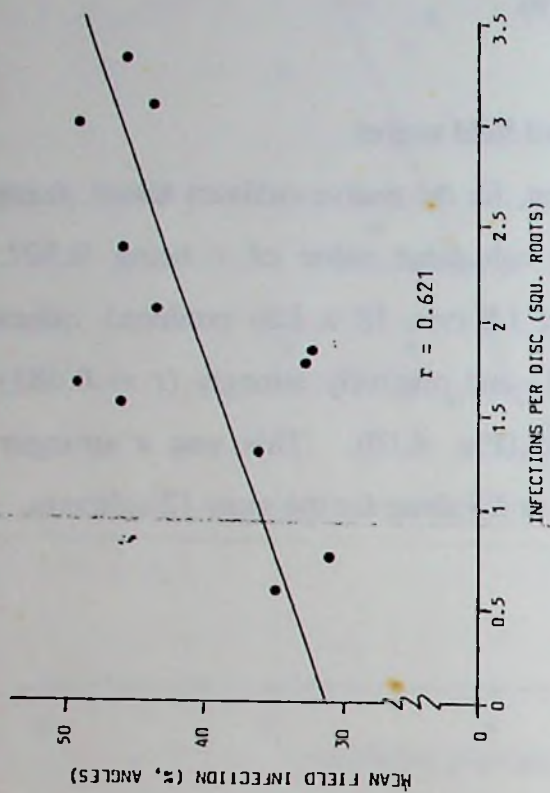
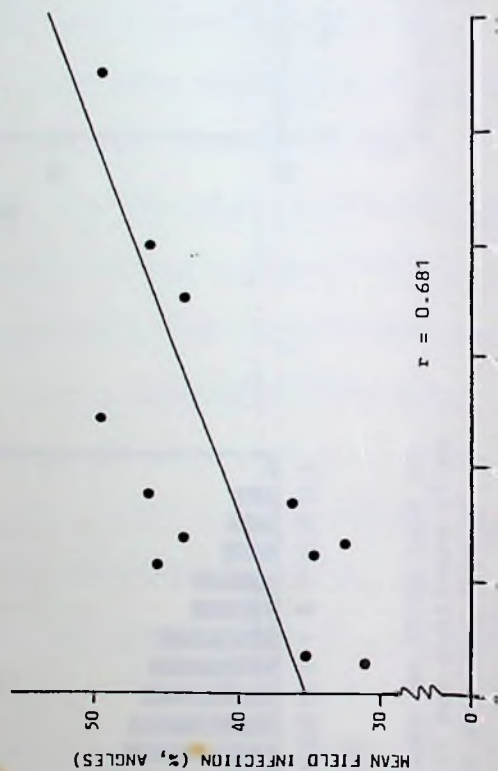


Fig. 4.9. Correlation between infections per leaf disc and infection in the field



Discussion

None of the twenty-five commercial cultivars used in these experiments possessed a really useful level of resistance to *M. pinodes*, but there were differences in susceptibility. Moreover, it is worth noting that under the conditions of the field experiment (very small (1m²) plots in close proximity to one another) that the less susceptible cultivars would be relatively disadvantaged. Inoculum transfer from more to less susceptible cultivars would tend to increase disease in the latter, a point made succinctly by Vanderplank (1963) who observed that horizontal resistance 'gains from uniformity'. When grown in large fields the difference between more and less susceptible cultivars would be expected to increase.

Variation in horizontal resistance, now more commonly termed partial resistance, is basically attributable to differences in three features of the host pathogen interaction viz. infection frequency (IF), latent period (LP) and sporulation (SP). Resistance is enhanced with low IF, long LP and low SP.

In these experiments no attempt was made to quantify LP or SP since these pose difficult problems which have yet to be addressed. LP is normally defined as the interval from infection to onset of sporulation. Measurement of the interval from infection to pycnidiospore production could well be accomplished as indicated by work on *Septoria (Leptosphaeria) nodorum* on wheat (Griffiths & Jones, 1987). However, the more important interval for *M. pinodes* is from infection to ascospore production and this offers a technically challenging problem. Practical methods for quantifying SP (both pycnidiospores and ascospores) are also difficult to envisage. For the present, therefore, only values of IF and LS (lesion expansion rates) which are relatively easy to determine, are likely to be available.

IF and field scores for *M. pinodes* were significantly correlated ($r = 0.62$), differences in IF thus accounting for about 38% of the variation between cultivars in the field. LS was also significantly correlated ($r = 0.60$) with field scores but interestingly, was not correlated with IF indicating that the host characteristics influencing lesion growth are different from those influencing

infection and may be under different genetic control. Combining values of IF and LS improved the correlation with field scores to $r = 0.68$ accounting for 46% of the variation between cultivars.

Assessments of components of partial resistance can provide excellent prediction of field performance (Leonard & Mundt, 1984; Griffiths & Jones, 1987; Kari & Griffiths, 1993) in some host-pathogen combinations. They have a potential value in plant breeding for screening progenies without recourse to expensive field trials but to be really useful it is necessary to have strong correlations, probably of the order of $r = 0.90$. Evidently the correlations obtained in these experiments fall short of what is needed for reliable prediction. However, it is important to note that the strength of the correlations depends not only on the assessments of partial resistance but on the reliability of the field assessments. In this instance they were derived from a single field experiment, conducted, as indicated earlier, under conditions which were far from ideal. To validate the utility (or otherwise) of seedling tests for components of partial resistance as predictors of field performance there is need for further field experiments, preferably with larger plots and screens to minimise interplot interference.

Appendix Table 4.1

Cultivars of *Pisom sativum* used in the 1992 Field Experiment

Number	Name	Number	Name
1	Baroness *	14	Arena *
2	Orb*	15	Celeste
3	Solara*	16	Grafila *
4	Conquest	17	Montana *
5	Bohatyr	18	Baccara *
6	Countess *	19	Axe *
7	Princess *	20	Azur *
8	Bunting	21	Alfetta *
9	Maro	22	Juno *
10	Progretra	23	Kiri
11	Rex	24	Olivin
12	Guido	25	Echo *
13	Mascot		

* Semi-leafless

Appendix Table 4.2

Anovar of disease scores (Table 4.1) on Leaves, Stipules, Stems, and Pods on 25 cultivars at four sampling dates
(Frongoch Field Experiment 1992)

	LEAVES			STIPULES		STEMS		PODS		
	DF	MS	VR	MS	VR	MS	VR	DF	MS	VR
Total	399							299		
Replicate	3	118.02	3.19*	145.13	3.39*	82.69	3.34*	3	176.13	5.62***
Combinations	99	716.25	19.35***	693.52	16.20**	449.19	18.15***	74	381.67	12.18***
Cultivar (C)	24	517.69	13.98***	313.36	7.32***	137.91	5.57***	24	91.33	2.91***
Dates (D)	3	18,672.42	504.36***	19,185.69	448.07*	12,913.06	521.87***	2	12,385.44	395.19***
Interaction (Cx D)	72	34.26	0.93	49.74	1.16***	33.63	1.36	48	26.68	0.85
Error	297	37.02		42.82		24.74		222	31.34	

Appendix Table 4.4**Anovar of Number of Infections on 10 mm leaf discs (Sq. root) of 25 cultivars**

Item	DF	MS	VR
Total	199		
Cultivars	24	5.532	9.408 ***
Replicates	7	0.837	1.424 NS
Error (a)	168	0.588	

Appendix Table 4.5**Anovar of Lesion Size (mm) of Leaves/Stipules of 12 Cultivars**

Item	DF	MS	VR
Total	47		
Cultivars	11	4.639	7.362 ***
Replications	3	1.443	2.290 NS
Error (a)	33	0.630	

Appendix Table 4.3

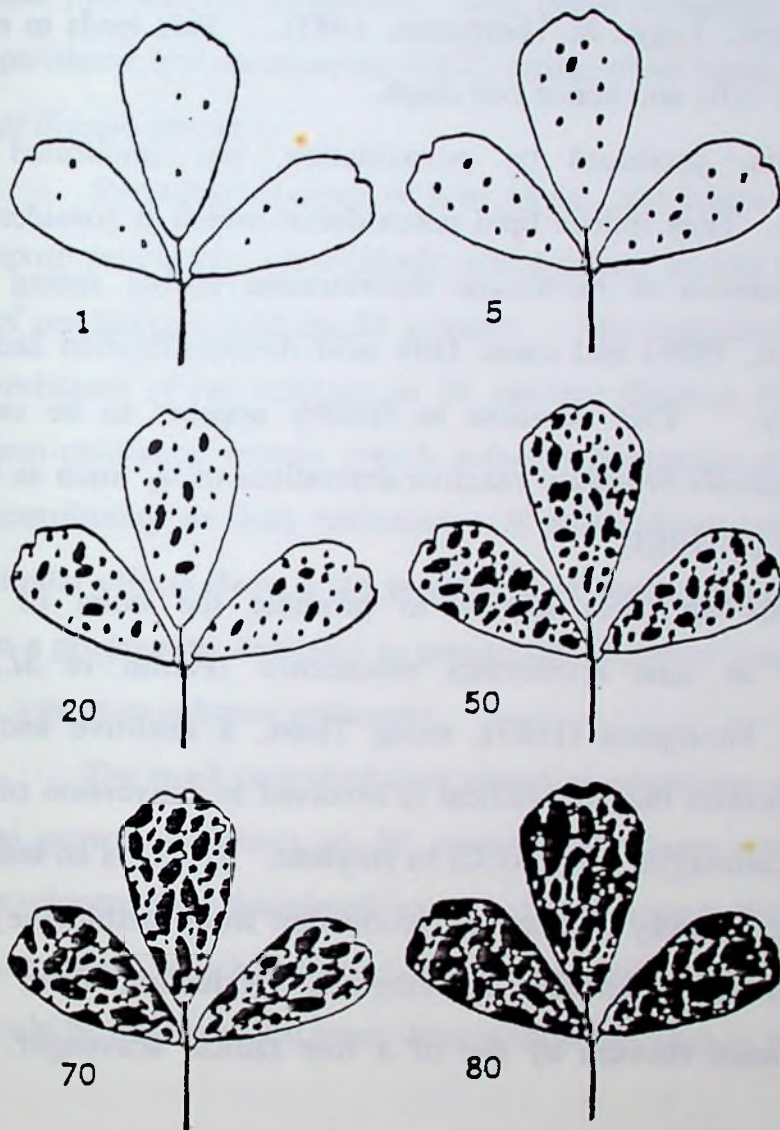
**Anovar of rates of disease progress on leaves and stems of 25 cultivars
(Frongoch Field Experiment 1992)**

Item	DF	MS	VR
Replications	3	0.009	2.10 NS
Cultivars	24	0.006	1.35 NS
Error (a)	72	0.004	
Plant Parts (leaves, stems)	1	0.180	103.77**
Cultivars x Plant Parts	24	0.004	2.18**
Error (b)	75	0.002	
Intervals	2	0.158	18.54***
Cultivar x Intervals	48	0.009	1.11 NS
Plant Parts x Intervals	2	0.090	10.60***
Cultivar x Plant Parts x Intervals	48	0.007	0.88 NS
Error (c)	300	0.009	

Appendix 4.4 Disease assessment key for common alfalfa leaf spot (James, 1971) used for disease assessment of pea blight (1992)

COMMON LEAF SPOT OF ALFALFA

Key No. 22



PERCENTAGE LEAF AREA COVERED

Use for:

Common leaf spot of alfalfa (*Pseudopeziza trifolii* (Biv.-Bern. ex Fr.) Fckl. f. sp. *medicaginis-lupulinae* Schmied.)

Procedure:

Assess individual leaves or plants, or plants in small sample areas (ft², yd², m²).

Growth stages:

Before first and second cuts and at any other appropriate stages (see growth stage key).

Assessing severity:

Assess percentage leaf area affected (including defoliation due to disease, if any).

Reference:

7

Introduction

Senescence of plant tissue is known to be accompanied by changes in membrane permeability brought about by physical changes in the lipid bilayer leading to decreased lipid fluidity and also chemical changes leading to the oxidation of phospholipids (Mayak, Legge & Thompson, 1983). This leads to efflux of electrolyte from the cells and hence cell death.

Free radicals, produced by peroxidation, are implicated in the senescence process. They induce lipid peroxidation which is considered to be an important mechanism of membrane deterioration during ageing (P. Droillard & Bureau, 1986) and cause fatty acid de-esterification and loss of membrane integrity. This decrease in fluidity appears to be caused by superoxide (O_2^-) radicals or highly reactive derivatives of O_2^- such as hydroxyl radicals (Mayak *et al.*, 1983).

Free radicals are also known to promote the burst of ethylene production which in turn accelerates senescence (Paulin *et al.*, 1983). McRae, Baker & Thompson (1983), using Tiron, a sensitive and specific detector of O_2^- , showed that this radical is involved in conversion of 1-aminocyclopropane, 1-carboxylic acid (ACC) to ethylene. (ACC is an intermediate in the biosynthetic pathway of ethylene production from methionine). They also demonstrated the inhibition of ethylene production from ACC in protoplasts of Ipomea flowers by use of a free radical scavenger, n-propylgallate.

There is more evolution of ethylene in diseased than healthy plants. Its production is more characteristic of diseases involving tissue necrosis. In diseases where a more stable host-pathogen relationship is established, for example, in a systemic virus infection (Archer & Hislop, 1975; 1988). Either the host or the pathogen may contribute to ethylene production.

Prusky (1988) used anti-oxidants to delay the onset of necrosis in anthracnose (*Colletotrichum gloeosporioides*) and stem end rot (*Diplo-*

natalensis) of avocado after harvest. Similarly, Elad (1992) achieved some success in the control of *Botrytis cinerea* and *Sclerotinia sclerotiorum* on vegetable crops using antioxidants.

Mycosphaerella pinodes, a necrotroph, infects all parts of the pea plant, and lesions, especially on leaves, quickly enlarge under favourable conditions and pycnidia are produced. Leaf death is rapidly followed by production of perithecia and ascospores, which being wind borne cause a rapid acceleration of disease spread.

Retarding necrosis of host tissue, and hence also retarding the onset of spore production (particularly ascospores), should achieve significant control of pea blight caused by *M. pinodes*. The evaluation of components of partial resistance of pea cultivars to *M. pinodes* (Section 4) has already indicated that host-resistance factors which retard colonisation appear to be important in contributing to field resistance. If antioxidants could be shown to produce similar effects there is the possibility of deploying them directly or using them in a programme designed to genetically engineer host plant physiology in such a way as to enhance resistance.

The work described here aimed at examining a range of antioxidants, as (a) growth inhibitors of *M. pinodes* in culture and (b) inhibitors of lesion development on inoculated leaves. It was hoped, that by using these two tests, compounds which achieved effects primarily through free radical scavenging could be distinguished from those with fungicidal or fungistatic effects.

Materials and Methods

Antioxidants

The following compounds were used. All except one (tBHQ) were obtained from Sigma Chemical Co. UK; tBHQ was obtained from Aldrich Chemical Co. UK.

(1) ascorbic acid (AA), (2) benzoic acid (BA), (3) butylated hydroxyanisole (BHA), (4) butylated hydroxytoluene (BHT), (5) tertiary butyl hydroquinone (tBHQ), (6) p-coumaric acid (pCA), (7) dimethyl sulphoxide (DMSO),

guaiaretic acid (NDGA), (11) propyl gallate (PG), (12) protocatechuic acid (PCA), (13) tannic acid (TA), (14) thiourea (TU), (15) tocopherol (T)

Four compounds (AA, DMSO, GAL and TU) were dissolved in distilled water and the remainder in ethanol, to produce molar stock solutions.

Agar media

The basal medium used for experiments on colony growth was 'C' PDA. Before pouring plates, 100 ml aliquots of sterilized medium were amended with 1000, 100 and 10 μ l of the stock solutions of the antioxidants thereby producing final concentrations of 10.0, 1.0 and 0.1 mM. Sterile plastic petri dishes (90mm diameter) each contained 20 ml medium.

Culture of *Mycosphaerella pinodes*

The stock culture of *M. pinodes* used in all previous experiments was used.

Inoculation of culture plates and measurement of colony growth

Plates were inoculated with single plugs (7.6mm diameter) cut from 10-day old stock cultures. After inoculation, plates were sealed with parafilm and incubated in darkness at 22°C for 10 days. Within the incubator, plates were stacked in groups of three (viz the 3 concentrations of each compound). Compounds and replicates (3) were randomised within the incubator.

At the end of the incubation period, colony diameter was measured by taking the mean of two measurements (minus diameter of the plug) at right angles to one another. Dividing by two gave the radial growth from the point of inoculation. Growth rate per day (mm) was calculated.

Lesion development on detached leaves of *Pisum sativum* (cv Conquest)

Suspensions of pycnidiospores (5×10^4 , ml⁻¹) of *M. pinodes* in water containing 0.025% 'Tween 20' were used. Detached leaves were taken

nodes 2 and 3 or 9 and 10 of glasshouse-grown plants at growth stage 104 (experiment 1) or 112 (experiment 2) respectively. Leaflets were placed on 0.25% water agar amended with antioxidants as described for colony growth experiments. Preliminary experiments had indicated that results were much influenced by differences between plants and nodes. To minimise these effects the four leaflets from each plant were distributed between four dishes for each compound i.e. one each for 0, 0.1, 1.0 and 10.0 mM. Leaflets from four plants were used for each set of plates.

After placing the leaflets on the agar surface, each was inoculated with a single 5 μ l droplet which was placed at the centre of the leaf but avoiding the midrib. The dishes were sealed with parafilm and incubated in a growth room (c. 21°C) with illumination (16 h day) provided by banks of fluorescent tubes set at 0.5 m above the dishes. The dishes were arranged in stacks of four (one for each treatment/concentration) and the stacks randomised within each of three replicate blocks. During incubation (9 days in experiment 1; 7 days in experiment 2) dishes were regularly re-randomised within both stacks and replicates.

At the end of the incubation period, lesions were measured using a pair of dividers and, after subtracting the diameter of the drop, the mean for the four leaflets in each dish was divided by two to obtain the mean radial growth. From these values the rate of lesion expansion per day was calculated.

Results

(i) Colony growth on agar

Three experiments were carried out: in each, the fifteen antioxidants were tested at three concentrations (0.1, 1.0, 10.0 mM) and there were three replicates of each compound/concentration combination. A control, (unamended PDA) provided a sixteenth treatment. Analysis of the data (Appendix Tables 5.1 and 5.2) showed highly significant differences between both antioxidants and concentrations in all experiments. The interaction,

compounds x concentrations, was also highly significant. Replicate variation was just significant ($P < 0.05$) in one experiment (3 (a), Appendix Table 5.2).

In experiment 1, the effects of individual antioxidants (Fig. 5.1 (a)) were surprising; most, apparently, stimulated growth at the lowest concentration, whilst some stimulated growth at the intermediate concentration. At the highest concentration most inhibited growth but a few were still stimulatory.

These stimulatory effects of antioxidants were unexpected and it was thought that they might have arisen from a combination of faulty sealing of the plates and an atypically low control value. In experiment 2, where particular care was taken to seal plates, no compound significantly stimulated growth (Fig. 5.1 (b)). However, the general pattern of growth responses was similar to that found in experiment 1 and the differences between the two experiments could largely be accounted for on the assumption that the control value in experiment 1 was too low.

In a third experiment a comparison was made between sealed and unsealed plates. This showed (Appendix Tables 5.1 and 5.2) that mean growth rates were higher in unsealed plates. Moreover, it is also evident (Fig. 5.1 (c) and (d)) that the lowest concentration often produced some stimulation in unsealed plates but rarely in sealed plates, indicating that imperfect sealing might have contributed to the results found in experiment 1.

A joint analysis of the four experiments (sealed and unsealed plates in experiment 3 were considered as separate experiments) (Appendix Table 5.3) showed that there were highly significant differences between experiments, compounds and concentrations, whilst replicate variation within experiments was not significant. The interactions, experiments x compounds and experiments x concentrations were, as may be deduced from Fig. 5.1, highly significant.

Considering the effects of the individual compounds averaged over all the experiments (Fig. 5.2) indicated that they fell into three groups (illustrated in Plate 5.1).

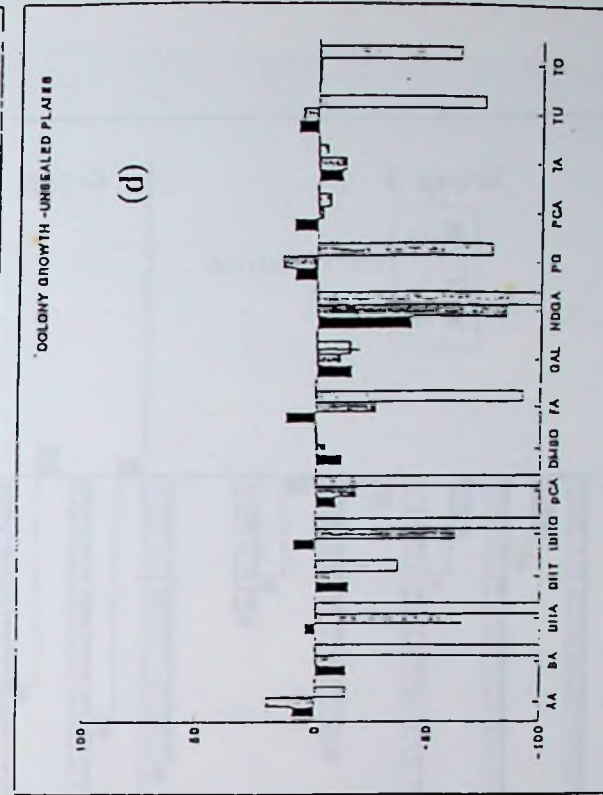
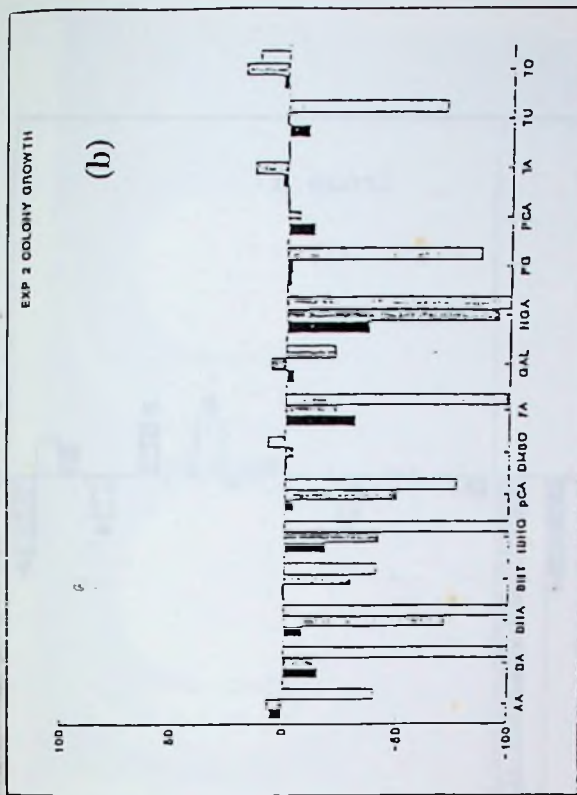
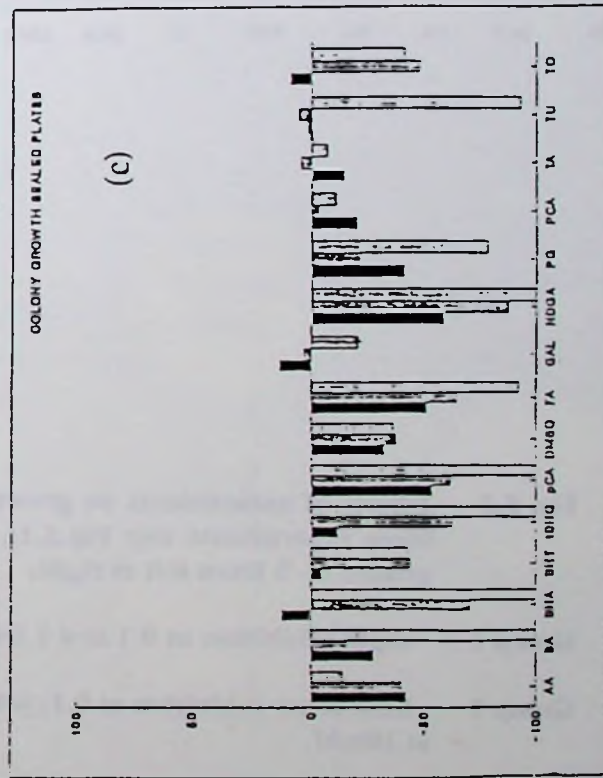
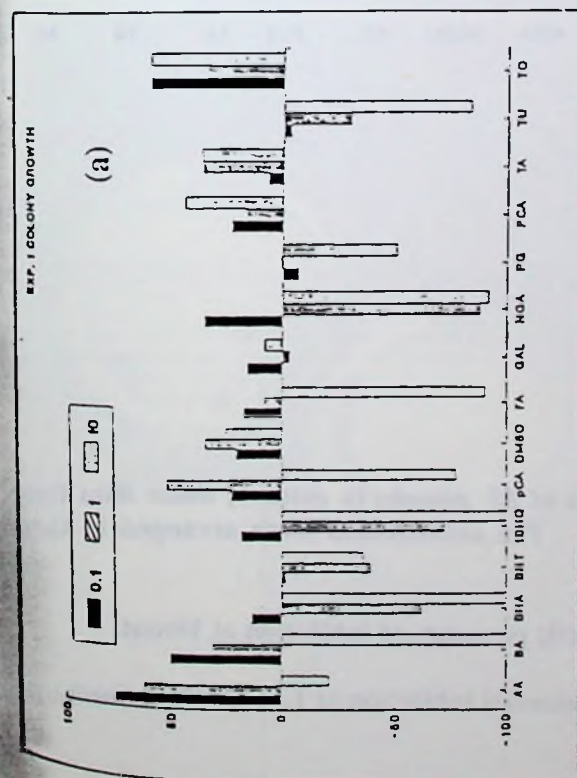


Fig. 5.1 Effect of antioxidants on colony growth of *M. pinodes* at three concentrations, 0.1, 1.0 and 10.0mM. Increase or decrease from control, %.

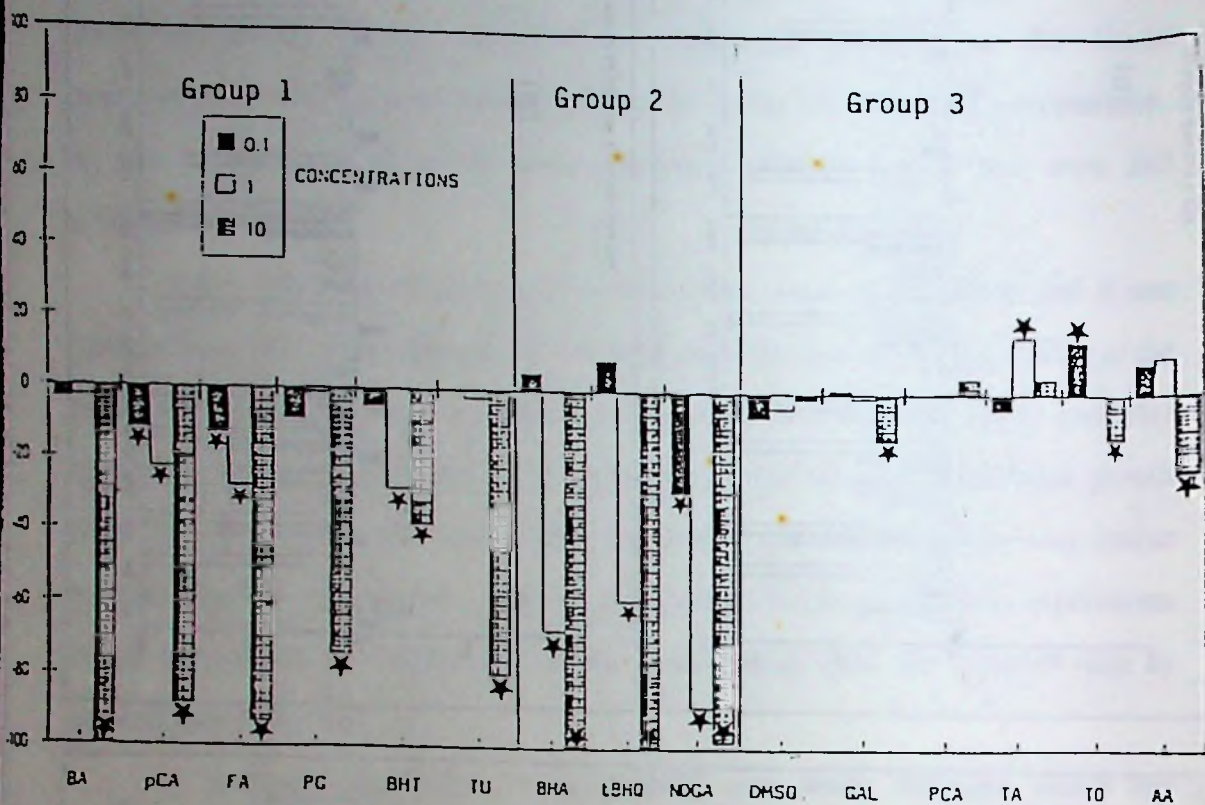


Fig. 5.2 Effects of antioxidants on growth of *M. pinodes* in culture; mean data from three experiments (see Fig.5.1). The antioxidants were arranged in three groups 1 - 3 from left to right.

Group 1 - slight inhibition at 0.1 and 1.0mM; pronounced inhibition at 10mM.

Group 2 - little or no inhibition at 0.1; substantial inhibition at 1.0; complete inhibition at 10mM.

Group 3 - negligible inhibition or stimulation at any concentration.

* = Significant increase or decrease ($P < 0.05$).



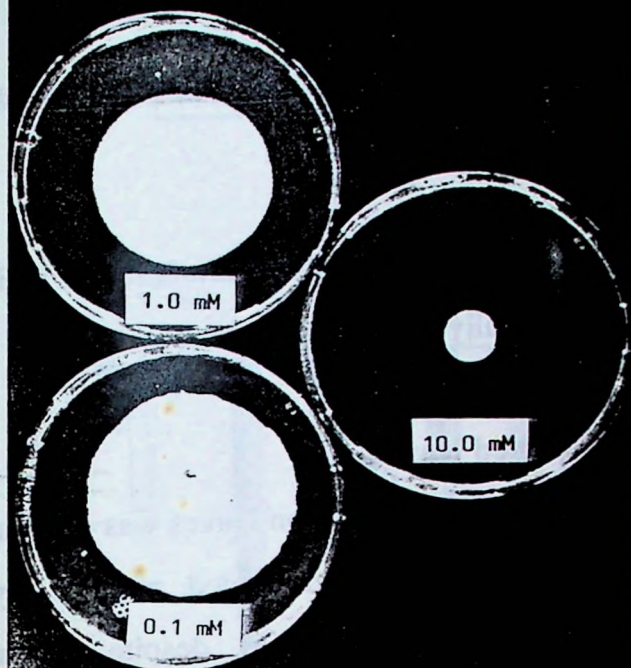
Figure 1. Life cycle of the...



Figure 2. Life cycle of the...

Plate 5.1

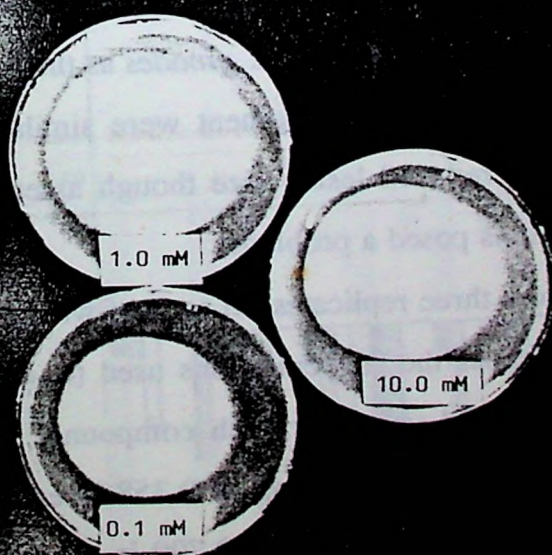
Effects of three antioxidants, at concentrations of 0.1, 1.0 and 10mM, on colony growth of *Mycosphaerella pinodes* in culture. The compounds shown (PG, NDGA and TO) are representative of three groups of compounds (see text) with similar effects on colony growth.



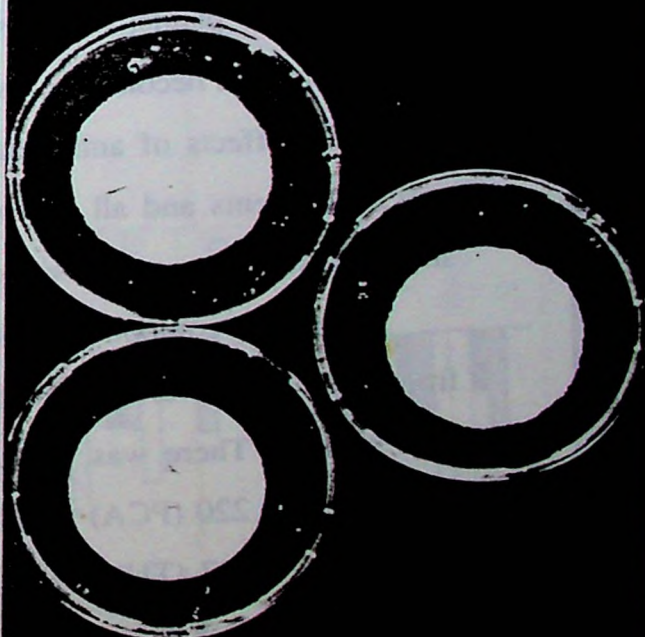
PROPYL GALLATE (PG)



NORDIHYDROGUAIARETIC ACID (NDGA)



TOCOPHEROL



CONTROL

Group 1 - BA, pCA, FA, PG, BHT, TU - where only slight inhibition was produced at concentration 0.1 and 1.0 mM and where at 10 mM inhibition was only complete in one instance (BA).

Group 2 - BHA, tBHQ, NDGA - where substantial inhibition was produced at 1.0 mM and complete inhibition at 10 mM.

Group 3 - DMSO, GAL, PCA, TA, TO, AA - where stimulation or inhibition was very small and usually not statistically significant.

(ii) Lesion development on detached leaves

Effects of antioxidants on lesion development on leaves was examined in two experiments (Fig. 5.3). Lesion size was recorded after 9 days in experiment 1 and after 7 days in experiment 2, but, despite the shorter incubation period in experiment 2, lesions were larger than in experiment 1 (mean lesion diameters, 0.254 mm and 0.498 mm in experiments 1 and 2 respectively; Appendix Tables 5.4 - 5.6). The difference is probably due to the age of the plants; those in experiment 2 were from older plants and it is known that peas become increasingly susceptible to *M. pinodes* as they mature.

The effects of antioxidants on lesion development were similar in both experiments and all compounds reduced lesion size though assessing statistical significance of the reductions posed a problem.

For each compound there were three replicates, in each of which leaves from four plants were distributed across the concentrations used (0, 0.1, 1, 10.0 mM). There was, thus, a control value for each compound. The values varied from 0.220 (PCA) to 0.532 (BHT) with a mean of 0.358 in experiment 1 and from 0.597 (TU) to 0.806 (FA) with a mean of 0.700 in experiment 2 (Appendix Table 5.4). In comparing the effects of different concentrations of individual compounds it is possible to use two different control values viz. a specific control for the compound or the mean control for the whole group.

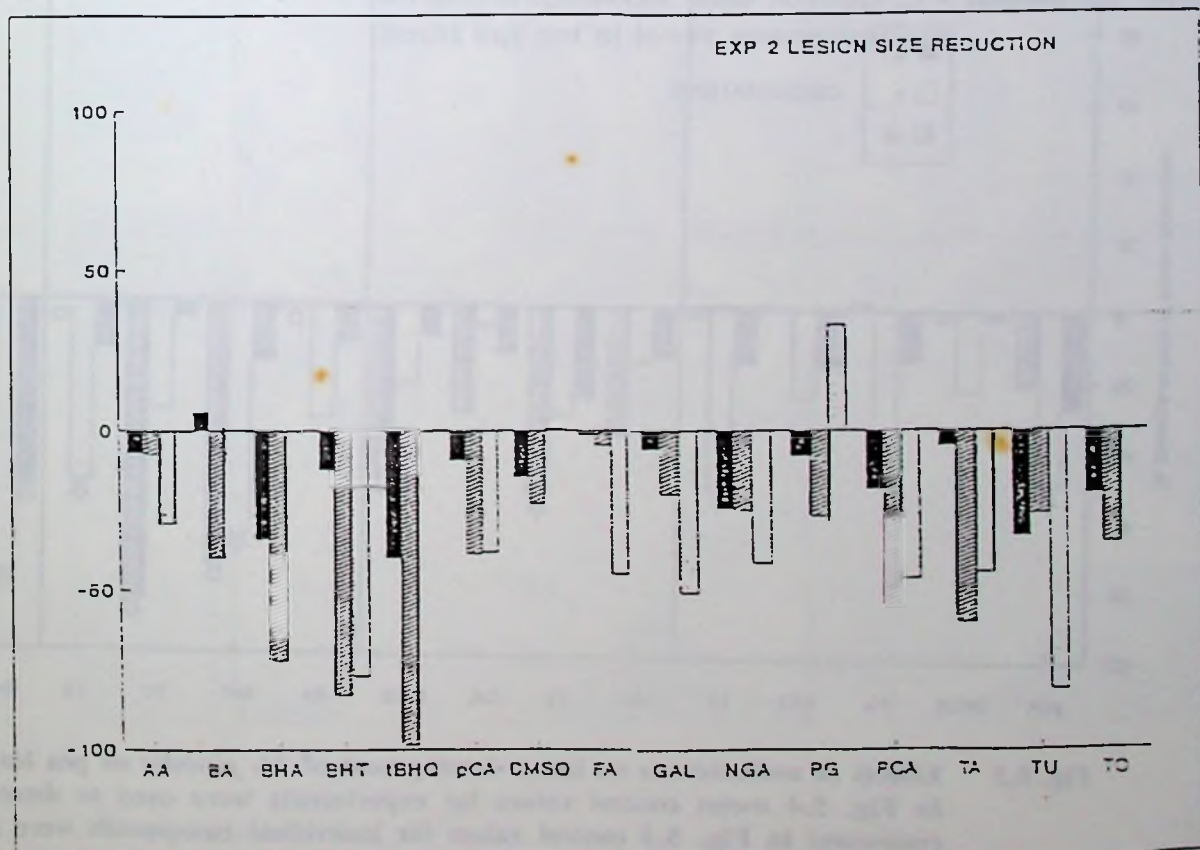
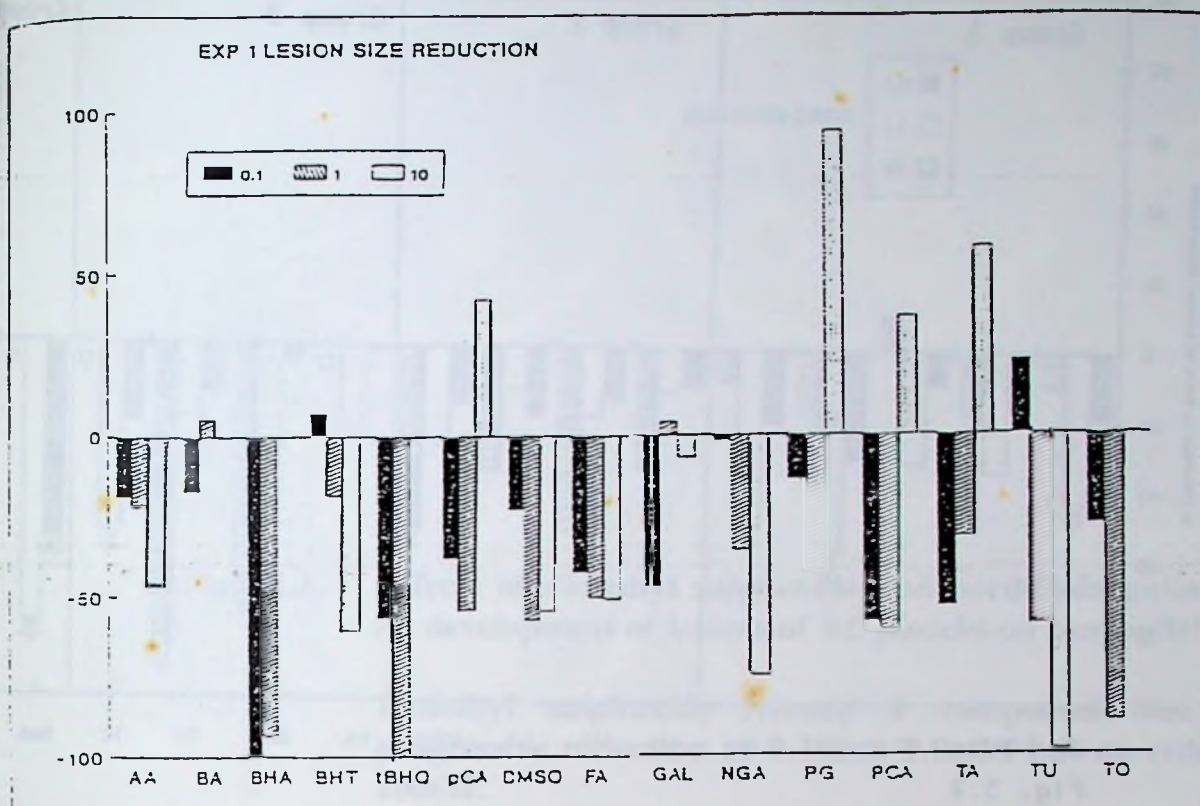


Fig. 5.3 Effects of antioxidants at three concentrations, 0.1, 1.0 and 10.0mM on lesion development of *ML pinodes* in two experiments. Increase and decrease from control, %.

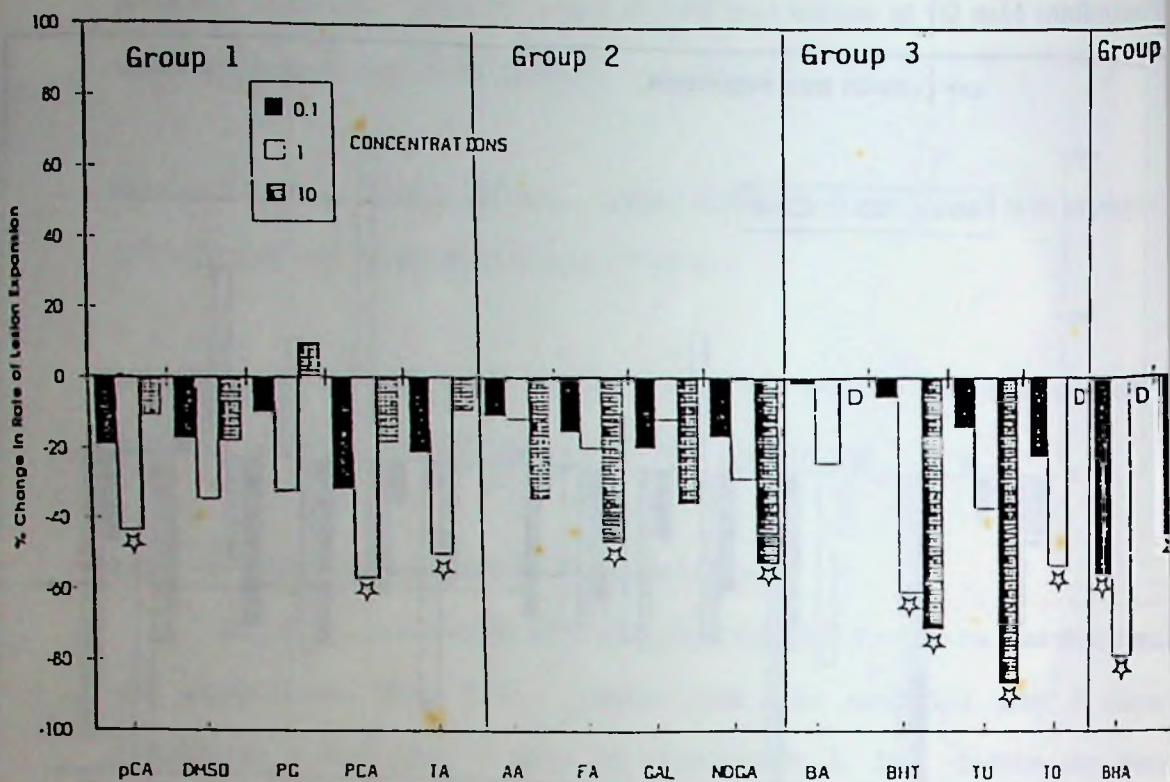


Fig. 5.4

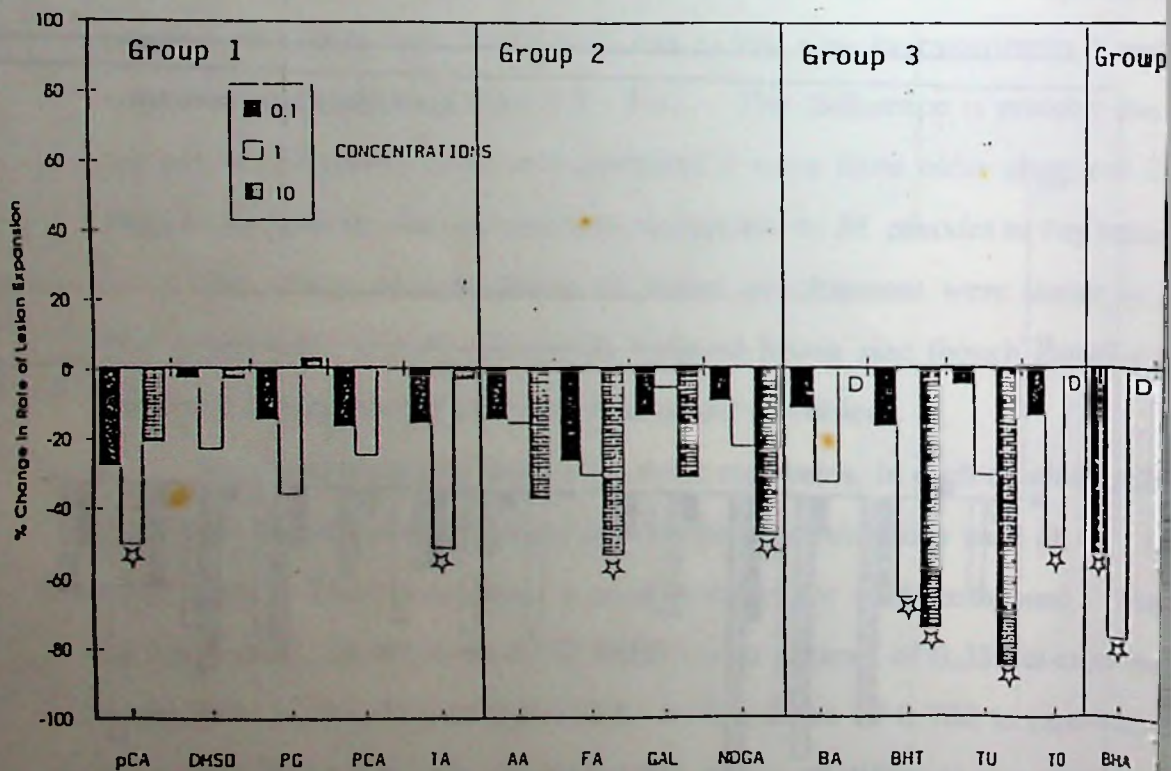


Fig. 5.5 Effects of antioxidants on lesion development of *M. pinodes* on pea leaflets. In Fig. 5.4 mean control values for experiments were used to determine responses; in Fig. 5.5 control values for individual compounds were used. Compounds are grouped according to common response patterns (see text).

☆ = significant increase or decrease ($P < 0.05$)

D = leaf died.

Plate 5.2. Effects of dimethyl sulphoxide and nordihydroguaiaretic acid on development of lesions of *M. pinodes* on pea leaflets

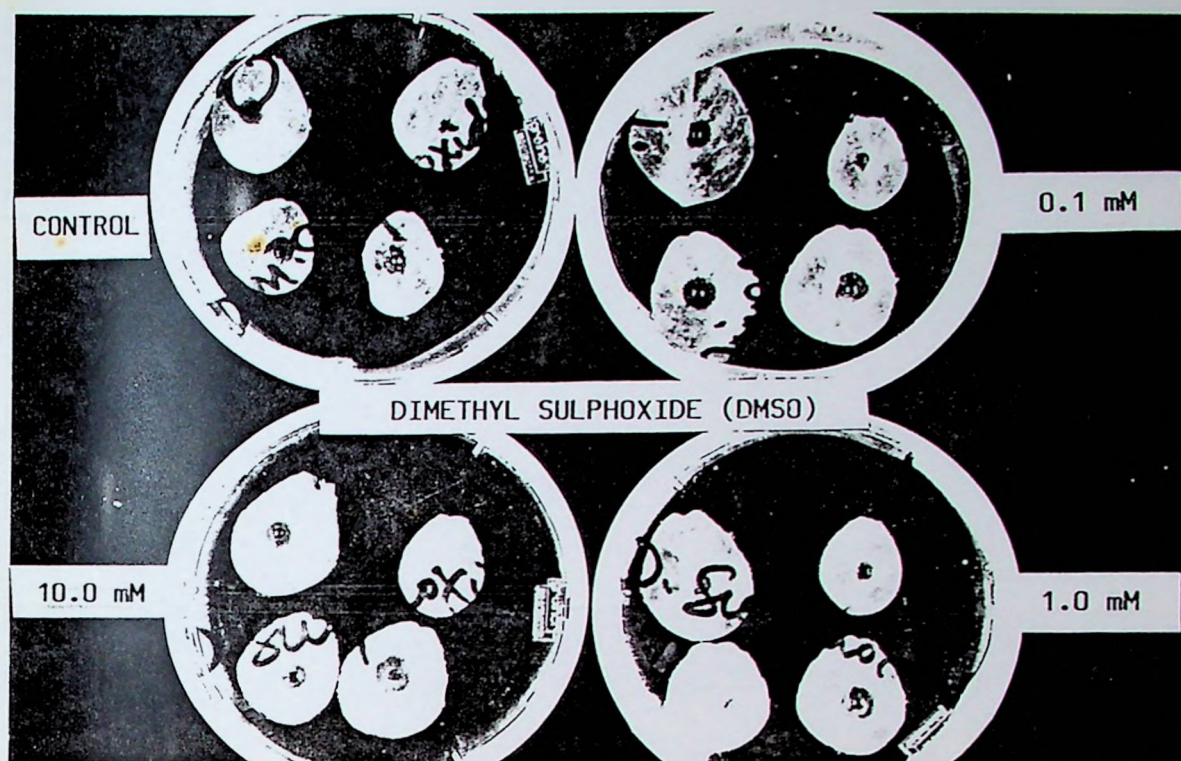
Dimethyl sulphoxide (Group 1 compound, see text) - progressive reduction at 0.1 and 1.0mM but no reduction at 10mM.

Nordihydroguaiaretic acid (Group 2) - significant reduction at 10mM but not at lower concentrations.

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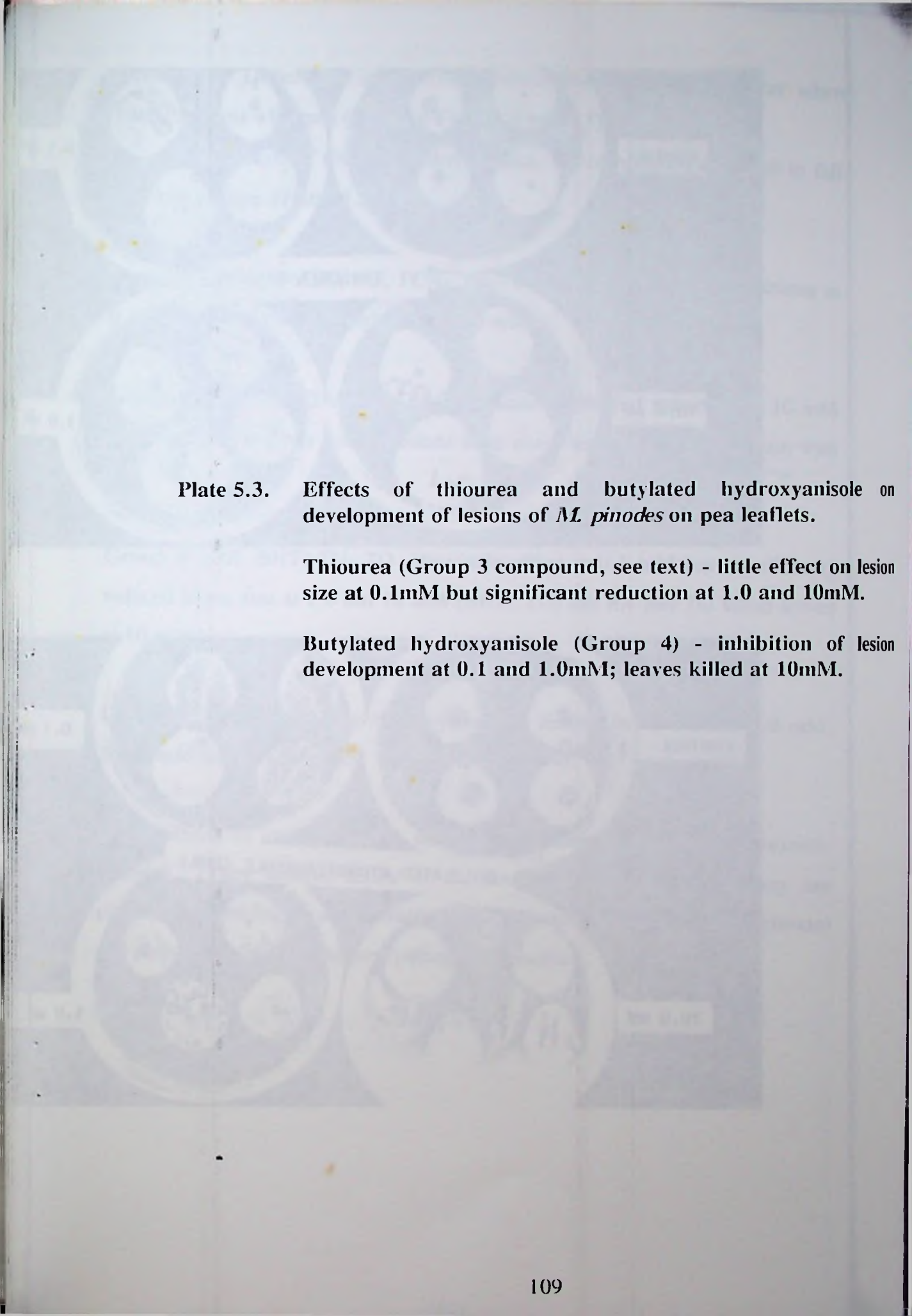
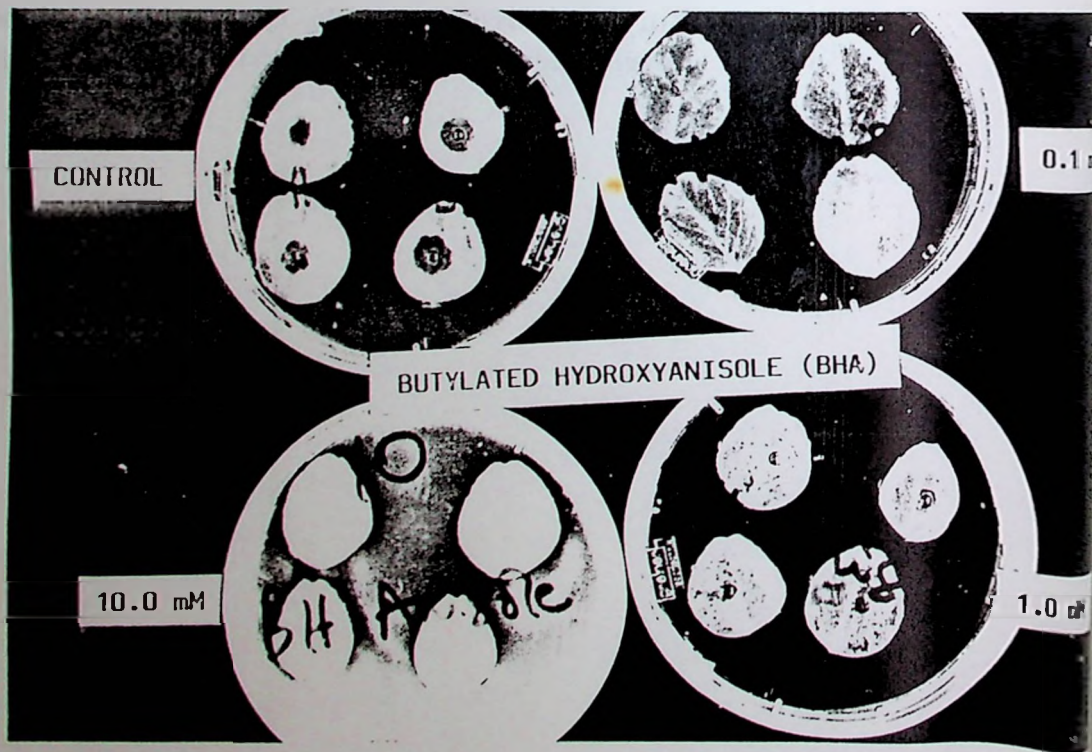
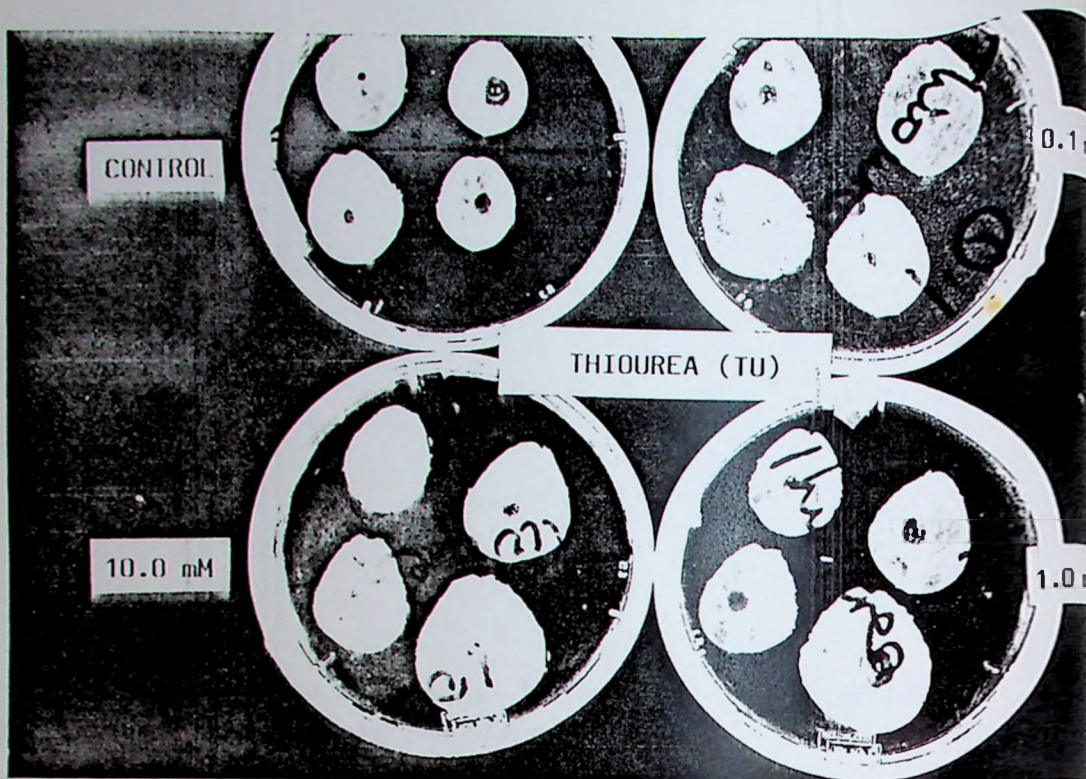
The image is a composite of six circular photographs arranged in a 3x2 grid. Each circle shows a pea leaflet with several small, dark, circular lesions. The lesions vary in size and number across the different leaflets, representing different treatments. The background of the plate is a light, mottled grey.

Plate 5.3. Effects of thiourea and butylated hydroxyanisole on development of lesions of *M. pinodes* on pea leaflets.

Thiourea (Group 3 compound, see text) - little effect on lesion size at 0.1mM but significant reduction at 1.0 and 10mM.

Butylated hydroxyanisole (Group 4) - inhibition of lesion development at 0.1 and 1.0mM; leaves killed at 10mM.



experiment. Differences in significant effects hardly varied, however when Tukey's D test was used (compare Figs. 5.4 and 5.5).

On the basis of the observed responses, the compounds appeared to fall into four groups: (Plates 5.2, 5.3)

Group 1 - pCA, DMSO, PG, PCA, TA - produced progressive reductions at 0.1 and 1.0 mM but little reduction at 10 mM.

Group 2 - AA, FA, GAL, NDGA - produced significant reduction at 10 mM but reductions at lower concentrations were much less and only significant with FA.

Group 3 - BA, BHT, TU, TO - had little effect at 0.1 mM but significantly reduced lesion size at 1.0 and 10 mM (BHT, TU) but BA and TO killed leaves at 10 mM.

Group 4 - tBHQ, BHA - significantly reduced lesion size at 0.1 and 1.0 mM. At 10 mM both killed leaves.

Effects of antioxidants on lesion development on leaves are evidently complex. They involve action of the compounds on the host (many are phytotoxic at the higher concentrations), on the pathogen (many are fungitoxic) and presumably also on the host/pathogen interaction.

(iii) **Comparison of effects of antioxidants on colony growth on agar and lesion development on detached leaves**

Few compounds had significant effects on either colony growth or lesion development at the lowest concentration, 0.1 mM. At the highest concentration, 10 mM, some were phytotoxic (BA, TO, BHA, tBHQ) and some virtually suppressed growth on agar media (BA, BHA, tBHQ, NDGA). In making a comparison between effects on colony growth and lesion development it seemed, therefore, that the intermediate concentration (1 mM) was the most suitable. Plotting values of rates of lesion expansion against rates of colony growth (Fig. 5.6) revealed that, although they were not significantly correlated, they did appear to fall into four groups.

Group 1 - BHA, tBHQ - (3 and 5 in Fig. 5.6 respectively) were strongly inhibitory to both colony growth and lesion development.

Group 2 - FA, NDGA (8 and 10 in Fig. 5.6 respectively) inhibited colony growth but had little effect on lesion expansion).

Group 3 - PCA, TO, BHT - (12, 15 and 4 in Fig. 5.6 respectively) had relatively little effect on colony growth but substantially reduced lesion development.

Group 4 - the remaining compounds had relatively small effects on either colony growth or lesion development though compound 13 (TA) might be considered to fit better in group 3.

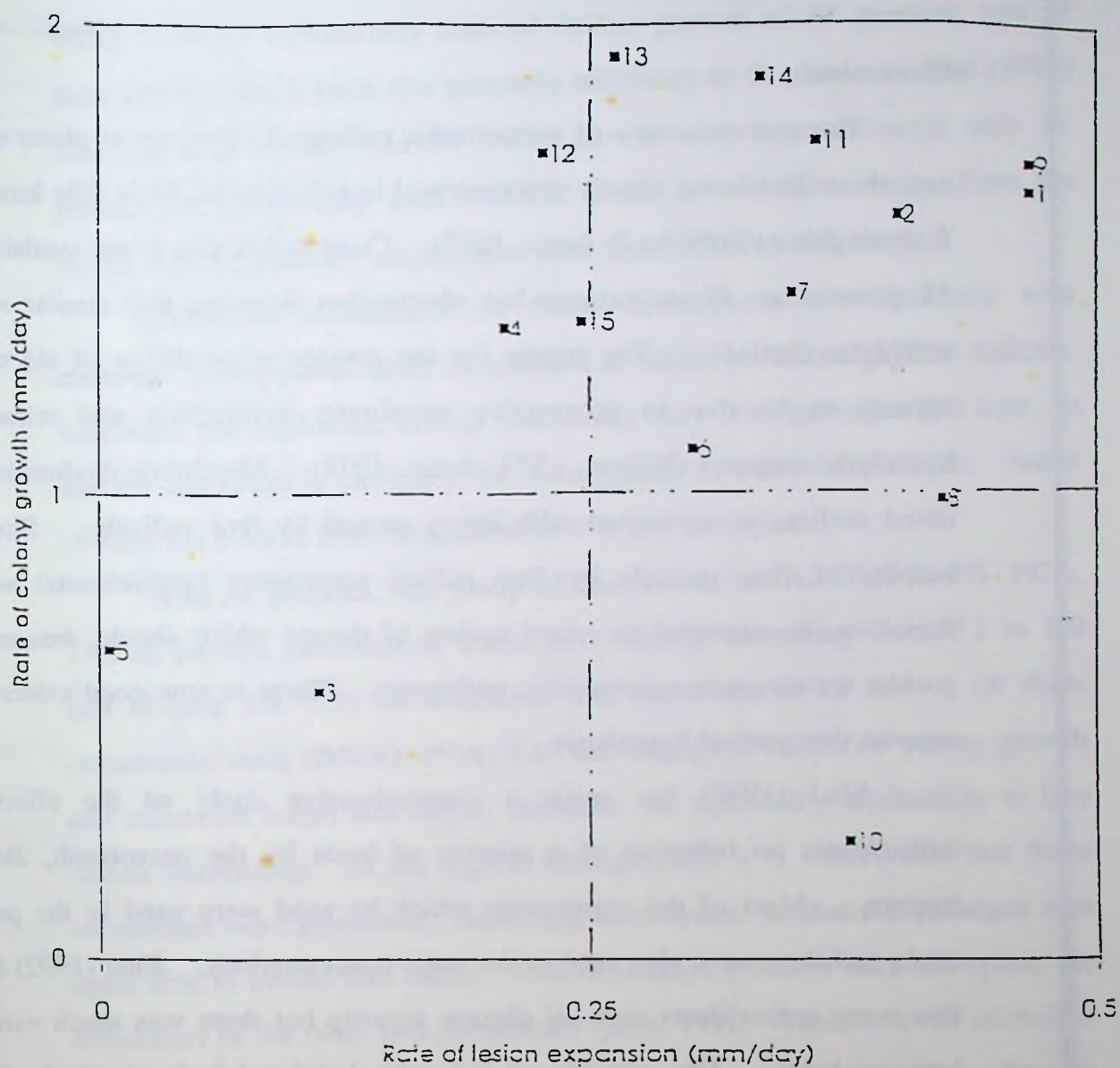


Fig. 5.6 Relationship between means of rates of lesion expansion and rates of colony growth with antioxidants (1-15) at 1.0mM.

(iv) Spore production

Lesions on inoculated leaves were checked for presence of both pycnid and perithecia but no quantitative assessments were made. In all treatments both pycnidiospores and ascospores were produced.

Discussion

The aggressiveness of necrotrophic pathogens increases as plants tissue age, this effect being clearly demonstrated in infection of *Vicia faba* leaves by *Botrytis fabae* (Griffiths & Amin, 1977). Comparable data is not available for *M. pinodes* or *Pisum sativum* but observation suggests that similar results would be obtained. The reason for the greater vulnerability of old tissue appears to be due to progressive membrane dysfunction and release of hydrolytic enzymes (Wilson, 1973; Amin, 1978). Membrane dysfunction, as noted earlier, is associated with injury caused by free radicals. Blocking the activity of free radicals by free radical scavengers (antioxidants) would therefore, be expected to retard ageing of tissues which should then exhibit greater resistance to necrotrophic pathogens. There is now good evidence to support this general hypothesis.

Elad (1992) has made a comprehensive study of the effects of antioxidants on infection of a number of hosts by the necrotroph, *Botrytis cinerea*. Most of the compounds which he used were used in the present study and they were also used at the same concentrations. Elad (1992) found that many antioxidants reduced disease severity but there was much variation between hosts. For example, at 0.1 mM, butylated hydroxyanisole (BHA) and nordihydroguaiaretic acid (NDGA) reduced infection of *Senecio* sp by 90% and 100% respectively but were less effective on tomato where reductions were 60% (BHA) and 25% (NDGA), the latter reduction not being statistically significant. It was also found that many compounds were phytotoxic, particularly at the highest concentration (10.0 mM). Fungicidal or fungistatic activity appeared to vary with the strain of *B. cinerea*. For example, with an isolate from cucumber flowers only four compounds (tBHQ, TA, thiopropion

acid and TO) significantly inhibited colony growth at 1.0 mM, while with an isolate from grape berries a further four compounds (AA, BHA, FA, PG) were inhibitory at 1.0 mM whilst one (TO) was not.

The effects of antioxidants on *M. pinodes* were, in many respects similar to those found by Elad (1992). Only three of the antioxidants (BHA, tBHQ, NDGA) substantially reduced colony growth of *M. pinodes*; two of these (BHA, tBHQ) were also generally inhibitory to *B. cinerea*. Elad (1992) used the same culture medium (PDA) as was used in experiments with *M. pinodes* but presumably used unsealed plates in which, to judge from the results with *M. pinodes*, antioxidants were less inhibitory to growth.

Effects of antioxidants on lesion development are obviously very complex. Compounds have, in addition to ability to scavenge free radicals, fungitoxic and phytotoxic effects which may vary with pathogen and host, so that the end result is inevitably the consequence of many interactions. Some of these are evident from consideration of a few examples.

With *M. pinodes*, one group of compounds (pCA, DMSO, PG, PCA, TA) all showed inhibition of lesion development increasing from 0.1 to 1.0 mM (Figure 5.4, 5.5) but inhibition was much less at 10 mM. Of these compounds, three (DMSO, PCA, TA) had negligible effects on colony growth and effects on lesion size might, therefore, be reasonably attributable to free radical scavenging. At the highest concentration it is conceivable that these compounds were phytotoxic, enhancing senescence, so that the pathogen was again able to invade host tissue. Unfortunately, it is less easy to explain the behaviours of the other two compounds (pCA, PG). These showed inhibition of colony growth at 10 mM but this fungicidal activity was not evident on lesion size at this concentration. It is just possible that phytotoxic effects were sufficient to enable the pathogen to grow despite the fungitoxicity of the compounds at this concentration but this is, admittedly, not a particularly convincing argument.

The interaction of free radical scavenging and of fungitoxicity and phytotoxicity are well illustrated by tBHQ and BHA. At 0.1 mM colony

growth was unaffected by lesion development was substantially reduced, presumably as a result of free radical scavenging; at 1.0 mM both colour and growth were reduced (? joint effects of fungitoxicity and free radical scavenging); at 10.0 mM leaves were killed, but there was no evidence of lesion development. At this concentration fungitoxic effects would appear to have suppressed pathogen growth even though the dead leaf would offer a favourable substrate for pathogen growth.

There is no doubt that antioxidants have potential to reduce diseases caused by necrotrophic pathogens and, at appropriate concentrations, disease reduction can reasonably be attributed to free radical scavenging. It remains to be seen whether they can be exploited in practice. The assertion (Elsner 1992) that "The practical use on field crops under field conditions is expected" seems somewhat overoptimistic at the present time. Specific responses to different host/pathogen combinations to individual antioxidants is very variable and might well require the use of specific antioxidants for different crops. In practice this would be a serious disadvantage. However, research on antioxidants as disease control agents is in its infancy: it may well be that useful products, suitable for a major crop or group of related crops may be developed in the future.

Appendix Table 5.1

Mean colony growth rate (mm per day) of *Mycosphaerella pinodes* when grown at 22°C on PDA amended with antioxidants at three concentrations (0.1, 1.0 and 10.0mM). In experiment 3, data for unsealed (a) and sealed plates (b) are shown separately.

Compound	Conc (mM)	Expt.1	Expt.2	Expt.3 (a)	Expt.3 (b)	Means	
1.AA	0.1	2.367	1.885	2.083	0.947	1.820	1.654
	1.0	2.192	1.907	2.292	9.981	1.843	
	10.0	1.065	1.067	1.643	1.420	1.299	
2.BA	0.1	2.035	1.503	1.685	1.197	1.605	1.076
	1.0	1.777	1.535	1.787	1.391	1.622	
	10.0	0.000	0.000	0.000	0.000	0.000	
3.BHA	0.1	1.542	1.619	1.986	1.852	1.750	0.765
	1.0	0.520	0.519	0.662	0.485	0.547	
	10.0	0.000	0.000	0.000	0.000	0.000	
4.BHT	0.1	1.330	1.791	1.620	1.568	1.577	1.263
	1.0	0.820	1.253	1.782	0.916	1.193	
	10.0	0.865	1.058	1.204	0.951	1.019	
5.tBHQ	0.1	1.608	2.106	2.088	1.451	1.813	0.834
	1.0	0.383	1.042	0.718	0.614	0.689	
	10.1	0.000	0.000	0.000	0.000	0.000	
6.pCA	0.1	1.668	1.702	1.731	0.754	1.464	0.979
	1.0	2.057	0.907	1.560	0.621	1.286	
	10.1	0.315	0.436	0.000	0.000	0.188	
7.DMSO	0.1	1.640	1.760	1.681	1.114	1.549	1.594
	1.0	1.828	1.708	1.819	1.025	1.595	
	10.1	1.700	1.920	1.889	1.045	1.639	
8.FA	0.1	1.593	1.234	2.153	0.792	1.443	0.918
	1.0	1.465	1.375	1.394	0.579	1.203	
	10.0	0.135	0.022	0.148	0.129	0.109	

Appendix Table 5.1 Continued....

Compound	Conc (mm)	Expt. 1	Expt. 2	Expt. 3 (a)	Expt. 3 (b)	Means	
9.GAL	0.1	1.572	1.711	1.606	1.871	1.690	1.55
	1.0	1.307	1.899	1.699	1.697	1.651	
	10.1	1.465	1.387	1.616	1.307	1.443	
10.NGA	0.1	1.835	1.134	1.099	0.674	1.186	0.40
	1.0	0.170	0.102	0.301	0.204	0.194	
	10.0	0.112	0.000	0.000	0.000	0.028	
11.PG	0.1	1.252	1.747	2.093	0.966	1.514	1.19
	1.0	1.362	1.737	2.190	1.299	1.647	
	10.1	0.672	0.245	0.426	0.356	0.425	
12.PCA	0.1	1.670	1.558	2.098	1.318	1.661	1.60
	1.0	1.580	1.673	1.856	1.591	1.675	
	10.1	1.957	1.766	1.792	1.466	1.745	
13.TA	0.1	1.452	1.830	1.690	1.405	1.594	1.73
	1.0	1.850	2.048	2.134	1.720	1.938	
	10.1	1.858	1.769	1.824	1.527	1.745	
14.TU	0.1	1.305	1.603	2.074	1.663	1.661	1.20
	1.0	0.950	1.795	2.028	1.735	1.627	
	10.1	0.230	0.538	0.491	0.106	0.341	
15.TO	0.1	2.170	1.830	1.889	1.796	1.921	1.60
	1.0	1.822	2.128	1.880	0.845	1.668	
	10.1	2.168	2.018	0.695	0.962	1.461	
16.Control	-	1.457	1.795	1.583	1.625	1.615	1.60
	-	1.202	1.741	2.023	1.666	1.658	
	-	1.397	1.795	2.092	1.636	1.730	
Mean		1.286	1.337	1.440	1.026	1.272	
SED		0.145	0.114	0.153	0.112	0.066	0.0
Error DF		64	64	64	64	256	12

Appendix Table 5.2

Anovar: effects of antioxidants on colony growth of *Mycosphaerella pinodes* (Appendix Table 5.1)

Item	DF	Experiment 1		Experiment 2		Experiment 3(a)		Experiment 3(b)	
		MS	VR	MS	VR	MS	VR	MS	VR
Replicates	2	0.026	0.65	0.016	0.89	0.172	4.41*	0.000	0.00
Compounds (A)	15	1.804	44.66***	1.911	103.49***	1.747	44.86***	1.582	85.54***
Error (a)	30	0.040		0.018		0.039		0.018	
Concentrations (b)	2	7.462	275.23***	8.210	409.34***	12.373	379.31***	4.898	262.36***
A x B	30	0.740	27.30***	0.579	28.86***	0.784	24.04***	0.493	26.39***
Error (b)	64	0.027		0.020		0.033		0.019	

N.B. In experiment 3, data for unsealed (a) and sealed plates (b) were analysed separately.

* (P < 0.05)

** (P < 0.01)

*** (P < 0.001)

Appendix Table 5.3

Anovar: effects of antioxidants on colony growth of *M. pinodes*: joint analysis of four experiments (Appendix Table 5.1)

Item	DF	MS	VR
Experiment (E)	3	4.4597	75.845***
Replicates within experiments	8	0.0539	
Compounds (C)	15	5.871	202.448***
C x E	45	0.391	13.476***
Pooled error (a)	120	0.028	
Concentrations (CT)	2	31.795	1292.480***
E x CT	6	0.382	15.529***
C x CT	30	2.026	82.370***
E x CT x C	90	0.190	7.715***
Pooled error (b)	256	0.025	

Appendix Table 5.4

Mean rate of lesion expansion (mm per day) of *M. pinodes* on leaves of *Pisum sativum* (cv. Conquest)

Compound	Conc (mM)	Expt. 1	Expt. 2	Means	
1. AA	0.1	0.291	0.651	0.471	0.459
	1.0	0.282	0.649	0.466	
	10.0	0.192	0.498	0.345	
	0	0.368	0.738	0.553	
2. BA	0.1	0.297	0.742	0.519	0.378
	1.0	0.377	0.423	0.400	
	10.0	0.000	0.000	0.000	
	0	0.381	0.806	0.593	
3. BHA	0.1	0.001	0.462	0.232	0.212
	1.0	0.024	0.196	0.110	
	10.0	0.000	0.000	0.000	
	0	0.292	0.720	0.506	
4. BHT	0.1	0.384	0.614	0.499	0.365
	1.0	0.292	0.119	0.206	
	10.0	0.141	0.161	0.151	
	0	0.532	0.673	0.602	
5. tBHQ	0.1	0.156	0.420	0.288	0.224
	1.0	0.001	0.009	0.005	
	10.0	0.000	0.000	0.000	
	0	0.417	0.789	0.603	
6. pCA	0.1	0.222	0.634	0.428	0.448
	1.0	0.165	0.430	0.298	
	10.0	0.509	0.434	0.471	
	0	0.419	0.768	0.594	
7. DMSO	0.1	0.276	0.598	0.437	0.417
	1.0	0.153	0.540	0.347	
	10.0	0.163	0.706	0.434	
	0	0.252	0.644	0.448	
8. FA	0.1	0.206	0.688	0.447	0.440
	1.0	0.178	0.667	0.423	
	10.0	0.176	0.384	0.280	
	0	0.472	0.747	0.610	
9. GAL	0.1	0.188	0.658	0.423	0.430
	1.0	0.373	0.560	0.466	
	10.0	0.333	0.343	0.338	
	0	0.345	0.643	0.494	

Appendix Table 5.4 Continued....

Compound	Conc (mM)	Expt. 1	Expt. 2	Means	
10.NGA	0.1	0.352	0.527	0.440	0.389
	1.0	0.229	0.524	0.376	
	10.0	0.089	0.408	0.248	
	0	0.338	0.642	0.490	
11.PG	0.1	0.308	0.643	0.476	0.495
	1.0	0.206	0.511	0.359	
	10.0	0.695	0.468	0.582	
	0	0.440	0.686	0.563	
12.PCA	0.1	0.149	0.571	0.360	0.363
	1.0	0.140	0.313	0.226	
	10.0	0.489	0.375	0.432	
	0	0.220	0.646	0.433	
13.TA	0.1	0.166	0.665	0.416	0.412
	1.0	0.244	0.278	0.261	
	10.0	0.567	0.389	0.478	
	0	0.285	0.702	0.493	
14.TU	0.1	0.440	0.468	0.454	0.333
	1.0	0.146	0.517	0.331	
	10.0	0.005	0.131	0.068	
	0	0.359	0.597	0.478	
15.TO	0.1	0.257	0.559	0.408	0.282
	1.0	0.037	0.452	0.245	
	10.0	0.000	0.000	0.000	
	0	0.248	0.699	0.474	
Mean		0.254	0.498		
Mean control value		0.358	0.700	0.529	

SED compound				0.043
Means				56
Error DF				
SED compound x Conc. Means (includes control)	0.089	0.121	0.075	
SED compound x Conc. Means (using mean control value)	0.091	0.125	0.077	
Error DF	90	90	180	

Appendix Table 5.5

**Anovar: Effects of antioxidants on lesion expansion of *M. pinodes*
(Appendix Table 5.4)**

		Experiment 1		Experiment 2	
Item	DF	MS	VR	MS	VR
Replicates	2	0.029	2.64	0.008	0.23
Compounds (A)	14	0.090	8.19***	0.125	3.72***
Error (a)	28	0.011		0.034	
Concentrations (B)	3	0.238	19.87***	1.529	83.03***
A x B	42	0.059	4.94***	0.056	3.07***
Error (b)	90	0.012		0.018	

Appendix Table 5.6

**Anovar: Effects of antioxidants on lesion expansion of *M. pinodes*: joint
analysis of two experiments (Appendix Table 5.4)**

Item	DF	MS	VR
Experiments (E)	1	5.342	239.41***
Reps. within expt.	4	0.018	0.82
Compounds (C)	14	0.167	7.48***
C x E	14	0.048	2.16*
Pooled error (a)	56	0.022	
Concentrations (CT)	3	1.365	87.79***
E x CT	3	0.402	26.46***
C x CT	42	0.081	5.34***
E x CT x C	42	0.035	2.27***
Pooled error (b)	180	0.015	

6. INFECTION OF PEA LEAVES BY ASCOSPORES AND PYCNIDIOSPORES OF *MYCOSPHAERELLA PINODES*.

Introduction

Infection involves a series of events, spore germination, penetration of host cells and establishment of the pathogen in the host tissues. Detailed descriptions of infection of peas by *M. pinodes* have been provided by Heath & Wood, (1969) and Nasir, Hoppe & Ebrahim-Nesbat, (1992). These observations, however, relate only to pycnidiospores and there have been no reports on the infection process by ascospores, despite their importance in the epidemiology of pea blight.

Pycnidiospores germinate, producing one or more germ tubes which penetrate directly (i.e. apparently without appressoria) or grow over the leaf before forming an appressorium from which infection hyphae penetrate through the cuticle or occasionally through a stomate. Penetration is followed by the formation of swellings or vesicles (Nasir, *et al.*, 1992) below the cuticle (and extending into the cell wall). From the vesicles, hyphae, which are thicker than those formed on the leaf surface, grow initially in the outer layers of the wall causing the cuticle to bulge outwards. Viewed from above with the scanning electron microscope (SEM) these appear as ridges. From these, thick hyphal branches are produced and eventually fungal colonies develop within the leaf (Heath & Wood, 1969; Nasir *et al.*, 1992).

Heath & Wood, (1969) reported penetration to be mechanical and not preceded by chemical alteration of the cell wall while Clulow *et al.*, (1991) reported extensive rupturing as hyphae tunnelled through cell walls of pea epicotyls.

It was noted earlier (see General Materials & Methods) that whereas pycnidiospores are readily wetted, enabling aqueous spore suspensions to be prepared, ascospores were hydrophobic, floating on water surfaces. This difference presumably reflects differences in surface characteristics between ascospores and pycnidiospores.

In this brief study, using an SEM, a comparison was made between infection processes of ascospores and pycnidiospores and attention was also given to the surface features of the two spore types.

Materials and Methods

Pycnidiospore suspensions were prepared from 2 week old cultures in the usual way (General Materials and Methods). They were sprayed on to pea leaves using a small atomiser (Humbrol Ltd., UK) and the leaves then place on 0.25% water agar plates (Oxoid Agar No. 2). The epidermal cells of pea leaves have a rough wax layer which effectively prevents observation of infection processes using the SEM. Leaf wax was, therefore, removed before inoculation, by spreading clear nail varnish over the leaf, allowing it to air dry and quickly peeling it off. This procedure was used for all the observations illustrated in Plates 6.1 and 6.2.

Agar plates, containing inoculated leaves, were sealed with parafilm and incubated (standard non-illuminated incubator) at 20°C. Observations were made after 10 and 20 h incubation. Ascospores were collected on detached leaves (dewaxed) which were placed on 0.25% water agar. The plates were placed in an Andersen sampler and ascospores drawn in on to the leaves from wetted infected pea debris (General Materials and Methods). Leaves were then sprayed with just sufficient water to wet the leaves, the plates sealed with parafilm and incubated in the same way as for pycnidiospores.

For examination of surface features, pycnidiospores and ascospores were placed on cellophane sheet. Pycnidiospores were sprayed on to the cellophane and allowed to air dry before microscopic examination. Ascospores were collected on to cellophane circles inside petri dishes using the Andersen sampler.

Inoculated leaf discs or spore bearing cellophane discs were attached to a copper stub using colloidal graphite and then frozen rapidly by introducing the stub to a cold stub holder (pre-cooled by immersion in liquid nitrogen slush to c. -196°C and transferring the assembly to the pre-cooled (-196°C) stage of

an Emscope SP 2000A sputter cryosystem. The material was viewed in a Joel JSM 840A scanning electron microscope with the cold stage at c. -165°C . If surface ice was present, this was sublimed by raising the stage temperature to -70°C for 1-5 min as necessary. The specimen was then sputter-coated with gold in the sputter cryosystem before detailed observation by SEM with 2.5 - 10kV electron accelerated voltage.

Results and Discussion

Examination of germinated spores after 10 h showed that one or more separate germ tubes arose from both ascospores and pycnidiospores. Germ tubes from both types of spore either penetrated directly after emerging (apparently without appressoria) (Plate 6.1 : 5, 6, 8) or grew over the leaf surface for some distance and then became swollen at their tips to form appressoria (Plate 6.1 : 1, 2, 4). After penetration, swellings were observed which were interpreted as vesicles (Plate 6.1 : 4, 5, 6). Penetration took place through the cuticle of epidermal cells or even guard cells (Plate 6.1 : 4, 6, 8) but penetration through stomata was not observed.

At 20 h both ascospores and pycnidiospores showed, at times, hyphae which appeared to be growing underneath the cuticle (possibly in the outer layers of the epidermal cell walls), forming ridges, before entering the parenchyma (Plate 6.2 : 3, 7).

Observations of the surface features of ascospores and pycnidiospores (Plate 6.3) showed prominent indentations on both but whether these were artefacts or actual features of the spore morphology was not established. Ascospores clearly had a rough surface in contrast to pycnidiospores which were smooth.

Evidently, the infection of leaves by both ascospores and pycnidiospores by *M. pinodes* is very similar, at least, as far as can be ascertained by observation with the SEM. Also, observations are consistent with those made by Nasir *et al.*, (1992).

It is also evident that the surfaces of ascospores and pycnidiospores are not the same. The rough surface of ascospores may well be due to waxy material which would explain their non-wettability; it may also aid attachment to the rough waxy surface of pea leaves (Plate 6.2 : 8).

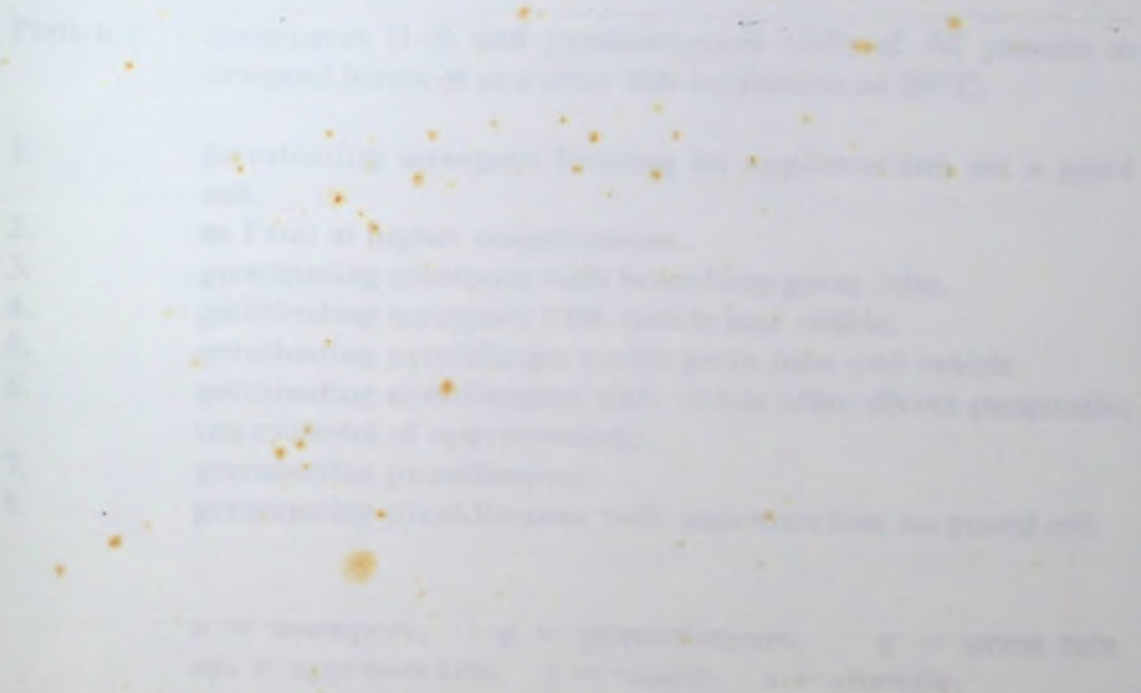
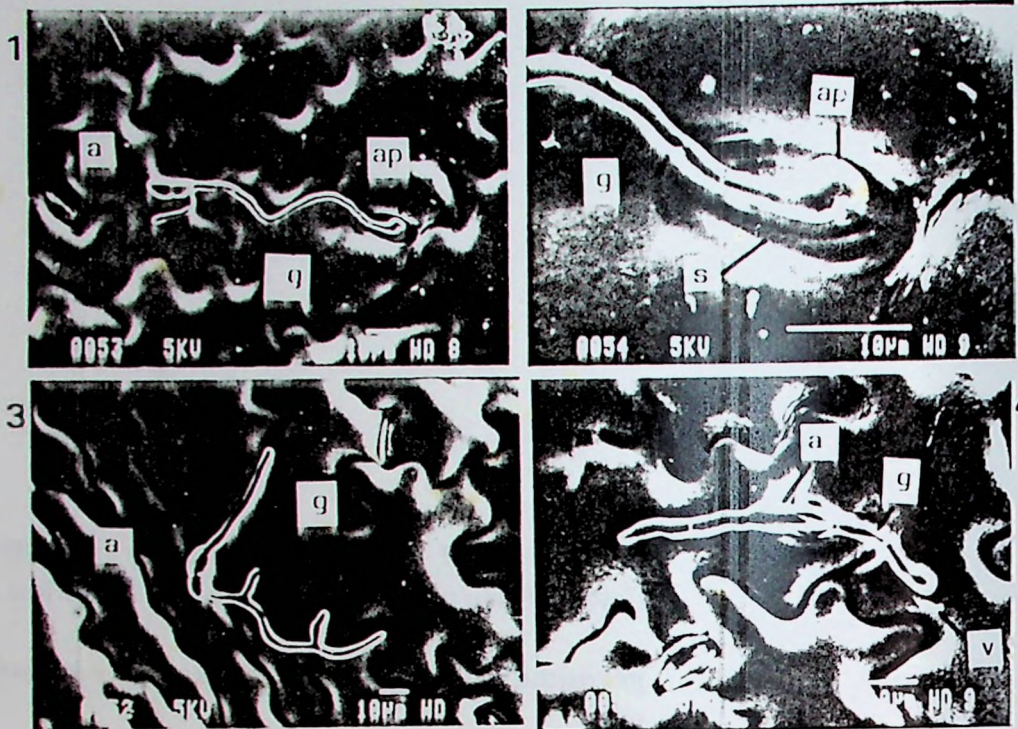


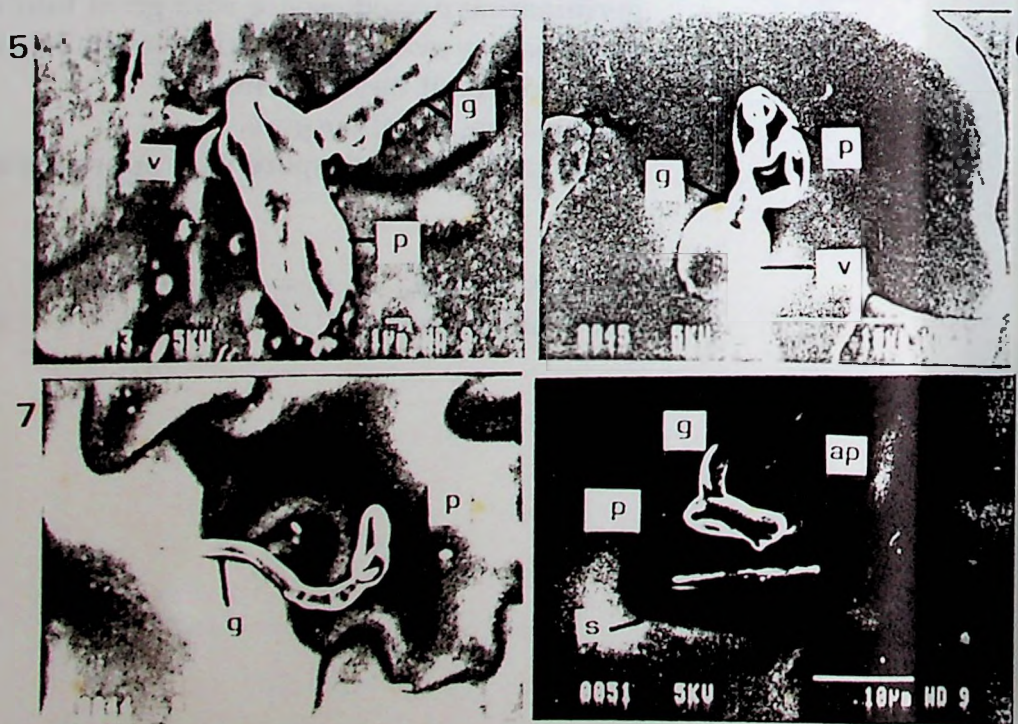
Plate 6.1 Ascospores (1-4) and pycnidiospores (5-8) of *M. pinodes* on dewaxed leaves of pea after 10h incubation at 20°C.

1. germinating ascospore forming an appressorium on a guard cell.
2. as 1 but at higher magnification.
3. germinating ascospore with branching germ tube.
4. germinating ascospore with vesicle just visible.
5. germinating pycnidiospore with germ tube and vesicle.
6. germinating pycnidiospore with vesicle after direct penetration (no evidence of appressorium).
7. germinating pycnidiospore.
8. germinating pycnidiospore with appressorium on guard cell.

a = ascospore, p = pycnidiospore, g = germ tube,
ap. = appressorium, v = vesicle, s = stomate.



ASCOSPORES

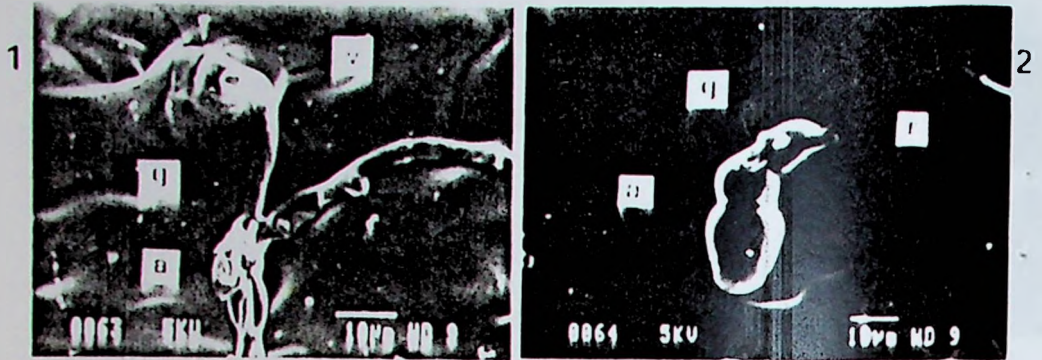


PYCNIDIOSPORES

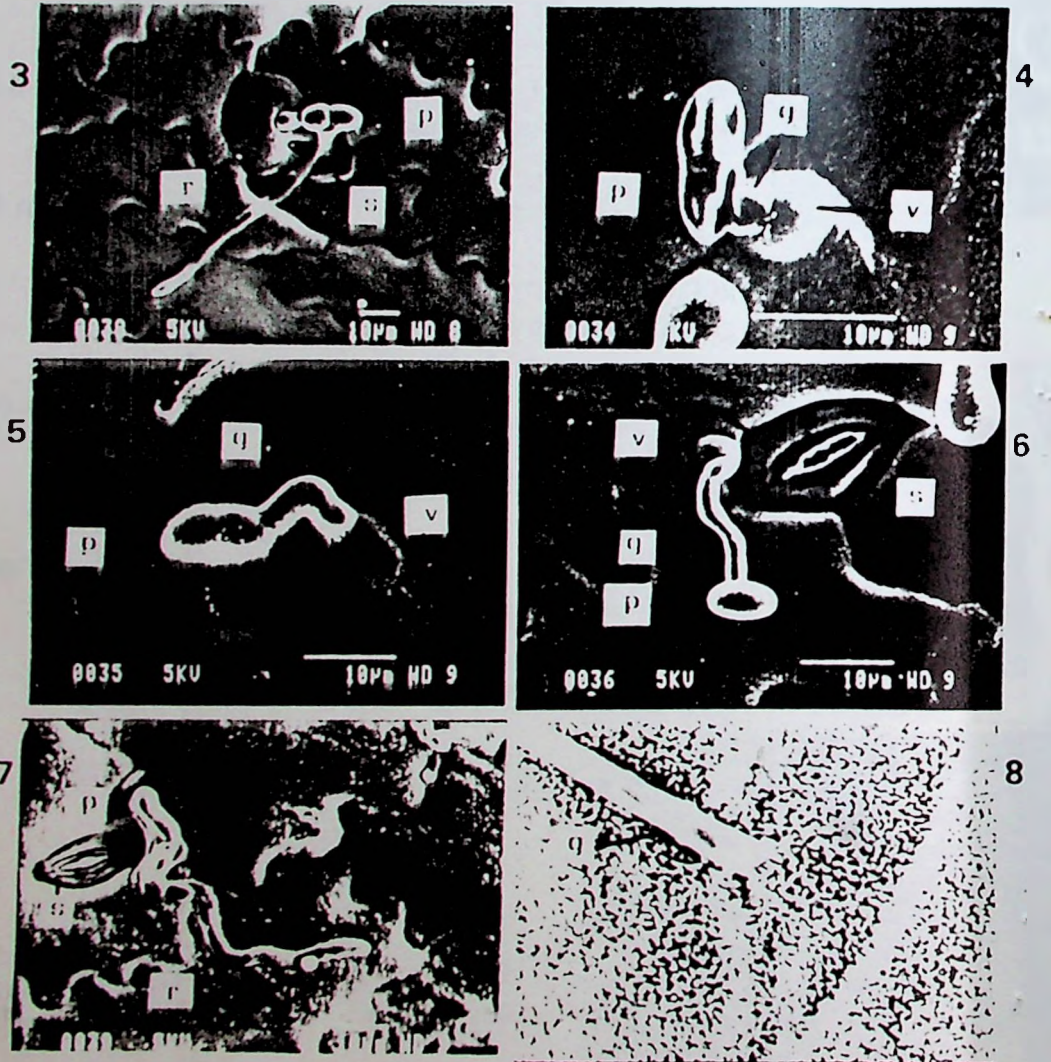
Plate 6.2 Ascospores (1-2) and pycnidiospores (3-7) of *M. pinodes* on leaves of pea after 20h incubation at 20°C.

- 1. & 2. germinating ascospores with vesicle apparent in 1 and hyphal ridge in 2.
- 3. germinating pycnidiospore with hyphal ridge.
- 4. germinating pycnidiospore with vesicle.
- 5. & 6. germinating pycnidiospores with vesicles.
- 7. germinating pycnidiospore with hyphal ridge in epidermal cell.
- 8. undewaxed leaf surface of pea leaf.

a = ascospore, p = pycnidiospore, g = germ tube,
v = vesicle, r = hyphal ridge, s = stomate.

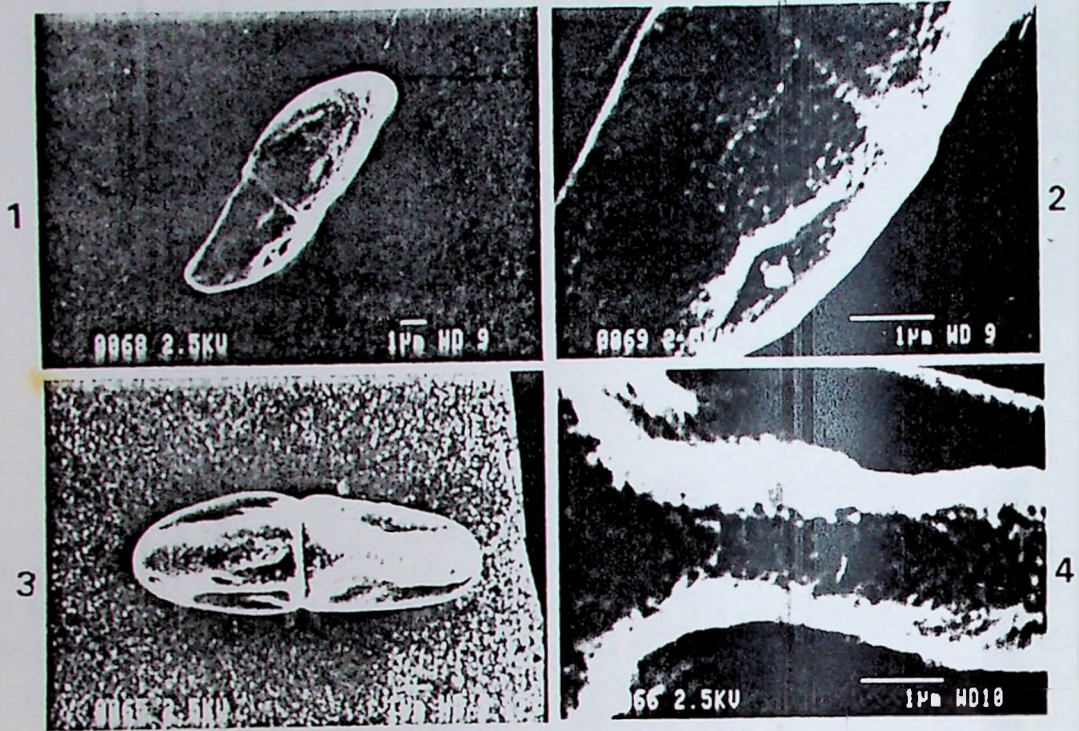


ASCOSPORES

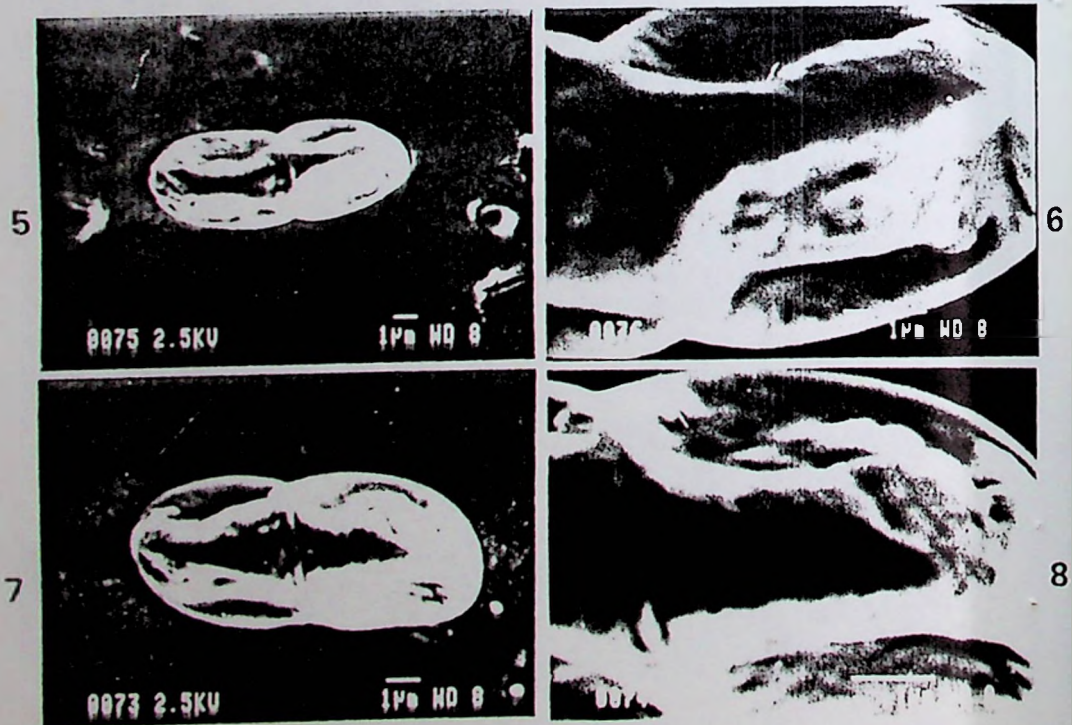


PYCNIDIOSPORES

Plate 6.3 **Ungerminated ascospores (1-4) and pycnidiospores (5-8).**
Note the indentations on both spores and rough surface of
ascospores compared to the smooth surface of pycnidiospores.



ASCOSPORES



PYCNIDIOSPORES

GENERAL CONCLUSIONS

Pea blight (*Mycosphaerella pinodes*) is, potentially, an extremely damaging foliar disease. Like other members of the 'Ascochyta complex' on peas, it is seed borne and this aspect of its epidemiology has received much attention. At the present time, however, seed transmission need not be a hazard. As indicated by Lawyer (1984) virtually complete control of this mode of transmission can be achieved, either by exploiting use of areas with low rainfall for seed production, or by the use of fungicides. Nevertheless, the disease remains a potential hazard in areas where peas are cultivated intensively because of the ability of the pathogen to survive on crop debris and its ability to disseminate its ascospores over considerable distances.

Economic control of pea blight is difficult. Chemical control is unlikely to be cost effective unless sprays can be accurately targeted in relation to both weather conditions and crop development. In seasons of low rainfall, pea blight is not a problem. Equally, if the epidemic develops late (expt. 1: section 3) losses do not seem to be particularly serious, whereas epidemics developing early in crop development seriously reduce yield of seed (expt. 2: section 3). More information is evidently needed if control measures, based on conventional crop protection chemicals, are to be efficient.

Ascospores occupy a central role in the epidemiology of pea blight. If their production could be blocked, pea blight would probably be relegated to the status of a minor disease. This was in mind when the study of the effects on antioxidants was undertaken. Unfortunately, although many compounds reduced lesion development of *M. pinodes* on leaves, none eliminated production of either ascospores or pycnidiospores.

Disease resistance remains the best long-term solution for control of pea blight. The demonstration of specific pathotypes of *M. pinodes* (Clulow *et al.*, 1991) indicates that resistance based on specific resistances may well be short-lived and more attention should, therefore, be paid to race-non-specific resistance (horizontal resistance). There is, as is evident from these studies, a

not insignificant element of horizontal resistance in pea cultivars currently grown in the UK and the level of this resistance could doubtless be improved. The studies on the relationships between field performance and seedling resistance, as expressed in infection frequency and lesion growth, are, in this connection, encouraging. If these results can be confirmed and techniques of seedling testing refined, breeding for horizontal resistance would be greatly simplified.

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