Final Report
HDC project FV53a

Studies into the biology, epidemiology and control of *Mycosphaerella brassicicola*, the ringspot pathogen of brassicas.

R Kennedy and J E Cullington

Horticulture Research International
Wellesbourne, Warwick, CV35 9EF

Final report April 1994

Project Number:	FV53a
Project Title:	Studies into the biology, epidemiology and control of *Mycosphaerella brassicicola*, the ringspot pathogen of brassicas.
Project leader:	Dr Roy Kennedy
Location of project:	Horticulture Research International Wellesbourne Warwick CV35 9EF
Project Co-ordinator:	Mr Arthur Whitlock
Date Project Commenced:	November 1990
Key Words:	Brussels sprouts, cauliflowers Brassica crops Disease forecasting, fungicide timing

Contents

Practical Section for Growers

The ringspot pathogen, *Mycosphaerella brassicicola*, which occurs on vegetable brassicas has been a major problem in many areas of production within the U.K. The disease has always been a problem in wetter areas but has recently become more prevalent in parts of Lincolnshire. Problems in controlling and forecasting economically important outbreaks of disease have resulted from a lack of knowledge about the biology of the pathogen. The presence and type of sources of inoculum occurring within the crop at different stages during the growing season is of critical importance in the formulation of any control strategy. Identifying critical stages in the life cycle of the pathogen is an essential first step in the development of a practical disease forecaster.

Studies in inoculated and uninoculated crops in the field in a number of brassica growing areas have shown the importance of ascosporic inoculum (infective stage) on disease development. Observations have shown that ascospore production from perithecia (sexual stage) which develop on the ringspot lesion was the only source of ringspot inoculum within the crop. No pycnidia (asexual stage) were observed on lesions. Although spermogonia were formed, especially when the pathogen was grown artificially, these seemed to function only as male cells. The duration and number of cycles of production of ascospores determined the final severity of the disease within the crop. Ascospore production was dependent on wetness which also promotes ascospore liberation. Wetness was not usually a limiting factor within the crop at the time of year when the disease had built up to potentially damaging levels.

The disease was characterised by two periods of ascospore production in most brassica growing areas. However in some areas of continuous cropping (notably Cornwall) epidemic development may be polycyclic in nature. Data suggested (notably in Cornwall) that at certain times of the year disease development in infected crops was not limited by availability of ascosporic inoculum. Studies also suggested that there are optimal conditions for air-borne transport and survival of ascospores.

Ringspot has a long period between occurrence of infection on the leaf and visibility of resulting lesions (latent period). This has been shown from the present study to cover up to a one month period under some environmental conditions. This characteristic may have some advantage to the grower in that it provides a greater window of opportunity during which the disease may be controlled through application of eradicant fungicides. However there are few fungicides which have shown eradicant activity against the ringspot pathogen. Benomyl (Benlate) has shown some activity against the disease and this study has shown that this chemical is particularly effective against ascosporic inoculum. Reductions in ascospore germination were observed after application of 1 - 10 μg ml^{-1}. The eradicant activity of this chemical has been previously reported. However the occurrence of benomyl-resistant strains of the pathogen has been reported indicating potential difficulties in the use of this chemical. Other workers have found that of the newer chemicals becoming available tebuconazole may have strong eradicant activity.

Many of these observations from the field can be further investigated in the laboratory. A method of inducing laboratory grown cultures of the pathogen to produce and liberate ascospores has been successfully developed. Using artificially produced ascospores it has been shown that the ascospore can germinate at temperatures of 5-25°C, although the rate of germination at 5°C is slow. Germination can also occur at relative humidities (r.h.) of 96.5 % and above. Although the ascospore is thin-walled, laboratory experiments suggest that it can survive exposure for 24 hours at 55 % r.h.. Survival of ascospores increased with exposure to higher levels of relative humidity for 24 hour periods. These experiments suggest that once ascospores have been produced they are capable of remaining viable until conditions become favourable for germination. Therefore it is likely that in the field inoculum produced within the crop during winter conditions will be able to survive until conditions favouring infection are present within the crop. During the winter, for example in crops of Brussels sprouts or on cauliflowers in Cornwall, wetness will not be limiting so it is likely that inoculum production will be the controlling factor determining the level of infection within the crop.

Laboratory studies, using artificially produced ascosporic inoculum, have also shown that

the pathogen does not directly penetrate the host tissues but enters through natural openings in the leaf surface. At temperatures of 15°C this process can occur within 18 hours at continuous wetness. Maximum infection occurs after 48 - 72 hours of wetness. From preliminary field and laboratory observation sporulation appears optimal at lower temperatures. These observations again suggest that inoculum production may be limiting due to wetness and temperature conditions during the early development of the disease in July and August. During this same period germination and infection are likely to be less limiting given periods of wetness but may become more limiting later in the season when temperatures are lower.

Temperature and wetness are important factors in determining the rate of inoculum production on lesions. Age of host tissue is another important factor with more rapid lesion development on senescing tissues than on young growing leaves. Continuous wetness may be found at the base of a Brussels sprout crop during winter conditions which will be conducive to inoculum production. Therefore the period of time between cycles of inoculum production will vary at different times of the year. Field studies designed to investigate the effect of different environmental conditions on the time period between ringspot first appearing in the crop and the onset of the next cycle of inoculum production have shown some similarities between different geographical locations if temperature and wetness conditions are calculated in terms of degree wetness. This crude measure has shown that it may be possible to model the production of inoculum from ringspot-infected plant material. Given the long period between infection and appearance of symptoms in the crop this approach to disease forecasting may have some potential. Crop growth factors would also have to be taken into consideration as a factor determining the rate of inoculum production. Field studies have shown that in most areas the initial cycle of inoculum production is not of economic importance. Predicting any further periods of inoculum production within the crop would appear to represent the most practical element necessary for ringspot forecasting.

Controlled environment experiments investigating the role of temperature and wetness on lesion development, perithecial initiation and ascospore liberation will be necessary to define periods of inoculum production more critically. It is unclear at present if this

could be done in culture or would be more appropriate using infected plants (given the complication of maintaining lesion development on young leaves over an eight week period). However any developed system which could forecast accurately ascospore production would be useful as a basis for other diseases where ascospore production plays an important role in disease development - notably blackleg (*Phoma lingam*), which is an increasing problem on vegetable brassicas.

Introduction

The ringspot disease of brassicas, caused by the fungal pathogen *Mycosphaerella brassicicola*, forms light green to almost black necrotic lesions on susceptible host tissue. These can develop on all green parts of the host plant, commencing as small brown spots visible on both leaf surfaces which expand up to 2-3 cm in diameter. All horticultural brassicas are susceptible, with the disease causing most problems on cabbage, cauliflower and Brussels sprouts. Although the pathogen will infect oilseed rape, levels of ringspot occurring in this arable crop in the United Kingdom are generally low (Evans *et al.*, 1984; Gladders, 1993). This is most probably due to the cultivation of oilseed rape in warmer, drier regions (away from horticultural brassicas) which are not favourable to the development of the disease. In the Federal Republic of Germany, oilseed rape cultivation occurs alongside horticultural brassica crops, leading to transfer of ringspot between the crops. In this country oilseed rape is considered to act as a reservoir of infection over the winter, leading to establishment of the disease in spring-planted cabbage crops (Zornbach, 1990).

Ringspot lesions occur on leaves, stems and seed pods of susceptible hosts, but opinions are divided as to the ability of the pathogen to colonise seed and cause infection on seedlings (Weimer, 1926; Huber & Gould, 1949; Pound *et al.*, 1951; Zornbach, 1990).

Outbreaks of ringspot generally have little effect upon crop yield, although severe infection upon cauliflower may lead to premature leaf abscision, reducing curd weight and quality (Weimer, 1926). The disease usually causes cosmetic damage to the crop. Infected cabbage plants require costly leaf trimming and spotting on Brussels sprout buttons and packing leaves around cauliflower curds result in down-grading or rejection of the crop at market.

There are few resistant cultivars for any brassica variety. Some cultivars possess a degree of resistance which generally breaks down under conditions of high disease pressure. Roscoff-type cauliflowers possess comparatively high levels of ringspot resistance when compared to other open-pollinated and F_1 hybrid cauliflower types. However when favourable weather conditions combine with high availability of inoculum, this resistance breaks down. A sudden explosive outbreak of ringspot upon crops of F_1 hybrids was observed in Cornwall during January 1993, resulting in heavy losses.

Ringspot is a disease favoured by wet weather (Frinking & Geerds, 1987) which explains its regional distribution; the most serious outbreaks occurring in the wetter, intensive brassica-growing areas of North-West and South-West England, where year-round brassica cultivation ensures a constant reservoir of infected plants. However, during wetter years, ringspot can cause problems in all areas of intensive brassica cultivation. For the same reasons, most serious outbreaks of ringspot occur from autumn through to late spring. The disease rarely causes problems during dry summers.

The life cycle of *M. brassicicola* (Appendix I) is prolonged and heavily influenced by climatic conditions (Nelson & Pound, 1959). Ringspot dispersal is by means of air-borne sexual ascospores alone; the pathogen produces no asexual conidia (Snyder, 1946; Dring, 1961). Ascospore development occurs within perithecia - visible as black specks on the surface of lesions following periods of leaf surface wetness. Prolonged leaf surface wetness is required before ascospores develop to maturity, with the rate of development dependent upon temperature (Nelson & Pound, 1959). Spore release occurs following rainfall or heavy dew (Hartill, 1977), with ascospores forcibly discharged into the air (Weimer, 1926). Germination of ascospores and subsequent infection also require periods of leaf surface wetness - of prolonged duration at low temperatures (Weimer, 1926). Following successful infection of the host by the germinating ascospore, a prolonged latent period occurs before symptoms are seen on the plant. The duration of the latent period varies with temperature; at 15°C symptom expression occurs approximately 14-21 days following inoculation. Few critical studies have been performed on the effect of climatic conditions on life cycle stages, hence the role of climatic conditions in disease development in the field is poorly understood.

Current control methods are heavily reliant upon routine fungicide applications once the disease is perceived to be a problem in the crop. Owing to the paucity of epidemiological information available on ringspot, fungicides are generally applied without regard to the likelihood of disease occurrence. Poor timing of sprays can result in inadequate control and inefficient usage of fungicides. Enhanced awareness of the role of climatic conditions in determining the onset of disease outbreaks should enable fungicide applications to be accurately timed to prevent disease development. Such a forecasting system, based upon the occurrence of climatic conditions favourable for

ringspot development, should improve control and may reduce the number of fungicide applications required.

From 1991 to 1993 epidemiological studies were performed in artificially-inoculated Brussels sprout crops in order to determine the overall pattern of ringspot development and its relationships with climatic conditions. In 1991 and 1992 disease development was monitored within one Brussels sprout plot in Warwickshire (HRI-Wellesbourne). In 1993 similar disease development studies were undertaken within an inoculated Brussels sprout plot in Wellesbourne, Yorkshire (HRI-Stockbridge House) and Lincolnshire (HRI-Kirton). From September 1993 to February 1994 ringspot development and climatic conditions were also monitored within a non-inoculated winter cauliflower crop, cv. Codebric Jan/Feb (a Roscoff type), on a grower's holding in Camborne, Cornwall. Laboratory and controlled environment studies were instigated to develop a greater understanding of the basic biology of the pathogen and the role of climatic conditions in important stages of the life cycle.

1 Materials and Methods

1.1 Epidemiological studies

1.1.1 Plant production

Studies were performed in inoculated plots of Brussels sprouts cv Golfer, a mid-season cultivar rated as susceptible to ringspot in NIAB classifications. In all years 15 x 15 m plots were employed, with the exception of 1992 when plot size was increased to 20 x 20 m. Plants were glasshouse-raised in Hassy 308 modules and planted out in early May, when 5-6 weeks old, at a spacing of 60 x 60 cm. Fertilizer regimes followed commercial practice, residual herbicides were applied post-transplanting with insecticides applied as required. No fungicides were applied to the crops.

1.1.2 Inoculation of crops

Crops were artificially inoculated by scattering ringspot-infected leaf trash among the plants. In 1991 an infector crop of spring cabbage cv First Early Market 218 was inoculated with dried ringspot-infected Brussels sprout leaf trash, collected from Hesketh Bank, Lancashire. Disease developed within the cabbage crop and transferred naturally to adjacent successive cabbage plantings, thus providing a continuous source of leaf inoculum which could be used to inoculate other plots.

In early July 1991 a polythene bag full (approx. 380 l) of infected "fresh" cabbage leaves was randomly scattered within the adjacent Brussels sprout crop. The 1992 Brussels sprout crop was similarly inoculated in late August - using two polythene bags full of ringspot-infected cabbage leaves. In 1993 a more rigorous method of inoculation was employed with a view to increasing the uniformity of disease development within the plot. Ringspot-infected cauliflower and cabbage leaves collected in early 1993 from Cornwall and Wellesbourne, respectively, were dried, broken into small fragments and thoroughly mixed to produce a homogeneous inoculum. This was uniformly scattered between each row of every plot at the rate of 19 g m^{-2}.

1.1.3 Measurement of crop microclimate

Half-hourly records of crop microclimate were obtained by using a data logger (Delta-T Devices LTD, Cambridge) which was linked to electronic sensors measuring air temperature, relative humidity and leaf wetness within the crop canopy. Half-hourly rainfall data were recorded from a rain-gauge outside of the crop.

1.1.4 Crop assessments

At first occurrence of disease, selected tagged plants within the crop were assessed at weekly or fortnightly intervals. Counts of numbers of ringspot lesions present on each leaf were taken. In 1991 and 1992 two parallel transects, of five plants each, were taken through the crop and assessed for disease. In 1993 all Brussels sprout plots were divided into four quadrants and four moderately-infected plants were selected per quadrant. The cauliflower trial in Cornwall was assessed using the same methods.
Weekly assessments were taken on a sample of 240 (192 for 1993 crops) randomly collected Brussels sprout buttons when ringspot lesions were first detected. Three buttons were randomly selected from the lower, middle and upper regions of the stem of each of the eight plants (four for 1993 crops) adjacent to those assessed for leaf disease. The percentage of infected buttons and mean lesion number per button were determined for each sample.

1.1.5 Trap plants

In each year trap plants were exposed within the inoculated Brussels sprout plots at Wellesbourne for consecutive 24 h periods during weekdays (72 h over weekends). These plants were disease-free 6-8 week old glasshouse-grown Brussels sprout plants, cv. Golfer. Following the period of exposure within the infected crop, trap plants were returned to the glasshouse and incubated for approximately four weeks under conditions non-conducive to infection. Occurrence of ringspot lesions on trap plants indicated that

during their period of exposure within the crop, ascosporic inoculum was available and climatic conditions favoured infection. Records of crop microclimate from the data logger allowed accurate determination of climatic conditions during each trap plant exposure period and provided empirical data on the relationship between specific climatic conditions, inoculum availability and occurrence of infection within the crop. Ten trap plants were placed within the Brussels sprout crops at Wellesbourne over each exposure period. In 1991 plants were sited together in the centre of the plot. In 1992 pairs of trap plants were exposed in five separate positions in the crop and in 1993 plants were placed individually at ten separate sites within the crop.

In Cornwall Brussels sprout trap plants were exposed for 14 day periods. Six plants were exposed for each period among cauliflower plants assessed for ringspot. A further six plants were exposed at the edge of a field of lettuce, sited approximately 30 m away from the nearest brassicas. This latter field had been free from brassicas for the past 7 years.

1.1.6 Spore trapping

A volumetric spore trap (Burkard Manufacturing Co. Ltd, Rickmansworth) operated continuously within the Brussels sprout crops at Wellesbourne and in the cauliflower plot in Cornwall, to determine aerial ascospore concentrations. Ascospores impact upon a mellanex tape, coated with petroleum-jelly-based adhesive, which is fixed to a rotating drum. Attached to the clockwork mechanism of the spore trap, the drum completes one revolution in seven days, after which the tape is removed, cut into sections representing consecutive 24 h periods and mounted on glass microscope slides in a gelvatol-lactophenol mixture. Slides were examined under the light microscope at a magnification of x400 and counts taken of numbers of ringspot ascospores present on transverse scans at intervals of 2 mm (corresponding to hourly periods).

1.2 Laboratory studies

1.2.1 Production of ascosporic inoculum

Sterile cultures of the ringspot pathogen were obtained by isolating the fungus from lesions excised from infected leaves. Lesions were surface-sterilised for 1 minute in 25 % aqueous sodium hypochlorite (14 % w/v available chlorine) and then dried on sterile paper towelling. Lesion fragments were placed on petri dishes of prune-lactose agar (see Appendix II) and incubated at 15°C under five warm-white fluorescent tubes (18 h day). Fungal colonies produced on agar and free from contamination from other micro-organisms were macerated in sterile distilled water and the resulting mycelial suspension was plated out on to prune-lactose agar (PLA), corn-meal agar (CMA), potato dextrose agar (PDA), vegetable juice agar (V8) and a decoction medium produced from senescing Brussels sprout leaves (SLD) (see Appendix 2). Each agar was adjusted to pH 5.5 with the exception of SLD which was maintained at its natural level (varying from 5.5 to 7.5). Cultures were incubated at 15°C under conditions of continuous darkness, 16 h warm fluorescent light / 8 h darkness, or 16 h warm fluorescent light + UV-B[1] / 8 h darkness. Ascospore development was monitored by periodic microscopic examination of samples of growth from each culture.

1.2.2 Ascospore germination

Ascospores were collected by lightly spraying sporulating *in vitro* cultures of the pathogen with distilled water and inverting over a Pyrex crystallising dish on crushed ice. The crystallising dish had been previously coated with a thin layer of silicone (Sigmacote®, Sigma Chemical Co.) and finely misted with distilled water containing Tween 20 (0.1% v/v) - into which ascospores discharged. Using this method it was possible to

[1] Spectral output maximal at 350 nm

produce up to 1 ml of ascosporic inoculum at a concentration of 10^6 spores ml^{-1} from each 9 cm diameter Petri plate.

The rate of ascospore germination at different temperatures was studied on water agar (10 g agar l^{-1} distilled water) inoculated with 0.01 ml of an ascosporic suspension, which was spread over the surface of the agar prior to incubation in growth cabinets in the dark at temperatures of 5, 10, 15, 20 and 25°C. Four plates were sampled from each temperature at intervals of 10-12 hours and percentage ascospore germination assessed. Ascospores were considered to have germinated when the length of the germ tube equalled or exceeded that of the length of the spore. The inhibitory effect of benomyl on ascospore germination was studied by amending water agar with benomyl (Benlate, 50 % w/p, Du Pont Chemical Co.), giving final concentrations of benomyl of 0.01, 0.1, 1, 10 and 100 μg ml^{-1}. Plates were inoculated with ascospores as above and incubated at 15°C in the dark.

Ascospore germination at a range of relative humidities was studied by discharging ascospores from sporulating cultures on to dry borosilicate glass coverslips (BDH Laboratory Supplies). Coverslips were incubated in saturated salt-solution humidity chambers at 20°C. Percentage ascospore germination was assessed after 24 hours incubation at each humidity. Ascospore survival at low relative humidities was determined by exposing spores deposited upon cover slips to a range of relative humidities for a 24 h period. Following this incubation period, cover slips were transferred to conditions of 100 % relative humidity. Percentage ascospores germinated was assessed by microscopic examination after a further 24 h incubation.

1.2.3 Infection

The infection process was studied using 15 mm diameter leaf discs, excised from mature leaves of 3 month-old glasshouse-grown Brussels sprout cv. Golfer and oilseed rape cv. Cobra. Discs were inoculated with a single droplet (0.01 ml) of an ascosporic suspension (containing 10^5 ascospores ml^{-1}), dispersed over the surface of the disc. Leaf discs were incubated on water agar in sealed Petri plates at 15°C, under a 12 h light regime. Leaf discs were sampled at 24 h periods, cleared of chlorophyll, and ascospores

on the leaf surfaces stained with lactophenol-trypan blue (according to Shipton & Brown, 1962). Ascospore germination and infection was followed by microscopic examination of the leaf disc surface.

1.2.4 Ascospore development *in planta*

In preliminary studies, wetness durations required for ascospore development at different temperatures were investigated. Infected, detached trap plant leaves with ringspot lesions of 10 mm diameter were incubated on mesh supports in controlled environment cabinets at temperatures of 5, 10, 15, 20 and 25°C. Lesions had developed on plants in the glasshouse which were bottom-watered only. Consequently leaves remained dry and no perithecia developed on the ringspot lesions. Leaves were wetted by misting within the cabinets at hourly intervals. This provided conditions favourable for perithecial development and ascospore maturation with subsequent discharge from perithecia. Successive exposures of trap plants within the cabinets detected the presence of viable discharged ascospores.

The time course of ascospore development was followed on attached infected leaves, bearing lesions of 10 mm diameter which had formed no perithecia. Six plants (bearing a mean of 18 lesions per leaf) were incubated in a controlled environment cabinet at 15°C with a 12 h light period. A humidifier maintained conditions of constant wetness on the leaves and a Burkard spore trap was set up within the cabinet to detect the presence of air-borne ascospores discharged from perithecia developed on the lesions.

2 Results

2.1 Epidemiological studies

2.1.1 Disease development

Development of ringspot followed a similar pattern in all years in the inoculated Brussels sprout crops and was comparable in the uninoculated cauliflower plot studied in Cornwall during 1993. Ringspot lesions first appeared on plants 6-8 weeks after inoculation. Most lesions occurred on older and senescing leaves, while younger leaves situated near the apex of the plant remained free from infection. As the plants produced new growth, many of the older, infected leaves had fallen to the ground and started to decay. No new infections were detected in the crop during this time period and plants often appeared virtually free from infection for periods of up to a month. Levels of ringspot then increased rapidly after this time - with all leaves becoming infected, including those not yet fully developed. During this growth stage of the Brussels sprout crop there was marked development of buttons which also became heavily infected. Two cycles of infection were observed within the crop - separated by a period in which levels of disease on the crop appeared to decline.

In 1991 initial infection occurred in the crop in late August, approximately six weeks after inoculation. Low levels of disease developed (Fig. 1a), with a mean of 22 lesions per plant, and remained constant for approximately six weeks although the number of visible lesions on the plants steadily declined due to the abscision of infected leaves. In early November disease levels increased on both leaves and buttons. The final disease assessment in the crop during early December gave mean disease levels of 55 visible lesions per plant with 33 % of marketable buttons infected (mean of 3.5 lesions per button).

In 1992 the Brussels sprout crop was inoculated in late August. Both leaves and buttons became infected in the initial infection cycle. The first symptoms were seen on the crop in late September (Fig. 2a), approximately four weeks after inoculation. Levels of leaf

Fig. 1 Brussels sprout crop, Wellesbourne 1991

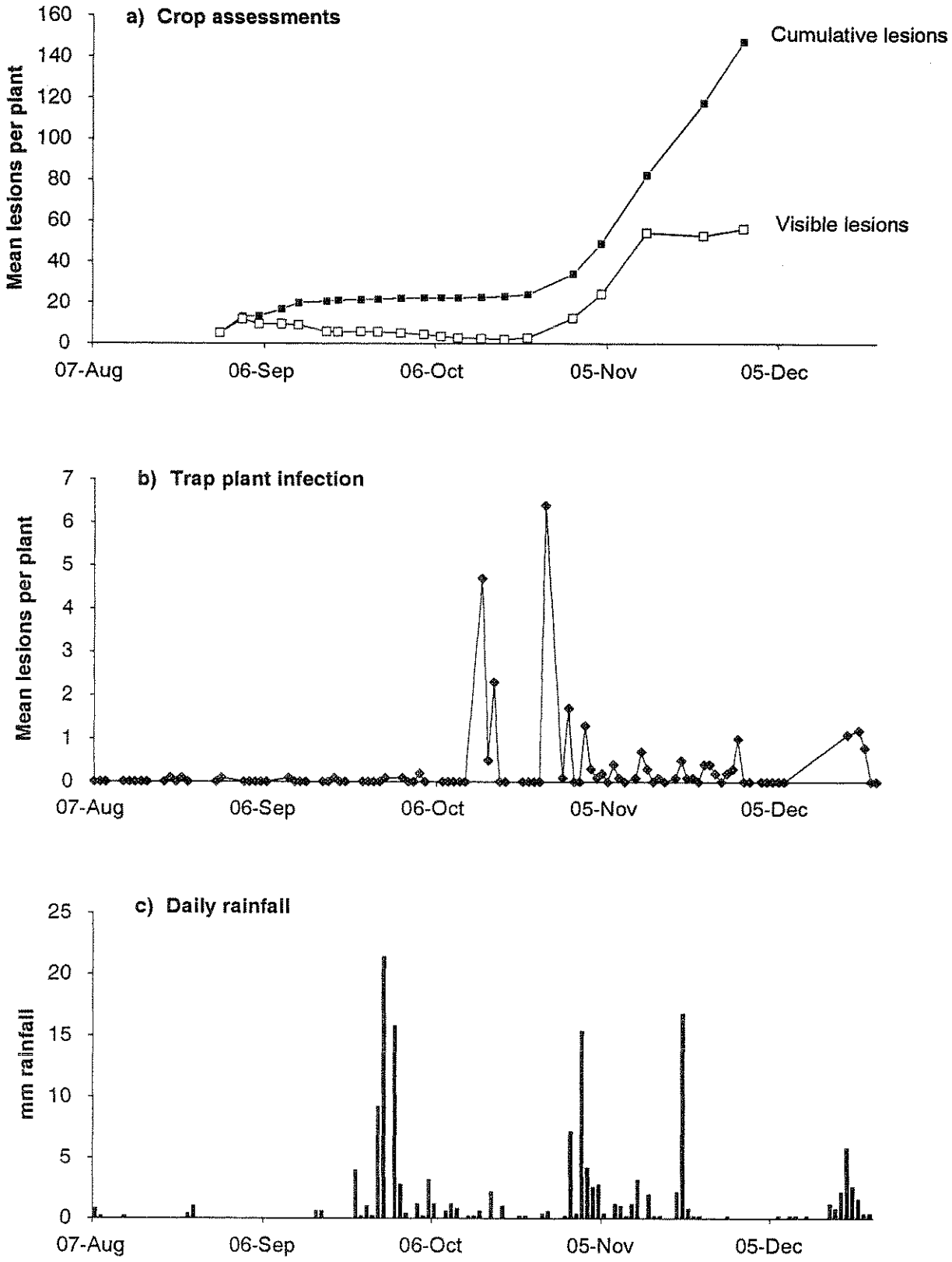


Fig. 2 Brussels sprout crop, Wellesbourne 1992

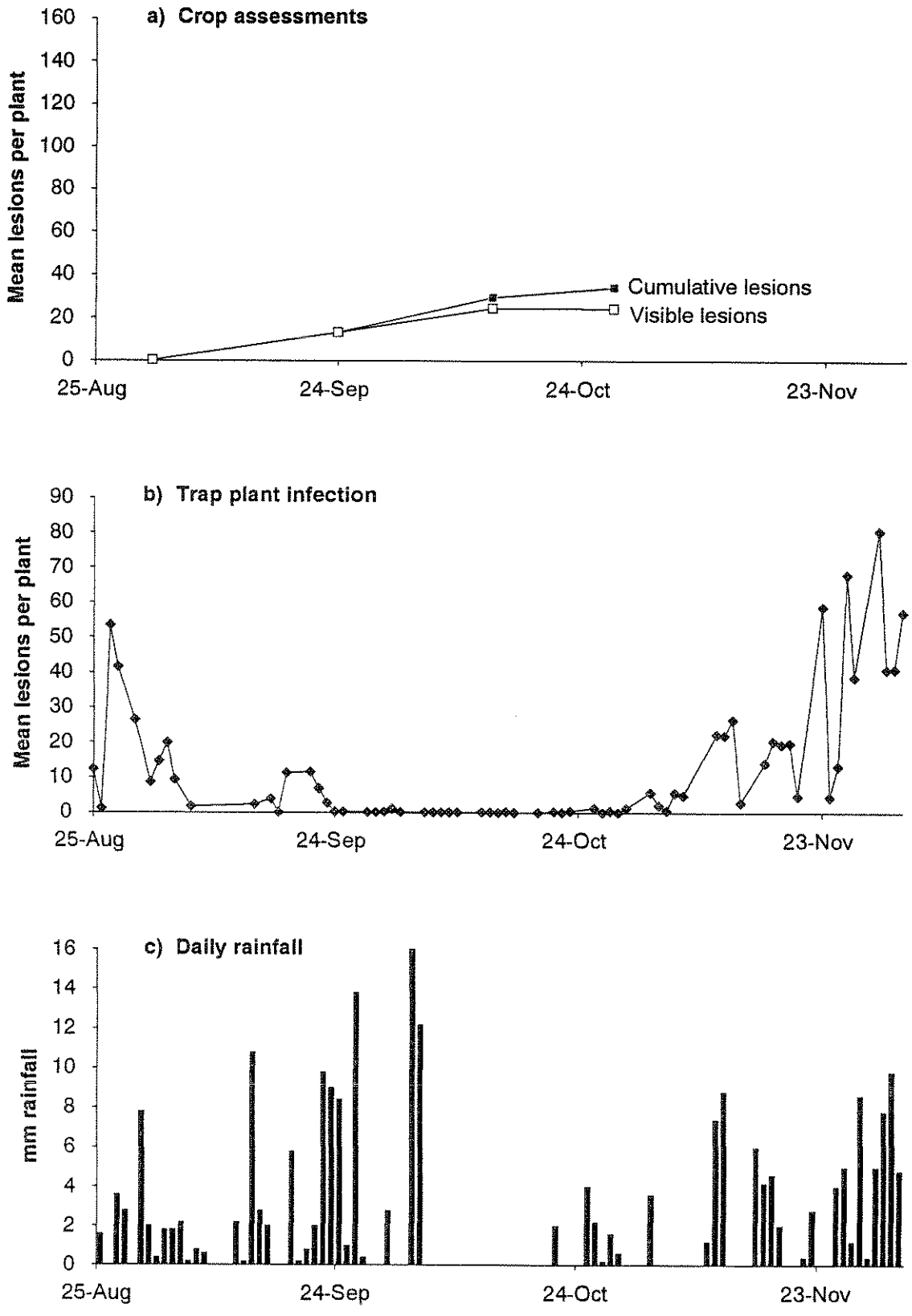
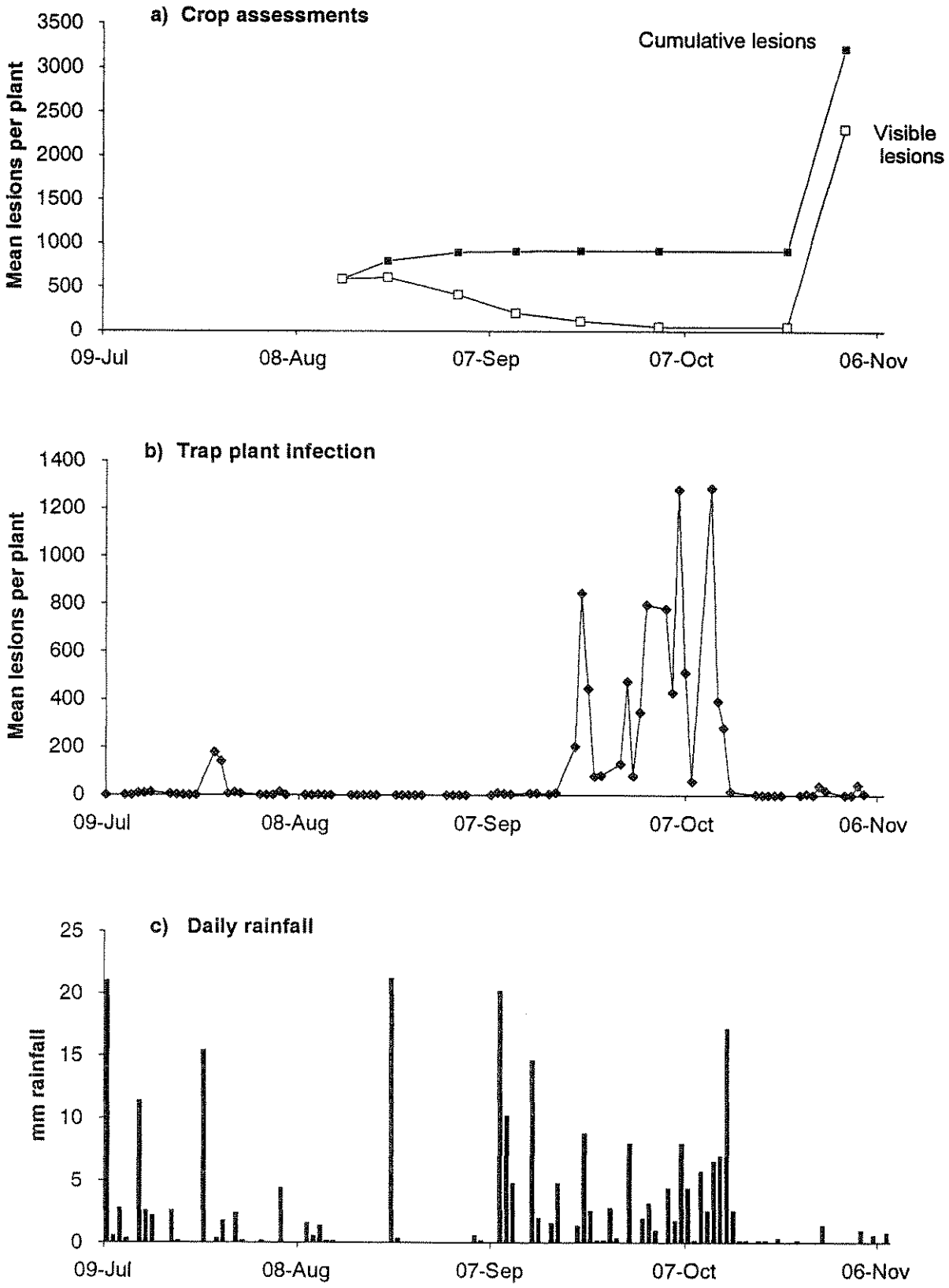


Fig. 3 Brussels sprout crop, Wellesbourne 1993



infection were similar to those observed after the initial infection cycle in 1991. However, levels of button infection were considerably lower, with 17 % of the sample infected (mean of 1.7 lesions per button). A second cycle of infection occurred later in 1992. This was detected by trap plants (Fig. 2b) but at this stage the crop was senescent, with few leaves remaining on assessed plants.

In 1993 the first ringspot symptoms on the Brussels sprout crop at Wellesbourne occurred in mid August (Fig. 3a), approximately seven weeks after inoculation. Plants became heavily infected, with a mean of 1010 cumulative lesions per plant in early September. By mid October, levels of disease had increased slightly to give 1030 cumulative lesions per plant. However, at this stage in the growth of the crop, many older infected leaves had abscised and plants appeared comparatively free from disease, with only 50 visible lesions per plant. In late October disease levels increased on both leaves and buttons. The final disease assessment in the crop during early November gave levels of 2314 visible lesions per plant, with 97 % buttons infected (mean of 19 lesions per button).

Disease development in the crops at Kirton and Stockbridge house followed a similar pattern to that in the crop at Wellesbourne (Fig. 4). Initial ringspot lesions were seen in both crops approximately seven weeks after inoculation. At the first assessment in mid August, plants at Kirton and Stockbridge House displayed a mean of 1340 and 2130 lesions per plant, respectively. By late September levels of cumulative infection in the crop at Kirton had increased to 1770 lesions per plant. However at this stage in the growth of the crop, heavy abscision of infected leaves had reduced levels of infection to eight visible lesions per plant (Fig. 4b). At the final disease assessment in late October the crop at Kirton displayed a mean of 3050 visible lesions per plant, with 82 % of buttons infected (mean of 36 lesions per button). Disease development in the crop at Stockbridge House followed a similar pattern. By mid October levels of cumulative infection had increased to 2600 lesions per plant. However levels of visible infection on the crop were extremely low, with less than one lesion per plant (Fig. 4c). During late October levels of infection increased rapidly on both leaves and buttons, giving a mean of 1900 visible lesions per plant at the final disease assessment in early November. At this stage 67 % of buttons sampled were infected (mean of 38 lesions per button).

Fig. 4 Disease development at different sites 1993

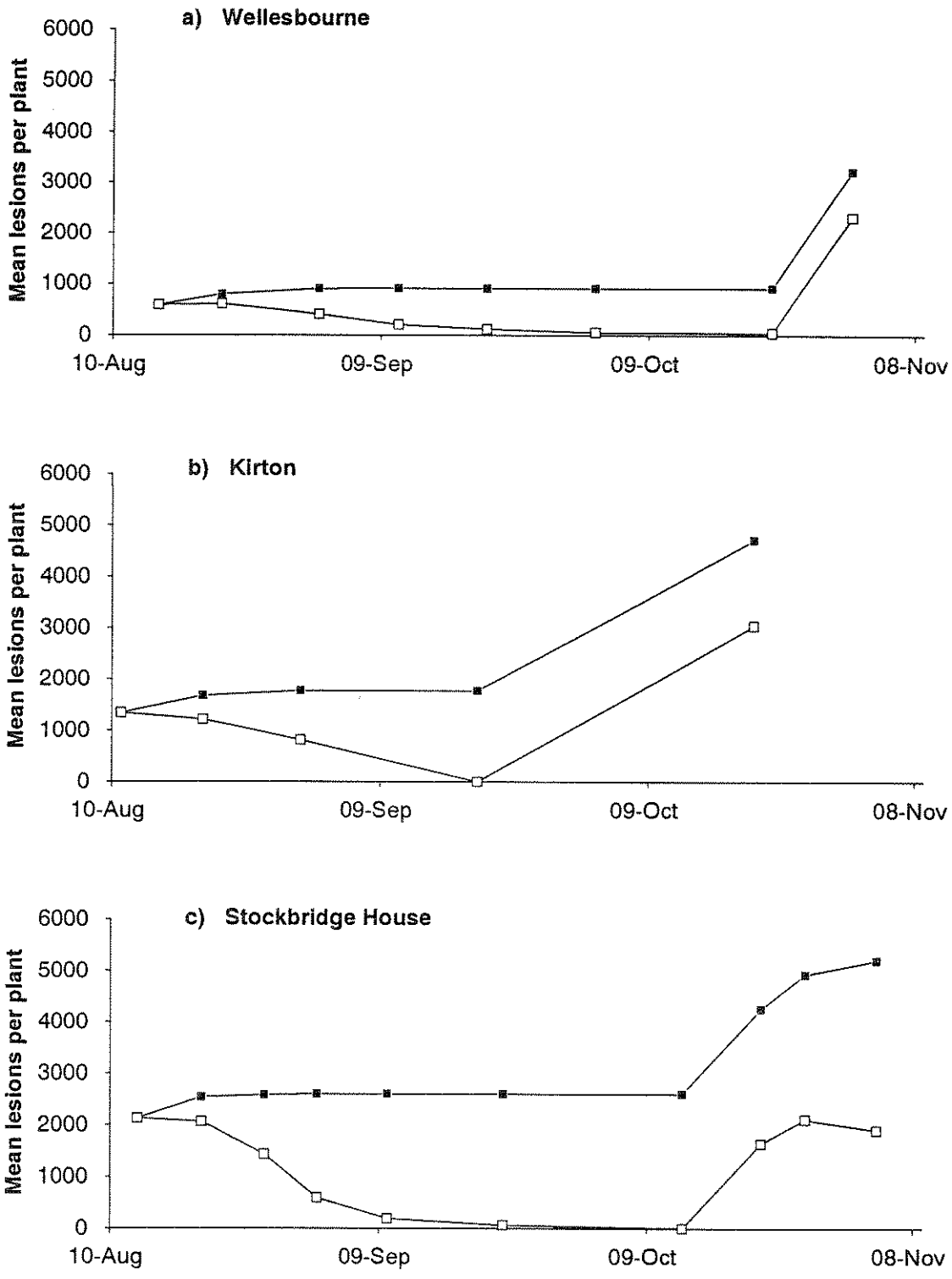
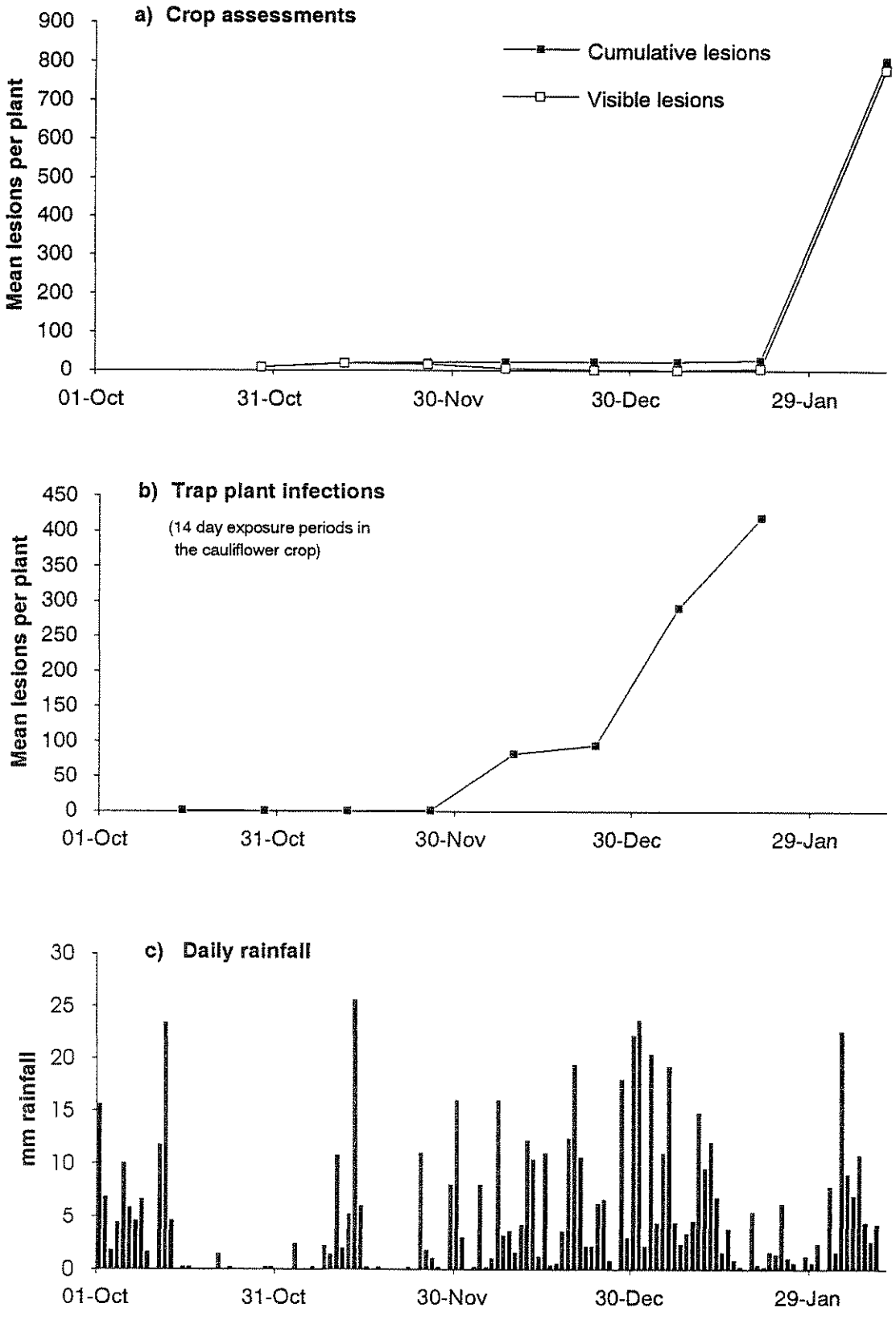


Fig. 5 Cauliflower crop, Cornwall 1993



The first ringspot lesions were detected on cauliflowers in the Cornwall field trial during late October (Fig. 5a). Infection levels were low, with a mean of 22 lesions on each assessed plant. Following abscision of the majority of infected leaves, plants appeared free from disease from late December until early February, after which disease levels increased to a mean infection level of 780 lesions per plant. This pattern of disease development is similar to that occurring in the inoculated Brussels sprout crops, providing evidence that the observed occurrence of two cycles of infection in the latter crops was not an artefact resulting from artificial inoculation.

2.1.2 Trap plants

Infections on trap plants exposed within the Brussels sprout crops at Wellesbourne reflected the pattern of disease development within the crops (Fig. 1b, 2b, 3b). Trap plants exposed for 24 h periods determined the days on which infection occurred. Lesions appeared on the crop (and the trap plants) approximately one month later, after the pathogen had completed its latent period. With the exception of the 1991 Brussels sprout crop, a cycle of infection occurred on successive exposures of trap plants following crop inoculation. In each year there then followed a prolonged period, over which very few infections occurred on trap plants. This was followed by a significant increase in infection, when successive exposures of trap plants developed high levels of disease. Trap plants exposed in Cornwall in 1993 (Fig. 5b) exhibited a similar pattern of infection over time - both within the cauliflower crop and on plants exposed some distance from infected crops.

2.1.3 Spore trapping

Few ascospores were detected using the Burkard spore trap - even on days on which trap plants became heavily infected. This may result from the low volume of air sampled by the trap, at only one location. In addition, the small dimensions and hyaline nature of ringspot ascospores does not facilitate their ready discrimination and visualisation on

spore tapes among the many other different spore types present.

2.1.4 Relationships with climatic conditions

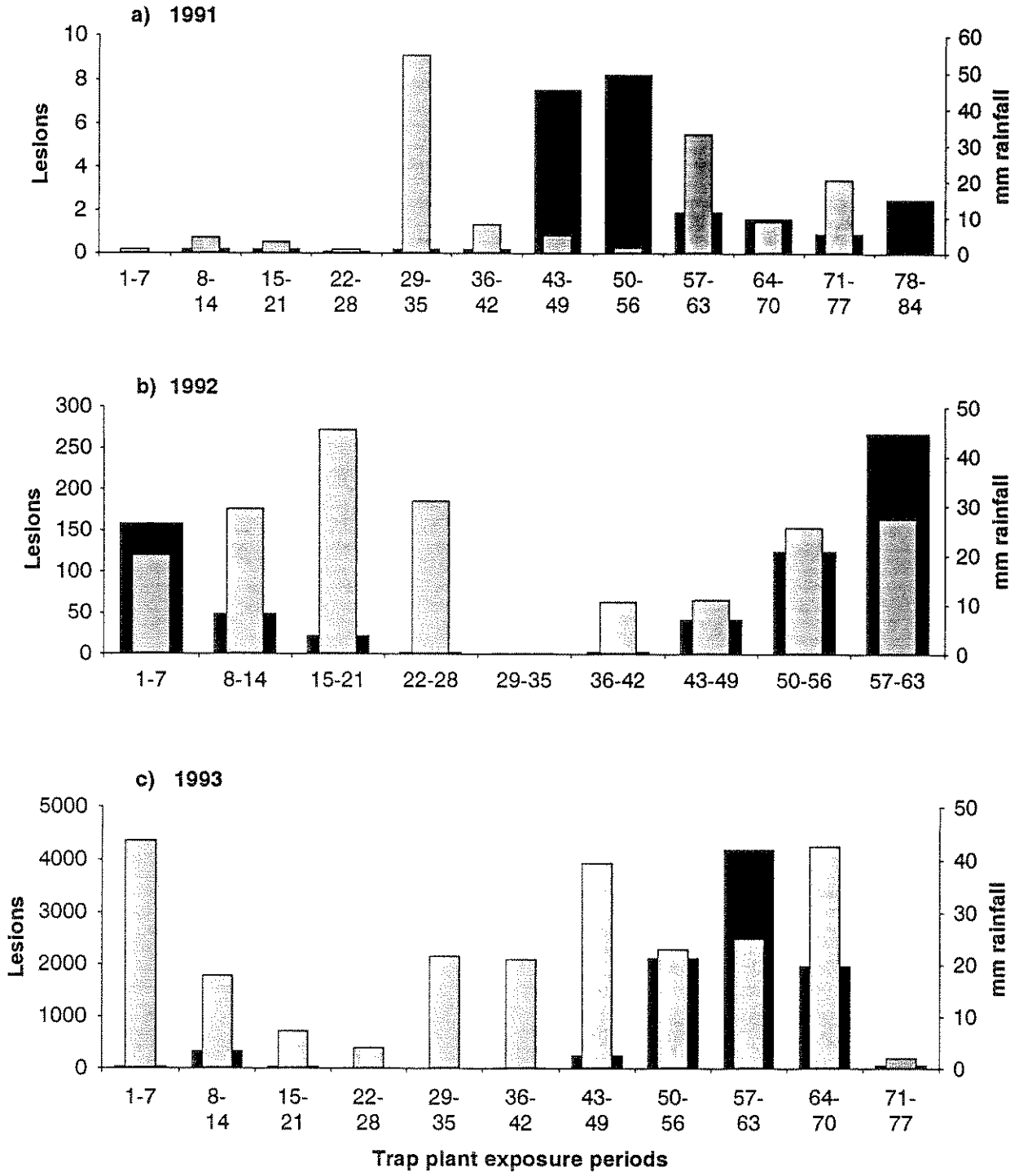
Attempts have been made to construe empirical relationships between climatic conditions and the pattern of disease development observed in the field.
Trap plant data provide a reliable test of when infection actually occurred. The cyclical pattern of infection on trap plants mainly reflects the pattern of disease development in the crop. (Section 1.2). Therefore it is important to determine whether periods of low trap plant infection (between cycles) result from the occurrence of climatic conditions unfavourable to infection or from unavailability of ascosporic inoculum.

2.1.4.1 Trap plant infection and rainfall

Previous studies have demonstrated that rainfall provides the necessary conditions of leaf wetness for ascospore release and also for infection. Trap plant (and crop) infection can be expected to occur only during or immediately after rainfall - provided that mature ascospores are present within perithecia and climatic conditions favour their discharge and dispersal. Data collected over three years show that there are many days on which rain fell however little to no infection occurred on trap plants (Fig. 1-3, Fig. 5). Cumulative rainfall and total lesions on all trap plants over groups of seven consecutive exposure periods is shown in Figure 6. Considerable rainfall occurred during numerous trap plant exposure periods - providing conditions of leaf wetness favourable for infection. However in many cases trap plants developed no lesions. The observed cyclical pattern of infection on trap plants therefore appears not to result from the pattern of occurrence of climatic conditions favourable for infection. This suggests that levels of airborne ascosporic inoculum were low over a prolonged period in the life of the crop, resulting in little to no infection on trap plants.

The initial cycle of infection in the inoculated crops is assumed to result solely from ascospores released from perithecia on lesions in the leaf-trash inoculum. This

Fig. 6 **Total rainfall and total lesions on trap plants over consecutive trap plant exposure periods at Wellesbourne**



assumption was supported by observations made on nearby uninoculated brassica crops - these showed virtually no ringspot infection. Once this supply of ascospores has been exhausted, in the absence of external sources of inoculum no more infection can occur until mature perithecia have developed on the first cycle of lesions in the crop. Prolonged periods of wetness are required for ascospore maturation (Section 3.2.4). Rainfall occurring during the "mid-cycle" trap plant exposure periods may initiate perithecial development and further maturation of ascospores but does not lead to substantial infection until considerable numbers of perithecia have developed mature ascospores. Therefore it is proposed that climatic conditions subsequent to the occurrence of the first cycle of lesions in the crop control the rate of perithecial development and ascospore maturation and hence the timing of the outbreak of a second major cycle of infection.

2.1.4.2 Perithecial development on lesions in the crop

A summary of meteorological data for the 1991-1993 Brussels sprout crops at Wellesbourne and the 1993 cauliflower field trial in Cornwall is presented in Table 1. The period examined is from the first recorded incidence of lesions in the crop until the first occurrence of significant infection on trap plants, which would indicate the start of the second cycle of infection in the crop. The timing of the second infection cycle in Cornwall was not precisely determined due to the 14 day trap plant exposure period. The first set of trap plants to develop heavy infection had been exposed from 26 November to 10 December. Infection occurred at any time during this 14 day period, however the meteorological summaries were calculated on the conservative assumption that ascosporic inoculum was only available at the end of the exposure period. In all crops 5 to 6 weeks elapse between the first occurrence of lesions and outbreak of heavy infection on trap plants. Total rainfall over this period is comparable for the three years at Wellesbourne, however much higher rainfall occurred in Cornwall. Total rainfall may be of lesser importance than the pattern of rainfall distribution over time. Rainfall distribution, in conjunction with temperature and wind-speed, determines the time period for which leaves remain wet, in the absence of dew, within the crop. Cumulative leaf

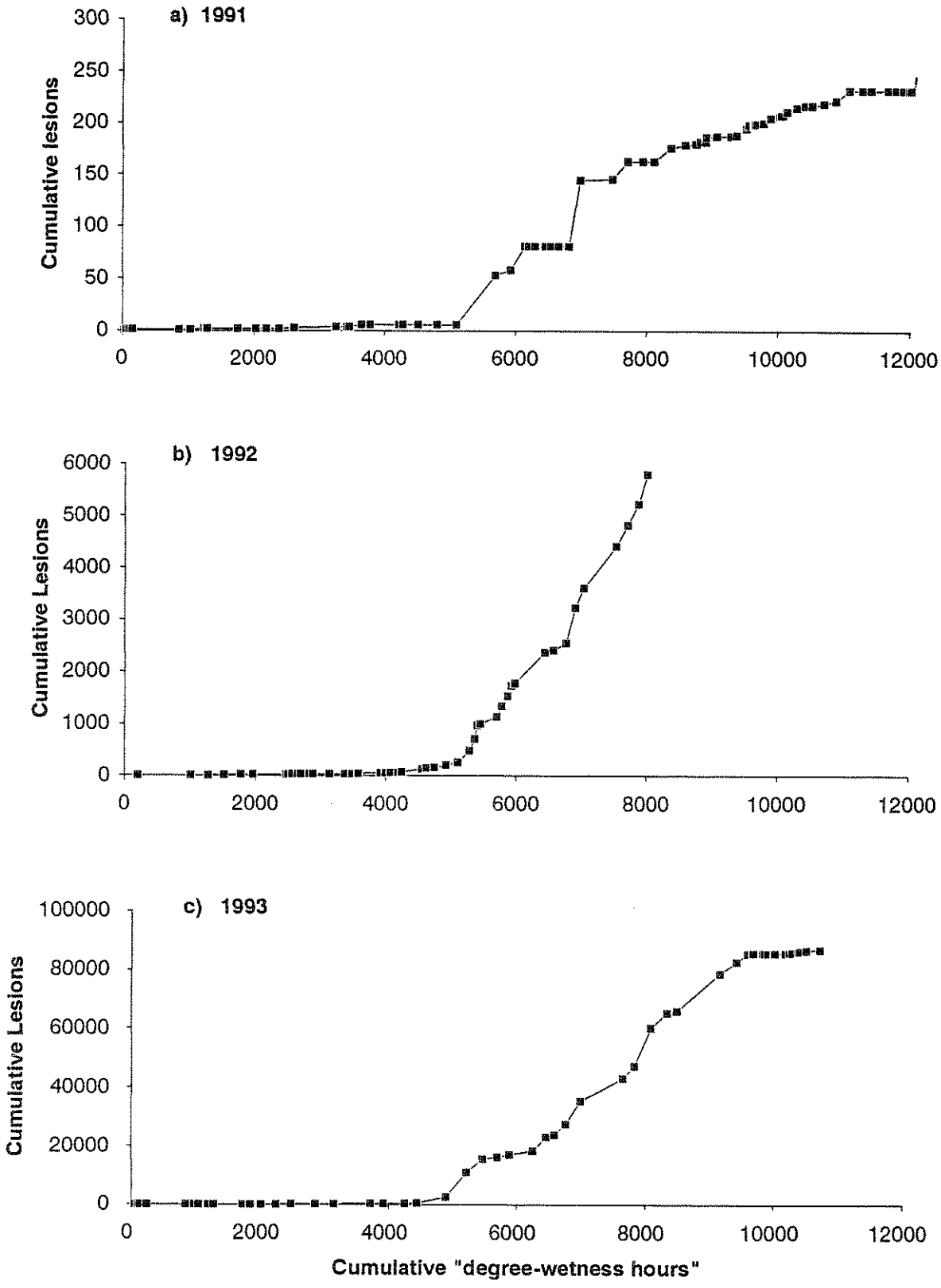
Table 1 Summary of climatic conditions from detection of initial lesions on the crop until first occurrence of heavy infection on trap plants.

	Wellesb.-91	Wellesb.-92	Wellesb.-93	Cornwall-93
Date first crop symptoms	29-Aug	24-Sep	15-Aug	29-Oct
Date heavy trap plant infection	14-Oct	2-Nov	20-Sep	10-Dec[1]
Days elapsed	46	39	36	42 (*29*)
Total rainfall (mm)	68.8	60.4	82	131.4 (*70.6*)
No. rainy days	21	13	12	28 (*17*)
Total leaf wetness (h)	517	557	347	533 (*347*)
Degree days (base 0°C)	570	328	520	340 (*221*)
Mean temperature (°C)	12.4	8.4	14.5	8.1 (*7.6*)
(Mean daily temp) x (daily leaf wetness period)	5644	4540	4813	4838 (*3120*)

[1] The exposure period was from 26 Nov until 10 Dec. Infection may have occurred at any time during this period. Figures in parenthesis denote values of climatic parameters if infection occurred at the start of the 14 day exposure period.

wetness is similar for Wellesbourne during 1991 and 1992 and in Cornwall in 1993, but is much lower for Wellesbourne during 1993. However mean temperature for Wellesbourne in 1993 is higher than in the other crops, which would accelerate perithecial development and diminish the total wetness duration required for production of ascosporic inoculum. Multiplication of mean daily temperature by daily hours of leaf-wetness generates a "degree-wetness hour" parameter which has been summed for each experimental crop. This parameter produces similar values for Wellesbourne 1992 and 1993. The higher value for Wellesbourne 1991 may result from occurrence of heavy infection over a three day trap plant exposure period (equivalent to 588 "degree-wetness hours"). A similar value is calculated for the un-inoculated cauliflower plot in Cornwall, assuming infection occurred only at the end of the 14 day exposure period. In Figure 7 cumulative trap plant lesions (sum of lesions on all ten plants over ensuing exposure periods) have been plotted against cumulative "degree-wetness hours" for trap plants exposed within the 1991-1993 Brussels sprout crops at Wellesbourne. In each year a large increase in trap plant infection occurs after approximately 5000 "degree-wetness hours".

Fig. 7 **The relationship between cumulative infection on trap plants and cumulative "degree-wetness hours" - from the first occurrence of lesions on the Brussels sprout crops at Wellesbourne**



2.2 Laboratory studies

2.2.1 Production of ascosporic inoculum

Growth and development of the pathogen varied with lighting regime and culture medium. When incubated in continuous darkness, the fungus produced downy white mycelial growth on all media types. No perithecia or ascospores developed in the culture. When incubated for 16 h per day under fluorescent lighting (with or without UV supplementation) cultures of the pathogen on SLD and V8 agar produced black, compact mycelium with fruiting bodies visible on the surface. Production of ascospores on these two media varied between individual plates and between subsequent inoculations, although perithecial production was generally greater on SLD agar than on V8 agar. Mature ascospores were detected on the former medium after 14 to 28 days incubation at 15°C. Ascospores developed when cultures were incubated under fluorescent lighting alone, however the magnitude and reliability of ascospore production was usually enhanced by the addition of UV-B light.

The fungus produced a thin base of black mycelium with an outer coating of downy white mycelium when grown on PLA. Such cultures occasionally produced low numbers of ascospores. When cultured on PDA and CMA agar, *M. brassicicola* produced a downy white mycelium - exhibiting a similar pattern of growth to that produced under conditions of continuous darkness. No perithecia or ascospores were produced on these two media.

2.2.2 Ascospore germination

Ascospore germination on water agar was rapid at 20°C and 25°C with 93 % and 99 % of ascospores, respectively, fully germinated after 10 h incubation (Fig. 8). At 15°C, 77 % of ascospores had germinated fully, whereas at lower temperatures of 10°C and 5°C the rate of germination was reduced to 28 % and 4 % respectively. After 22 h incubation 90 % of ascospores incubated at 10°C were fully germinated, however only 38 % of ascospores incubated at 5°C had germinated fully. Percentage germination of

Fig. 8 Ascospore germination on water agar at different temperatures Figures in legend denote incubation temperature (°C)

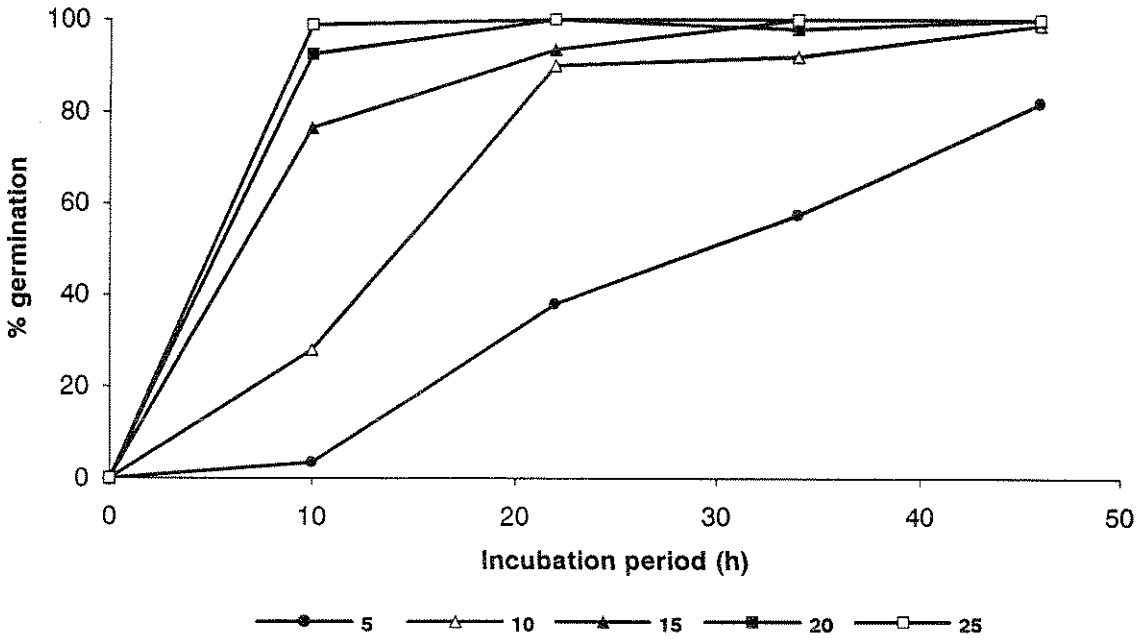
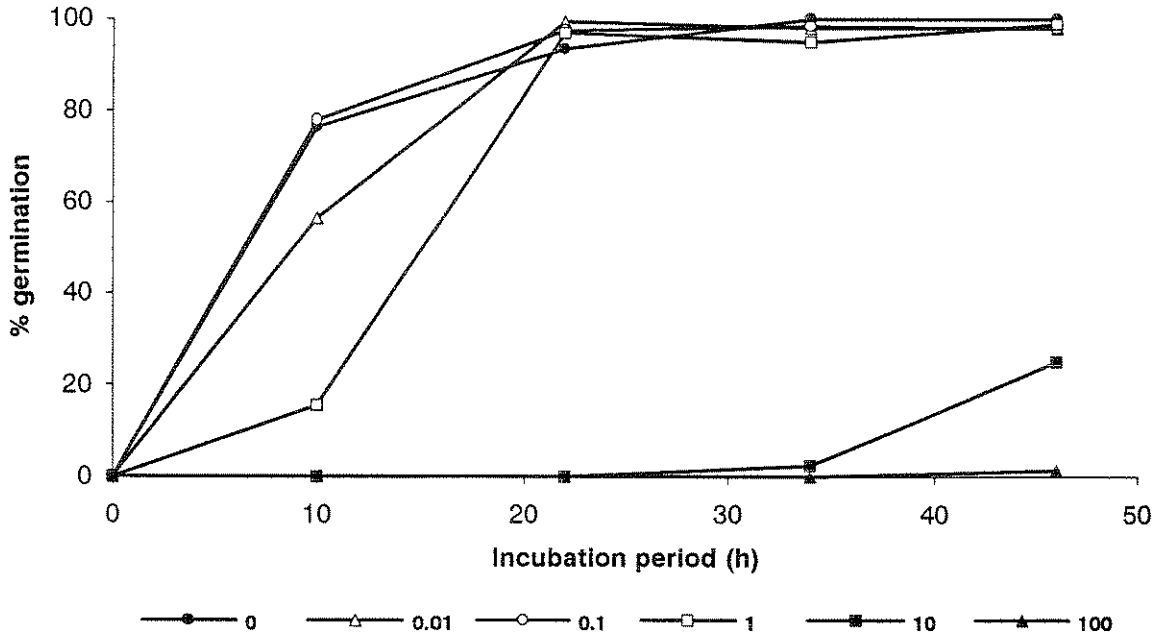


Fig. 9 Ascospore germination on water agar amended with benomyl at different concentrations. Figures in legend denote μg ml benomyl



ascospores increased to 82 % after 46 h incubation. At this final assessment time, ascospores at the higher temperatures were showing 100 % germination.

Incorporation into the water agar of the fungicide benomyl at concentrations of 10 $\mu g\ ml^{-1}$ and greater resulted in substantial reductions in the rate of ascospore germination (Fig. 9). After 46 h incubation at benomyl concentrations of 10 and 100 $\mu g\ ml^{-1}$ 25 % and 2 % of ascospores, respectively, had germinated. Benomyl concentrations of 1 $\mu g\ ml^{-1}$ and lower had little effect on the rate of ascospore germination.

When incubated for 24 h on glass coverslips at the range of relative humidities (r.h.), 86 % of ascospores germinated at 100 % r.h., 76 % at 98 % r.h. and 21 % at 96.5 % r.h. (Table 2). Ascospore germination was reduced to zero at relative humidities of 93.5 % and below, but ascospores were shown to remain viable at low relative humidities, giving high rates of germination when incubated for 24 h at 100 % r.h. (Table 2). Following 24 h exposure to 55 % r.h., there was 75 % ascospore germination on subsequent exposure to 100 % r.h..

Table 2 Ascospore germination and survival at different relative humidities

RH (%)	Ascospores germinated after 24 h incubation		Ascospores germinated after further 24 h incubation at 100 % RH	
	%	Sample size	%	Sample size
55	0	693	75	947
76	0	639	72	1175
90	0	608	64	929
93.5	0	631	71	764
96.5	21	854	91	1133
98	76	614	95	1109
100	86	525	-	-

2.2.3 Infection

Rate of germination and germ tube growth of ascospores deposited onto susceptible leaf discs and incubated under humid conditions at 15°C was similar to that on water agar at the same temperature. After 24 h incubation many germ tubes had grown to four times the length of the spore. At this stage a few germ tubes were commencing growth into stomatal pores on the leaf disc. After 48 h moderate numbers of germ tubes were observed to have penetrated stomatal pores, with some beginning to develop branching hyphae within the sub-stomatal cavity. After 72 h many germ tubes that had penetrated stomata had produced hyphae of considerable length which were observed to grow among the mesophyll cells of the leaf tissue. In all studies performed there was no evidence of infection occurring by any method other than by random growth into stomatal pores. There was no discernable difference in the pattern of germination and infection between leaf discs from oilseed rape and Brussels sprouts. However, inoculation of similar plants of Brussels sprout cv. Golfer and oilseed rape cv. Cobra with *in vitro* ascospores resulted in the development of many more lesions upon the Brussels sprout plants than on the oilseed rape plants. In addition lesion formation upon the oilseed rape plants was retarded by approximately seven days when compared to the Brussels sprout plants - with lesions on the former developing atypical, irregular margins.

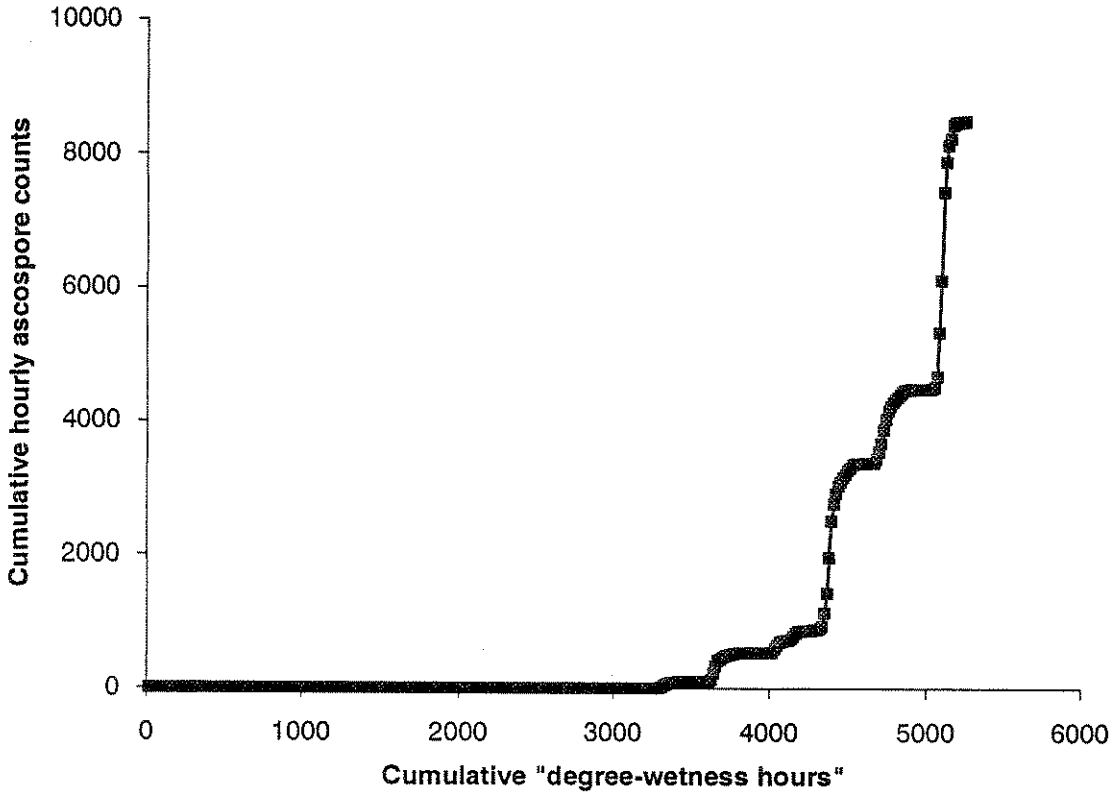
2.2.4 Ascospore development *in planta*

When detached ringspot infected leaves were incubated in controlled temperature cabinets under conditions of constant wetness, trap plants placed within the cabinets first became infected after 7 days at 20°C. At 15°C, high levels of trap plant leaf infection first occurred after 9 days incubation. However at lower temperatures of 10°C and 5°C, only very low levels of infection were seen upon trap plant leaves after termination of the experiment at 10 days incubation. No lesions occurred on trap plants placed within the cabinet operating at 25°C.

In the second experiment, in which leaves bearing ringspot lesions were still attached to plants, ascospores were again detected only after 9 days incubation at 15°C. This

incubation period corresponds to 3240 "degree-wetness hours" (Fig. 10).

Fig. 10 **Time course of ascospore release from attached ringspot-infected leaves incubated at 15°C under controlled conditions of constant wetness**



Discussion

The pattern of disease development in 1993 was similar in Brussels sprout crops at Wellesbourne, Kirton and Stockbridge House. Lower initial levels of disease in the crop at Wellesbourne may reflect differences in climatic conditions between sites and their effect on the magnitude of production of ascosporic inoculum from the leaf trash inoculum or the severity of infection. Alternatively, the crop at Wellesbourne may have been less susceptible to infection.

The origin of ascosporic inoculum responsible for initial infection in the cauliflower field trial was not established. Possible sources include ascospores released from infected remains of the previous crop or ascospores produced by lesions resulting from infection in the nursery.

The second cycle of infection in the inoculated Brussels sprout crops was believed to result solely from ascospores produced within the crop as neighbouring brassica crops remained virtually free from ringspot infection. In Cornwall occurrence of heavy infection on trap plants exposed some distance from infected cauliflower crops suggested that significant aerial ascospore concentrations occurred outside infected crops. The second cycle of infection in the cauliflower trial may therefore have resulted partly from ascospores disseminated from infected neighbouring crops. Germination of ascospores following exposure to *in vitro* conditions of low r.h. suggests that ascospores may survive long distance aerial transport, leading to infection in other brassica crops.

In Brussels sprout and cauliflower crops, timing of occurrence of cycles of ringspot infection in relation to developmental stage of the crop will determine the extent of damage to the marketable yield. Late inoculation of the 1992 Brussels sprout crop at Wellesbourne led to development of low levels of disease on buttons - resulting from the first cycle of infection. Levels of disease on buttons were considerably higher in Brussels sprout crops studied in other years, when earlier inoculation resulted in buttons developing lesions in the second cycle of infection. In the cauliflower field trial in 1993, curd development was advanced when high levels of disease developed on the leaves. There was no effect on curd size and quality, with mature leaves shielding packing leaves, maintaining them free from ringspot lesions. In 1992 high levels of infection

occurred earlier in the growth of the cauliflower crop, leading to substantial abscision of mature leaves and weakened plants. Curd size and quality was severely affected, with exposed packing leaves developing unacceptable levels of disease.

Under controlled environment conditions, ringspot-infected plants produced ascospores after 3240 "degree-wetness hours". However in the Brussels sprout crops at Wellesbourne high levels of airborne ascosporic inoculum were detected by trap plants only after 5000 "degree-wetness hours" from initial development of lesions in the crop. The comparatively rapid rate of ascospore maturation in the controlled environment may result from the early senescence of leaves observed on the pot-grown plants - possibly providing conditions more favourable for ascospore maturation (Götz *et al.*, 1993). Alternatively, interruption of periods of leaf wetness in the field or fluctuations in temperature may retard the rate of ascospore maturation when compared to the rate under conditions of constant temperature and wetness.

Studies on leaf discs suggest that the infection process is prolonged, requiring a minimum of 72 h wetness at 15°C. Similar studies performed by Götz *et al.* (1993) found that 10-20 days were required between germination of ascospores and penetration of leaves of white cabbage plants by germ tubes. These observations contradict those from the trap plants exposed in the Brussels sprout crops at Wellesbourne; high levels of infection developed on trap plants exposed to periods of wetness of 24 h duration, after which plants were moved to dry glasshouse conditions. This discrepancy may possibly be explained by occurrence of high relative humidities in the boundary layer at the leaf surface, providing conditions favourable for continued growth of germ tubes of germinated ascospores after free water on leaves has evaporated.

Conclusions

1. In all inoculated Brussels sprout crops studied, ringspot developed in a similar fashion - with two distinct cycles of infection, separated by a period of 6-8 weeks during which little infection occurred. A similar pattern of disease development occurred in an un-inoculated cauliflower field trial in Cornwall.

2. Timing of occurrence of each cycle of infection in relation to the developmental stage of the crop plays an important role in determining the extent of damage to the marketable yield.

3. The period of low infection between cycles of infection is believed to result from unavailability of ascosporic inoculum, rather than occurrence of climatic conditions unfavourable to infection.

4. The second cycle of infection occurred when mature ascospores had developed within perithecia on lesions in the crop. The timing of onset of the second cycle of infection is a function of the rate of ascospore maturation - a process dependent upon temperature and occurrence of prolonged periods of leaf wetness.

5. Application of an eradicant fungicide following successful prediction of the occurrence of the second cycle of infection should prevent development of lesions, resulting in effective control of the disease. The prolonged latent period of the pathogen renders the disease amenable to post-infection control.

6. A technique has been developed for producing large numbers of ascospores of *Mycosphaerella brassicicola* in culture, providing inoculum for critical studies into ascospore germination, survival and infection.

7. Infection occurs only as a result of random growth of ascospore germ tubes into

stomata. For a given density of ascospores on leaf surfaces, development of maximum infection requires periods of leaf wetness of at least 72 h duration at 15°C. With shorter periods of leaf wetness, lower numbers of germ tubes will reach stomata and levels of infection will be reduced.

References

Dring, D.M. (1961). Studies on *Mycosphaerella brassicicola* (Duby) Oudem. *Transactions of the British Mycological Society* 44 (2), 253-264.

Evans, E.J., Gladders, P., Davies, J.M.L., Ellerton, D.R., Hardwick, N.V., Hawkins, J.H., Jones, D.R., Simkin, M.B. (1984). Current status of diseases and disease control of winter oilseed rape in England. *Aspects of Applied Biology* **6**, 323-334.

Frinking, H.D., Geerds, C.F. (1987). The effect of temperature and moisture on the development of *Mycosphaerella brassicicola* in Brussels sprouts. *Gewasbescherming* **5**, 133-143.

Gladders, P. (1993). Observations on ringspot (*Mycosphaerella brassicicola*) in winter oilseed rape in South West England. *Bulletin OILB/SROP (1993)* **16** (9), 9-14.

Götz, M., Zornbach, W., Boyle, C. (1993). Life cycle of *Mycosphaerella brassicicola* (Duby) Lindau and ascospore production *in vitro*. *Journal of Phytopathology* **139**, 298-3.

Hartill, W.F.T. (1977). Epidemiology and control of ringspot in cabbages and cauliflowers. *Proceedings of the 30th New Zealand Weed and Pest Control Conference*, 91-95.

Huber, G.A., Gould, C.J. (1949). Cabbage seed treatment. *Phytopathology* **39**, 869-875.

Pound, G.S., Cheo, P., Calvert, O.H., Rabbe, R.D. (1951). Extent of transmission of certain cabbage pathogens by seed grown in Western Washington. *Phytopathology* **41**, 820-827.

Shipton, W.A., Brown, J.F. (1962). A whole-leaf clearing and staining technique to demonstrate host-pathogen relationships of wheat stem rust. *Phytopathology* **52**, 1313.

Snyder, W.C. (1946). Spermogonia versus Pycnidia in *Mycosphaerella brassicicola*. *Phytopathological Notes*. **36**, 481-484.

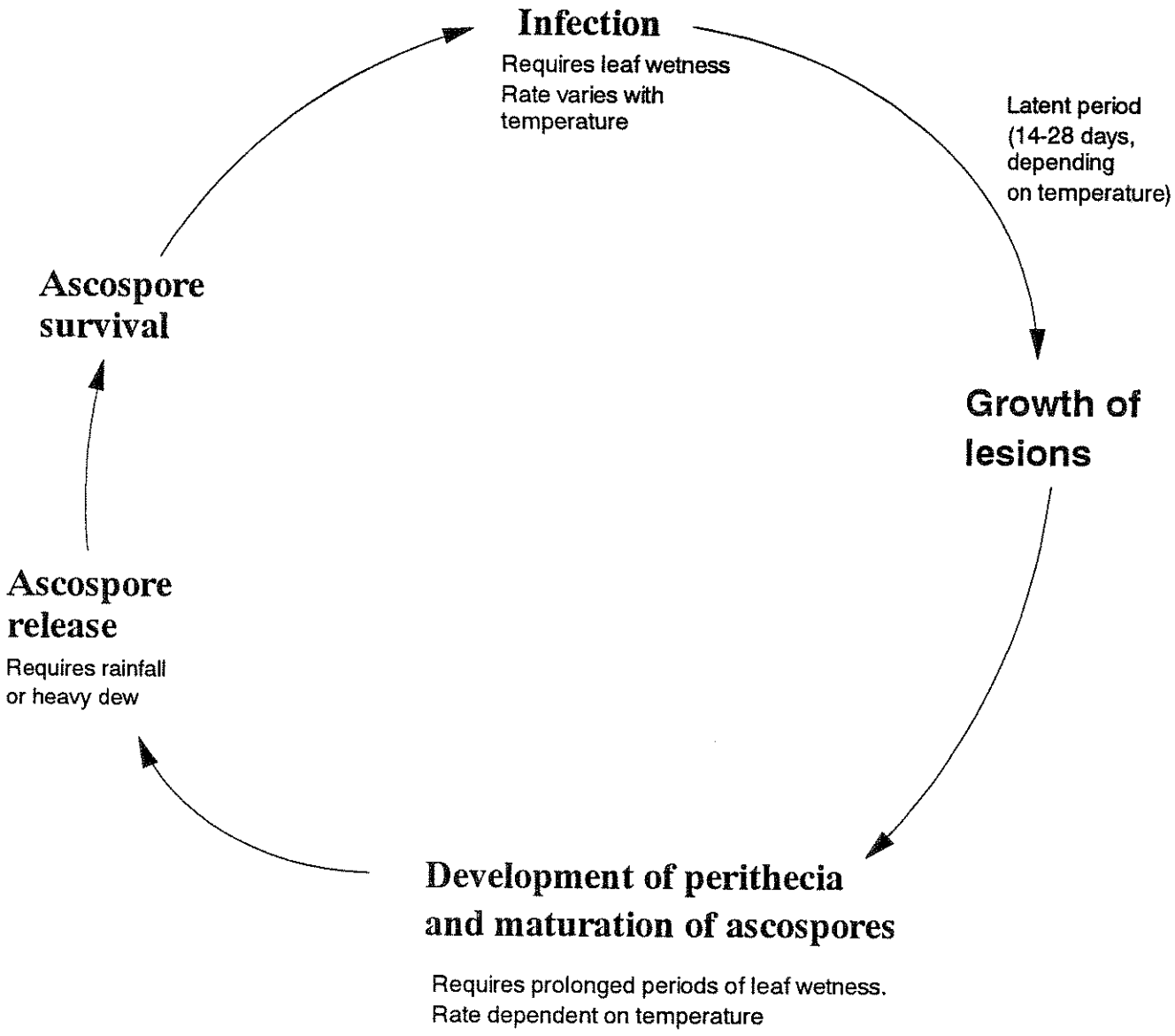
Weimer, J.L. (1926). Ringspot of crucifers caused by *Mycosphaerella brassicicola* (FR.) Lindau. *Journal of Agricultural Research* **32** (2), 97-131.

Zornbach, W. (1990). Studies on the pathogenicity, epidemiology and control of *Mycosphaerella brassicicola* (DUBY) Lindau, the cause of the ringspot disease of crucifers. *PhD Thesis*, University of Hamburg: 105 p.

Glossary

Ascospore	A sexually-produced spore - the only type of infective spore produced by *M. brassicicola*.
Cumulative lesions	The sum of all new lesions recorded in the crop from the first assessment. Expression of disease development as cumulative lesions against time allows the occurrence of new lesions to be detected when numbers of lesions visible on the plants are declining due to abscision of earlier-infected leaves.
Degree-wetness hours	The product of mean daily temperature and hours of wetness occurring within the same 24 h period.
Latent period	The time interval between successful penetration of stomatal pores by ascospore germ tubes and the development of lesions. For *M. brassicicola* the latent period is 3 to 4 weeks in duration, during which time the pathogen is growing between the cells of the host.
Perithecia	Fruiting bodies developing after occurrence of leaf wetness - visible as minute black specks on the surface of lesions of *M. brassicicola*. Ascospores are produced within perithecia.
Packing leaves	Fringe of leaves surrounding the curd of cauliflowers. These are retained at market and if blemished may lower the value of the produce.
Trap plants	Glasshouse-grown disease-free plants exposed within the infected crop for a 24 h period, after which they are returned to disease-free glasshouse conditions. Following a suitable incubation period, occurrence of lesions on the plants indicates that during their period of exposure within the crop, ascosporic inoculum was available and climatic conditions favoured infection.

Appendix I Life cycle of *Mycosphaerella brassicicola*



Appendix II Composition of mycological culture media (l^{-1})

Prune lactose agar (PLA)

Prune extract	100 ml
Lactose	5 g
Yeast extract	1 g
Agar	20 g
Distilled water	900 ml

Corn-meal agar (CMA)

Maize	30 g
Agar	20 g
Distilled water	1000 ml

Potato dextrose agar (PDA) Obtained from OXOID

Potato extract	4 g
Glucose	20 g
Agar	15 g

Vegetable juice agar (V8)

Campbells V8 juice	200 ml
Agar	20 g
Distilled water	800 ml

The above media were prepared according to the methods described in the Plant Pathologist's Pocketbook (Commonwealth Mycological Institute, Kew, Surrey). Medium pH was adjusted to 5.5 with 1 M NaOH prior to autoclaving at 121°C for 15 minutes.

Brussels sprout leaf decoction (SLD)

Senescent Brussels sprout leaves	1000 g
Agar	20 g
Distilled water	to 1000 ml

Senescent leaves (yellow in colour, but retained on the plant) were collected from field-grown Brussels sprout plants cv. Golfer. Leaf blades were macerated in distilled water in a Wareing blendor. The leaf extract was filtered through muslin, amended with agar and autoclaved as for the other media. Medium pH varied from 5.5 to 7.5 and was not adjusted prior to autoclaving.