PROJECT REPORT No. OS56

SCLEROTINIA CONTROL IN OILSEED RAPE: PROGRESS WITH QUANTITATIVE DIAGNOSIS AND DEVELOPMENT OF A WEB-BASED FORECASTING SCHEME

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SCLEROTINIA CONTROL IN OILSEED RAPE: PROGRESS WITH QUANTITATIVE DIAGNOSIS AND DEVELOPMENT OF A WEB-BASED FORECASTING SCHEME

by

J TURNER[1], C YOUNG[1], A RIDING[2] AND P GLADDERS[3]

[1]Central Science Laboratory, Sand Hutton, York YO41 1LZ
[2]ADAS Wolverhampton, Woodthorne, Wergs Road, Wolverhampton, WV6 8TQ
[3]ADAS Boxworth, Battlegate Road, Boxworth, Cambridge CB3 8NN

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ABSTRACT

The aims of this four-year project were to improve the effectiveness of strategies for control of sclerotinia stem rot of oilseed rape by (a) development of a rapid diagnostic test for identification of crops at risk of infection to allow optimisation of fungicide requirement and timing (b) obtaining fundamental information on the relationship between sclerotinia inoculum, fungicide timing and final disease levels and yield and (c) development of a decision guide to assist with assessment of disease risk.

A number of monoclonal antibodies with potential for incorporation in rapid immunodiagnostic assay formats have been produced which are highly specific and sensitive to *Sclerotinia sclerotiorum* and appear to be significantly in advance of those produced in other countries. As yet, the assay formats tested have not had the qualities of ease of use and accuracy required for a laboratory service or in-field methodology to be developed. The report identifies strategies for overcoming or avoiding problems such as the inhibition of the test caused by the presence of oilseed rape petal extracts in test samples through further development of the current protocols.

Spatial analysis of sclerotinia infected crops showed that the disease can occur in gradients across a field indicating an external inoculum source, or in 'hot spots' within the crop indicating a source of infection within the field. Data clearly emphasise the requirement for sampling at several points in each field for reliable determination of disease risk using agar or immunodiagnostic petal tests. A sampling strategy involving taking samples on a 100 m grid pattern was able to detect the spatial variability present within the fields monitored. This is equivalent to approximately 15 samples for a 10 ha field. Further work is needed to confirm minimal, but statistically reliable, sampling strategies. Accurate prediction of inoculuum and disease within a field may allow spraying to be targeted only in areas at risk. Field experiments showed that fungicide treatments were very effective (95% control) for sclerotinia control but timing of applications was critical. In 1999 and 2000, the optimum timing for sprays to control sclerotinia was between late April and early May. However, the timing of infection was related to weather conditions favouring petal sticking and disease development and this was the critical factor determining the optimum growth stage within the flowering period for treatment. A difference of only 10 days between treatments had a large effect on the level of disease control and this clearly identifies the value of a rapid test to guide spray applications.

A range of risk factors for sclerotinia stem rot have been investigated and together with data gathered during the project, have been incorporated into an web-based Decision Guide for determination of sclerotinia disease risk. These factors include previous history of disease, weather and secondary crop factors. The Guide also has options for inputting results of petal testing for estimation of inoculum potential. The Decision Guide calculates a numeric value for each set of data entered and assigns a value for risk of sclerotinia, which is displayed as Low, Medium or High. The Decision Guide is available and now requires validation on farms.

SUMMARY REPORT

Introduction

The importance of diseases in winter oilseed rape has been quantified by application of disease-yield loss relationships to disease severity data available from the annual surveys of winter oilseed rape carried out by ADAS and CSL. Stem rot caused by *Sclerotinia sclerotiorum* was particularly severe in 1991 when 46% of surveyed crops were affected and some severe attacks continue to occur annually. Yield loss from sclerotinia is estimated to be up to 50% on affected plants (i.e. 10% plants infected would result in a 5% yield loss) and economic responses to fungicide treatment can be expected where more than 10% of plants are affected. Losses in 1991 due to sclerotinia, after fungicides had been applied, were in excess of £5M. In subsequent years the numbers of oilseed rape crops sprayed during flowering, predominantly for control of sclerotinia, rose dramatically but have now started to decline due to a reduction in perceived and actual risk of the disease. Clearly, there will be an economic and environmental penalty from such increased spraying where disease would not have developed or where control was ineffective through inappropriate timing.

The sclerotia (resting bodies) produced by the fungus on oilseed rape are capable of long term survival in soil, posing a threat to future crops of oilseed rape and other broad-leaved crops. Under favourable conditions in spring the sclerotia produce apothecia (spore bearing structures) which release ascospores. These can be wind dispersed onto the petals during flowering. Infection of the stem occurs if colonised petals adhere to the stems during moist conditions at petal fall. Farmers are concerned not only about the direct effects of stem rot on yield, but also about the longer term consequences of a build up of sclerotinia in the rotation.

Current fungicides have very little curative activity and need to be used as protectants to achieve good disease control. Accurate prediction of stem rot infection is not possible at present and decisions to spray are based on an assessment of risk. Crucially this depends on estimating the amount of inoculum in the crop and taking account of recent and future weather conditions which favour crop infection. At present in the UK, prediction of sclerotinia stem rot on winter oilseed rape and decisions to spray fungicides are based mainly on farm history of stem rot and weather conditions which favour infection. The presence of sclerotinia on petals, as determined by agar plate testing, is another useful indicator of risk, and has been used successfully in Canada with weather factors also taken into account. The relationship between petal infection and stem rot in the UK is less clear cut, partly because of the presence of botrytis in the field, a problem which does not apparently occur in Canada. Petal testing is a useful technique but the agar method takes 10 days to complete and is therefore potentially unreliable because delays may result in the optimum timing for the spray being missed and also levels of petal infection can change during the flowering period.

In order to implement use of diagnostic tests to estimate disease levels in crops, sampling strategies need to be developed to ensure accurate estimation of disease incidence and severity. This situation clearly highlights the need for a rapid test to determine petal infection in time for spray decisions to be made more timely and effective.

The aims of this project were to:

1. Develop a rapid diagnostic test for detection of sclerotinia spores on oilseed rape petals.
2. Compare the new diagnostic test with the standard agar method.
3. Develop a crop sampling protocol for use with the diagnostic test.
4. Study optimum timing of fungicides and thresholds for control of sclerotinia in field experiments.
5. Develop a forecasting/risk assessment scheme for sclerotinia in oilseed rape incorporating results from the project.

Development of a rapid diagnostic test for sclerotinia stem rot

Methods

Development of the rapid diagnostic test for sclerotinia focused on the use of specific monoclonal antibodies to detect ascospores on oilseed rape petals at flowering. Monoclonal antibodies with target specificities for the mycelium, spores or germinating spores of *S. sclerotiorum* were produced using standard methods. Antibodies were screened for specificity to sclerotinia and for cross reactivity to almost thirty other fungal species. Antibodies showing specific reactions only to *S. sclerotiorum* were selected for incorporation into assay formats with the intention of developing a test which could be carried out within one day. Three assay formats were investigated: a plate trapped antigen (PTA) ELISA, a double antibody sandwich (DAS) ELISA and an immunofluorescence (IF) assay.

Results

Antibodies targeted at ascospores proved to be the most specific and those with high specificity for sclerotinia and showing no detection of other fungal species were selected for further development. The antibodies were all capable of the specific detection of *S. sclerotiorum* ascospores in solution in all three formats tested. However, the addition of oilseed rape petal extract to ascospore suspensions resulted in the total inhibition of spore detection by the plate trapped antigen format. The problem of inhibition by petal extract was less significant in the double antibody sandwich ELISA and immunofluorescence assay. Of these two assay formats immunofluorecence was the most sensitive, capable of detecting 4 ascospores on a single fresh petal. However, high background fluorescence from the petal material, particularly if petals had

been previously frozen, hindered visual detection of spores and the test was more time-consuming than the other formats.

The DAS format would be the most suitable format for high throughput testing of samples and for further development into an in-field lateral flow test. Unfortunately, time within the project did not allow investigation into additional adjustments to the DAS protocols which may have improved sensitivity and reduced inhibition by petal material. An additional solution, which could lead to the production of a successful DAS format and in-field test in the future, would be incorporation of an antibody from another source as a partner antibody to the highly specific antibodies developed in this project.

Use of agar plate and rapid diagnostic tests to quantify petal infection

The newly developed immunofluorescence (IF) test was selected for further development and was validated against the standard petal culturing method using plant material from a range of sources to determine levels of accuracy and ease of use.

Methods

1. Disease monitoring on farms

The relationship between farm history, levels of sclerotinia detected on petals and final stem rot levels was investigated using agar and immunodiagnostic petal testing to examine the potential of a diagnostic test in improving oilseed rape management decisions.

In 1993-1998, petal samples were taken at early, mid and late flowering from 50 crops of winter oilseed rape chosen annually from farms in England with different histories of stem rot. The farm disease risk was assessed as nil (no stem rot seen previously), low (1-9% plants affected), moderate (10-19% plants affected) or high (20% or more plants affected) based on infection levels in the previous year.

Agar tests

Petals were sampled during dry weather and cultured immediately or within 2-3 h. Advice was given on whether to spray the crop during flowering, based on the history of that field or an adjacent field, and on petal culturing results from early flowering petal tests and forecast weather conditions. Prior to harvest, 100 plants in the monitored area, and the adjacent treated area if fungicide was applied, were assessed for stem rot.

ELISA plate samples

In 1996 and 1997 at each visit to each of the 50 farms, 40 petals per crop were placed in individual wells of a standard 96-well ELISA plate to form a parallel sample to that used for agar testing. Samples were sent to CSL or ADGEN and stored at -30°C. Sub-samples from the frozen store of field samples were tested to determine the presence/absence of sclerotinia spores using the IF test. Thirty samples were selected which were sampled at mid-flowering in 1996 and comprised equal numbers of samples determined to have low, medium or high levels of sclerotinia spores on the petals using the agar method. Samples were tested using the immunofluorescence assay and the results compared with those from agar culturing.

2. Field trials

Samples from trials undertaken at Boxworth in 2000 to investigate spatial distribution of the disease were also selected for analysis as part of the assessment of the IF test. Ten flower racemes were collected from 13 points around the field. Samples were sent to CSL where they were frozen at –30˚C until required. Two flower racemes were chosen at random from each of the sample points and from each raceme four flowers sampled from along the length of the raceme. One petal from each flower was placed into a well of a 96 well ELISA plate. The process was repeated for the remaining three petals/flower, making sure that the petals taken from each flower were placed in equivalent wells on a plate.

The four replicate plates were tested as follows:-

1. Cultured on agar – to compare pre- and post-freezing results.
2. Cultured on agar – to indicate similarity of infection by sclerotinia on mirrored petals taken from the same flower.
3. Subjected to IF analysis (CSL) – for comparison with petal culturing.
4. Subjected to IF analysis (ADGEN) – allowed comparison of methodology between laboratories

3. Plot trials

Experiments were carried out on plots established at CSL in 1999/2000 to investigate the relationship between inoculum, petal infection and stem disease.

Plots (12m x 9m) were sown with winter oilseed rape culivar Alpine in autumn 1999. Twenty sub-plots representing five treatments with four replicates, were marked out and inoculated at GS 4,5 with differing levels of ascospore inoculum: 0, 1, 100, 1000, 6000 spores per ml. Petals were sampled one hour after inoculation and plated onto PDA for determination of petal colonisation. A misting system was switched on two hours after inoculation using a regime of 30 seconds misting per hour for 7 days to provide conditions

which would allow petals to adhere to stems during petal fall. Sclerotinia stem rot assessments were carried out 40 days post-inoculation.

Results

Petal testing of samples collected from crops in 1996 and 1997 showed considerable variation in petal infection by sclerotinia between sites. Data show that relationships between farm risk, level of petal infection at flowering and severity of stem rot disease were not consistent and illustrated the need for a more accurate test to determine the level of petal infection at flowering. Results from immunodiagnostic tests carried out on previously frozen samples taken from farms showed no agreement with results from agar tests carried out on fresh petals. Levels of background fluorescence from the thawed petal material were high and identifying spores was difficult. Further experiments using material from field trials showed that levels of infection detected by petal culturing or the IF test were considerably reduced if samples had been frozen. Detailed comparison of levels of infection on petals from the same flower using frozen samples showed that there was little or no relationship between petals in the level of colonisation by *S. sclerotiorum*. Results indicated that agar tests on fresh material detected presence of sclerotinia as spores, germlings or mycelium whereas, after freezing, only ungerminated spores present at the time of freezing were detected. Data also showed that the assumption that petals within the same flower will have an equivalent level of infection present might not be reliable, particularly when levels of petal infection are low. These factors affected the ease of comparison of the immunodiagnostic method with the agar test. However, results indicated that the IF test was not suitable for routine screening of large numbers of samples for sclerotinia spores on oilseed rape petals due to background fluorescence levels obscuring detection of the spores. Despite the unsuitability of the IF test for routine detection on oilseed rape petals, the test may have value in detecting sclerotinia infection on other crops where the plant material may not produce such significant fluorescence. Data collected from field trials indicated that the level of air-borne inoculum needed to produce threshold levels of petal infection (~41%) was between 1000 and 6000 spores per ml if deposition was in the form of a fine mist. The IF test could be used to detect air-borne spores collected using a spore trap where discrimination between sclerotinia and botrytis/other fungi would be critical and background fluorescence would not be an issue.

Spatial distribution of sclerotinia spore inoculum on petals

In order to develop sampling protocols to exploit diagnostic tests, field trials were carried out in 1999 and 2000 to collect information on the spatial distribution of ascospores of sclerotinia on oilseed rape at flowering.

Methods

Two field experiments were carried out in 1999, at Crowland (Lincolnshire) and Much Wenlock (Shropshire), to quantify spatial variation of sclerotinia infection at a range of spatial scales. The sampling plan was based on a 50 m grid to cover the whole of each field, plus additional samples located around baseline points to provide information on spatial variation at a scale smaller than 50 m. Petal samples were collected at early, mid and late flowering for testing. In 2000 two further field trials, at Boxworth and Syerscote, were carried out to develop practical sampling strategies for use on farm using a 100 m grid pattern. Petal samples were collected at early flowering for agar testing.

Results

Levels of infection detected by agar testing were low in 1999, mainly due to the samples having been frozen prior to analysis. No discernible spatial pattern was evident for any sampling occasion for either trial site in 1999. In 2000, where agar testing was carried out immediately after sampling, there were two 'hotspot' areas of infection at the Boxworth site surrounded by lower infection levels and an area in one corner without infection. At Syerscote, a gradient of infection was seen across the site. This distribution was almost certainly due to spread from an adjacent field known to have been affected by sclerotinia in the past. Data clearly emphasise the requirement for sampling across the whole field for reliable determination of disease risk. The 2000 data showed similarity between neighbouring sampling points suggesting that the 100 m strategy was able to detect the spatial variability present within the field. Further work is needed to define minimal sampling requirements statistically and to investigate other spatial distributions.

Evaluation of fungicide programmes for control of sclerotinia stem rot

Timing of the fungicide spray is critical for control of sclerotinia and early to mid-flowering (or early petal fall) is usually the most effective. As yet, petal testing is not used by farmers and many adopt an insurance spray approach, arguing this is justified to prevent a continuing build of the disease. With the changing economics of oilseed rape production, prices of £100-120/tonne and increasing environmental pressure to reduce pesticide use, inputs need to be re-appraised regularly. The use of a diagnostic test should enable the timing of fungicide applications to be improved. These experiments were carried out to provide guidance on thresholds for fungicide treatment and to provide samples as part of the development and validation of a rapid test for sclerotinia on petals.

Methods

Fungicide treatments at a range of timings during flowering were evaluated for control of sclerotinia stem rot in winter oilseed rape cv. Pronto at ADAS Boxworth (Cambridgeshire) and cv. Orkan at Syerscote (Staffordshire), in 2000. At Boxworth, treatments compared a single dose application of Compass (2 l/ha) + Stefes C-Flo 2 (0.5 l/ha) at early flowering (27 April), full flower (9 May) and late flowering (16 May) and a

two spray programme at early and late flowering. At Syerscote, single dose applications only were compared; Compass (2 l/ha) + Bavistin DF (1 kg/ha) at early (10 April), full (28 April) or late (10 May) flowering.

Results

The standard early flowering spray of Compass + MBC used at Boxworth gave excellent control of sclerotinia (97%) which affected 3.4% of untreated plants. Full flowering sprays gave moderate control (72%), whilst the late flowering timing was ineffective (14% control). The two-spray programme gave almost complete control (99%). At Syerscote, where stem rot levels were at 4% plants affected in untreated plots, all sprays gave good control (80- 98%). In contrast to Boxworth, later timings were more effective than the early flowering spray. Data from both sites indicate that the standard optimum timing for sprays to control sclerotina was between late April and early May. These experiments also highlight the importance of the weather for infection and in determining the optimum timing for treatment within the flowering period. There was no significant effect of treatment on yield at either site, though benefits are anticipated in the longer term from control of sclerotinia inoculum.

Development of a risk assessment scheme

Experimental work detailed above has shown that use of farm history alone does not give a reliable indication of sclerotinia risk. A range of risk factors for sclerotinia stem rot have been investigated and together with data gathered during the project, have been incorporated into a decision guide for determination of sclerotinia disease risk.

Previous disease

Severe attacks of sclerotinia (>20% plants affected) are uncommon in the UK and recent studies highlight the importance of previous history of the disease on the same farm in assessing risk. History of sclerotinia problems has therefore been selected as one of the most important and useful factors for prediction of disease risk. Entry of a value for previous sclerotinia history is mandatory in the decision guide. Where this is not known, a default value indicating that there have not been severe problems on the farm, may be used.

The availability of inoculum would also be indicated by the presence of any apothecia within the oilseed rape crop or nearby. In the UK, apothecia are usually produced over a long period from late March onwards and numbers are low during the critical stages of flowering.

Weather

Previous disease history is an indicator of presence of inoculum in the form of sclerotia, but weather and crop factors must be suitable for ascospore release and infection to occur.

A sequence of events must be completed for stem rot to occur: (1) Sclerotia near the soil surface germinate to produce ascospores. (2) Ascospores are released, dispersed and reach the petals; (2) Petals fall and stick to leaves or stems; (3) The fungus spreads from petal to leaf/stem and causes stem lesions.

The precise weather factors which are conducive to disease development have not been well defined. In general, high temperatures and unsettled weather with light rain (alternating wet and dry days) are likely to be most conducive to sclerotinia development.

However, qualitatively, rainfall and temperature factors contribute to disease development as follows:

1. Rainfall or dew which leads to petal sticking to petioles and stems
2. Heavy rain
   - floods apothecia (and hence reduces spore release)
   - washes petals off the foliage and stems and hence reduces risk of disease
3. Sclerotinia has a high temperature optimum and growth is slow at low temperatures (<10°C), conditions which may prevail during flowering in the UK.
4. Conversely, high temperatures (>15°C) during flowering will favour rapid disease development and enable infection to take place in shorter periods of leaf wetness than at low temperatures.

Temperature observations are thought to be important at the time of risk assessment and are categorised in the range >15^{o}C, between 10 and 15^{o}C, or < 10^{o}C. These temperature parameters have been incorporated in the decision guide and increased risk can be expected if temperatures exceed 15°C.

Weather forecasts have been used instead of rain, e.g., low pressure, changeable or high pressure. In the Canadian petal test manual for sclerotinia in oilseed rape, weather data are not required, but, together with petal infection levels, an assessment of weather pattern is asked for, i.e. wetter, drier, or constant.

Current fungicides have very limited curative activity against sclerotinia and therefore decisions must take account of spore inoculum, current weather and forecast weather. Current weather allows judgement to be made about sclerotinia being able to produce its air-borne spores, whilst forecast weather is used to predict whether petals will stick and infection take place. Clearly there are uncertainties about forecast weather, but ideally fungicides should be applied to petals before they fall in order to maximise disease control. Entry of weather details is also mandatory and decisions cannot be made selectively without rainfall and temperature inputs.

Other factors

Most farmers and advisers should be able to make decisions based on history of sclerotinia and weather factors. A range of other factors can influence the disease but these are usually considered of secondary importance to inoculum and weather factors. These include: crop height, crop density, onset of lodging and interactions with other susceptible crops in rotation.

Inoculum levels

The presence of apothecia in the oilseed rape crop itself or in adjacent (e.g. cereals) fields would also constitute an important observation and identify local risk. Whilst previous history is a useful factor in decision making, better decisions can be made if inoculum can be quantified in individual oilseed rape crops. It has been apparent from field observations that severe attacks can occur on farms with no previous problems and severe attacks do not always occur on badly affected farms. The use of agar petal tests has enabled high risk situations to be identified more effectively and could be used to reduce prophylactic spraying of winter oilseed rape by 50% (as reported in Section 2). Agar test thresholds (>50% petals with sclerotinia) have been incorporated into the Decision Guide as an option rather than a mandatory component because few farmers are using agar tests. Agar petal test results are weighted so that decisions can still be made using only previous disease and weather factors. The Decision Guide can be easily updated in the future to use data from the new diagnostic test.

System output

The Decision Guide has been developed as a web-based system which calculates a numeric value for each set of data entered and assigns a value for risk of sclerotinia which is displayed as Low, Medium or High. High risk situations are likely to suffer >10% plant infection and loss of yield so fungicide treatment is advisable. Moderate risk indicates that there will be obvious signs of sclerotinia infection which is likely to give a build up of the disease on the farm but direct yield losses are likely to be less than 5%. The benefits of fungicides are therefore considered to be from longer-term disease management. Low risk crops may well show some plants (<2%) with sclerotinia which can be tolerated. The Decision Guide is available and now needs validation on farms.

Conclusions and implications for growers

Antibodies have been developed which have strong potential for use in a rapid diagnostic test for sclerotinia ascospores in oilseed rape crops. Subject to further development of the assay format, and validation of sampling protocols, the test could be offered as a laboratory-based service and could provide a valuable aid to growers. The development of an in-field kit such as a lateral flow device using these reagents may still be pursued as the antibodies developed during the project are extremely specific to sclerotinia. The potential value of such a test has been clearly demonstrated by field experiments showing that the optimum timing for

spray application is very much dependent on the timing of the infection event. These field trials have also demonstrated that fungicides can be extremely effective in controlling sclerotinia when timed appropriately.

Use of a diagnostic test would enable spray decisions to be made in time for optimal spray timings to be achieved. Also the use of spray thresholds to interpret results from the agar petal test have the potential to save up to 50% of sprays in an average year. Using data generated by the project, a web-based Decision Guide has been developed which, following validation on farms, would assist with identification of sclerotinia risk on individual farms. This is considered to be an effective and efficient method of disseminating information to growers. With further development the Decision Guide could use data from diagnostic tests to substantially improve decision making for control of sclerotinia.

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Published papers and articles arising from the project

Anon. 1999. Diagnosis and Control of Sclerotinia Stem Rot in Oilseed Rape. Agriculturelink newsletter – October 1999.

Anon. 2000. Diagnosis and Control of Sclerotinia Stem Rot in Oilseed Rape. Agriculturelink newsletter - October 2000.

Davies JMLl, Gladders P, Young C, Dyer C, Hiron L, Locke T, Lockley D, Ottway C, Smith J, Thorpe G, Watling M, 1999. Petal culturing to forecast sclerotinia stem rot in winter oilseed rape: 1993-1998. *Aspects of Applied Biology* **56**,129-134.

Section 1: Development of a monoclonal antibody-based diagnostic assay for detection of *Sclerotinia sclerotiorum* on oilseed rape petals

SUMMARY

Monoclonal antibodies with specificity to either ascospores, mycelium or germlings of *Sclerotinia sclerotiorum* were produced with the aim of developing a rapid immunodiagnostic for the detection of *S. sclerotiorum* on oilseed rape petals. Antibodies targeted at ascospores proved to be the most specific and those with high specificity for sclerotinia and showing no detection of other fungal species were selected for further development. Three monoclonal antibodies (74/2.E10.D9.D10, 74/4.B4.F3.A12 and 44/2.H8.G10.A10) were tested in three assay formats: a plate trapped antigen (PTA) ELISA, double antibody sandwich (DAS) ELISA and an immunofluorescence (IF) assay. The antibodies were all capable of the specific detection of *S. sclerotiorum* ascospores in solution in all three formats. However, the addition of oilseed rape petal extract to ascospore suspensions resulted in the total inhibition of spore detection by the plate trapped antigen format. The problem of inhibition by petal extract was less significant in the double antibody sandwich ELISA and immunofluorescence assay. Of these two assay formats immunofluorecence was the most sensitive, capable of detecting 4 ascospores on a single petal. However, high background fluorescence from the petal material hindered visual detection of spores (particularly when using frozen petal material) and the test proved time-consuming. Further validation and testing of the IF assay is described in the next section of this report. The DAS format would be the most suitable format for high throughput testing of samples and for further development into an in-field lateral flow test. Unfortunately, time within the project did not allow investigation into additional adjustments to the DAS protocols which may have improved sensitivity and reduced inhibition by petal material. An additional solution, which could lead to the production of a successful DAS format and in-field test in the future, would be incorporation of an antibody from another source as a partner to the highly specific antibodies developed in this project.

INTRODUCTION

Since the first report of plant virus detection by enzyme linked immunosorbant assay (ELISA) (Clark and Adams, 1977), the incorporation of serological methods into routine diagnosis for plant pathogens has improved the sensitivity and reliability of disease diagnosis. ELISA has generally superseded most other immunological approaches such as gel diffusion and agglutination assays to become the standard laboratory method. ELISA is particularly suited to large scale testing of field samples and antibodies have been developed for many major plant pathogens.

There is a range of formats available for rapid immunodiagnostic testing in the laboratory. Plate trapped antigen (PTA) or indirect ELISA is a simple and convenient method where antigen is physically absorbed on to the surface of the well and generally works most efficiently where a soluble antigen is being detected. The double antibody sandwich (DAS) ELISA format is a common approach, often used to reduce matrix effects. It relies on a coating antibody absorbed to the plate surface, which is capable of selecting specific target proteins from a complex matrix. The antibody used to coat the plate can be the same or a different antibody, to the one that is used to detect and measure the antigen. An alternative approach is the immunofluorescence (IF) assay which is commonly used in the detection of bacterial pathogens such as *Ralstonia solanacearum* (brown rot) of potatoes. The specific detection of targets is obtained using specific monoclonal antibodies (Mabs) labelled using an anti-species fluorochrome label (FITC) which can be visualised using a fluorescence microscope.

There is also practical advantage in the detection of pathogens on-site, under field conditions, using a test that can rapidly and reliably confirm the presence or absence of a particular pathogen in asymptomatic tissue. The development of a test for laboratory use is relatively simple, as the end user is likely to have the required facilities and expertise, enabling new reagents and protocols to be easily integrated. The characteristics required for an on-site test are very different. The end user may have no appropriate expertise, may be unfamiliar with the handling of diagnostic equipment and have no facilities or equipment to hand. They will not be prepared to wait a long time for the test result and will require a clear and unambiguous result (Holmes, 1996). The lateral flow device, developed at CSL, fulfils these criteria of speed, accuracy and ease of use and would be a suitable format for in-field testing for sclerotinia ascospores on oilseed rape petals.

There are a number of reports of the development of polyclonal antibody-based immunoassays for detection of sclerotinia on oilseed rape petals (Jamaux and Spire, 1994; Jamaux-Despreaux and Spire, 1999) but the antibodies developed to date have not had the required specificity and sensitivity for further development into successful diagnostic formats.

The aim of this project was to develop a reliable laboratory immunodiagnostic test for the detection of ascospores of *Sclerotinia sclerotiorum*, based on the use of monoclonal antibodies. The suitability of the antibodies produced would then be assessed for their use in a lateral flow device for use in the field.

MATERIALS AND METHODS

Antigen preparation

Three antigen targets were identified for antibody development (spores, germlings and mycelium) covering three stages in the process of infection of the oilseed rape petal by *S. sclerotiorum*. In order to carry out effective cross-reactivity screening, antigen preparations of infected and uninfected petals, and a range of fungi likely to be present on the petal, were also established.

Production of ascospores and germling antigens

Thirty-seven isolates of *S. sclerotiorum* were collected from sites across England over a period of two years. These were maintained as sclerotia until required for use in production of ascospores and germlings for monoclonal antibody screening. The method of ascospore production was based on that of Sansford and Coley-Smith (1992).

Sclerotia of each *S. sclerotiorum* isolate were produced by growing mycelium in 250 ml conical flasks containing 12.5 g wheat grain, 25 g coarse grade Perlite and 30 ml distilled water. The wheat-Perlite medium was autoclaved at 120°C for 30 minutes, cooled and inoculated with mycelial disks taken from 7 day old cultures of *S. sclerotiorum*. Flasks were incubated in the dark for 3 weeks at 20°C with agitation of the medium carried out every two to three days during incubation to prevent clumping. Sclerotia were subsequently subjected to a cold treatment at 4°C for four weeks before being removed from the medium.

Carpogenic germination of sclerotia was induced by placing cold treated sclerotia into clear plastic sandwich boxes containing sterile moistened coarse grade Perlite and covering with a 2 cm layer of the Perlite. Lids were placed on the boxes and incubated in the dark at 10°C for 7-8 weeks until stipes were produced. The surface of the perlite was periodically misted with sterile distilled water to prevent drying out of the sclerotia. Once germinated the sclerotia were placed under near UV light at 20°C until mature apothecia were produced, normally 7-8 days later.

Ascospores were released from the apothecia by removing the mature caps and agitating them in sterile distilled water. The ascospore suspension was filtered through a double layer of sterile lens tissue, to remove

apothecial debris, and frozen at –30°C until required. Germination of ascospores to produce germlings occurred between 3-12 hours after thawing of the spore suspension.

Production of mycelial antigen

Mycelial antigen was prepared in shake culture. Spore suspensions were seeded in to a Czapeck Dox (with 10 % sucrose) medium and incubated on a rotary shaker in the dark for 7 days at 25°C. The resulting fungal pellets were collected, washed in SDW and phosphate buffered saline (PBS), freeze dried, snap frozen in liquid nitrogen and ground to a fine powder. The powdered mycelium was added to PBS (200 mg ground mycelium in 1.5 ml PBS) and centrifuged. The pellet was separated from the supernatant and saved. Total protein estimations were performed using the Bradford assay (Bradford, 1976).

Other fungal antigens

A culture collection was established of fungal species including isolates of *Monilinia*, *Botrytis*, *Cladosporium*, *Penicillium*, *Fusarium*, *Microdochium*, *Alternaria*, *Phoma*, *Pyrenopeziza*, *Epicoccum*, *Rhizopus* and *Aspergillus* species which represented a range of typical oilseed rape petal microflora (Table 1). These were used in cross-reactivity studies to determine the specificity of the antibodies. Antigens were prepared as described above. All fungi used for cross-reactivity studies were freeze dried in phials or on slopes and added to the CSL culture collection.

Petal extracts

Oilseed rape plants were cultivated under glass to prevent natural infection by *S. sclerotiorum*. At flowering, petals were harvested and placed in 96 well microplates (one petal per well). These plates were either frozen immediately at –30°C (uninfected) or sprayed with a fine mist of a spore suspension containing 10^6 ascospores ml^{-1} before freezing (infected). Extracts were prepared by grinding the thawed petals until fine in distilled water at a ratio of one petal to 200 µl water.

Table 1. Fungal species used in cross reactivity testing

	Antigen tested		
Fungal Isolate	Mycelia	Conidia	Ascospores
Alternaria species	*	*	
Aspergillius flavus	*	*	
Aspergillius multicolor	*	*	
Botrytis cinerea	*	*	
Cladosporium species	*	*	
Colletotrichum acutatum	*	*	
Epicoccum nigram	*	*	
Eurotium amstelodami	*	*	
Fusarium avenaceum	*	*	
Fusarium culmorum	*	*	
Fusarium equiseti	*	*	
Fusarium poae	*	*	
Leptosphaeria maculuns (Phoma lingam)			*
Microdochium nivale	*	*	*
Monilinia fructicola	*	*	
Monilinia fructigena	*	*	
Monilinia laxa	*	*	
Mucor species	*	*	
Mycosphaerella brassicicola	*	*	
Mycosphaerella capsellae	*	*	
Mycosphaerella graminicola		*	
Penicillium aurangtiogrisieum	*	*	
Penicillium thornii	*	*	
Penicillium verrucosum	*	*	
Phytophthora infestans	*	*	
Puccinia recondita		*	
Puccinia striiformis		*	
Pyrenopeziza brassicae	*	*	
Rhizoctonia cerealis	*		
Stagonospora nodorum	*	*	

Antibody Development

Immunisation

Mycelial preparations were adjusted to 1 mg ml^{-1} and spore and germling suspensions to 10^6 ml^{-1}. Six week-old balb/c mice were injected intraperitoneally with a 0.1 ml emulsion of antigen mixed with an equal volume of adjuvant. A total of five injections were given at two-week intervals. The adjuvant used for the first injection was Freund's complete adjuvant; Freund's incomplete adjuvant was used for the 2^{nd}, 3^{rd} and 4^{th} injections and PBS for the final injection.

Blood samples were collected 7 days after the 4^{th} injection to monitor the immune response of each mouse.

Fusion

At least 4 days after the last injection the mice selected for monoclonal antibody (MAb) production were sacrificed under anesthesia and their spleens removed for fusion. Splenocytes from the immunised mice were fused with the myeloma cell line P3-NS/1-Ag4 at a ratio of $1x10^8$: $5x10^7$. Fusion was carried out by the gentle addition of 2 ml of 30 % (w/v) polyethylene glycol (PEG, MW 1500) over 60 seconds. Then, 10 ml of warm serum-free RPMI 1640 medium (Gibco) was added over the next 60 seconds with gentle stirring. A further 20 ml of warm RPMI was added before centrifugation at 400 g for 3 min at room temperature. The resulting pellet of cells was re-suspended in 50 ml of growth medium [RPMI 1640 with 20% (v/v) Myoclone foetal calf serum (FCS) (Gibco)] and distributed into five 96-well microplates at 100 µl per well. After 24 hours, 100 µl of hypoxanthine/aminopterin/thymidine (HAT) medium (50x strength, Gibco), diluted 1:50 in growth medium was added to each well of the fusion plates. All wells were subsequently fed with growth medium plus HAT on day 2, 4, 7 and 10 by removing 100 µl and replacing with a fresh equivalent volume. Screening of supernatants by indirect ELISA was carried out after 10 days to identify cell lines producing antibody to the homologous antigen. These cell lines were expanded into growth medium containing HT supplement (x50, Gibco) diluted 1:50 and spleen lymphokine preparation diluted 1:20. Healthy growing hybrids were then cloned twice by limiting dilution in non-selective medium and preserved by freezing slowly in 7.5 % (v/v) dimethyl sulphoxide (DMSO) in FCS using a Kryo 10 (Planer Biomed), and stored in liquid nitrogen. Cultures of each clone were then allowed to overgrow and the supernatant collected for antibodies.

Coating of ELISA plates with antigen

ELISA plates were coated with antigen using two methods:

(i) Gluteraldehyde trapped antigen (used for spore and germling preparations)
Ascospore and germling antigen preparations were adjusted to 10^6 ml^{-1} and 10^7 ml^{-1}. ELISA plates were coated with 50 µl per well of 0.005 % poly-L-lysine (PLL) and incubated for 45 min at 25°C. Plates were washed with phosphate buffered saline + Tween 20 (PBST), blot dried and 50 µl of 2% gluteraldehyde added per well. After 15 min incubation at 25°C plates were washed in PBST, blot dried and loaded with 50 µl of adjusted antigen added. Plates were incubated overnight at 25°C, washed with PBST and blocked with 200 µl of a 3 % solution of bovine serum albumin per well.

(ii) Plate trapped antigen (used for all antigen preparations)
Mycelial antigen preparations were adjusted to 2 µg ml^{-1}. Plates were loaded with 50 µl of antigen preparation per well. Plates were incubated open overnight at 4°C, washed with PBST and blocked with 200 µl of a 3 % solution of bovine serum albumin per well.

Antibody screening

A plate trapped antigen ELISA was used to identify positive hybridomas and to screen antibodies for cross-reactivity. An antibody previously developed by Rhone Poulenc which showed high specificity for germinating spores of *S. sclerotiorum* was used as a positive control in all screening tests (antibody B2).

Tissue culture supernatant or anti-sera (50 µl) was added to plates previously coated with mycelia, spore or germling preparations and incubated for 1 hr at 25°C. Plates were washed 3 times with PBST, blot dried and alkaline phosphatase labeled anti-mouse conjugate (100 µl) added to each well and incubated for 1 hr at 25°C. Following a final washing step 200 µl of p-nitrophenol phosphate in substrate buffer (1 mg ml^{-1} of substrate buffer) was added to the wells and the plates incubated in the dark at 25°C for 45 min. The reaction was stopped by adding 50 µl of a 3 M NaOH solution to the plate and the absorbance read at 405 nm against air.

Development of assay formats

Three assay formats were investigated for suitability for use with antibodies demonstrated to have specificity for sclerotinia. These were a plate trapped antigen (PTA) or indirect ELISA, a double antibody sandwich (DAS) or direct ELISA and an immunofluorescence (IF) assay.

Plate trapped antigen ELISA

These assays were carried out as described above.

Double antibody sandwich ELISA

(i) Enzyme conjugation

Antibody was purified by passing it through a protein G Hi Trap affinity column and the concentration altered to 1 mg ml^{-1}. 1 ml of the purified antibody was into added to 100 µl of alkaline phosphatase (EIA grade, Roche) and 11 µl of 5% gluteraldehyde (Sigma), and mixed at room temperature for 4 h. The antibody/conjugate solution was then dialysed against 1 litre of PBS overnight. The buffer solution was replaced and the exchange continued for a further 2 h. Finally the antibody/conjugate solution was dialysed against PBS + 50 mM Glycine dialysis buffer for 2 h. The alkaline phosphatase conjugated antibody was then preserved with 10 µl of a 1% sodium azide solution and 5 mg of bovine serum albumin powder (Sigma).

(ii) DAS ELISA

ELISA plates were coated by adding 100 µl of purified sclerotinia antibody (diluted 1:1000 in coating buffer) to each well of an ELISA plate and incubating overnight at 4°C. Plates were washed three times in PBST and non-specific sites blocked by adding 250 µl of 5 % skimmed milk to each well and incubating for 1 h at 33°C. 50 µl of a sclerotinia ascospore suspension containing 10^4 spores ml^{-1} was added to each well and incubated at 33°C for 1 h. Plates were washed three times in PBST and 100 µl of sclerotinia conjugated antibody (diluted 1:1000 in PBST) was added to each well and incubated at room temperature for 1 h. Plates were washed three times in PBST and 100 µl of p-nitrophenol phosphate in substrate buffer (1 mg ml^{-1} of substrate buffer) added to each well and incubated at room temperature for 1 h. The absorbance of the plate was read at 405 nm.

Immunofluorescence assay (IF)

Antigen sub-samples (25 µl) were added to each well on a teflon coated microscope slide and air dried over night. The slide was washed twice with PBST, soaked in PBST for 30 seconds and air-dried. Antibody (25 µl) was added to each well and the slide incubated at 33°C for 1 hour (wash, soak and dry as before). 25 µl of anti-mouse FITC (diluted 1:250 in water) was applied to each well and the slide incubated at 33°C for 1 hour (wash, soak and air-dry). A few drops of fluorescent mounting fluid were added to each well and a glass cover slip placed across the surface. Slides were observed using a microscope with UV illumination.

Assay format sensitivity

Limits of detection

(i) Plate trapped antigen ELISA

Ascospores of *S. sclerotiorum* were coated on ELISA plates using the plate trapped antigen method at concentrations ranging from 800 to 10^5 spores/ml. The limits of ascospore detection for MAbs 74/2.E10.D9.D10, 74/4.B4.F3.A12 and 44/2.H8.G10.A10 were determined.

(ii) Immunofluorescence assay

Oilseed rape petals (16) were ground until fine in 1 ml distilled water and diluted 1:5 with distilled water to give a working dilution. An equal volume (100 µl) of petal extract and a spore suspension containing spores of *S. sclerotiorum* and *Botrytis cinerea* at either, 10^3,10^4 or 10^5 spores ml^{-1} were mixed well. IF assays were carried out on these antigen samples as described before.

Effect of petal extracts on detection

Extract of oilseed rape petals was obtained by grinding petals in 2 ml of sterile distilled water. Two extracts were produced, one from 10 petals and the other from 20 petals. Both extracts were allowed to settle and the liquid fraction removed and saved. The saved extracts were divided into two equal volumes and to one *S. sclerotiorum* ascospores added to give a final concentration of 10^5 spores ml^{-1}. Two ELISA plates were coated with the spiked and unspiked petal extracts, one by the plate trapping method and the other by the gluteraldehyde method.

RESULTS

Production of monoclonal antibodies

Over 35 different cell lines targeted for either spores, mycelium or germlings of *S. sclerotiorum* were produced during the project. These included cell lines from nine different immunizations and fusions. During all screening procedures, the candidate antibodies were selected for positive detection of *S. sclerotiorum* and negative detection of *B. cinerea*. Following the second cloning of cell lines, they were further tested against 37 isolates of *S. sclerotiorum* and a wide range of other fungal species. Antibodies raised to mycelial or germling antigen showed low specificity for sclerotinia and therefore only antibodies showing specificity to sclerotinia ascospores were selected for further development. Only cell lines producing antibodies that successfully detected all *S. sclerotiorum* isolates and showed no detection of the other fungal species were used in the assay development stage.

Three MAbs (74/4.B4.F3.A12, 44/2.H8.G10.A10 and 74/2.E10.D9.D10) were selected for further assay development based on their specificity and performance in the three assays (Table 2).

Table 2. Summary of specificity of monoclonals in different formats

Number	Cell line	PTA	DAS	IF
1	74/4.B4.F3.A12	++	++++	+
2	26/4.H9.E2.G9	-	nt	+
3	26/4.H8.C5.D7	-	nt	-
4	26/4.H8.C5.E12	-	nt	-
5	26/4.H9.E2.C9	-	nt	-
6	27/1.D7.B9.G3	+	nt	+
7	27/1.D7.A11.C2	+	nt	+
8	44/2.H8.G10.G7	+	nt	+ + + +
9	44/1.H1.F7.A10	+	nt	+
10	44/5.H9.G11.G12	+	nt	+ + +
11	74/2.E10.C10.F3	+++	++++	-
12	44/3.A4.E8.A10	+	nt	-
13	44/5.H9.G11.H12	+	nt	+ + +
14	72/2.H3.G8	-	nt	-
15	44/1.H1.A11.D7	+	nt	-
16	44/2.H8.G10.A10	+	nt	+ + + +
17	44/3.A4.G8.C12	+	nt	-
18	27/1.D7.B9.E3	+	nt	-
19	26/2.C8.G1.H5	-	nt	-
20	26/2.C11.H9.B6	-	nt	-
21	74/2.E10.D9.D10	++++	nt	-

nt = not tested

- = negative result

Development and sensitivity of assay formats

(i) Plate trapped antigen ELISA

Using a PTA ELISA method, MAb 74/2.E10.D9.B10 was found to be the most sensitive of the three antibodies selected, detecting less than 800 spores per/ml (Figure 1).

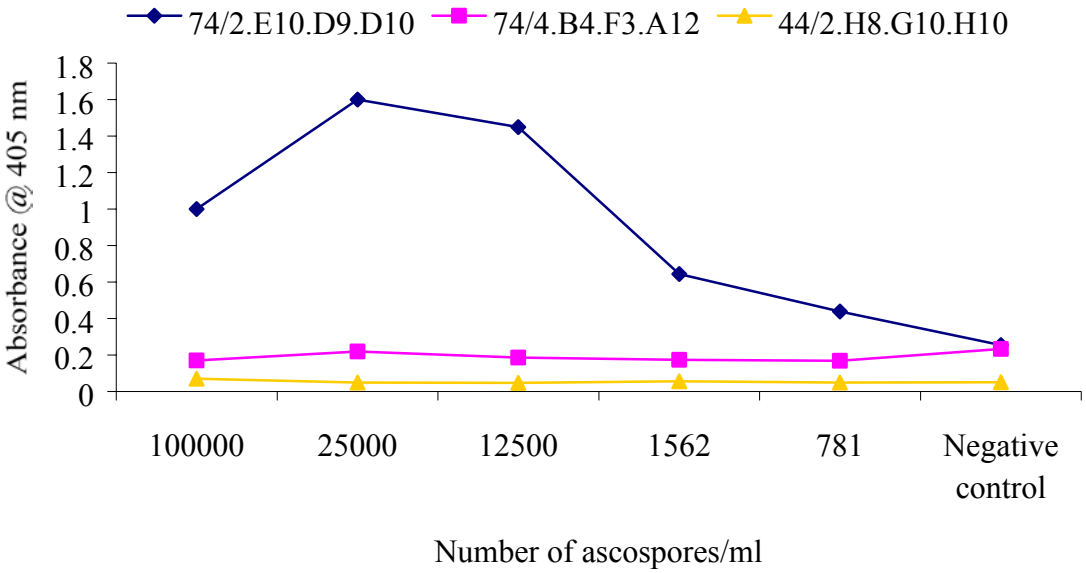


Figure 1. Range of detection of *S. sclerotiorum* ascospores by MAbs 74/2.E10.D9.D10, 74/4.B4.F3.A12 and 44/2.H8.G10.A10

The addition of plant antigens in the form of oilseed rape petal extracts significantly reduced the detection of ascospores by MAb 74/2.E10.D9.D10 and by the positive control MAb (B2) irrespective of the method of spore attachment used (Figures 2 and 3).

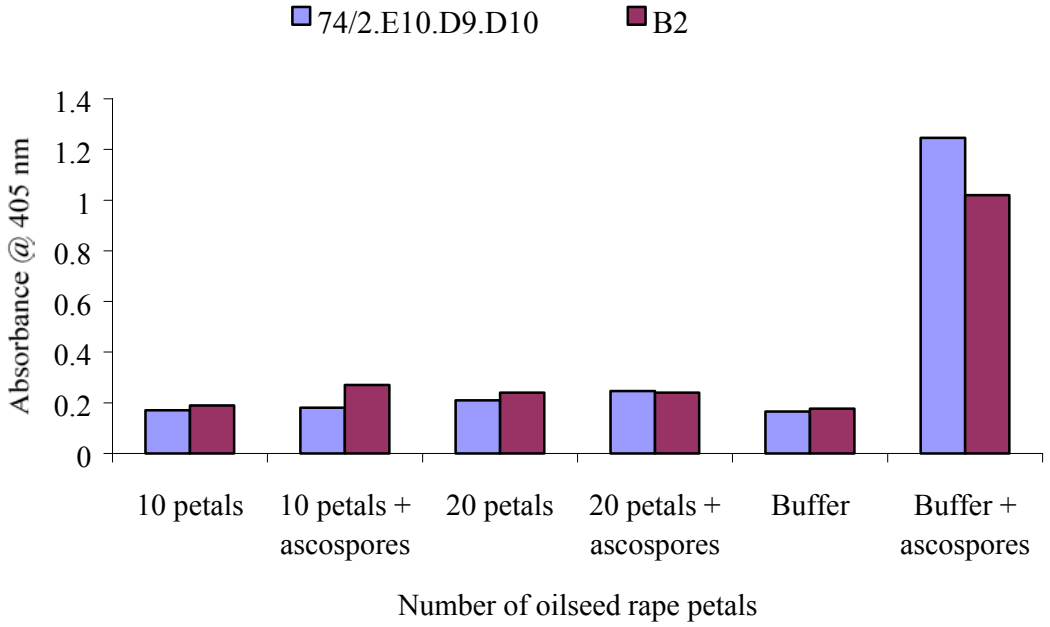


Figure 2. Effect of petal extract on spore detection in a PTA ELISA by MAb 74/2.E10.D9.D10 and B2 - using plate trapped adhesion of spores

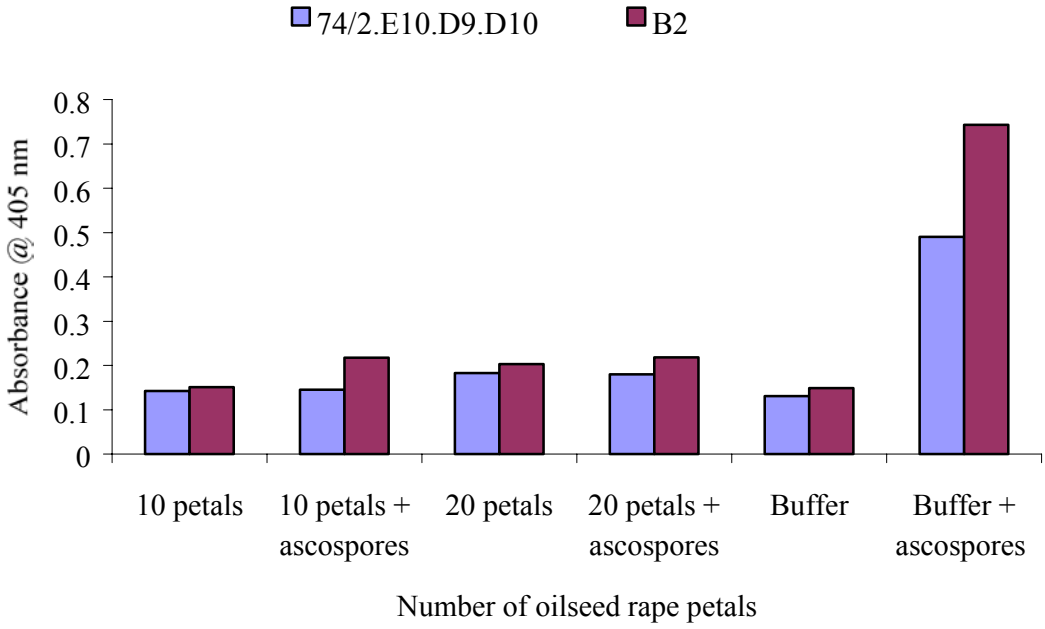


Figure 3. Effect of petal extract on spore detection in a PTA ELISA by MAb 74/2.E10.D9.D10 and B2 - using PLL attachment of spores

Double Antibody Sandwich ELISA (DAS)

Several MAb antibody combinations were evaluated for their effectiveness at detecting *S. sclerotiorum* ascospores in solution using the DAS format (Figure 4). The use of B2 + 74/2.E10 and 74/4.B4.F3.A12 + 74/4.B4.F3.A12 (where the first antibody is the coating antibody and the second is the capture antibody) gave the best detection of *S. sclerotiorum* ascospores with the least background detection.

The use of DAS ELISA appeared to remove the inhibition problems encountered through the addition of petal extract in the plate trapped antigen ELISA format (Figure 5). However, significant levels of detection were achieved only at high spore and antibody concentrations. Unfortunately, use of high antibody concentrations gave rise to high background signals. Further adjustments to the DAS protocols to resolve these problems were not possible within the time-scale of this project.

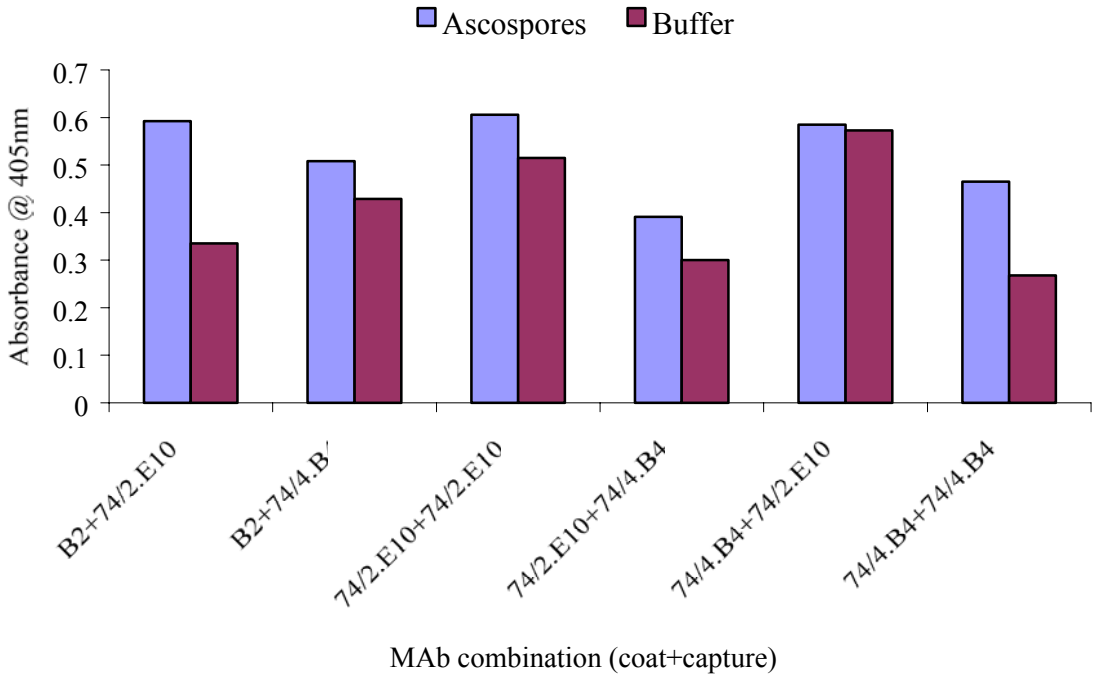


Figure 4. Specificity of MAb combinations in the DAS ELISA format for the detection of *S. sclerotiorum* ascospores

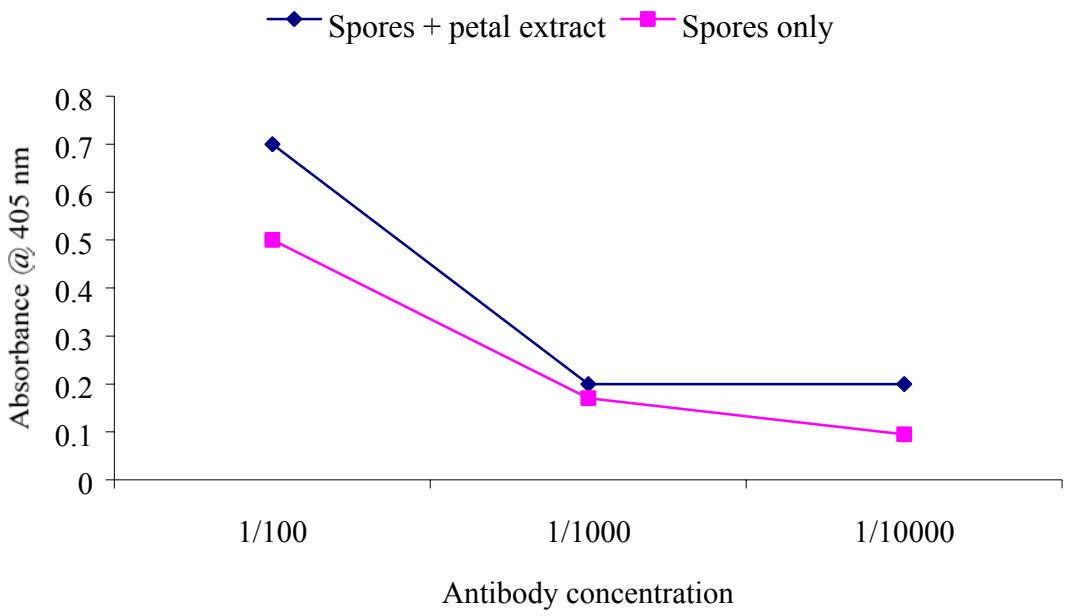


Figure 5. Detection of *Sclerotinia* ascospores (10^4 ml^{-1}), both in the presence and absence of petal extract, using MAb 74/4.B4.F3.A12 as the coating and capture antibody

Immunofluorescence Assay (IF)

The antibody characteristics required for this format are different to those for the other methods and therefore antibodies not performing well in ELISA may show more specificity with IF. The optimum fluorescence was obtained with MAb 44/2.H8.G10.A10, which gave a very intense fluorescence all over the spore coat (Figure 6). Use of MAb cell line 74/4.B4.F3.A12 produced intermittent spotting of the spore coat (Figure 7) whilst no fluorescence was obtained with MAb cell lines such as 74/2.E10.D9.D10.

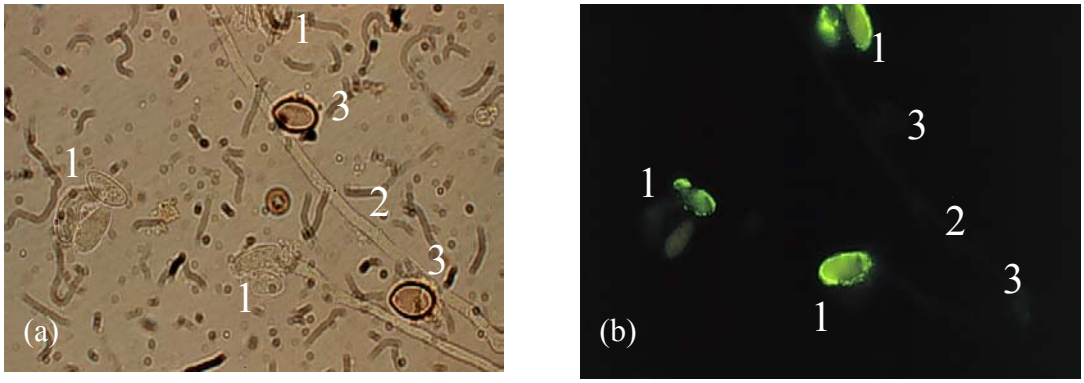


Figure 6. Detection of (1) *S. sclerotiorum* ascospores, (2) *S. sclerotiorum* mycelium and (3) *B. cinerea* spores by a) light microscopy and b) immunofluorescence

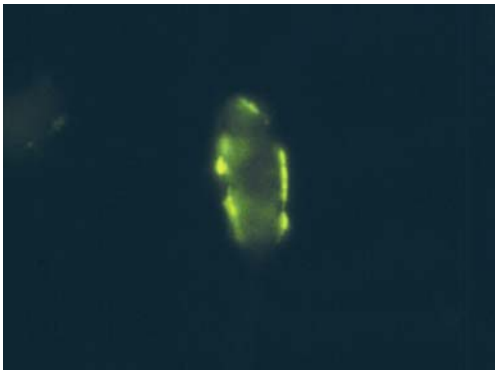


Figure 7. Fluorescent spotting associated with coating ascospores of *S. sclerotiorum* with MAb from cell line 74/4.B4.F3.A12

Spores from a number of other field fungi used in earlier cross-reactivity studies (Table 1) were also tested using the IF assay and no cross reactivity was obtained.

The IF assay successfully detected ascospores in the presence of petal extracts when spores were artificially inoculated onto fresh petals. The visualization of spores was hindered by slight background fluorescence resulting from concentration of the petal matrix. Some parts of the matrix also showed a degree of fluorescence, however shape and size could be used to confirm spores but this was time-consuming. The assay was less successful at detecting ascospores from previously frozen petal material obtained from the field due to high background fluorescence and crumpling of petals

The overall performance of the antibodies in the different formats is shown in Table 3. The IF assay was clearly the most effective method for detecting low numbers of spores on oilseed rape petals.

Table 3. Summary of performance of sclerotinia-specific antibodies in different assay formats

	Assay format					
	PTA		DAS		IF	
Number of spores	With Petals	Without Petals	With Petals	Without Petals	With Petals	Without Petals
36,000	X	✓	✓	✓	✓	✓
18,000	X	✓	✓	✓	✓	✓
9,000	X	✓	✓	✓	✓	✓
4,500	X	✓	X	X	✓	✓
2,250	X	✓	X	X	✓	✓
1,125	X	✓	X	X	✓	✓
563	X	✓	X	X	✓	✓
281	X	X	X	X	✓	✓
141	X	X	X	X	✓	✓
70	X	X	X	X	✓	✓
35	X	X	X	X	✓	✓
17	X	X	X	X	✓	✓
8	X	X	X	X	✓	✓
4	X	X	X	X	X	✓
2	X	X	X	X	X	X
1	X	X	X	X	X	X

DISCUSSION

Highly specific monoclonal antibodies have been developed for the detection of *S. sclerotiorum*. These were tested in three assay formats, plate trapped antigen (PTA) ELISA, double antibody sandwich (DAS) ELISA and immunofluorescence (IF). The detection limits and suitability of use with petal extracts varied between the assays and the different MAbs tested and this gave an insight into the nature of the epitope for each of the monoclonals. The nature of the epitope for each of the MAbs developed is indicated by the performance of the antibody in different formats and gives indications as to why certain antibodies performed poorly in some formats.

74/2.E10.D9.D10 appears to be specific for a soluble protein associated with the spore, hence was very effective in PTA ELISA, as soluble proteins bind very efficiently to plate surfaces. No fluorescence was obtained using 74/2.E10.D9.D10 probably as a result of the washing steps used in the preparation of the IF slide, removing the soluble antigen.

74/4.B4.F3.A12 appears to be specific for an insoluble protein, or glycoprotein, found within the spore coat, however the epitope is only repeated occasionally, as indicated by the spot fluorescence obtained using IF. The infrequent occurrence of the epitope on the spore surface would lead to inefficient or loose immunocapture of the spore on the plate surface. The inefficient spore capture may help to explain the limited sensitivity of the DAS ELISA.

44/2.H8.G10.A10 appears to be specific for a very common insoluble protein, or glycoprotein, associated with the spore coat. Hence the fluorescence obtained is found all over the spore surface. This antibody would have been expected to work well in the DAS ELISA, but did not. This was probably due to it being an IgM antibody as opposed to an IgG. IgMs are much larger immunglobulins and are difficult to purify and conjugate effectively to enzymes.

PTA assays carried out using MAb obtained from cell line 74/2.E10.D9.D10 were able to detect ascospores in suspensions containing less than 800 spores per ml, however the addition of petal extract to the spore suspension reduced detection dramatically. Solutions of petal extract contain large amounts of soluble protein which compete with the spores for binding sites on the well surface thus inhibiting spore detection. This is likely to be the reason for the reduction in spore detection observed. Use of dilution series showed that the inhibitory effect of petal extract on spore detection was not removed until a single petal was diluted in 32 ml of extraction buffer (results not shown). Using such high dilution rates makes the detection of spores from petals by PTA ELISA unrealistic, as spore concentrations of 25,600 spores per petal would be required to achieve even the lowest level of spore detection (800 spores/ml). Such spore loads are unlikely to be seen under natural inoculum conditions. An alternative approach to improve the format of the PTA assay involving use of poly-L-lysine to coat the plate and gluteraldehyde to chemically bind the spores to the plate surface, did not improve levels of detection. The PTA ELISA was therefore identified as limited to use with spore preparations in the absence of petal extract.

Using the DAS approach eliminated the inhibition of spore detection associated with the inclusion of petal extract in the PTA ELISA. However, significant levels of detection were only achieved at high spore and antibody concentrations. Further work on development of the DAS protocol for use with these antibodies is required but was not possible within the time-scale of the project.

The IF assay was the only format able to detect low numbers of spores in the presence of petal extract. In experiments using artificial inoculation of petals spore detection as low as 4 spores/petal was achieved. The effective detection of 4 spores on single petals was only achieved on fresh petal material. Where pre-frozen samples were analysed by IF spore detection was variable (see Section 2).

Previous work by other research groups on development of antibodies for detection of sclerotinia has focused on use of polyclonal antisera (Jamaux and Spire, 1994; Jamaux-Despreaux and Spire, 1999) but these antisera have shown significant cross-reactivity with *Botrytis cinerea* and other fungal species. Considerable improvements in specific detection of *Sclerotina sclerotiorum* have been achieved in this project through the use of monoclonal antibodies. These antibodies have not only proved highly specific but also have the intrinsic advantage of infinite existence, unlimited volume availability and consistent performance. The antibodies have a detection limit of between 4 spores per ml (IF) and 800 spores per ml (PTA) which is significantly better than the 1000 spores per ml limit for the polyclonal antibodies developed by the French team. With the exception of the IF assay, the limits of detection of the assays developed in this project were too great to allow the detection of a few spores per petal. These results are in accordance with those of other groups working on immunodetection of fungal pathogens (Bossi and Dewey, 1992).

Due to time limitations this project has focused on the development of the IF assay for detection of sclerotinia ascospores (see next section). However, the double antibody sandwich assay as a format is the most suitable approach for high throughput testing of samples, significantly faster than IF, and also for applications such as lateral flow device development for in-field detection of spores. The current DAS format developed during the project did not have the desired sensitivity for the detection of sclerotinia on oilseed rape petals. Further work on the protocol for the assay may resolve these problems. Also, the methods developed during this project, and the successes obtained, could enable the development of a second generation monoclonal or adoption of an existing monoclonal for incorporation into a DAS ELISA and ultimately a lateral flow device. The project team is still active in trying to locate a suitable antibody developed by other research teams for testing as a partner to the CSL antibodies in the DAS format.

Section 2: Use of agar and rapid diagnostic tests to quantify petal infection

SUMMARY

The newly developed immunofluorescence test was carried out on frozen plant material from a range of sources to determine levels of accuracy and ease of use. Petal testing by agar plating of samples collected from crops in 1996 and 1997 showed considerable variation in petal infection by sclerotinia between sites. Data show that relationships between farm risk, level of petal infection at flowering and severity of stem rot disease were not consistent and illustrate the need for a more accurate test to determine the level of petal infection at flowering. Results from immunodiagnostic tests carried out on previously frozen samples taken from farms showed no agreement with results from agar tests carried out on fresh petals. Levels of background fluorescence from the thawed petal material were high and identifying spores was difficult. Further experiments using material from field trials showed that levels of infection detected by petal culturing or the IF test were considerably reduced if samples had been frozen. Detailed comparison of levels of infection on petals from the same flower using frozen samples showed that there was little or no relationship between petals in level of colonisation by *S. sclerotiorum*. Results indicate that agar tests on fresh material detected presence of sclerotinia as spores, germlings or mycelium whereas after freezing, only spores ungerminated prior to freezing were detected. Data also show that the assumption that petals within the same flower will have an equivalent level of infection present may not be reliable. These factors affected ease of validation of the immunodiagnostic method. The IF test was found not to be suitable for routine screening of large numbers of samples for sclerotinia spores on oilseed rape petals due to background fluorescence levels obscuring the spores. Despite the unsuitability of the IF test for routine detection on oilseed rape petals the test may have uses to detect sclerotinia infection on other crops where the plant material may not produce such significant fluorescence. Data collected from field trials indicate the level of air-borne inoculum needed to produce threshold levels of petal infection (~41%) is between 1000 and 6000 spores per ml. The IF test could be used to detect air-borne spores collected using a spore trap where discrimination between sclerotinia and botrytis/other fungi would be critical and background fluorescence would not be an issue.

INTRODUCTION

At present in the UK, prediction of sclerotinia stem rot on winter oilseed rape and decisions to spray fungicides are based mainly on farm history of stem rot and weather conditions which favour infection. The presence of sclerotinia on petals as determined by agar plate testing is another useful indicator of risk, and has been used successfully in Canada (Morrall and Thomson, 1991; Turkington *et al.*, 1991) with weather factors also taken into account (Turkington and Morrall, 1993). The relationship between petal infection and stem rot in the UK is less clear cut, partly because of contamination of plates with botrytis species, a problem which does not apparently occur in Canada. Davies *et al*. (1999) sampled petals annually from 1993 to 1998 from farms with different histories of stem rot (301 sites in total), and found a poor relationship between petal infection and stem rot (R^2 18%) when data from all sites was analysed. Nevertheless, it did allow more effective targeting of fungicide sprays and had the potential to reduce unnecessary sprays by 50%. Petal testing is a useful technique but the agar method takes 10 days to complete and is therefore potentially unreliable because petal infection in the can change during that period. A rapid test might be even more effective and demonstration that it produced comparable results would speed up its acceptance for field use.

The newly-developed rapid immunodiagnostic test was tested and validated using petal samples from crop monitoring, field trials and plot experiments.

OBJECTIVES

1. To determine the incidence of sclerotinia on petals using agar and immunodiagnostic tests
2. To investigate the accuracy of both the agar and IF tests

METHODS

Disease monitoring on farms

In 1993-1998, 50 crops of winter oilseed rape were chosen annually from farms in England with different histories of stem rot (this work was undertaken as part of a sister project funded by DEFRA). The farm disease risk was assessed as nil (no stem rot seen previously), low (1-9% plants affected), moderate (10-19% plants affected) and high (20% or more plants affected).

Agar plate tests

From 1996 – 1998, from 50 sites per year, four petals from intact flowers were removed from each of ten plants taken at random at early (GS 4.1 - 4.2), mid (GS 4.6 - 5.1) and late flowering (GS 4.7 - 5.9) from an area in each crop 50 m long by one spray boom wide. This area was marked by canes at each end and was not treated with fungicide. Petals were sampled during dry weather and cultured immediately or within 2-3 h. Four petals per plate were cultured onto potato dextrose agar (PDA) plus 50 mg kg^{-1} streptomycin sulphate (Sigma Chemical Co. Ltd., Poole, Dorset, UK). Fungal colonies were identified after 8-10 days incubation. Advice was given on whether to spray the crop during flowering, based on the history of that field or an adjacent field, and in some instances on petal culturing results from early flowering petal tests. Prior to harvest, 100 plants in the monitored area and the adjacent treated area if fungicide was applied, were assessed for stem rot. The results have been reported by Davies *et al*. (1999).

Immunodiagnostic tests

In 1996 and 1997, at each visit to each of the 50 farms, 40 petals per crop were placed in individual wells of a standard ELISA plate to form a parallel sample to that used for agar testing. Plates were sprayed with a mist of water (e.g. Evian Le Brumisateur sprayer), wrapped in cling film and sent by overnight delivery to ADGEN to be deep frozen. In 1996, a random selection of petals representing top, centre and base of flowering racemes was sampled. In 1997, petals were paired from individual flowers (e.g. upper petal to agar, lower petal to an ELISA well), again reflecting the range of flowering on individual racemes. Individual petals were numbered so that direct comparisons at individual flower level could be made. In total, samples from 100 farms at three sampling times (early, mid and late flowering) provided 12,000 petals.

Plates were sent to CSL and ADGEN for freezing prior to testing using the immunodiagnostic assay.

A subset of samples collected from farms in 1996 was selected for experiments to compare the immunodiagnostic test with the petal test method. Thirty samples taken at mid-flowering and representing a wide regional spread with equal proportions of high, medium and low risk farms were thawed and tested using the immunofluorescence method.

Antigen sub-samples (25 µl) were added to each well on a teflon coated microscope slide and air dried over night. The slide was washed twice with PBST, soaked in PBST for 30 seconds and air-dried. Antibody (25 µl) was added to each well and the slide incubated at 33°C for 1 hour (slide washed, soaked and dried as before). 25 µl of anti-mouse FITC (diluted 1:250 in water) was applied to each well and the slide incubated at 33°C for one hour (wash, soak and air-dry). A few drops of fluorescent mounting fluid were added to

each well and a glass cover slip placed across the surface. Slides were observed down a microscope with UV illumination.

Field trials

High levels of sclerotinia on petals were identified from the petal testing undertaken following spatial sampling experiments carried out at Boxworth in 2000 (described in Section 3 of this report). As a result racemes taken from this experiment were used in a comparison study of the petal culturing and IF methods. Samples were also used to investigate the variability of infection on the same flower and the influence of freezing on the level of detection by the agar method.

The spatial sampling at Boxworth involved the collection of 10 flower racemes from 13 points around the field. Samples were sent to CSL where they were frozen at -30˚C until required.

Two flower racemes were chosen at random from each of the sample points and from each raceme four flowers were sampled along the length of the raceme. One petal from each flower was placed into a well, on a 96 well plate. The process was repeated for the remaining 3 petals/flower, making sure that the petals taken from each flower were placed in equivalent wells on a 96-well plate.

The four replicate plates were tested as follows:-

1. Cultured on agar - to compare pre- and post-freezing results
2. Cultured on agar - to indicate similarity of infection by sclerotinia on mirrored petals taken from the same flower
3. Subjected to IF analysis (CSL) - for comparison with petal culturing
4. Subjected to IF analysis (ADGEN) - allowed comparison of methodology between laboratories

Plot trials

Experiments were carried out on plots established at CSL to investigate the relationship between inoculum, petal infection and stem disease.

Plots (12m x 9m) were sown with winter oilseed rape culivar Alpine in autumn 1999. Twenty sub-plots (1m x 1m), representing five treatments with four replicates, were marked out and inoculated at GS 4,5 (4/5/00) with spore treatments as described below:

1. No spores (control)
2. 1 ascospore/ml
3. 100 ascospores/ml
4. 1000 ascospores/ml
5. 6000 ascospores/ml

Ascospore suspension (200 ml) comprising ascospores from four different sclerotinia isolates (Sc4, 5, 7 and 10) was applied to each replicate plot.

Petals were sampled one hour after inoculation. Ten flowers (40 petals) were sampled at random from each replicate plot and plated onto PDA (4 petals /plate). Plates were incubated at room temperature for 10 days. In addition, one main flowering raceme was sampled from each replicate plot and frozen at -30˚C.

A misting system suspended above the crop was switched on two hours after inoculation to raise plot moisture levels using a regime of 30 seconds misting per hour for 7 days, thereby aiding adhesion of petals to stems and leaf axils.

Sclerotinia stem rot assessments were carried out 40 days post-inoculation.

RESULTS

Disease monitoring on farms

Agar tests

There was considerable variation in petal infection by sclerotinia between sites and this provided useful material for use in development and validation of the rapid diagnostic test. Relationships between farm risk, level of petal infection at flowering and severity of stem rot disease were not consistent (Table 1).

From all farms sampled from 1993 to 1998, 18 sites had high stem rot levels ($\geq$20%), and the mean petal infection at each flowering time was determined: early, 47.4%; mid, 36.6% and late 39.3% (Davies *et al.,* 1999). These infection levels were used as thresholds to predict which crops justified spray treatment in 1996 and 1997, i.e. which crops were predicted to have 20% or more stem rot in 1996 and 1997 (Table 2).

Table 1. Incidence of petal infection by sclerotinia at sites monitored by agar plate method in 1996 and 1997, and final stem rot incidence

Year	Farm Risk	Mean petal infection %			Stem rot %
		Early	Mid	Late	
1996	Nil	12.1	8.9	13.2	0
	Low	10.3	7.5	9.5	0.5
	Moderate	13.1	13.5	12.8	0.7
	High	10.6	4.7	16.4	0.6
1997	Nil	3.3	0.8	0.8	0
	Low	2.9	2.2	4.9	1.4
	Moderate	2.0	3.0	3.8	3.9
	High	2.6	5.5	20.8	5.3

Table 2. Crops in 1996 and 1997 which justified spray treatment based on petal test thresholds determined from crops in 1993-1998.

Farm disease risk	No. of crops	No. of high risk crops based on petal thresholds			
		Early flower	Mid flower	Late flower	Total no. of sites at risk
Nil	13	1	1	1	1
Low	33	0	1	1	1
Moderate	33	0	2	1	2
High	21	0	0	3	3
Total	100	1	4	6	7

The thresholds determined from all six years of the study indicated a low number of farms at risk in 1996 and in 1997. In these two years, infection was generally low, but with occasional very high infection levels. Overall, it was possible to obtain a set of petals with a wide range of infection levels for testing with the rapid antibody test.

Immunodiagnostic tests

Results from immunodiagnostic tests carried out on previously frozen samples showed no relationship with results from agar tests carried out on fresh petals (Table 3). Levels of background fluorescence from the thawed petal material were high and identifying spores was difficult.

Table 3. Comparison of agar tests on fresh petals and IF methods on frozen petals for detection of sclerotinia

Site no.	County	Risk based on previous disease	Sample date	% infected - agar	% infected - IF
152	Cambs	Moderate	13/05/96	0	25.0
154	Suffolk	High	15/05/96	0	37.5
156	Essex	Moderate	15/05/96	0	50.0
157	Essex	Low	15/05/96	0	50.0
158	Essex	None	15/05/96	0	0.0
159	Essex	None	15/05/96	0	62.5
160	Cornwall	None	04/06/96	0	37.5
172	Oxon	Low	14/05/96	0	25.0
174	Kent	Low	13/05/96	0	No data
176	Kent	Moderate	13/05/96	0	12.5
185	Kent	Moderate	16/05/96	5.0	62.5
189	Worcs	Low	16/05/96	5.0	0.0
195	Yorks	Moderate	21/05/96	5.0	12.5
201	Lincs	Low	19/05/96	5.0	No data
161	Devon	Low	13/05/96	7.5	75.0
177	Sussex	Low	14/05/96	7.5	25.0
181	Surrey	High	14/05/96	7.5	37.5
184	Gloucs.	Low	13/05/96	7.5	50.0
187	Hereford	None	13/05/96	7.5	12.5
165	Devon	Low	13/05/96	10.0	50.0
183	Gloucs.	Moderate	13/05/96	10.0	50.0
162	Dorset	Moderate	21/05/96	12.5	12.5
167	Staffs.	Moderate	16/05/96	15.0	75.0
168	Staffs.	Moderate	16/05/96	17.5	50.0
171	Hereford	Moderate	15/05/96	17.5	62.5
197	Lincs	High	19/05/96	20.0	37.5
198	Lincs	None	19/05/96	50.0	No data
164	Somerset	Low	13/05/96	67.5	37.5
170	Shrops.	Moderate	14/05/96	70.0	12.5
169	Hereford	Moderate	15/05/96	77.5	62.5

Field trials

Using the agar plate method for detection of *S. sclerotiorum* from fresh petals indicated differences between the sample points within the field trial at Boxworth, with point 12 having the highest level of petal colonisation (Table 4). However, agar plate testing of samples following freezing showed a reduction in the number of petals testing positive for *S. sclerotiorum*, with the highest level at any sample point being 12.5%. No petal infection was detected by either CSL or ADGEN using the IF test (Table 4).

Table 4. Comparison of agar plate method (pre- and post-freezing) and IF analysis for detection of *S. sclerotiorum* presence on oilseed rape petals

	% petal colonisation by *S. sclerotiorum*				
	Agar plate method			IF test	
Spatial sample point	Pre-freezing	Post-freezing Plate 1	Post-freezing Plate 2	CSL Post-Plate 3	ADGEN Freezing Plate 4
1	37.5	0	12.5	0	0
2	37.5	0	0	0	0
3	25	0	0	0	0
4	12.5	0	0	0	0
5	0	12.5	0	0	0
6	0	0	0	0	0
7	0	0	0	0	0
8	25	0	0	0	0
9	25	0	0	0	0
10	25	12.5	0	0	0
11	37.5	0	0	0	0
12	62.5	0	12.5	0	0
13	37.5	0	12.5	0	0

Detailed comparison of levels of infection on petals from the same flower using frozen samples showed that there was little or no relationship between petals in level of colonisation by *S. sclerotiorum* (Tables 5a and b) though incidence of viable sclerotinia spores was very low.

Tables 5a & 5b. **Agar plate comparison for *S. sclerotiorum* detection on mirror petals after freezing.**

5a.

Petal	Spatial sampling point												
	1	2	3	4	5	6	7	8	9	10	11	12	13
A1	X	X	X	X	X	X	X	X	X	X	X	X	X
B1	X	X	X	X	X	X	X	X	X	X	X	X	X
C1	X	X	X	X	X	X	X	X	X	X	X	X	X
D1	X	X	X	X	√	X	X	X	X	√	X	X	X
E1	X	X	X	X	X	X	X	X	X	X	X	X	X
F1	X	X	X	X	X	X	X	X	X	X	X	X	X
G1	X	X	X	X	X	X	X	X	X	X	X	X	X
H1	X	X	X	X	X	X	X	X	X	X	X	X	X

√ indicates the presence of *S. sclerotiorum*

5b.

Petal	Spatial sampling point												
	1	2	3	4	5	6	7	8	9	10	11	12	13
A2	X	X	X	X	X	X	X	X	X	X	X	X	X
B2	X	X	X	X	X	X	X	X	X	X	X	X	X
C2	X	X	X	X	X	X	X	X	X	X	X	X	X
D2	X	X	X	X	X	X	X	X	X	X	X	X	X
E2	X	X	X	X	X	X	X	X	X	X	X	X	X
F2	X	X	X	X	X	X	X	X	X	X	X	X	√
G2	√	X	X	X	X	X	X	X	X	X	X	√	X
H2	X	X	X	X	X	X	X	X	X	X	X	X	X

√ indicates the presence of *S. sclerotiorum*

Plot trials

Petal culturing showed that levels of petal infection/colonisation were closely linked to the amount of inoculum applied with a concentration of one spore per ml resulting in 11% of petals colonised. The mean petal infection threshold determined from on-farm monitoring was 41% petals infected (above). Plot trials indicate that inoculum levels need to be between 1000 and 6000 spores per ml for this to occur (Figure 1).

No stem rot symptoms were recorded in any of the plots. Sparse crop, dry conditions and high winds during petal fall resulted in most petals falling to the ground rather than sticking to the leaf axils.

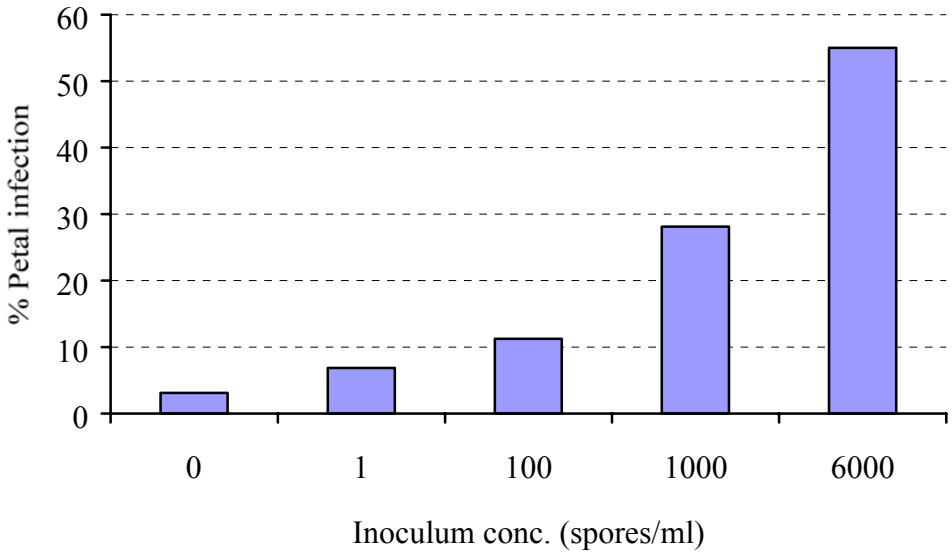


Figure 1. Effect of inoculum concentration on petal infection

DISCUSSION

Results from petal testing on samples collected during 1996 and 1997 highlight the scope for improvement of risk forecasting using a diagnostic test over previous history alone. Using data from 301 farms monitored between 1993 and 1998, it can be estimated that sprays for sclerotinia could be reduced by 50% compared to current practise using a petal test and forecast weather (Davies *et al.,* 1999). However, some farmers would justify sprays to stop a build up of the disease in the rotation and not base a decision merely on the economic losses in any one crop. The role of a rapid test would be to assist in the development of threshold models for prevention of both economic losses and build up of sclerotinia in the rotation.

Despite the improvement in risk analysis through use of the petal test there was still major inconsistency in the relationships between farm risk, level of petal infection at flowering and severity of stem rot disease. This is due to many factors which include accuracy of the petal testing method, the timing of the petal test in relation to timing of petal infection and the influence of the weather on petal stick and the consequent development of stem rot disease. Experiments using the petal test show that a number of factors can affect accuracy, principally storage of samples pre-testing. Storage of petals in humid conditions may encourage spore germination and freezing of samples resulted in loss of viability of germinated spores and mycelium. Detailed comparison of levels of infection on petals from the same flower using frozen samples showed that there was little or no relationship between petals in level of colonisation by *S. sclerotiorum*. This indicated that the assumption that petals within the same flower will have an equivalent level of infection present may not be reliable in all situations. However, sclerotinia incidence was low in these tests and identical results from mirror petals would imply that inoculum was aggregated on individual flowers or plants rather than randomly distributed on petals. The loss of viable sclerotinia after freezing affected ease of validation of the immunodiagnostic test. A test which relies on detection of pathogen DNA might still be able to provide useful data on frozen samples as it would not rely on the integrity of spores or mycelium.

Results from immunodiagnostic tests carried out on previously frozen samples taken from farms showed no agreement with results from agar tests carried out on fresh petals. Additional to the factors described above, the IF test differs from the agar test in highlighting all spores present on the petal whereas the agar test will detect only viable spores. Despite this, there was no trend in the comparison between the two tests probably due to the over-riding influence of high levels of fluorescence from the oilseed rape petal material which obscured detection of the sclerotinia spores. Use of dilution and filtering techniques was unsuccessful in improving visualisation without loss of spore detection.

In conclusion, the IF test was found not to be suitable for routine screening for sclerotinia spores on oilseed rape petals due to background fluorescence levels obscuring the spores. Despite the unsuitability of the IF test for detection on oilseed rape petals the test may have uses to detect sclerotinia infection on other crops where the plant material may not produce such significant fluorescence. Data collected from field trials indicate the level of air-borne inoculum needed to produce threshold levels of petal infection (~41%) is between 1000 and 6000 spores per ml if deposition was in the form of a fine mist. The IF test could be used to detect air-borne spores collected using a spore trap where discrimination between sclerotinia and botrytis/other fungi would be critical and background fluorescence would not be an issue. This would require field calibration, as air-borne inoculum would not necessarily reflect spore deposition at that location.

Section 3: Spatial distribution of sclerotinia spore inoculum on petals

SUMMARY

In order to develop sampling protocols for use of diagnostic tests, field trials were carried out in 1999 and 2000 to collect information on the spatial distribution of ascospores of sclerotinia on oilseed rape at flowering. Two field experiments were carried out in 1999 to quantify spatial variation of sclerotinia infection at a range of spatial scales. Levels of infection detected by agar testing were low, mainly due to the samples having been previously frozen prior to analysis. No discernible spatial pattern was evident for any sampling occasion for either trial site. In 2000 two further field trials, at Boxworth and Syerscote, were carried out to develop practical sampling strategies for use on farm using a 100 m grid pattern. At the Boxworth site, there were two 'hotspot' areas of infection surrounded by lower infection levels and an area in one corner without infection. At Syerscote a gradient of infection was seen across the site. This distribution was almost certainly due to spread from an adjacent field known to have been affected by sclerotinia in the past. Data clearly emphasised the requirement for sampling at several points in each field for reliable determination of disease risk. The 2000 data showed similarity between neighbouring sampling points suggesting that the 100 m strategy was able to detect the spatial variability present within the field. Further work is needed to confirm sampling methods which are practical and statistically reliable. Linking spatial variation inoculum with accurate prediction of disease levels within a field may allow spraying to be targeted only on areas at risk.

INTRODUCTION

In previous DEFRA funded projects the incidence of *Sclerotinia* on petals of winter oilseed rape on different farms has been determined from samples taken from a small area (50 m by one spray width) within each field (Davies *et al.*, 1999). Since the aim was to examine the relationship between petal infection and stem rot within the one area, this sampling approach was justified. However, for predicting the risk of stem rot in a field, spatial variation in petal contamination by sclerotinia needs to be understood and quantified. Field observations suggest that the distribution of spores across many fields is unlikely to be uniform, particularly if there are variations in crop density or obvious sources of infection in nearby fields (Turkington *et al.*, 1988). Information on the spatial distribution of petal infection is important for defining protocols for sampling of petals to determine disease risk. This is particularly important in relation to the development of a rapid antibody test for sclerotinia, where the need for accurate infection assessment has to be balanced against the cost of processing individual samples. The objective of the current study was to characterise the spatial distribution at two sites in each of two years, and to interpret the results in terms of guidelines for sampling of petals for determination of disease risk.

OBJECTIVES

The objectives of the project were:

1. To gain information on the spatial distribution of ascospores of *Sclerotinia sclerotiorum* on petals of winter oilseed rape, at two sites on three sampling occasions during 1999.
2. To conduct spatial analysis to characterise the nature of spatial variation found at each site and sampling time.
3. To interpret the results of the spatial analysis in terms of guidelines for sampling of petals of oilseed rape for determination of disease risk.

METHODS

In 1999 there were two fields sampled: Crowland, Peterborough (23.5 ha) and Much Wenlock, Shropshire (8 ha). In 2000 there were also two fields sampled: Boxworth, Cambridgeshire (10.7 ha), and at Syerscote, near Tamworth, Staffordshire (8.3 ha). The fields were designated as moderate to high risk for sclerotinia infection, based on stem rot levels seen on the farms in previous years.

In 1999, the sampling plan for each field was specifically designed to quantify spatial variation at a range of spatial scales. The sampling plan was based on a 50 m grid to cover the whole of each field, plus additional samples located around baseline points to provide information on spatial variation at a scale smaller than 50 m. Sample points were first marked out with ringots in tramlines according to the appropriate plan, by measuring from the same end of the field each time with a landwheel. Sampling points and field boundaries were georeferenced with a Leica Differential GPS (Global Positioning System).

Petals were sampled at each point, at early, mid and late flowering. At Crowland, petals were sampled at 264 locations, on 13th April, 26 April and 10 May 1999. At Much Wenlock, petals were sampled at 187 locations (except for mid flower, when 101 points were sampled, due to bad weather) on 28 April, 12 May and 20 May 1999. On each occasion, at each sample point, one petal was removed from the main raceme on each of eight randomly selected plants located from an area of 1m radius around the sample point. Each petal was placed in an individual well of a labelled 96-well microtitre plate. Plates were then wrapped in cling film and sent to CSL, York, for testing for presence of sclerotinia, using the antibody test being developed by CSL. The plates were stored in the freezer until required. These petals were plated on agar in 2000 and the plates examined for the presence of sclerotinia colonies.

In 2000, sampling was done on a 100 m grid pattern, at early flower only. The rationale for this sampling strategy was to mimic what may be practical for a field assessment and to quantify the spatial variation across a field to emphasise the need for whole-field sampling. At Boxworth, 13 points were sampled, and at Syerscote, 16 points. From each point, 8 petals were taken from 8 randomly selected racemes within 1 m of the marker cane, and plated on agar. Plates were examined after 6 – 8 days for the presence of sclerotinia. In addition, 10 racemes from within 1 m of the marker cane were sampled, and placed together in a bag and sent the same day to CSL for testing with the rapid antibody test.

RESULTS

1999 data

In 1999, at Much Wenlock and Crowland, petal infection was determined by agar plating of frozen samples that were originally intended for testing by the rapid antibody test. The results from culturing on agar gave generally low infection levels across the two fields, with the highest infection being 25% (Table 1, Figures 1 and 2). The freezing of petals appears to have adversely affected the viability of spores and/or mycelium of sclerotinia on the petals and the results probably reflect the distribution of ungerminated ascospores. There is no reason to believe that this differed from the initial distribution of ascospores.

For both the Crowland and Much Wenlock sites the percentage of petals infected ranged from 0 to 25 % (Table 1). At all sites and sampling occasions, the majority of the sampling points had no infected petals. There was little change in the overall level of infection between sampling occasions for either site. However, no temporal consistency was observed between sampling occasions; the percentage of petals infected at a sampling point did not remain the same between samples.

Table 1. Frequency distribution of data from Crowland and Much Wenlock

Petal infection	Crowland			Much Wenlock	
(% petals infected)	13/4/99	26/4/99	10/5/99	28/4/99	20/5/99
0	95.0%	96.5%	96.5%	82.5%	82.5%
12.5	4.0%	3.0%	3.5%	15.5%	16.0%
25.0	1.0%	0.5%	0.0%	2.0%	1.5%

Data on the number of petals infected at each georeferenced sampling point were visualised using ArcView GIS and are shown in Figures 1 and 2. No discernible spatial pattern was evident for any sampling occasion for either Crowland or Much Wenlock. The locations where infected petals were found were randomly distributed within the fields. Given the random nature of the distributions, no further spatial analysis was carried out which would have allowed more detail characterisation of spatial patterns.

Figure 1 Spatial variation in % petals infected at the Crowland Site on three sampling occasions in 1999

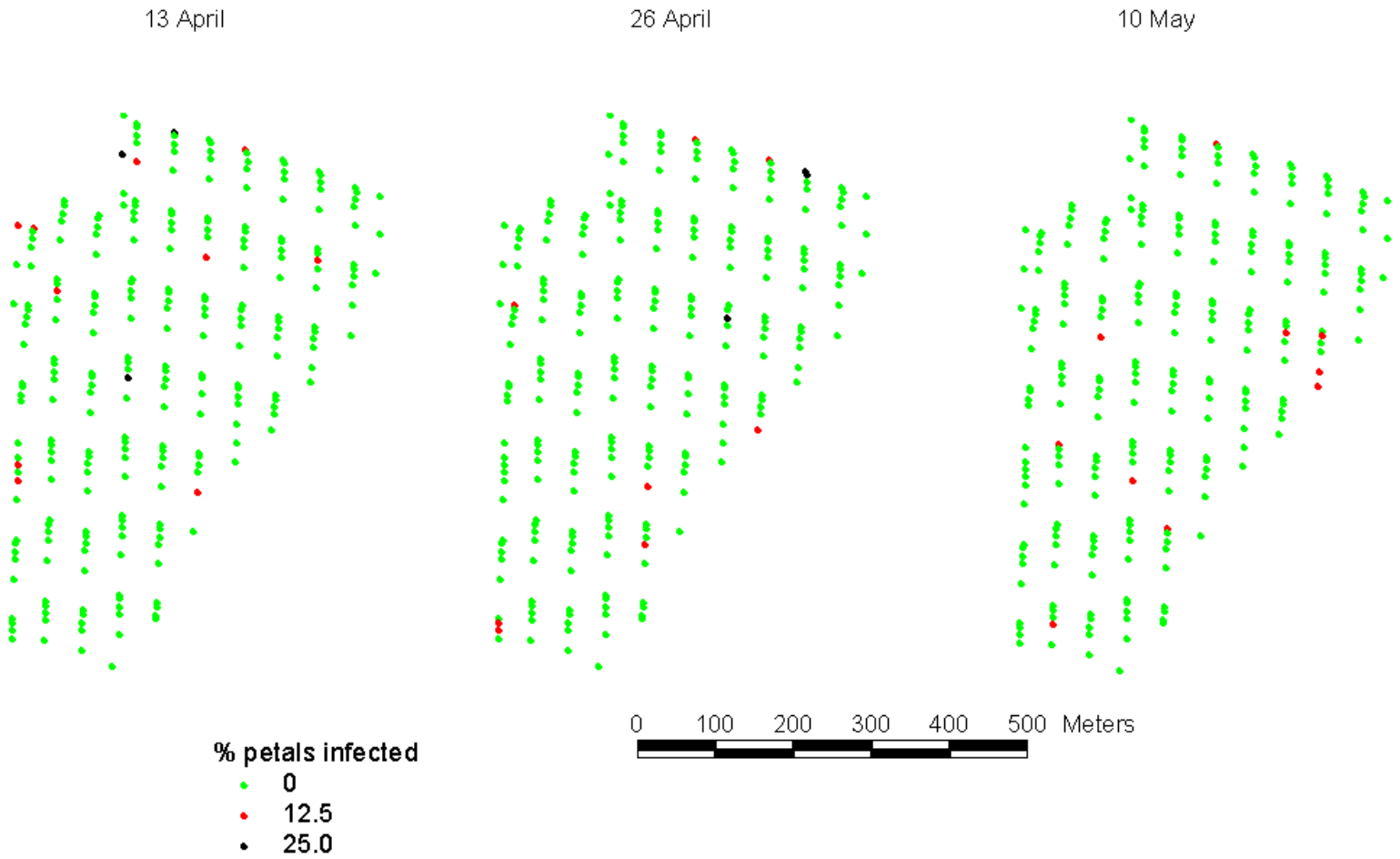
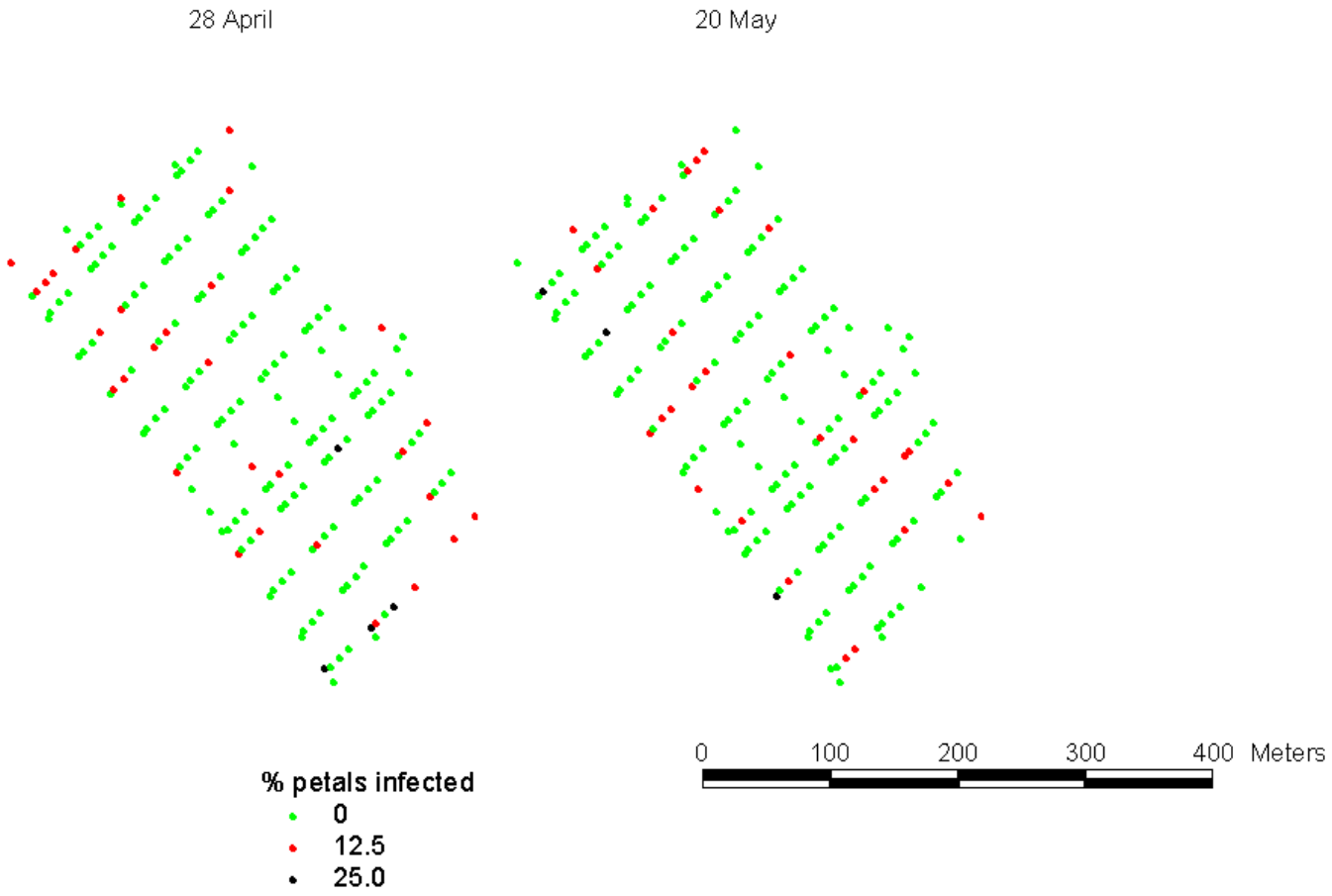


Figure 2 Spatial variation in % petals infected at the Much Wenlock site on three occasions in 1999

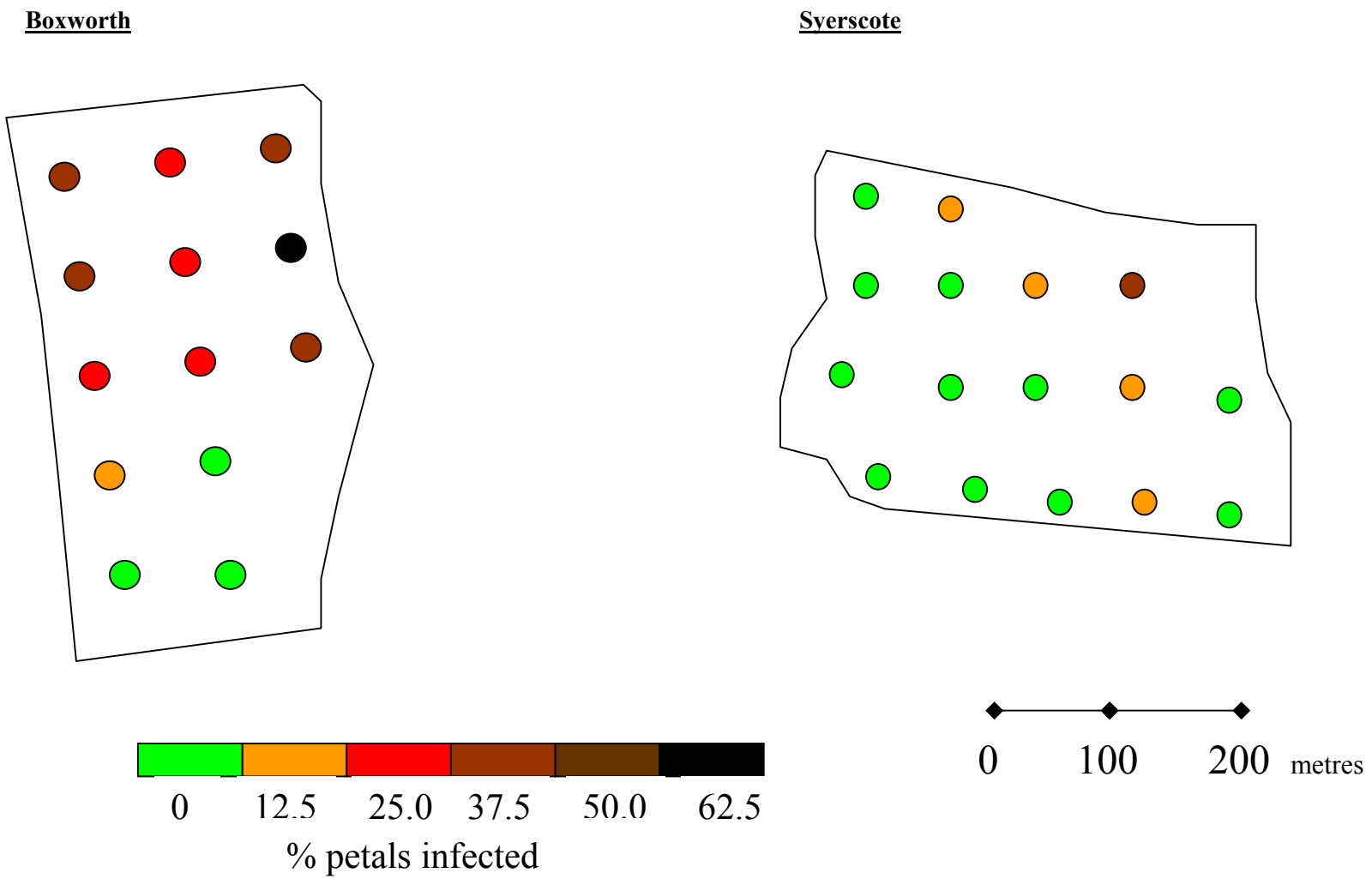


2000 data

In 2000 at Boxworth and Syerscote, petal infection was tested immediately after sampling by agar plating. Infection at Boxworth ranged from 0 to 62.5 %, and at Syerscote, from 0 to 37.5%.

Spatial pattern in the percentage of petals infected was evident in these data and is shown in Figure 3. At the Boxworth site, an area without infection was seen at the bottom right hand corner of the field. The rest of the field had petals infected with two 'hotspot' areas having slightly higher percentages. At Syerscote, a gradient in percent petals infected was seen across the field with more than half of the field having no infection and an increase in levels of infection towards the top right hand corner of the field. The Syerscote distribution is almost certainly due to spread from an adjacent field known to have suffered sclerotinia problems.

Figure 3. **Spatial variation in % petals infected at Boxworth and Syerscote in 2000 using a 100m grid**



CONCLUSIONS

The lack of distinct spatial variation in petal infection and the low levels of petal infection observed in the 1999 data were not surprising. As the samples were not frozen immediately prior to dispatch to CSL, spores present on the petals may have germinated due to humid conditions during transport. These germinated spores would not have survived the freezing process thus leaving low numbers of ungerminated spores which were the only viable ones detected after freezing. The lack of spatial variation is consistent with inoculum being produced within the field and uniform distribution of sclerotia within the soil.

The observed spatial variation in petal infection seen in the 2000 data emphasises the requirement for sampling at several points within a field, ideally across the whole field, for reliable determination of disease risk. In practice, the distribution of samples within a field may be determined by the effort required to sample different parts of individual fields and cost of tests per sample. Farmers are only likely to sample at accessible field margins because of the difficulties of walking through a flowering crop. It is possible that a sampling strategy based on walking the field perimeter and sampling at intervals from points at a defined short distance into the crop may be acceptable in terms of practicality and statistical viability.

The 2000 data showed similarities between neighbouring points at both sites which suggests that the 100 m sampling strategy was able to detect spatial variability present within the field. It is not possible to show statistically that the level of sampling was detailed enough to detect the spatial variation present without doing more intensive sampling (as in 1999) which would allow spatial statistical analysis for characterisation of spatial structure and quantification of the range of spatial dependence. While these maps do show distinct spatial pattern, there is no statistical basis to advise use of this density of sampling.

Further work is needed to determine the minimum size and number of samples needed to obtain an accurate picture of infection in a field. These studies did not investigate the spatial distribution of disease within fields. The linkage between inoculum and stem rot severity will be an important issue to understand if sprays are targeted in relation to measures of inoculum. Variation in crop and weed density, for example, may interact with inoculum to produce a patchy disease distribution even though inoculum is uniformly distributed. Some of these factors are considered in Section 5.

Section 4: Evaluation of fungicide programmes for control of sclerotinia stem rot

SUMMARY

Fungicide treatments at a range of timings during flowering were evaluated for control of sclerotinia stem rot in winter oilseed rape cv. Pronto at ADAS Boxworth and cv. Orkan at Syerscote, in 2000. At Boxworth, treatments compared a single dose applications of Compass (2 l/ha) + Stefes C-Flo 2 (0.5 l/ha) at early flowering (27 April), full flower (9 May) and late flowering (16 May) and a two spray programme at early and late flowering. The standard early flowering spray of Compass + MBC gave excellent control of sclerotinia (97%) which affected 3.4% of untreated plants. Full flowering sprays gave moderate control (72%), whilst the late flowering timing was ineffective (14% control). The two spray programme gave almost complete control (99%). At Syerscote, single dose applications only were compared; Compass (2 l/ha) + Bavistin DF (1kg/ha) at early (10 April), full (28 (April) or late (10 May) flowering. All sprays gave good control (98%) but stem rot levels were low (4%) in untreated plots. There was no significant effect of treatment on yield at either site, though benefits are anticipated in the longer term from control of sclerotinia inoculum.

INTRODUCTION

The importance of diseases in winter oilseed rape has been quantified using disease - yield loss relationships derived from fungicide experiments carried out under DEFRA and HGCA funding (Fitt *et al.,* 1997). Disease severity data were available from the annual surveys of winter oilseed rape carried out by ADAS and CSL. Stem rot caused by *Sclerotinia sclerotiorum* was particularly severe in 1991 when 46% of survey crops were affected and some severe attacks occur each year (Davies *et al*., 1999). Yield loss from sclerotinia is estimated to be up to 50% on affected plants (i.e. 10% plant infection would result in a 5% yield loss) and economic responses to fungicide treatment can be expected where more than 10% plants are affected.

The sclerotia (resting bodies) produced by the fungus on oilseed rape are capable of long term survival in soil, posing a threat to future crops of oilseed rape and other broad-leaved crops. Farmers are therefore concerned not only about the direct effects of stem rot on yield, but also about the longer term consequences of a build up of sclerotinia in the rotation. Fungicides have very little curative activity and are used as protectants to achieve good disease control. Accurate prediction of stem rot infection is not possible at present and decisions to spray are based on an assessment of risk. Crucially this depends on estimating the amount of inoculum in the crop and taking account of recent and future weather conditions which favour crop infection. A history of stem rot problems on a farm is a useful indicator of risk and decision making can be further improved by testing petals for the presence of sclerotinia.

Timing of the fungicide spray is critical and early to mid-flowering (or early petal fall) is usually the most effective. Early flowering is often the most cost-effective as ground sprayers cause little crop damage at this stage compared with later applications. This may have disadvantages if alternaria is present as sprays applied during the first half of flowering will not protect the pods against alternaria during pod ripening. As yet petal testing is not used by farmers and many adopt an insurance spray approach, arguing this is justified to prevent a continuing build of the disease. With the changing economics of oilseed rape production and prices of £100-120/tonne, the value of inputs needs to be re-appraised regularly. The use of a diagnostic test should enable the targeting of fungicide inputs to be improved. This experiment was carried out as part of project to develop a rapid test for sclerotinia on petals of oilseed rape and to provide guidance on thresholds for fungicide treatment.

OBJECTIVES

To obtain fundamental information on the relationship between sclerotinia inoculum as determined by petal culturing, rapid diagnostic testing, fungicide timing and final disease levels and yield.

METHODS

The investigation was carried out in winter oilseed rape, grown with standard farm inputs, on cultivar Pronto at ADAS Boxworth, and on cultivar Orkan at Syerscote near Tamworth, West Midlands. At Boxworth, the field had a history of sclerotinia problems in sunflowers which were cropped in 1997 and 1994. At Syerscote, the field had a history of moderate sclerotinia disease. Details of site, crop and treatments applied to the crops are given in Appendices II and III. Natural inoculum was relied upon as the source of sclerotinia at both sites.

Treatments

Fungicide treatments were scheduled to be applied at early, mid- and late flowering stages in a randomised block design with five replicates. Treatments, including an untreated control, are listed in Tables 1a and 1b for Boxworth, and Tables 2a and 2b for Syerscote.

Table 1a. Fungicides, active ingredients and rates of application, Boxworth

Product	Active ingredient(s)	Formulation	Rate of use	
			Active ingredient	Product
Stefes C-Flo 2	carbendazim	500 g/l, SC	250 g a.i./ha	0.5 l/ha
Compass	iprodione + thiophanate methyl	167 + 167 g/l, SC	333 +333 g a.i./ha	2.0 l/ha

Table 1b. Details of rates and timings of fungicide applications in 2000, Boxworth

Trt. No.	Fungicide	Early flowering 27 April	Full flowering 9 May	Late flowering 16 May
1	Untreated	-	-	-
2	Compass 2 l/ha + Stefes C-Flo 0.5 l/ha	+	-	-
3	Compass 2 l/ha + Stefes C-Flo 0.5 l/ha	-	+	-
4	Compass 2 l/ha + Stefes C-Flo 0.5 l/ha	-	-	+
5	Compass 2 l/ha + Stefes C-Flo 0.5 l/ha	+	-	+

Table 2a. Fungicides, active ingredients and rates of application, Syerscote

Product	Active ingredient(s)	Formulation	Rate of use	
			Active Ingredient(s)	Product
Bavistin DF	Carbendazim	50% w/w, WG	0.5 kg/ha	1.0 kg/ha
Compass	Iprodione + Thiophanate methyl	167 + 167 g/l, SC	333 +333 g a.i./ha	2.0 l/ha

Table 2b. Details of rates and timings of fungicide applications in 2000, Syerscote

Trt. No.	Fungicide	Early flowering 10 April	Full flowering 28 April	Late flowering 10 May
1	Untreated	-	-	-
2	Compass 2 l/ha + Bavistin DF 1 kg/ha	+	-	-
3	Compass 2 l/ha + Bavistin DF 1 kg/ha	-	+	-
4	Compass 2 l/ha + Bavistin DF 1 kg/ha	-	-	+

Table 3a. Treatment dates and growth stage at application, Boxworth

Target treatment timing	Actual application date	Actual growth stage
Early flowering (EF)	27 April 2000	4,0 -4,3
Full flowering (FF)	9 May 2000	4,5-4,9
Late flowering (LF)	16 May 2000	4,8-6,1

Table 3b. Treatment dates and growth stage at application, Syerscote

Target treatment timing	Actual application date	Actual growth stage
Early flowering (EF)	10 April 2000	4.2
Full flowering (FF)	28 April 2000	4.5 - 4.6
Late flowering (LF)	10 May 2000	4.8 - 4.9

Dates of spray applications are detailed in Tables 3a and 3b. Treatments were applied to plots (Boxworth: 24m x 6m; Syerscote: 12m x 4m) by farm sprayer in 200 litres of water/ha using 03 F110 nozzles operated at 2.2 bars pressure.

At both sites, conditions at spraying were generally favourable with low windspeed (<3 kph) and only slight spray drift. At Boxworth, the foliage was damp when sprays were applied on 27 April and 9 May and dry at application on 16 May. The spray on 9 May followed wet weather, but other sprays followed dry conditions. At Syerscote, the foliage was dry when sprays were applied on 10 April and 10 May but damp at application on 28 April. The spray on 28 April followed wet weather, but other sprays followed dry conditions.

Plots were harvested at Boxworth on 22 July 2000 using a Sampo 2025 plot combine. The harvest length of individual plots (c. 20 m) was recorded and harvest width was 2.44 m. At Syerscote, plots were harvested on 20 July 2000 using a Sampo 580 plot combine. The harvest length of individual plots (c. 10 m) was recorded and harvest width was 2.25 m.

At Boxworth, seed moisture content was determined by a Dickey-John GS2000 moisture meter and seed yield adjusted to 91% dry matter. At Syerscote, seed moisture content was determined using the iso moisture method (oven drying samples at 130^{o}C for 2 hours) and seed yield adjusted to 91% dry matter.

Petal tests for sclerotinia

Petals were tested for the presence of sclerotinia using the agar plate method (Davies *et al*., 1999) on each date when sprays were applied. At Boxworth, four petals were selected from each of 10 racemes (two racemes per control plot). Similarly, at Syerscote, four petals were selected from each of 10 racemes, but in a spiral pattern descending down the flowering head. Petals were placed on agar and examined after at least 10 days incubation for the presence of sclerotinia and botrytis on individual petals. Results are expressed as the percentage petals affected.

Disease assessments

Disease assessments were made at crop maturity on 200 plants per plot at Boxworth, and 100 plants per plot at Syerscote. At Boxworth, plants with lesions on the main stem were distinguished from those with infection confined to minor lateral stems. At Syerscote, lesions were categorised as being present within the bottom third of the stem, the middle third of the stem or the top third (including side racemes) of the stem. Results are expressed as the percentage plants affected. Pod and other stem diseases were present at very low levels and were not assessed.

Statistical analyses

Disease and yield data were subjected to analysis of variance using GENSTAT.

RESULTS

Petal infection in untreated plots.

At both Boxworth and Syerscote sites, agar tests revealed a low incidence of sclerotinia on petals (15%) throughout the flowering period, with some indication that inoculum declined towards the end of flowering (Tables 4a and 4b).

Table 4a. Incidence of sclerotinia and botrytis on petals, Boxworth

Target treatment timing	Actual application date	Growth stage	% petals with sclerotinia	% petals with botrytis
Early flowering (EF)	27 April 2000	4,0 -4,3	15	52.5
Full flowering (FF)	9 May 2000	4,5-4,9	15	32.5
Late flowering (LF)	16 May 2000	4,8-6,1	5	20

Table 4b. Incidence of sclerotinia on petals, Syerscote

Target treatment timing	Actual application date	Growth stage	% petals with sclerotinia
Early flowering (EF)	10 April 2000	4.2	0
Full flowering (FF)	28 April 2000	4.5 - 4.6	7.5
Late flowering (LF)	10 May 2000	4.8 - 4.9	15

At Syerscote, sclerotinia was detected on more petals from the middle region of the flowering head than for petals from the top or bottom (Table 5).

Table 5. Incidence of petals with sclerotinia from different locations on the flowering head, Syerscote

	% petals with sclerotinia			
Culturing Date	Top	Middle 1	Middle 2	Bottom
Early flowering (10 April)	0	0	0	0
Full flowering (28 April)	0	5	2.5	0
Late flowering (10 May)	2.5	5	5	2.5

Disease progress and weather

At both sites there were few petals sticking to leaves at early flowering (Boxworth, 27 April; Syerscote, 10 April). There was considerable petal stick at Boxworth by full flowering (9 May) when 10-15% leaf area was covered by petals. At Syerscote, petal stick was moderate by full flowering (28 April).

At Boxworth, April was a very wet month with rainfall recorded on 27 days out of 30 at Boxworth. In May, there was light rain on 6 days in the first half of the month followed by rain each day from 17-31 May. Minimum temperatures only exceeded 7°C on 25 and 26 April and 13 days in May. Maximum temperatures reached 18.5 °C on 29 April and were >20°C on 8 days in May.

At Boxworth, the crop showed some variation in flowering across the trial area with blocks I and II being more advanced than the other three replicates. This was attributed to differential pigeon grazing during the winter. Subsequent analyses indicated no significant differences in the incidence of sclerotinia between blocks, though the first two blocks had the highest stem rot. There was a significant difference between blocks for seed yield with the first two blocks producing 0.4 t/ha higher yield than the other three replicates. The first signs of sclerotinia infection were seen on 30 May, when the last few flowers were still present and seeds in the lower pods were translucent but full sized (GS 6,2).

At Syerscote, there were problems with the weather data logger such that there were gaps in the data, but it was possible to determine that, as at Boxworth, April was also a wet month with rainfall recorded on 13 days in the last two weeks of April. May was similarly wet, but data was complete only for the last week, with rain on 6 days of this week. Minimum temperatures in April reached 9.4°C on 27 April and 12.3°C on 17 May. Maximum temperatures in April reached 25.1°C on 24 April and were 31.4°C on 17 May.

At Syerscote, the crop showed very little variation in flowering across the trial area. Subsequent analyses showed no significant differences in the incidence of sclerotinia between blocks. There was a significant difference between blocks for seed yield with the highest yield from block 2 and the other blocks producing sequentially less yield. The first signs of sclerotinia infection were seen in mid June, when most seeds in the pods were green (GS 6.3).

The crop remained standing and the pod layer formed a leaning canopy late in the season. There was limited secondary spread of stem rot through plant to plant contact. Stem rot lesions were found mainly at the base or middle of the main stem and these caused premature ripening at crop maturity. There was phoma canker in the crop and about 2% stems were severely affected.

Table 6a. Effect of fungicide treatments on stem rot and yield, Boxworth 2000

Trt. No.	Fungicide	% plants with stem rot (% control)	% plants with main stem infection	Yield at 91% dry matter (t/ha)
1	Untreated	3.45	3.30	3.81
2	Compass 2 l/ha + Stefes C-Flo 0.5 l/ha EF	0.10 (97)	0.10	3.85
3	Compass 2 l/ha + Stefes C-Flo 0.5 l/ha FF	0.95 (72)	0.85	3.82
4	Compass 2 l/ha + Stefes C-Flo 0.5 l/ha LF	2.95 (14)	2.55	3.81
5	Compass 2 l/ha + Stefes C-Flo 0.5 l/ha EF+FF	0.05 (99)	0.05	4.15
	SED (36 df)	0.537	0.531	0.125
	CV(%)			5.1
	F test significance	<0.1%	<0.1%	NS

NS = not significant

Table 6b. Effect of fungicide treatments on stem rot and yield, Syerscote, 2000

Trt. No.	Fungicide	% plants with stem rot TOTAL (% control)	% plants with stem rot top	% plants with stem rot middle	% plants with stem rot bottom	Yield at 91% dry matter (t/ha)
1	Untreated	4.0	0.20	2.60	1.20	4.19
2	Compass 2 l/ha + Bavistin DF 1 kg/ha EF	1.6(80)	0.20	1.20	0.20	4.47
3	Compass 2 l/ha + Bavistin DF 1 kg/ha FF	0.6 (85)	0	0.60	0	4.51
4	Compass 2 l/ha + Bavistin DF 1 kg/ha LF	0.2 (95)	0	0.20	0	4.33
	SED (12 df)	0.772	0.208	0.624	0.277	0.111
	CV(%)					4.0
	F test significance	1%	NS	1%	1%	5%

NS = Not significant

Disease control

At Boxworth, sclerotinia affected 3.5 % plants in the untreated control (Table 6a) and most of this (3.3%) was infection of the main stem. A spray at early flowering gave very effective control (97%) and this contrasted with later timings which were less effective, with 72% control (at mid-flowering) and 14% control (at late flowering). The two spray programme gave almost complete control of sclerotinia and was comparable to the single early spray treatment. The late flowering treatment showed the highest incidence of lateral stem infection, but as this only affected 0.4% plants, few firm conclusions can be drawn.

At Syerscote, sclerotinia stem rot affected 4 % plants in the untreated control (Table 6b) and most of this (2.6%) was infection of the middle section of the main stem. A spray at early flowering gave the lowest level of control (60%) and this contrasted with later timings which were more effective, with 85% control (at mid-flowering) and 95% control (at late flowering). The early flowering treatment showed the highest incidence of stem infection on the top and bottom sections of the stem but as this affected <1% plants few firm conclusions can be drawn.

Yield

At both Boxworth and Syerscote (Tables 6a & 6b respectively), there were no significant effects between treatments on yield. No treatment yield was less than the untreated control. Given control of only 3% sclerotinia infection, yield responses of about 1.5% (0.06 t/ha) might have been expected.

DISCUSSION

Spray timing was very critical to achieve effective control of sclerotinia stem rot.

At Boxworth, a late April treatment at this site gave almost complete control of sclerotinia stem rot up to harvest. The late flowering spray in mid-May gave very little control of sclerotinia and the full flowering application gave partial control. These results suggest that late April and early May were the main window for infection. This is supported by observations that petals were sticking in large numbers in early May and the appearance of symptoms at the end of May. Normally, visible signs of stem infection occur 4-6 weeks after infection.

At Syerscote, a late flowering spray in May gave almost complete control of sclerotinia up to harvest. The early flowering spray in early April gave little control of sclerotinia and the full flowering application gave partial control. These results also suggest that late April and early May were the main window for infection. This is further supported by observations that petals were sticking in early May and the incidence of petals with sclerotinia also increased in May. Visible appearance of symptoms occurred in mid June.

The optimum period for spraying in late April/early May at both sites is indicative of the importance of weather factors for disease development. Early to mid-flowering is usually the most effective timing and the poorer control experienced at Syerscote at early flowering suggests that few petals stuck to leaves and temperatures were too low for disease activity. In 2000, weather conditions appear to have been very conducive for petal sticking and stem rot developed despite low petal infection.

There were small differences between test treatments and the standards at individual timings. The control of a low incidence of disease was expected to produce small effects on yield (e.g. 2-3%) and this proved to be the case. Prematurely ripened plants were clearly visible in the crop canopy and would cause concern to many farmers. In this case, the benefits of disease control must be appraised in terms of managing sclerotinia in the rotation and reducing the risks to future cropping. Nevertheless, under low disease pressure, lower cost fungicide treatments will remain attractive.

In future, fungicide use should be targeted more effectively. Petal tests in this crop showed only 15% petals infected with sclerotinia, well below the provisional threshold (for economically damaging attacks) of 40% petals affected (Davies *et al*., 1999). In 2000, weather conditions favoured petal sticking and this may have resulted in a higher sclerotinia incidence than would usually be the case with 15% infected petals. More detailed understanding of the interaction of petal inoculum, petal sticking, plant infection and weather factors will be required to improve risk assessment at the crop level.

Section 5: Development of a risk assessment scheme for sclerotinia in oilseed rape

SUMMARY

Information from published research and practical experience has been collated to produce a web-based Decision Guide for determining risk of sclerotinia in UK oilseed rape crops. Published data from Europe has formed a framework for developing guidelines in the UK and advisory experience has been utilised where specific research was unavailable. The Decision Guide calculates a numeric value for each set of data entered and assigns a value for risk of sclerotinia which is displayed as Low, Medium or High. High risk situations are likely to suffer >10% plant infection and loss of yield so fungicide treatment is advisable. Moderate risk indicates that there will be obvious signs of sclerotinia infection which is likely to give a build up of the disease on the farm but direct yield losses are likely to be less than 5%. The benefits of fungicides are therefore considered to be from longer-term disease management. Low risk crops may well show some plants (<2%) with sclerotinia which can be tolerated. In future, petal testing using a rapid diagnostic test could be part of an improved Decision Guide. The diagnostic should reduce the need to consider factors affecting spore production. The Decision Guide is now available for validation by farmers and their advisers. Threshold values for fungicide treatment will be needed and could be readily incorporated into the Guide.

INTRODUCTION

There have been various schemes produced in Europe to provide assessment of risk of sclerotinia in oilseed rape. There is considerable diversity in oilseed rape cropping within the EU and regions prone to frequent infections have been most active in formulating schemes, notably Denmark, France, Germany and Sweden (see Twengstrom *et al*., 1998). The schemes have taken account of a range of factors which are important for sclerotinia development and these factors have been given numeric values (Maisonneuve *et al*., 1997; Twengstrom *et al*., 1998; Yuen *et al*., 1996). The total of these values for a specific crop provides an indication of risk and hence the need for fungicide treatment. The schemes are based on local experimental and survey work and have been validated in some cases.

Local weather, field and crop factors are generally considered important, though it is not clear to what extent these apply to the UK as quantitative models for individual factors are rarely available. Where relationships are available, analysis is retrospective rather than predictive (Gohari and Ballester, 1991).

European experiences form a valuable framework for developing guidelines in the UK and should allow advisory experiences to be utilised where specific research is unavailable. However, there may well be significant differences between the UK and other regions in the relative importance of individual factors. For example, systems developed for spring rape in Sweden may not include a temperature component because temperature was not a limiting factor in June epidemics (Twengstrom *et al*., 1998). However, temperatures may be limiting for epidemics in March or April in the UK as they are sub-optimal for the pathogen (Willetts and Wong, 1980)

UK Scheme

Previous disease

Severe attacks of sclerotinia (>20% plants affected) are uncommon in the UK and recent studies highlight the importance of previous history of the disease on the same farm in assessing risk (Davies *et al*., 1999). Analysis of sclerotinia infection in unsprayed areas on 300 farms showed that mean level of infection was 7.3% on farms which had suffered at least 20% stem rot infection previously. In contrast, farms showing 0, <10% or <20% infection showed rather similar and low sclerotinia incidence (0.3-1.6%). History of sclerotinia problems has therefore been selected as one of the most important and useful factors for England and Wales. This is consistent with schemes in France (Maisonneuve *et al*., 1997) and Sweden (Twengstrom *et al*., 1998) though they also placed emphasis on infection in the last crop and numbers of susceptible crops in the previous 10 years (detailed long-term cropping records were not available for survey sites in the UK). As it is now almost 10 years since the last major sclerotinia epidemic, records of previous history prior to 1991 may be unavailable or be of decreasing significance (Davies *et al*., 1999). Entry of a value for previous

sclerotinia history is mandatory in the decision guide. Where this is not known, a default value indicating that there have not been severe problems on the farm, may be used.

The availability of inoculum would also be indicated by the presence of any apothecia within the oilseed rape or nearby crops. Low numbers of apothecia are difficult to find and an alternative technique is to bury natural sclerotia from oilseed rape in small grid within the crop in autumn and monitor appearance of apothecia during flowering. Such 'depots' take account of local and seasonal weather differences and provide guidance on whether the pathogen is likely to active during flowering (Twengstrom *et al*., 1998). This would have the advantage of decreasing reliance on rainfall and temperature data before flowering which are likely to affect apothecial production. To date only a few 'depots' have been monitored and most farmers will need to check for local apothecia to improve awareness of seasonal risk. In the UK, apothecia are usually produced over a long period from late March onwards and numbers are low during the critical stages of flowering.

Weather

Previous disease history is an indicator of presence of inoculum in the form of sclerotia, but weather and crop factors must be suitable for ascospore release and infection to occur.

A sequence of events must be completed for stem rot to occur: (1) Sclerotia near the soil surface germinate to produce ascospores. (2) Ascospores are released, dispersed and reach the petals; (2) Petals fall and stick to leaves or stems; (3) The fungus spreads from petal to leaf/stem and causes stem lesions.

The precise weather factors that are conducive to disease development have not been well defined. In general, high temperatures and unsettled weather with light rain (alternating wet and dry days) are likely to be most conducive to sclerotinia development (Willetts and Wong, 1980). These would produce periods of high humidity (>95%) and temperature (20°C or more) for infection (Hannusch and Boland, 1996) and be favourable for petals sticking to leaves. However, for oilseed rape there is disease activity at much lower temperatures and low temperatures may be a limiting factor for disease development. Whilst rainfall appears to be a requirement for severe stem rot epidemics, excessive rainfall may reduce disease risk if petals are washed off the plant or apothecia are flooded.

However, qualitatively, rainfall and temperature factors contribute to disease development as follows:

1. Rainfall or dew which leads to petals sticking to petioles and stems
2. Heavy rain
   - floods apothecia (and hence reduces spore release)
   - washes petals off the foliage and stems and hence reduces risk of disease
3. Sclerotinia has a high temperature optimum and growth is slow at low temperatures (<10°C), conditions which may prevail during flowering in the UK.

4. Conversely, high temperatures (>15°C) during flowering will favor rapid disease development and enable infection to take place in shorter periods of leaf wetness than at low temperatures.

Examples of rainfall measures used for risk prediction in winter oilseed rape include 1-10 mm rain 10-14 days, 5-9 days, less than 5 days or on no days previous to the time of risk prediction in France (Maisonneuve *et al.* 1997). The French scheme also takes account of rainfall in the 3-4 weeks before flowering and soil moisture. For spring oilseed rape risk factors, Twengstrom *et al.* (1998) asked for the amount of rain in June (<10 mm, 10-30 mm or >30 mm), and rain in the last two weeks (same categories as for June rain). Temperature observations are thought to be important at the time of risk assessment and are categorised in the range >15^{o}C, between 10 and 15^{o}C, or < 10^{o}C (Maisonneuve *et al.,* 1997; Ahlers, 1989). These temperature parameters have been incorporated in the decision guide and increased risk can be expected if temperatures exceed 15°C.

Weather forecasts have been used instead of rain, e.g., low pressure, changeable or high pressure (Maisonneuve *et al.,* 1997, Ahlers, 1989). In the Canadian petal test manual for sclerotinia in oilseed rape, weather data are not required, but, together with petal infection levels, an assessment of weather pattern is asked for, i.e., wetter, drier, or constant (Morrall and Thomson, 1991).

Fungicides have very limited curative activity against sclerotinia and therefore decisions must take account of spore inoculum, current weather and forecast weather. Current weather allows judgment to be made about sclerotinia being able to produce its air-borne spores, whilst forecast weather is used to try and predict whether petals will stick and infection take place. Clearly there are uncertainties about forecast weather, but ideally fungicides should be applied to petals before they fall to achieve disease control. Entry of weather details is also mandatory and decisions cannot be made selectively without rainfall and temperature inputs.

<u>Other factors</u>

Most farmers and advisers should be able to make decisions based on history of sclerotinia and weather factors. A range of other factors can influence the disease but these are usually considered of secondary importance to inoculum and weather factors

These include:

- Crop height - short plants are more at risk than tall plants (Sweet *et al*., 1992) though the opposite trend was reported from Canada, albeit with many short (<1 m) crops (Turkington and Morrall, 1993)

- Crop density - dense crops are more severely affected than thin crops (Turkington and Morrall, 1993; Twengstrom *et al*., 1998)
- Presence of weeds - provides more humid microclimate for spore production and infection (Maisonneuve *et al.,* 1997; Twengstrom *et al*., 1998)
- Onset of lodging - favours secondary spread of sclerotinia through plant to plant contact
- Cultivar differences are inconsistent and effects may be related to differences in height and time of flowering (Sweet *et al*., 1992)

Interactions with other susceptible crops should also be considered. Sclerotinia can build up during crop senescence and be unnoticed or cause direct problems in a wide range of broad-leaved crops. Such crops can help maintain sclerotinia populations on the farm even if they are not adversely affected themselves. The most frequently involved susceptible crops include:

Potato
Peas
Carrots
Vegetable crops e.g. celery, lettuce

The presence of apothecia in the oilseed rape crop itself or adjacent (e.g. cereals) fields would also constitute an important observation and identify local risk (Twengstrom *et al*., 1998).

Inoculum levels

Whilst previous history is a useful factor in decision making, better decisions can be made if inoculum can be quantified in individual oilseed rape crops. This has been apparent from field observations that severe attacks can occur on farms with no previous problems and severe attacks do not always occur on badly affected farms. Petal testing has been developed successfully in Canada (Turkington *et al*., 1991; Turkington and Morrall, 1993) where results are available within 5 days. In the UK, agar tests would also be beneficial although they take longer because botrytis (which is uncommon in Canadian samples) needs to be differentiated from sclerotinia (Davies *et al.*, 1999). The use of agar petal tests has enabled high risk situations to be identified more effectively and could be used to reduce prophylatic spraying of winter oilseed rape by 50% (Davies *et al,* 1999). Agar test thresholds (>50% petals with sclerotinia) have been incorporated into the Decision Guide as an option rather than a mandatory component because few farmers are using agar tests. Agar petal test results have been weighted so that decisions can be made using only previous disease and weather factors.

Outline of the UK Decision Guide

Factor	Options
*What has been the highest level of sclerotinia in oilseed rape on your farm?	>20% plants affected <20% plants affected Don't know
*Rainfall - what is the forecast weather for the next week?	Dry and settled Unsettled, light rain or showers on 2-3 days Very heavy rain or thunderstorms on 2 or more days
*Temperature - what is mean temperature likely to be next week?	>15°C 10-15°C <10°C
Apothecial activity at early to mid flower. How many apothecia are present?	High numbers, >25% germination in depots or apothecia found in field or nearby <25% germination in depots None Don't know
Petal tests - on agar	>50% with sclerotinia <50% Don't know
Years since last oilseed rape crop grown on the field	1-2 years 3-5 years 6 or more years
Other susceptible crops in rotation - identify each one from list	Dwarf beans Peas Carrots Other vegetables Other susceptible crops None

Crop height	<1.2 m
	1.2-1.8 m
	>1.8 m
Weed cover	High
	Moderate
	Low
Lodging onset	Up to early flowering
	Late flowering onwards
	None
Lodging extent	>50% crop area
	<50% crop area
	Slight - pod layer only

* Mandatory components providing minimum details to guide decisions.

System output

The decision guide calculates a numeric value for each set of data entered and assigns a value for risk of sclerotinia which is displayed as Low, Medium or High. High risk situations are likely to suffer >10% plant infection and loss of yield so fungicide treatment is advisable. Moderate risk indicates that there will be obvious signs of sclerotinia infection which is likely to give a build up of the disease on the farm but direct yield losses are likely to be less than 5%. The benefits of fungicides are therefore considered to be from longer-term disease management. Low risk crops may well show some plants (<2%) with sclerotinia which can be tolerated.

The Decision Guide is now available for validation by farmers and their advisers. It will require updating in future to accommodate changes in fungicide recommendations.

In future, petal testing using the rapid diagnostic test would be part of an improved Decision Guide. The diagnostic should reduce the need to consider factors affecting spore production. Threshold values for fungicide treatment will be needed and could be readily incorporated into the Guide.

REFERENCES

Ahlers D, 1989. Integrated plant protection for fungus diseases in winter oilseed rape. *Gesunde Pflanzen* **41**, 306-311.

Bossi R, Dewey FM, 1992. Development of a monoclonal antibody-based immunodetection assay for Botrytis cinerea. *Plant Pathology* **41**, 472-482.

Bradford MM, 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein dye binding. *Annals of Biochemistry* **72**, 248-254.

Clark MF, Adams AN, 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay (ELISA) for the detection of plant viruses. *Journal of General Virol*ogy **34**, 475-483.

Davies JMLl, Gladders P, Young C, Dyer C, Hiron L, Locke T, Lockley D, Ottway C, Smith J, Thorpe G, Watling M, 1999. Petal culturing to forecast sclerotinia stem rot in winter oilseed rape: 1993-1998. *Aspects of Applied Biology* **56**,129-134.

Fitt BDL, Gladders P, Turner JA, Sutherland KG, Welham SJ, Davies JMLl, 1997. Prospects for developing a forecasting scheme to optimise use of fungicides for disease control on winter oilseed rape in the UK. *Aspects of Applied Biology* **48**, *Optimising pesticide applications*, 135-142.

Gohari H, Ballester F, 1991. Regles de decision pour la reduction des traitements contre *Sclerotinia sclerotiorum* sur colza en fonction des conditions climatique. *EPPO Bulletin* **21**, 507-514.

Hannusch DJ, Boland GJ, 1996. Influence of air temperature and relative humidity on biological control of white mold of bean (*Sclerotinia sclerotiorum)*. *Phytopathology* **86**, 156-162.

Holmes SJ, 1996. The validation and commercial development of immunological diagnostic technology for non-medical applications. *Proceedings 1996 BPC symposium: Diagnostics in Crop Production.* **1,** 333-342.

Jameaux I, Spire D, 1994. Development of a polyclonal antibody-based immunoassay for the early detection of *Sclerotinia sclerotiorum* in rapeseed petals. *Plant Pathology* **43**, 847-862.

Jameaux-Despreaux I, Spire D, 1999. Comparison of responses of ascospores and mycelium by ELISA with anti-mycelium and anti-ascospore antisera for the development of a method to detect Sclerotinia sclerotiorum on petals of oilseed rape. *Annals of Applied Biology* **134**, 171-179.

Maisonneuve C, Penaud A, Auclert B, Arjaure G, Jung L, Lamidieu L, 1997. *Sclerotinia sclerotiorum* Lib. De Bary: Mise au point d'un outil de decision pour le traitement du colza d'hiver. *ANPP Cinquieme Conference Internationale Sur Les Maladies Des Plantes tours – 3-4-5 Decembre 1997,* pp. 731-737.

Morrall RAA, Thomson JR, 1991. Petal test manual for sclerotinia in canola. Dept Biology. University of Saskatchewan Saskatoon SN7 0W0.

Sansford CE, Coley-Smith JR, 1992. Production and germination of sclerotia of *Sclerotinia sclerotiorum*. *Plant Pathology* **41**, 154-156.

Sweet JB, Pope SJ, Thomas JE, 1992. Resistance to Sclerotinia sclerotiorum in linseed, oilseed rape and sunflower cultivars, and its role in integrated control. *Proceedings of Brighton Crop Protection Conference - Pests and Diseases*, **1**, 117-126.

Turkington TK, Morrall RAA, 1993. Use of Petal Infestation to forecast Sclerotinia stem rot of canola: the influence of inoculum variation over the flowering period and canopy density. *Phytopathology* **83**: 682-689.

Turkington TK, Morrall RAA, Baker RJ, 1988. Sample sizes in relation to forecasting sclerotinia stem rot of canola. *Canadian Journal of Plant Pathology* **10**: 159-165.

Turkington TK, Morrall RAA, Gugel RK, 1991. The use of petal infestation to forecast Sclerotinia stem rot of canola; evaluation of early bloom sampling, 1985-90. *Canadian Journal of Plant Pathology* **13**:50-59

Twengstrom E, Sigvald R, Svensson C, Yuen J, 1998. Forecasting sclerotinia stem rot in spring sown oilseed rape. *Crop Protection* **17**, 405-411.

Willetts HJ, Wong JAL, 1980. The biology of *Sclerotinia sclerotiorum*, *S trifoliorum*, and *S. minor* with emphasis on specific nomenclature. *Botanical Review* 46, 101-165.

Yuen J, Twengstrom E, Sigvald R, 1996. Calibration and verification of risk algorithms using logistic regressions. *European Journal of Plant Pathology* **102**, 847-854.

RECOMMENDATIONS FOR FURTHER WORK

1. Further validation and improvement of the IF diagnostic test on fresh petal samples.
2. Production of second monoclonal as coating antibody in a DAS ELISA. This would lead to the development of a rapid in-field test, possibly a lateral flow device.
3. Use of the IF method to investigate sclerotinia risk through monitoring of spores collected by spore trapping (avoids inhibition problems encountered with petals). This could be extended to crops other than oilseed rape.
4. Further fundamental studies on the spatial distribution of sclerotinia stem rot are essential to develop sampling protocols to allow accurate estimation of infection levels using rapid diagnostic methods. Without these critical data, accurate use of rapid diagnostic techniques to quantify disease levels in field situations will not be possible.
5. In 2000, weather conditions favoured petal sticking and this may have resulted in a higher sclerotinia incidence than would usually be the case with 15% of petals infected. More detailed understanding of the interaction of petal inoculum, petal sticking, plant infection and weather factors will be required to improve risk assessment at the crop level.
6. Fundamental studies of sclerotinia and weather interactions should be continued with the aim of producing improved risk assessment guidelines in a decision support system. The Decision guide produced in this project now requires validation on farms. The use of fungicides during flowering should remain flexible and take in account the threat from pod diseases as well as sclerotinia.

APPENDIX

Appendix I. Site details – CSL Sand Hutton 1999/2000

Site:	CSL, Sand Hutton, York		
Previous cropping:	1997	Fallow	
	1998	Winter wheat	
Crop:	Winter oilseed rape		
	Cultivar:		Alpine
	Sowing date:		25/08/1999

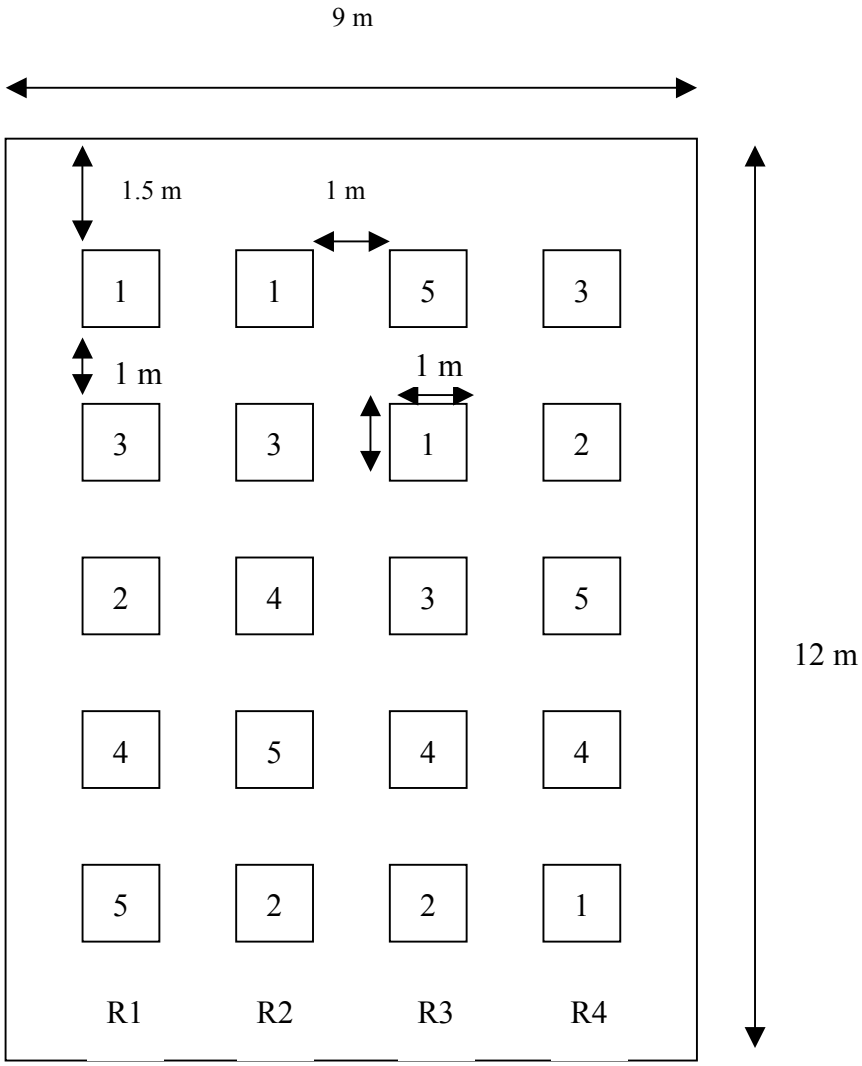
Seed treatment: Lindex Plus FS

Herbicides:	27/08/1999	Butisan S	1.5 l/ha
	04/09/199	Butisan S	1.0 l/ha
Fungicides:	None		
Insecticides:	None		
Molluscicides:	None		

Trial Layout for Inoculated plots at CSL in 1999/2000

Replicate plot size = $1m^2$, with 1m walkways (see diagram below)

20 plots (5 treatments x 4 replicates)



Appendix II. Site details ADAS Boxworth 1999/2000

Site:	ADAS Boxworth		
Field name:	Samsons East		
Soil texture:	Clay		
Drainage:	Good		
Soil analysis:	PH	:	8.0
	P mg/l (index)	:	14 (1)
	K mg/l (index)	:	258 (3)
	Mg mg/l (index)	:	97 (2)
Previous cropping:	1997 Sunflowers		
	1998 Winter wheat		
	1999 Winter wheat		
Previous cultivation:	27/08/99 Disced		
	01/09/99 Pig tail cultivated		
	02/09/99 Power harrowed × 2		
	03/09/99 Rolled		
Crop:	Cultivar	:	Pronto
	Sowing date	:	02/09/99
	Seed rate (kg/ha)	:	14.0
	Fertiliser (kg/ha)	:	28/09/99 40 kg/ha Nitrogen
			12/02/00 50 kg/ha Nitrogen
			29/03/00 100 kg/ha Nitrogen
			21/03/00 70 kg/ha Phosphate
Herbicides:	04/09/99 Katarmaran 1.35 l/ha		
	12/10/99 Falcon 0.3 l/ha + Sprayprover 1 l/ha		
Fungicides:	Experimental treatments only at flowering. Farm crop and plots received Punch C 0.4 l/ha on 27/10/99 and 27/01/00		
Insecticides:	27/01/00 Toppel 0.25 l/ha		
Molluscicides	10/09/99 Lynx slug pellets 10 kg/ha		
	28/09/99 Draza 5.5 kg/ha		
Desiccant:	06/07/00 Glyphosate 3 l/ha		
Harvest date:	24/07/00		

Apendix III. Site details Syerscote, Tamworth 1999/2000

Site:	Syerscote		Tamworth, West Mids
Field name:	Stonebridge Field		
Soil texture:	SL (sandy loam)		
Drainage:	Good / free		
Soil analysis:	PH	:	6.3
	P mg/l (index)	:	53 (4)
	K mg/l (index)	:	247 (3)
	Mg mg/l (index)	:	66 (2)
Previous cropping:	1997 Winter Barley		
	1998 Winter Wheat		
	1999 Combinable Peas		
Previous cultivation:	Conventional plough		
	Combined cultivator + drill		
Crop:	Cultivar	:	Orkan
	Sowing date	:	27 August, 1999
	Seed rate (kg/ha)	:	5.22
	Fertiliser (kg/ha)	:	15/08/99 242 Kg/ha 0:26:26 (62.5 Kg P, 62.5 Kg K)
			26/08/99 87 Kg/ha Olympia A.N 34.5%
			07/03/00 198 l/ha Nuram 35+7 SO_3
			22/03/00 277 l/ha Nuram 35+7 SO_3
Herbicides:	20/08/99 Roundup 1.19 l/ha		
	26/10/99 Mithras (Kerb) 0.94 Kg/ha		
Fungicides:	26/10/99 Plover 0.3 l/ha		
	21/03/00 Caramba0.6 l/ha		
Insecticides:	26/10/99 Permasect 0.25 l/ha		
Molluscicides	None		
Desiccant:	Crop not desiccated (Reglone (diquat) applied to trial plots, 10/07/00)		
Harvest date:	20 July, 2000		