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DISCLAIMER

The results and conclusions in this report are based on an investigation conducted over a one-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.

AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

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Signature .... Date ....06/10/2015................

Table of Content

GROWER SUMMARY

Headline

This project focuses on the determination of the population structure of the causal agent of light leaf spot, *Pyrenopeziza brassicae*. It will be determined whether the same *P. brassicae* strains can infect both, oilseed rape and vegetables. Gene-for-gene interactions between pathogen strains and plant cultivars will be studied.

Background

Light leaf spot, caused by the fungal pathogen *Pyrenopeziza brassicae*, is currently the major disease problem in oilseed rape (*Brassica napus* L.) production in the UK and also affects vegetable brassicas such as cabbage, cauliflower and Brussels sprouts. The disease was considered a problem in Scotland and North England but has substantially increased importance in all parts of England over the last decade. Due to the polycyclic (fulfils its life cycle more than once in a cropping season) nature of the disease, the pathogen has the potential to adapt to an environment (McDonald & Linde, 2002). Effective control of light leaf spot to reduce yield and economic losses is difficult to achieve. Fungicide control of the disease in crops is difficult since fungicides must be applied when the pathogen is growing asymptomatically (without visible symptoms) in plant tissues (Figueroa *et al.* 1994). Additionally, decreased sensitivity to azole fungicides has been reported (Carter *et al.* 2013). Exploiting plant resistance against the pathogen could help control the disease but current commercial oilseed rape cultivars show poor resistance and more information on resistance of vegetable cultivars would be beneficial. Although light leaf spot affects vegetables and oilseed rape it is not yet clear if the same strains of the pathogen can infect both or are specific to a crop. The potential spread between host species may have an influence on epidemics of the disease.

Summary

The aim of the project is to identify the pathogen population structure, to determine if the same strains are able to infect oilseed rape and other brassicas, and to gain a better understanding of the plant-pathogen interactions. This project will support breeders with regard to breeding better light leaf spot resistance into cultivars and therefore, give farmers and growers better material to choose from in the long term.

In the 2013/14 and 2014/15 oilseed rape cropping seasons, four field trials were established across the UK to distinguish potential differences in the *P. brassicae* population structure between locations. The oilseed rape cultivars have shown varying performance at different locations, which suggested the presence of different pathogen populations at the different locations. Selected oilseed rape cultivars were also tested with *P. brassicae* populations under controlled environment conditions. Interactions between cultivars and pathogen populations were identified and differences between populations from different locations recorded. These findings indicate that the pathogen forms races and may interact in a gene-for-gene manner with cultivars.

Furthermore, cross-infection experiments were done to determine if *P. brassicae* isolates originated from oilseed rape are able to infect Brussels sprouts and other vegetable brassicas (cabbage, broccoli, cauliflower and romanesco) and vice versa. All tested species, oilseed rape and vegetables, showed light leaf spot symptoms for *P. brassicae* populations from oilseed rape and Brussels sprouts. Brussels sprouts were less susceptible to light leaf spot than oilseed rape, broccoli and cabbage (Figure 1, Figure 2). This could be due to thickness of waxy layer of the host plant, other structural differences or secondary metabolites (e.g. higher glucosinolates). More Brussels sprouts cultivars should be tested to confirm the result. Nevertheless, cross-infections are generally possible. With the presented experiments it cannot be excluded that there are *P. brassicae* isolates that are limited to only one or a few hosts.
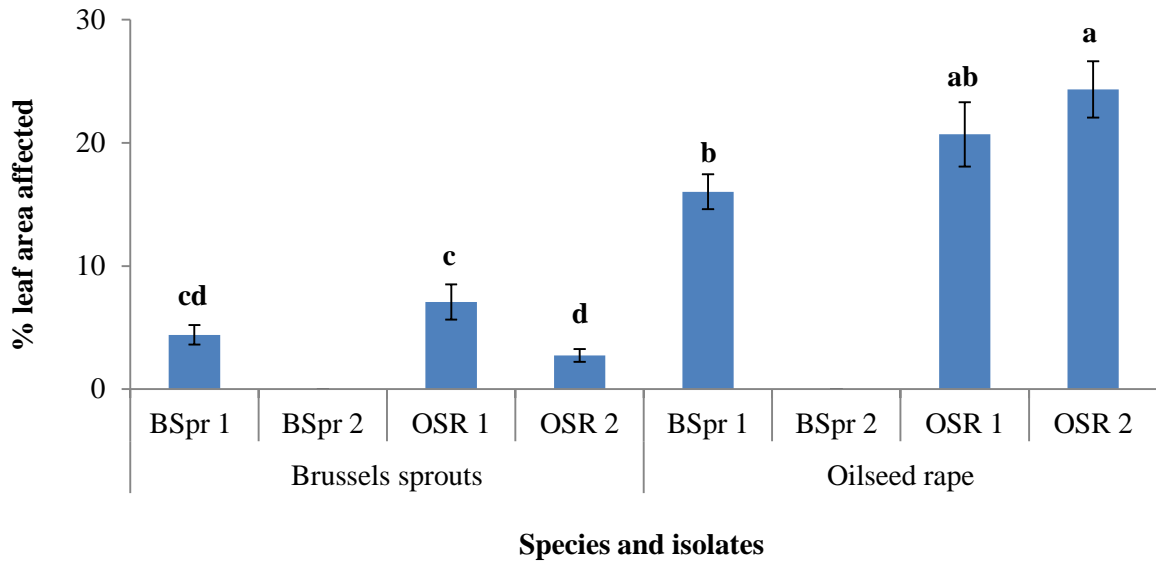
Figure 1. Light leaf spot severity (in % leaf area affected) of Brussels sprouts and oilseed rape with populations of *Pyrenopeziza brassicae*

Populations are originated from Brussels sprouts (BSpr 1, BSpr 2) and oilseed rape (OSR 1, OSR 2). The inoculation with *P. brassicae* population BSpr2 failed. Bars show mean and standard error. Different letters indicate significant differences at α=0.05.
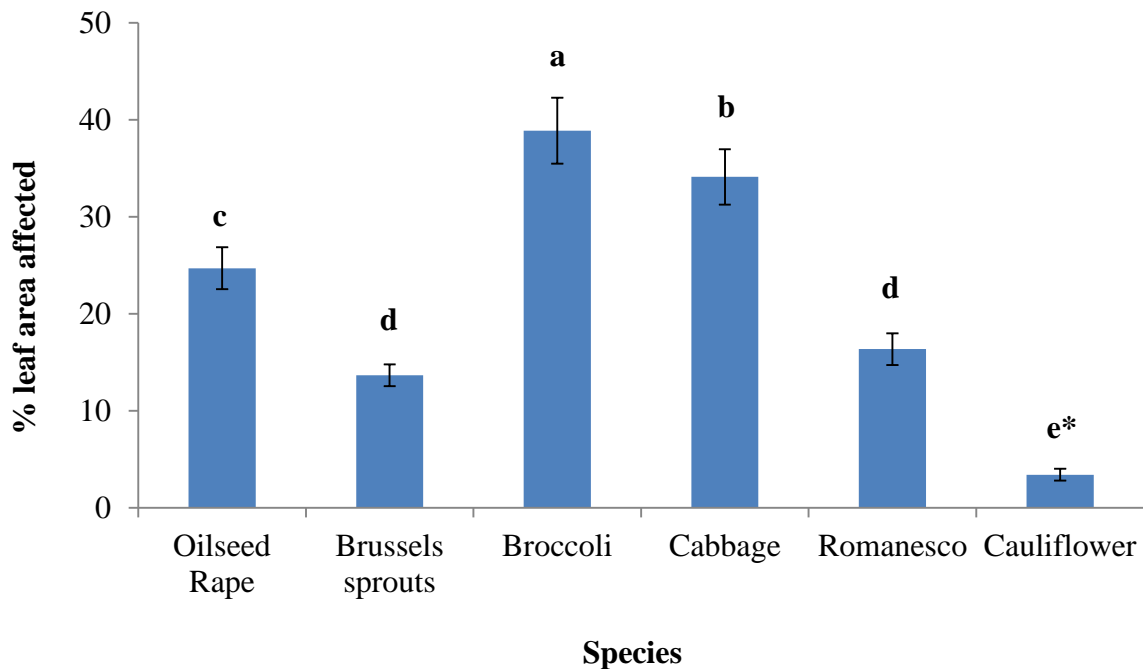
Figure 2. Light leaf spot severity (in % leaf area affected) of different Brassica species with populations of *Pyrenopeziza brassicae*

Bars show mean and standard error. Different letters indicate significant differences at α=0.05. *Result of cauliflower not comparable due to extensive loss of infected leaves

Future work for the determination of the *P. brassicae* population structure includes sequencing of a *P. brassicae* strain, the development of microsatellite (SSR) markers and *in planta* testing of oilseed rape and Brussels sprouts cultivars to study potential gene-for-gene interactions with the pathogen.

SCIENCE SECTION

Importance of oilseed rape production

Oilseed rape (*Brassica napus* L.) belongs to the family Brassicaceae, formerly known as Cruciferae, and originated from a spontaneous interspecific hybridization between *B. rapa* and *B. oleracea*, illustrated by the triangle of U that describes the relationship between members of the *Brassica* genus (Figure 3).

The importance of this crop has been achieved due to breeding of cultivars with special qualities. The quality of these cultivars is called double zero ('00'), i.e. low content of erucic acid and low content of glucosinolates, allowing the use of oilseed rape as food and feed (Abbadi & Leckband 2011). Erucic acid has been shown to cause myocardial lipidosis in pigs and rats and also reduced growth rate in rats (Kramer et al. 1973, Nesi et al. 2008). Therefore, *B. napus* oil was considered to be unusable for human consumption (Nesi et al. 2008). The spring oilseed rape cultivar "Liho" showed a single mutation in the pathway of the synthesis from oleic acid to erucic acid, which resulted in seeds with a low erucic acid content (Bao et al. 1998, Hasan et al. 2008). This mutation was the basis for the production of single zero cultivars ('0').

Furthermore, the breeding of cultivars that also had low glucosinolate content contributed to the current success of the crop. Glucosinolates are secondary metabolites localised in vacuoles that can be present in all tissues of the plants, such as leaves and seeds (Velasco et al. 2008). For human consumption glucosinolates are believed to reduce risk for cancer, in particular colon cancer (Verkerk et al. 2009). However, glucosinolates in animal nutrition leads to reduced food intake, decreased iodine uptake, a change in thyroid activity and hypertrophy of the liver and kidney (Tripathi & Mishra 2007). Therefore, use of oilseed rape in animal nutrition was limited. In the late 1960's a Polish cultivar was identified that showed a low glucosinolate content (Hasan et al. 2008). This genetic material then was used to improve the quality of oilseed rape cultivars.

After the introduction of these two traits with major effects and a continuous improvement in yield of oilseed rape, it is now the fourth most important crop for oil production with a worldwide production of 65M tonnes in 2012, after oil palm

(249.5M tonnes), soybean (241.8M tonnes) and seed cotton (76.5M tonnes) (FAOSTAT 2014). The cropping area of oilseed rape has been increasing greatly since the introduction of the first double zero cultivar in 1974 (Figure 4) (Hasan et al. 2008).
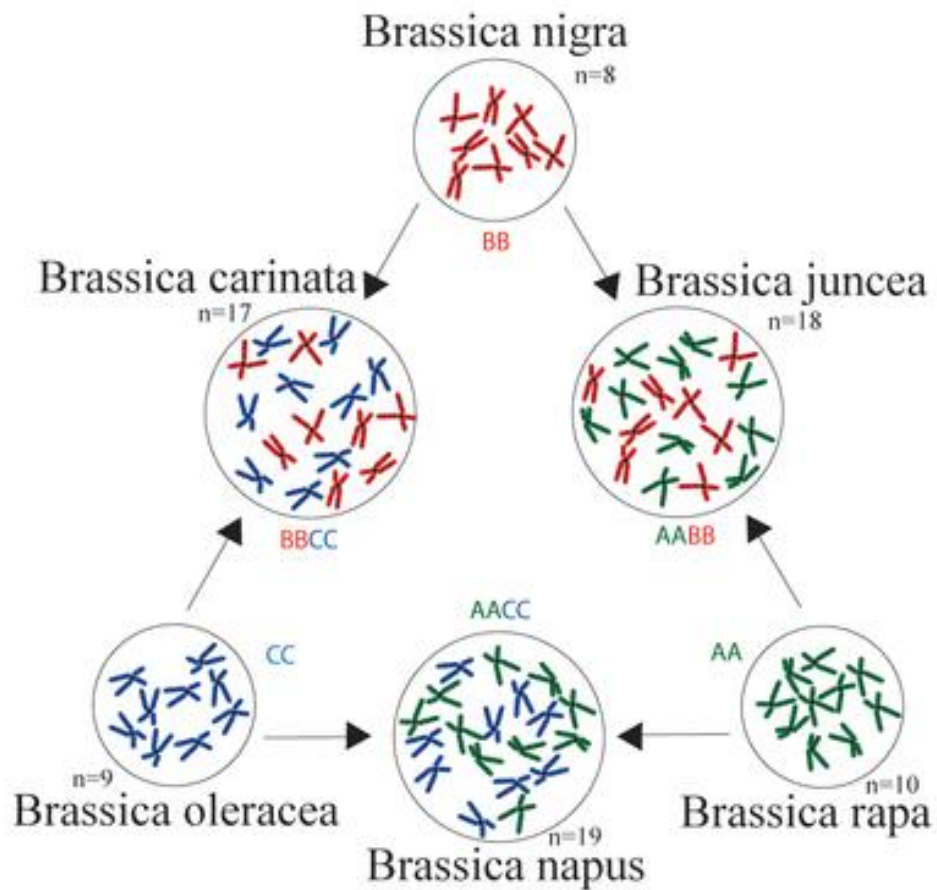
Figure 3. Triangle of U showing the relationship between *Brassica oleracea*, *B. rapa* and *B. nigra* forming allotetraploid species *B. napus*, *B. carinata* and *B. juncea* (Anonymous 2015b)
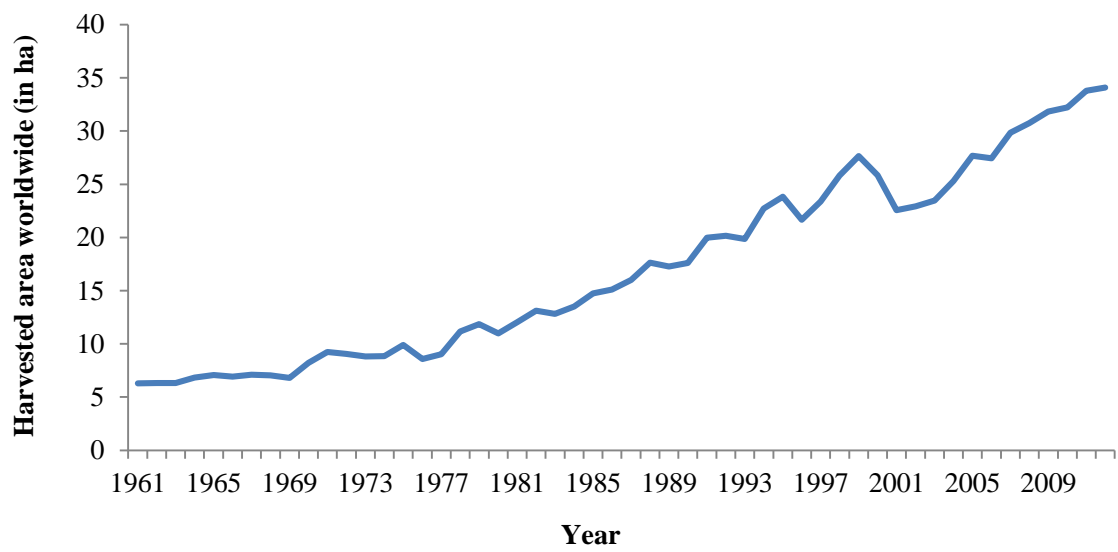
Figure 4. Harvested area of oilseed rape worldwide (in ha) from 1961 until 2012 (FAOSTAT 2014).

Importance of vegetable brassicas

Brassicas are species belonging to the genus *Brassica* in the family Brassicaceae. Vegetable brassicas, mainly produced for human consumption, include species such as *Brassica olereacea* including different convarieties (convar.) and varieties (var.) (Table 1) as well as *Brassica rapa* (turnip rape, *Brassica rapa* subsp. *rapa*).

The overall cultivated area of vegetable brassicas in Europe decreased from 247 000 ha in 2003 to 213 000 ha in 2012 (Figure 5). The United Kingdom is the fourth largest producer of vegetable brassicas after Italy, Spain and France (Figure 5).

However, the production of Brussels sprouts, for example, is greatest in the UK, with about 3000 ha cultivated (Figure 6).

Table 1. List of Latin and common names of *Brassica oleracea* convarieties and varieties

Latin name	Common name
Brassica oleracea convar. *capitata* L.	Headed cabbage
Brassica oleracea convar. *capitata* var. *alba* L.	White cabbage
Brassica oleracea convar. *capitata* var. *rubra* L.	Red cabbage
Brassica oleracea convar. *capitata* var. *sabauda* L.	Savoy cabbage
Brassica oleracea var. *botrytis* L.	Cauliflower
Brassica oleracea convar. *botrytis* var. *botrytis* L.	Romanesco
Brassica oleracea var. *italica* Plenck	Broccoli
Brassica oleracea var. *gemmifera* DC.	Brussels sprouts
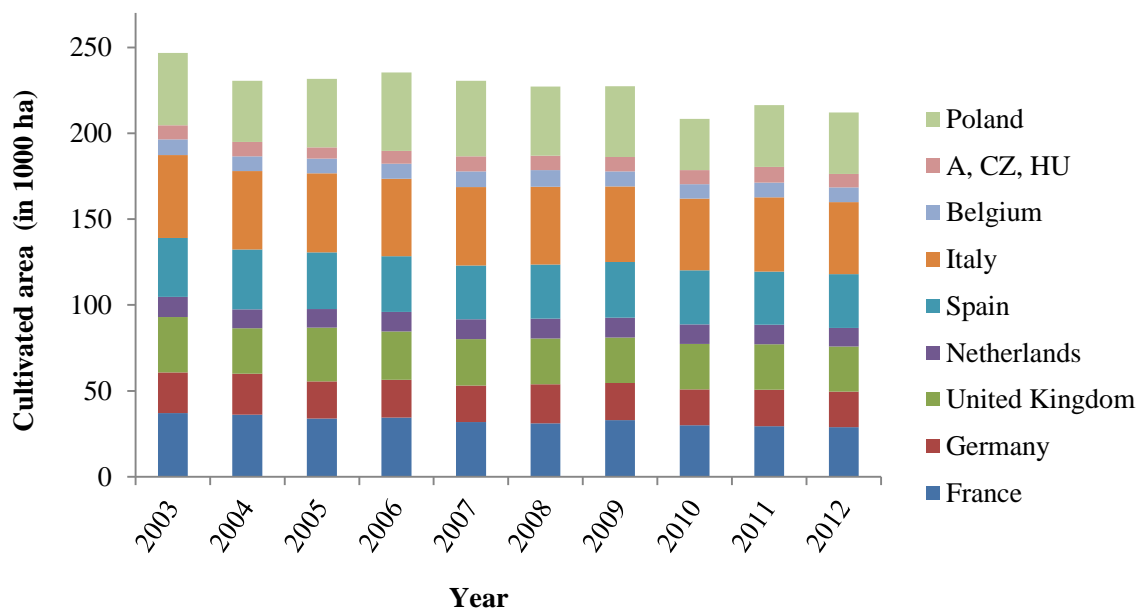
Figure 5. Cultivated areas of brassicas in European countries (in 1000 ha) from 2003 until 2012 (Behr 2014). Abbreviation A: Austria, CZ: Czech Republic, HU: Hungary
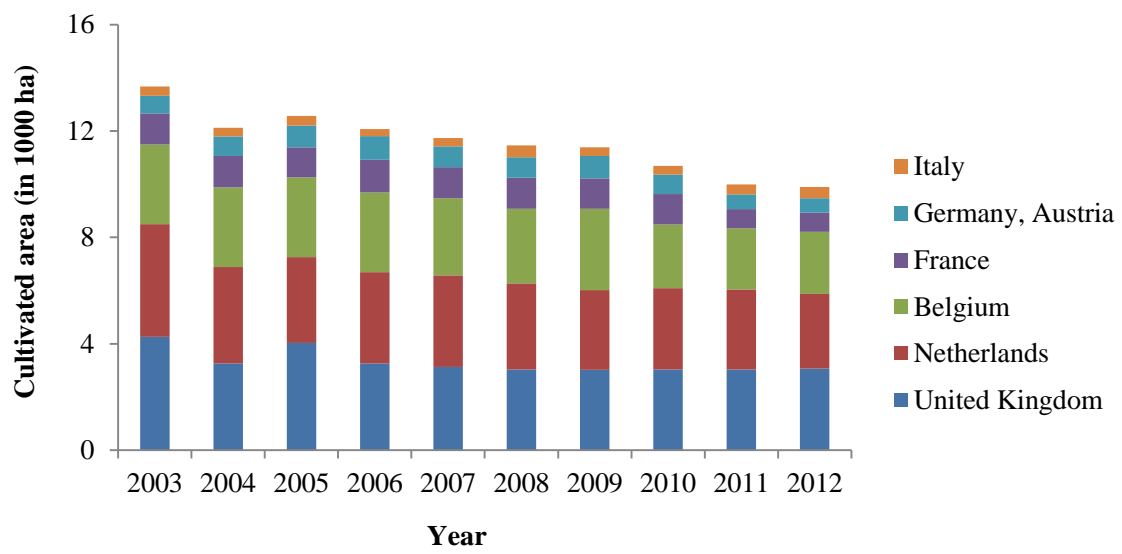
Figure 6. Cultivated area of Brussels sprouts in European countries from 2003 until 2012 (Behr 2014)

Pyrenopeziza brassicae and light leaf spot

Pyrenopeziza brassicae (teleomorph) is the causal agent of light leaf spot in oilseed rape and vegetable brassicas. The fungal pathogen was first recorded on cabbage and its anamorphic stage was named as *Cylindrosporium concentricum* by Greville in 1823. The natural occurrence of the perfect stage (teleomorphic stage) of the pathogen was first observed in Ireland in 1966 (Staunton & Kavanagh 1966 cited from Cheah et al. 1980) before the teleomorphic stage was described as *Pyrenopeziza brassicae* by Rawlinson, Sutton and Muthyalu (1978), who observed formation of apothecia in culture. The involvement of two mating types for the production of apothecia (i.e. heterothallism) was then studied by Illot & Ingram (1984).

P. brassicae is a hemibiotrophic pathogen, causing a polycyclic disease affecting *Brassica* species, such as oilseed rape and vegetable brassicas. In the last decade light leaf spot has become to be the major disease problem in oilseed rape in the UK. In 2014 it caused an annual yield loss of approximately £ 140 million (Neal Evans, CropMonitor). The main reasons for yield loss due to light leaf spot in oilseed rape are a reduction in photosynthetic area and increased susceptibility to frost (Baierl et al. 2002). Furthermore, pod infection causes pod shatter, which leads to additional yield loss (Fitt et al. 1998). In Brussels sprouts losses are estimated to be 10% mainly because of a reduction in quality (Simon Jackson, personal communication).

Disease cycle of light leaf spot

Epidemics of light leaf spot on UK winter oilseed rape crops start with the release of ascospores from apothecia of *P. brassicae* in autumn (Figure 7, Figure 8). These apothecia are formed on infested plant debris from the previous cropping season (Gilles et al. 2001). The ascospores germinate on the leaf and penetrate the cuticle directly (Li et al. 2003). Li et al. (2003) have described the requirement for an extracellular cutinase that enables *P. brassicae* to penetrate the leaf cuticle. When the pathogen has infected the plant tissue it grows within the sub-cuticular niche without producing visible symptoms (Boys et al. 2007). The first signs of the disease after a period of symptomless growth are *P. brassicae* acervuli, which are formed in infected leaves. Under wet weather conditions, the acervuli disintegrate

13

into spore suspensions and conidia are washed off or splash-dispersed, causing secondary infections of crop plants (Evans et al. 2003). Secondary infection by splash-dispersed conidia explains the patchy distribution of light leaf spot in crops (Evans et al. 2003). Conidia and ascospores, which are developed on senescing tissue/leaves, can both infect stem and pod tissues. When plant tissues begin to senesce, the pathogen starts its sexual stage by producing the sexual fruiting bodies, apothecia, which serve as an inoculum source for the following cropping season (Boys et al. 2007).
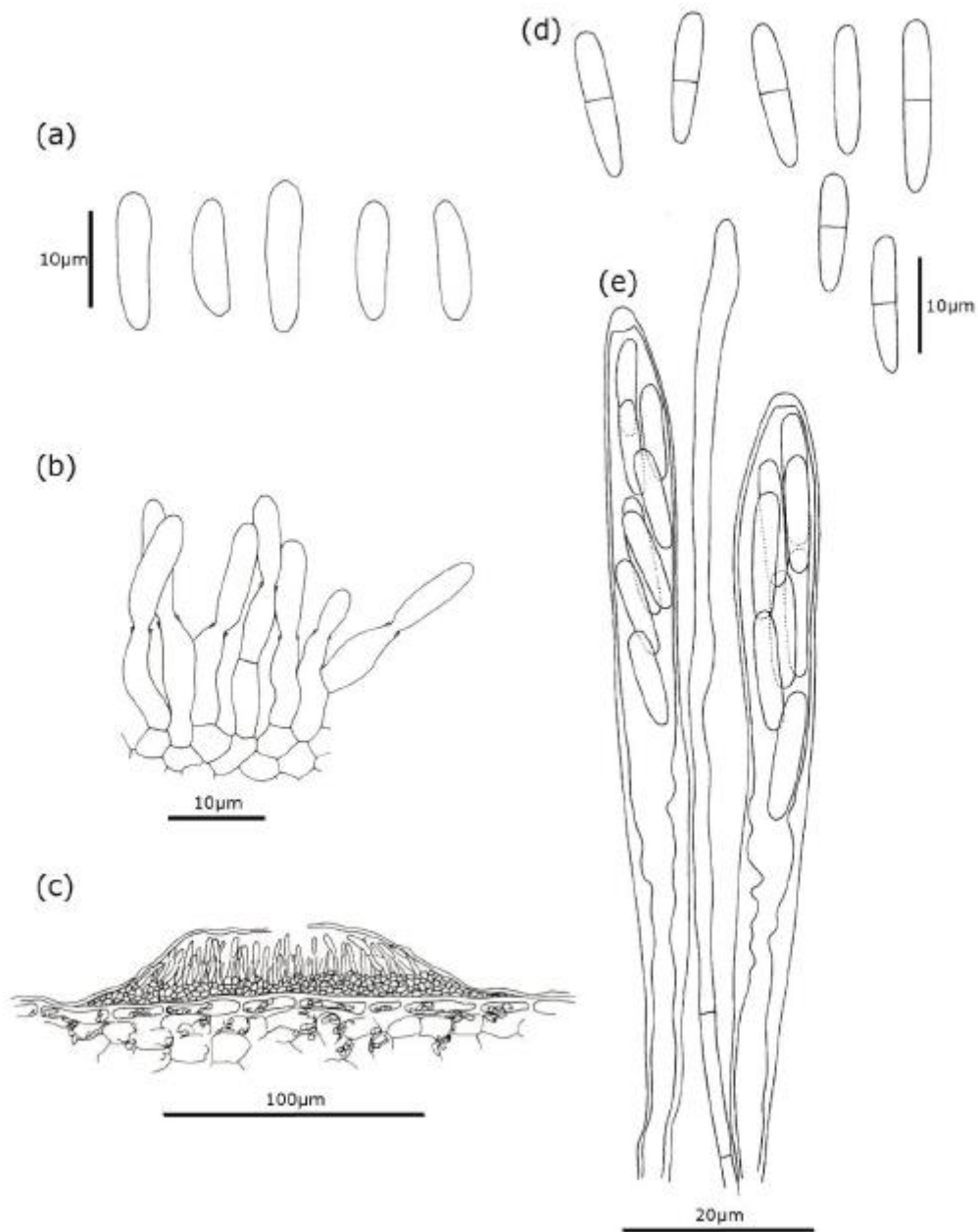
Figure 7. Spores of Pyrenopeziza brassicae: asexual forms: conidia (a), conidiophores (b) and acervuli (c); sexual forms ascospores (d) and asci (e) (CMI Descriptions of Pathogenic Fungi and Bacteria No. 536, Rawlinson et al. 1978, Boys 2009)
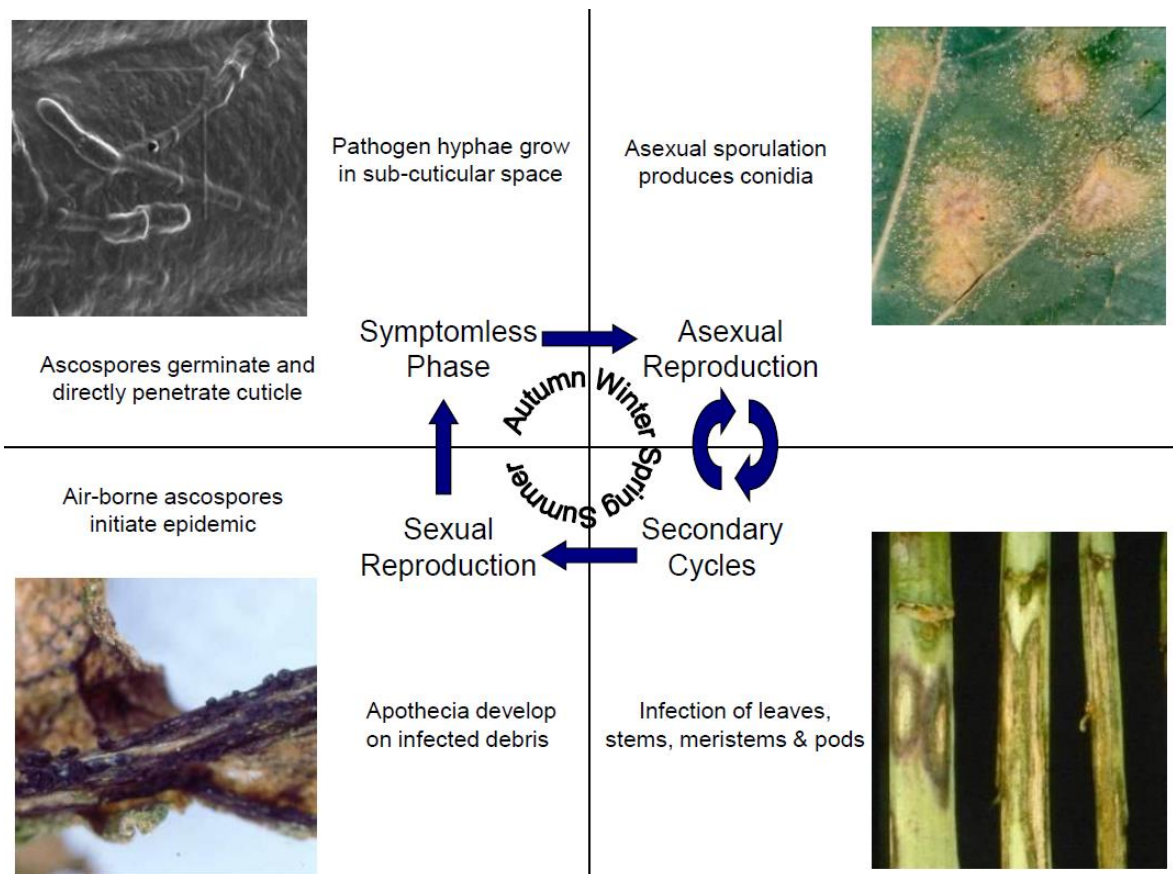
Figure 8. Life cycle of *Pyrenopeziza brassicae* on winter oilseed rape (graph: Bruce Fitt).

Diagnosis and symptom development on plants

Symptom development of light leaf spot on oilseed rape

After infection of oilseed rape by *P. brassicae,* the pathogen grows symptomlessly in leaf tissues until the occurrence of white pustules (acervuli), which erupt through the leaf surface (Fitt et al. 1998) (Figure 9). Affected leaves may show a slight yellowing of infected areas, which become more bleached and brittle (Fitt et al. 1998). Leaves may also show distortion and plants can be stunted when extremely colonized (Figure 9).

Light leaf spot symptoms other than the typical white pustules can easily be confused with those of abiotic symptoms such as frost damage or damage due to fertilizer treatments (Sue et al. 1998). Later in the cropping season, *P. brassicae* also infects stems and pods.

Symptom development of light leaf spot on vegetable brassicas

Light leaf spot symptoms are easier to diagnose in vegetable brassicas. As well as the sporulation/acervuli on leaves and buds, obvious dark concentric rings are formed on plant tissues (Figure 9). Symptoms start to develop in late October and continue on leaves and other organs of the vegetables, e.g. buds of Brussels sprouts.

Molecular diagnosis of *Pyrenopeziza brassicae*

Visual diagnosis is impossible during a symptomless growth phase of a pathogen. Therefore, early detection methods on a molecular basis are helpful. For *P. brassicae*, primer pairs have been developed to diagnose pre-symptomatic colonisation firstly by Foster et al. (1999, 2002). They also generated primers for discrimination between the two mating types of *P. brassicae.* Subsequently, Karolewski et al. (2006) sequenced the ITS (internal transcribed spacer) region and produced more sensitive primers for detecting *P. brassicae* colonisation of leaf material.
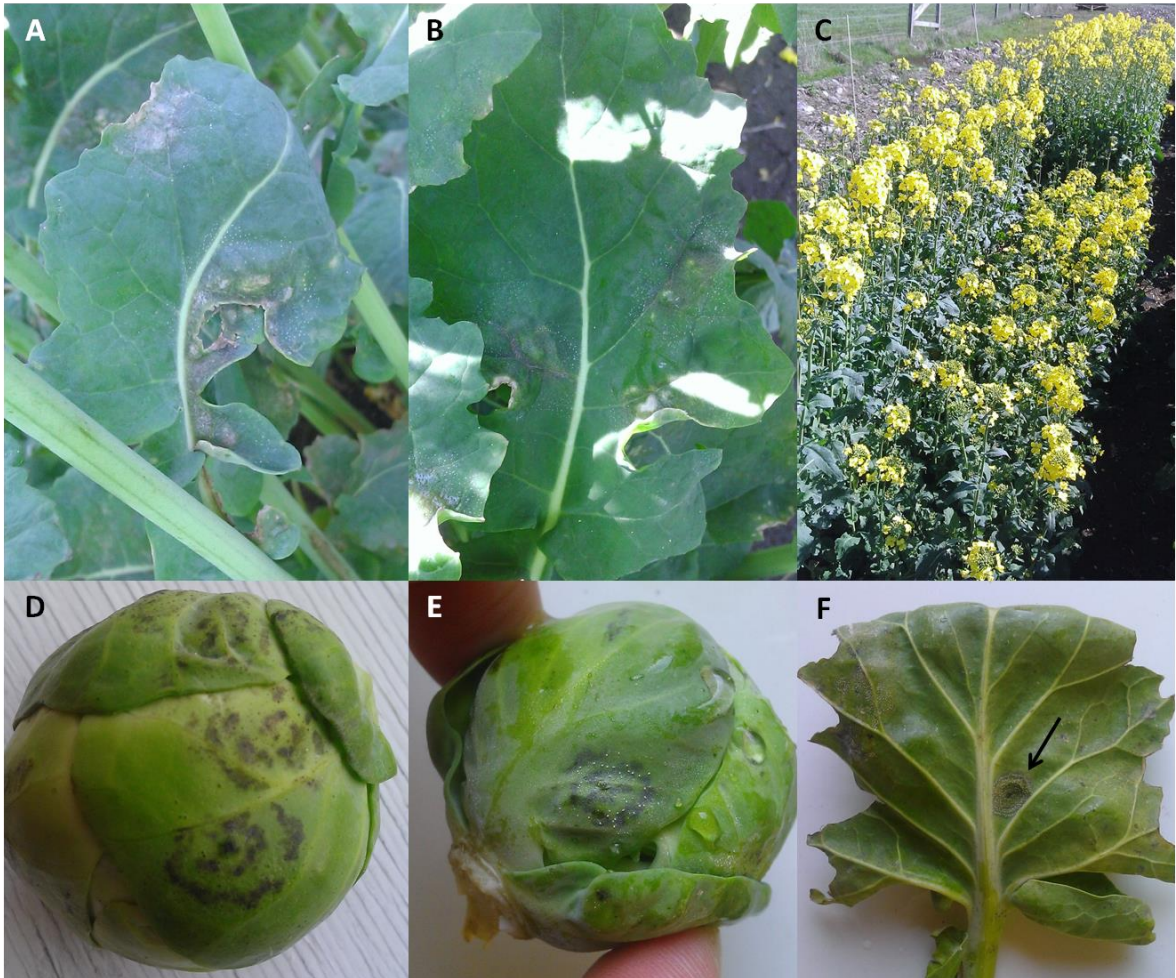
Figure 9. Light leaf spot symptoms on oilseed rape (A-C) and Brussels sprouts (D-F). A) distortion of a leaf and light leaf spot symptoms with sporulation, B) light leaf spot symptoms with sporulation, C) oilseed rape cultivar stunted (right hand side) due to light leaf spot compared with a more resistant cultivar (left hand side), D) light leaf spot symptoms on Brussels sprouts bud, E) sporulating symptom on Brussels sprouts bud, F) Brussels sprouts leaf with sporulating lesion (arrow).

IPM strategies to control light leaf spot

Integrated pest management (IPM) is defined by the FAO as: "*Integrated Pest Management (IPM) means the careful consideration of all available pest control techniques and subsequent integration of appropriate measures that discourage the development of pest populations and keep pesticides and other interventions to levels that are economically justified and reduce or minimize risks to human health and the environment. IPM emphasizes the growth of a healthy crop with the least possible disruption to agro-ecosystems and encourages natural pest control mechanisms." (FAO 2014).*

Farmers have the opportunity to influence the outcome of a disease event in the crop by decisions about cultural practices, cultivars and by the use of fungicides.

Cultural practices

Infested plant debris provides a source of inoculum of fungal pathogens. Fungi can survive on the debris and are able to infect crops in the following cropping season. On senescent plant debris *P. brassicae* forms apothecia, which release ascospores, the inoculum for initial infections in newly emerging crops (Gilles et al. 2001). With regards to soil cultivation, disease problems can be decreased by removing infected plant debris by ploughing (Bailey & Lazarovits 2003). Soil cultivation practices also removes volunteer plants. Volunteers can act as a "green bridge" between crops and therefore operate as another inoculum source. Maddock & Ingram (1981) first stated the importance of volunteers as a habitat for *P. brassicae* to survive over the uncropped period between harvest and establishment of new crop. A build-up of diseases can also caused by short crop rotations (Krupinsky et al. 2002). Intensification of agricultural environments has greatly decreased crop diversity and resulted in increased occurrence of diseases in crops (Tilman et al. 2002). Figueroa et al. (1994) observed a substantial increase in severity of light leaf spot in oilseed rape crops when oilseed rape was grown in two successive cropping seasons. Inclusion of a wider range of crops in a rotation programme can result in a decrease in disease problems.

Postponing the date for drilling oilseed rape by about two weeks can decrease severity of light leaf spot due to a change in coincidence of pathogen with a more

19

susceptible stage of the plant host or more likely the peak of ascospore release may have already happened (Welham et al. 2004). However, drilling an oilseed rape crop later can cause problems with phoma stem canker (causal agents: *Leptosphaeria maculans* and *L. biglobosa*). Infection of smaller plants can lead to a considerable increase in canker formation because smaller leaves and shorter petioles enable the pathogen to reach stem tissues quicker (Sun et al. 2001, Aubertot et al. 2004). An early sowing date for a rapid plant development is more advantageous for control of phoma stem canker, whereas later drilling may reduce development of light leaf spot.

Resistance against *Pyrenopeziza brassicae*

The use of resistant cultivars as measure for controlling diseases is usually the most efficient and environmentally friendly strategy.

Farmers have the opportunity to choose cultivars from the AHDB Cereals & Oilseeds recommended list that includes information about different traits, such as average seed yield, agronomic factors, seed quality and resistance information such as resistance against *P. brassicae* (AHDB Cereals & Oilseeds 2015a). Disease resistance is indicated on a scale from 1 to 9 with the higher numbers indicating better resistance. On the recommended list for the previous cropping season, the mean light leaf spot resistance ratings for listed cultivars recommended for the East/West and North region are 5.7 (SD = 0.74) and 6.36 (SD = 0.87) and for the current cropping season it is 6.0 for both regions (East/West SD = 0.69; North SD = 0.68), respectively. Therefore, the resistance in cultivars is generally moderate. Whereas in 2014/15 cultivars with a score of 8 or more were available with the cultivar Cracker (score = 8) and Cuillin (score = 8.7), in the current recommended lists the maximum resistance score for light leaf spot is 7. It seems that resistance of Cracker has been rendered ineffective in areas of Scotland (score = 7) and the cultivar Cuillin is no longer marketed. Therefore, there is a need for improved resistance against *P. brassicae* in cultivars.

To date, not many scientific studies on resistance against *P. brassicae* have been published. Pilet et al. (1998) first described quantitative resistance against the pathogen, which generally results in reduced disease progress. Qualitative resistance (major *R* gene resistance) has got a greater potential to restrict

pathogen growth but is usually less durable than quantitative resistance. However, Bradburne et al. (1999) have studied introgression of major resistance genes from wild *Brassica* material into oilseed rape cultivars and found two resistance genes, one located at the N1/A1 chromosome and the other on N16/C6. Boys et al. (2012) used the cultivar "Imola" for studies on resistance. This cultivar was derived from the material Bradburne et al. (1999) used. Boys et al. (2012) identified a single *R* gene on chromosome A1 which leads to the recognition of *P. brassicae* effectors and causes an atypical hypersensitive response with black necrotic flecking. This particular resistance gene (*R* gene) limits asexual sporulation but does not prevent sexual reproduction (Boys et al. 2012). A second major gene has not been identified in "Imola". The disappearance of the second *R* gene detected by Bradburne et al. (1999) could be result of a loss during the process of breeding for "Imola" (Boys et al. 2012).

Another mapping population, the Q population, has been derived from the same synthetic cross that was studied by Bradburne et al. (1999) and then backcrossed with a different cultivar "Tapidor". Segregation for resistance against *P. brassicae* was observed for the mapping population (Rachel Wells, personal communication). More work is needed to determine if the same *R* gene(s) can be detected as in the Bradburne material and cv. "Imola".

The existence of major *R* genes and the rapid observed loss of resistance in UK cultivars (example "Cracker") provide evidence for the existence of gene-for-gene interactions between *R* genes in oilseed rape cultivars and *P. brassicae* strains. Simons & Skidmore (1988) seem to have found differential interactions for *P. brassicae* and cultivars of *Brassica* species that indicate a gene-for-gene relationship. The interaction of *P. brassicae* with different *Brassica* species and cultivars should be investigated more closely.

Fungicide application

Chemical control of pests and diseases is a common tool to reduce yield losses. The timing of chemical applications is crucial for effective disease control. Fitt et al. (1999) have suggested a spraying regime of three applications during the growing season in the UK. The crop should receive the first application during the symptomless phase of pathogen growth in autumn followed by a second spray in

late winter that decreases the occurrence of secondary spread of the disease (Fitt et al. 1999). A third spray post-flowering should control pod infections which can lead to pod shatter and but is rarely necessary and may also increase losses through mechanical damage from equipment. The autumn spray is very important to substantially decrease light leaf spot disease incidence (Figueroa et al. 1994, Gilles et al 2000) but accurate timing of the first spray is very difficult because the farmer is not able to see the disease in the crop at that time.

Therefore, forecasting models have been developed to support farmers in their spraying decisions. Gilles et al. (2000) discussed possibilities for light leaf spot forecasts based on different sources of information; e.g inoculum based, disease assessment based or spore biology based forecasting. Currently, a forecasting model is provided by Rothamsted Research that predicts expected light leaf spot incidence and severity for the next spring (Anonymous 2015a). This forecast includes regional mean rainfall and summer temperature data from 30 years as well as data for pod disease incidence of the previous cropping season and is updated in spring for the deviation of winter temperatures from the 30 years mean (Anonymous 2015a).

Nevertheless, fungicide applications may still be not effective although they have been timed properly because of reduced fungicide sensitivity to *P. brassicae* strains to certain fungicide groups. Carter et al. (2013) found reduced sensitivity to methyl benzimidazole carbamate (MBC) and identified an amino acid substitution in the β-tubulin gene. MBCs bind to β-tubulin and interrupt the cell division process. Changes at the target site therefore, lead to decreased efficacy of the particular fungicide. Moreover, reduced sensitivity of azole fungicides, including imidazole and triazole, has been reported due to mutations in the *CYP51* gene encoding for sterol 14α-demethylase (Carter et al. 2014).

Due to the pathogen's evolutionary potential an increase of fungicide insensitivity may be considered which will cause more problems for the control of light leaf spot.

Ineffective control strategies make it necessary to understand the pathogen population better.

Genetic structure of plant pathogen populations

The field of population genetics focuses on evolutionary forces that lead to genetic change and diversity. Genetic variation and population structure of plant pathogen populations result from these evolutionary forces, coevolution with host plant species, and the biology of the pathogen. The genetic structure of pathogen species can generally be determined as the genetic diversity within and among populations (McDonald & Linde 2002). Determining the genetic structure of a plant pathogen population is crucial to the development of strategies to control the pathogen and can be used for improved disease management and resistance breeding.

Influences on pathogen population genetics and structure

Evolutionary forces affect the population genetics and, therefore, the structure of plant pathogen populations. These forces are mutation, genetic drift, gene flow, selection and pathogen biology (McDonald & Linde 2002, Barrett et al. 2008). Therefore, coevolution of pathogens with host plant species greatly influences pathogen population structures.

Coevolution of species describes the response of one species A to a change in a certain trait of the other species B, which than will respond to that new trait of species A (Janzen 1980). This can be applied to the gene-for-gene interactions of plant *R* genes and pathogen effector (*Avr*) genes. If a new *R* gene is introduced in an environment and widely exploited, the pathogen population is likely to change, so that isolates that are avirulent against that *R* gene are replaced by isolates that are virulent, according to the coevolutionary theory. The evolutionary potential of the pathogens is usually greater than that of plants because they have shorter generation times, greater population sizes, higher rates of mutation, and larger migration rates (Gandon & Michalakis 2002). However, host resistance genotypes can be very diverse and complex and can interact with the pathogen to influence the pathogen population structure to high extent (Barret et al. 2008). Therefore, both host and pathogen can show polymorphisms for *R* and *Avr* genes, respectively (Frank 1992). The change in a trait of the species can be initiated by mutations that lead to immediate changes in the genomic sequence of, for instance, the pathogen and introduce new alleles, such as these for virulence

23

(Gandon & Michalakis 2002, McDonald & Linde 2002). Rates of mutation are usually small but mutations appear more abundantly with increasing population size. Furthermore, enhanced frequencies of virulent alleles occur more often in agro-ecosystems when *R* genes are exploited on a large-scale and can result in so-called boom-and-bust cycles (McDonald & Linde 2002, Stukenbrock & McDonald 2008). If a certain *R* gene/cultivar is used extensively over a large area ("the boom"), the pathogen can change from avirulent to virulent due to a mutation. Natural selection for the virulent strains may occur and the effectiveness of the *R* gene decreases, which is followed by "the bust" when the cropping area of that *R* gene/cultivar reduces considerably.

Moreover, crop diversity in agro-environments is decreased which implies great availability of suitable host resources that leads to decreased between-population diversity (Barrett et al. 2008). In contrast, wild plant populations that are often partially isolated show less migration between pathogen populations and genetic drift and selection occurs more frequently, this can result in divergence among the pathogen populations (Barrett et al. 2008). Small populations are vulnerable to genetic drift (McDonald & Linde 2002). Genetic drift can be described as a random fluctuation of allele frequencies in consecutive generations, this means that alleles can be either fixed or lost in the population (Masel 2011). Gene flow counteracts the divergence of populations by breaking down boundaries (e.g. geographical) by exchange of alleles between populations, this leads to an increase in similarity between the populations (Slatkin 1985, McDonald & Linde 2002). The mode of dispersal of the pathogen is the main driver for gene flow between fungal pathogen populations (Barrett et al. 2008). Populations with the ability to spread their propagules over long distances (e.g. by wind-borne spores) tend to homogeneity between populations (Barrett et al. 2008). The spread of infective material is more limited when a pathogen depends on rain-splash, seedborne or soilborne dispersal (Barrett et al. 2008).

In addition to the spatial distribution of propagules, the type of propagules (i.e. sexual or asexual spores) is just as important and, therefore, the reproduction system plays a role for gene and genotype diversity of pathogens (McDonald & Linde 2002). Genotype diversity, which means the variation in the combination of alleles that occur across all loci, can be used to gain information about genetic

structure for both asexual pathogens and pathogens that use mixed reproductive systems (McDonald & Linde 2002). Sexual pathogens display high genotype diversities due to recombination and the measurement of gene diversity is more informative (McDonald & Linde 2002). Pathogens with mixed reproductive systems combine the advantages of sexual and asexual reproduction. Due to sexual reproduction, new allele combinations and genotypes occur and the most favourable combinations will be fixed and propagated through asexual reproduction. Consequently, pathogens with the ability to reproduce both sexually and asexually have the greatest potential for evolution (McDonald & Linde 2002). High complexity of the pathogen in terms of its life cycle leads to high diversity in genetic composition (Barrett et al. 2008).

The more diverse the pathogen population and the greater the potential of the pathogen to adapt to its environment the more difficult the control of the disease.

Aims of the study

Light leaf spot is a major problem in oilseed rape production at the moment due to a knowledge gap on both, the plant and the pathogen site. This PhD project focuses mainly on understanding the pathogen but also investigates the interactions with host plants. The aims of this project are:

- to study the *P. brassicae* population structure molecularly with the use of neutral markers
- to study the *P. brassicae* population structure morphologically
- to identify differential interactions between *P. brassicae* strains and oilseed rape/Brussels sprouts
- to determine if *P. brassica* is able to cross-infect *Brassicas*

General Material & Methods

Preparation of *Pyrenopeziza brassicae* inoculum from infected plant material

Plant material showing light leaf spot symptoms with erupting acervuli was selected from field sites. Plant material with symptoms of other fungal diseases was discarded. *P. brassicae* conidia were washed off by shaking the plant material in distilled water in polyethylene bags. The spore concentration of the resulting spore suspensions was counted using a Bright-Line™ haemocytometer slide and subsequently stored in -20 °C until use.

Growing conditions of plants

Plants used for the experiments were grown in a soil mixture containing 60% potting soil (Fruhstorfer Topferde Typ T, Hawita Gruppe GmbH, Vechta), 30% steamed compost and 10% sand. Seeds were sown in a seed tray which was covered by a glass plate for 3 days. Seedlings were transplanted into 7 x 7 x 8 cm^3 pots 6 days after sowing and grown at 21 °C day and 18 °C night temperature with a 16 hours photoperiod.

Five days before inoculation, plants were transferred into a controlled environment room with 16 °C day and 14 °C night temperature and a 12 hour photoperiod.

Plants were watered daily. Hakaphos® Blau including 15% nitrogen, 15% potassium dioxide, 10% diphosphorous pentaoxide and 2% magnesium oxide was used as fertilizer. A concentration of 3 g/l was applied every week beginning at 14 days after transplanting.

Inoculation of plants with *Pyrenopeziza brassicae* populations

Plants were spray-inoculated (Boots Travel Spray Bottle 50 ml) at BBCH 14 with mixed spore suspensions at a concentration of 10^5 spores/ml. Silwet® Gold, 0.1% concentrated, was added to the spore suspension as a wetting agent to prevent run-off. After leaves were evenly covered by spore suspension, plants were

packed into polyethylene bags for 48 h to ensure high humidity. Plants were maintained until assessment as described above.

Determination of the *Pyrenopeziza brassicae* population structure with molecular markers

Introduction

Information on population structure of plant pathogens can help improving control strategies against the disease the pathogen is causing.

Population genetics and structure can be determined by molecular markers to provide useful information for breeding of durable resistance (McDonald & McDermott 1993, McDonald & Linde 2002). Spatial distribution and the change of the pathogen population over time can be determined, that information can be used for deciding on strategies for resistance breeding (Peever et al. 2000, McDonald & Linde 2002).

Generally, molecular markers can be distinguished between dominant/recessive and co-dominant markers. Dominant/recessive markers only generate information about presence and absence of an allele whereas co-dominant markers also distinguish if the individual is homo- or heterozygous at the given locus and, therefore, provides information about allele frequencies (Mueller & Wolfenbarger 1999, Chail 2008, Allan & Max 2010).

For studies on population structure of pathogens diverse marker approaches are available. The first wide-spread marker technique used was RFLPs (restricted fragment length polymorphism). Firstly, DNA must be digested by restriction endonucleases that cleave the DNA into fragments of different length. Secondly, the fragments are electrophoretically separated before the hybridization of a probe and various other steps, involved in the method called Southern blotting (Southern 1975). RFLPs are co-dominant markers but this technique is very time-consuming and has lost importance because of high costs. These markers have been replaced (Nguyen & Wu 2005) by more efficient markers such as RAPD markers (random amplified polymorphic DNA). This marker technique is a PCR (polymerase chain reaction) based method. It involves primers that anneal randomly in the genome at multiple loci and amplify fragments that are shown as bands after gel electrophoresis (Kumar & Gurusubramanian 2011). The main advantage using RAPD markers is that no sequence information is needed for this

technique but the major disadvantage is their low reproducibility (Kumar & Gurusubramanian 2011). In combination with other markers considerable results could be produced. King et al. (2013) have studied the evolutionary relationships of *Rhynchosporium* species with RAPD markers and have confirmed their results with rep-PCR (repetitive sequence-based PCR) methods. King et al. (2013) were able to identify a new subspecies of *Rhynchosporium*, *R. lolii*, by the combination of pathogenicity tests, morphological studies and molecular markers.

AFLP (amplified fragment length polymorphism) markers combine characteristics of RFLPs and RAPDs; they are involving digestion of DNA by restriction enzymes and are based on PCR (Mueller & Wolfenbarger 1999). AFLPs do not allow conclusions on heterozygosity or allele frequencies because of their dominant mode of inheritance but they are reproducible, are high-resolution and do not require sequence information (Mueller & Wolfenbarger 1999). For example, Majer et al. (1998) have studied the population structure of 79 *Pyrenopeziza brassicae* isolates with the help of AFLP markers and found a high level of genetic diversity especially within regions.

With the improvement of sequencing techniques and increased availability of sequence data other marker types have been developed, for example minisatellites and microsatellites. Mini- and microsatellites characterised by tandem repeats of base pairs in genome sequence (Carter et al. 2004). The difference between the two marker types is the length of the repeat units. Minisatellites consist of six to 100 bp in tandem repeat units, whereas microsatellites are defined to consist of one to six bp motifs and both marker types are, therefore, summarised under the designation of variable number of tandem repeats (VNTR) (Vergnaud & Denoeud 2000, Carter et al. 2004). They are co-dominantly inherited, highly variable and are widespread in the eukaryotic genome (Carter et al. 2004). Mini- and microsatellites are used for DNA fingerprinting, phylogenetic studies and for determination of population structure (Vergnaud & Denoeud 2000, Carter et al. 2004). Both mini- and microsatellite markers have been described for the oilseed rape pathogen *Leptosphaeria maculans* that allow studies on pathogen population structure (Eckert et al. 2004).

There are many other marker approaches available and the type of marker to choose for a study depends on the questions to be answered. As an example, Allan & Max (2010) illustrated the decision-making process for selected markers based on taxonomic level in a diagram (Figure 10).
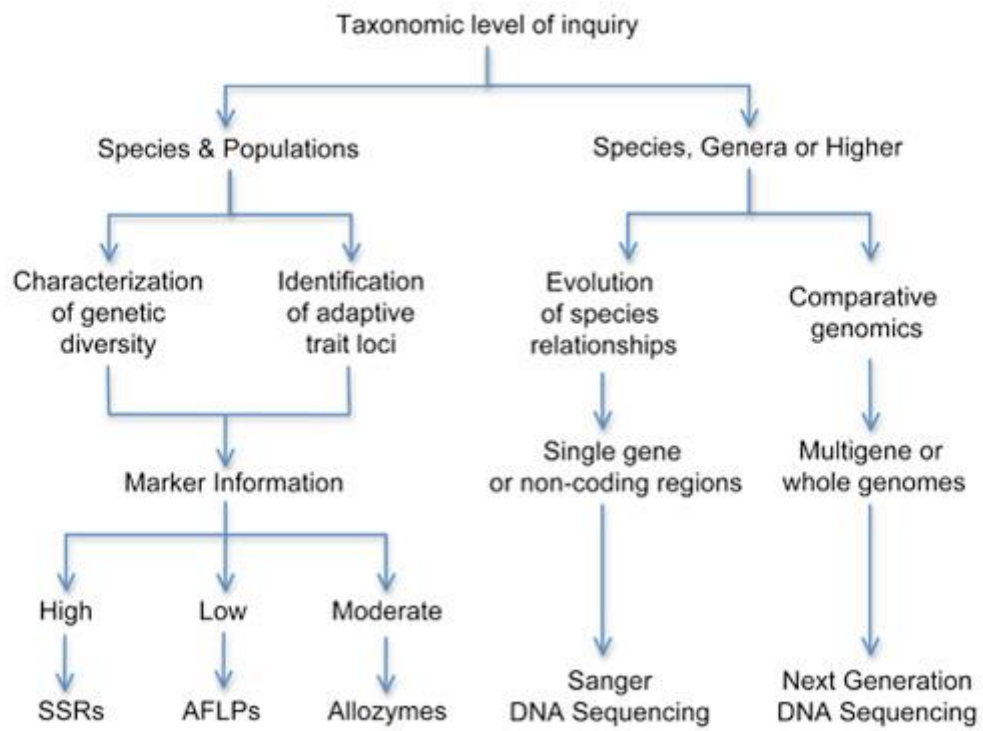
Figure 10. Decision tree for the use of molecular markers based on taxonomic level (Allan & Max 2010), Abbreviations: SSR = simple sequence repeats, AFLPs = amplified fragment length polymorphism

Due to the very little sequence information on *P. brassicae* the options for determining the population structure are limited to RAPD or AFLP markers. This project is planning on using RAPD markers as a start to determine the relationship between selected *P. brassicae* isolates. However, as RAPD markers are considered to be less reliable and AFLPs as alternative are difficult to work with it would be of interest to produce more sequence information to be able to choose state of the art marker techniques.

For the determination of differences between *P. brassicae* populations by molecular markers it was essential to collect a representative number of isolates.

The aim of this chapter is:

- To develop a diverse library of *P. brassicae* for the molecular studies on population structure

Material & Methods

Collection of *Pyrenopeziza brassicae* isolates

For the collection of *P. brassicae* isolates ten oilseed rape cultivars were chosen according to different light leaf spot disease ratings on the AHDB Cereals & Oilseeds recommended list (AHDB Cereals & Oilseeds 2015a). The cultivars were drilled at four different locations (Cambridgeshire, North Yorkshire and Herefordshire) by ADAS Ltd. and Elsoms Seeds Ltd in 2013/14. Younger leaves showing clear light leaf spot symptoms were sampled and wrapped into absorbent paper. Samples from other locations, especially vegetable samples, were kindly provided by various people.

Sampled leaves were incubated in polyethylene bags with a wet tissue at 10 °C for 4 days to enhance sporulation according to the method of Fitt et al. (1998). Leaves, suspected to be colonised by *P. brassicae*, which did not show sporulation after 4 days of incubation were incubated for up to 8 days longer.

Single acervuli were isolated with a sterile needle and placed onto PDA plates. After 7 days in a growth incubator at 15 °C the germinated non-contaminated

isolates were transferred onto MA plates and subcultured if they showed later contamination.

Results

Collection of *Pyrenopeziza brassicae* isolates

So far 734 isolates have been collected from 15 locations, nine in England and six in Scotland (Appendix, Table 4 - Table 6). 469 oilseed rape isolates have been collected from 17 different cultivars and 213 Brussels sprouts isolates from 14 cultivars. Furthermore, 11 isolates from white cabbage, 10 isolates from red cabbage, 29 cauliflower and 2 isolates from calabrese have been maintained.

Discussion

A substantial number of isolates were obtained which will be a good basis for molecular studies to determine the genotypic variation between isolates. Work on the population structure of *P. brassicae* by Majer et al. (1998) revealed high genetic diversity within regions but not between Scotland and England. However, more than 50% of the tested isolates were from Cambridge or Aberdeen. The present *P. brassicae* collection includes isolates from a wider range of locations.

Furthermore, with the increasing problems to manage the disease it is important to gain information about the current *P. brassicae* populations in the different areas. A change of the population since the study in 1998 is very likely as the pathogen has a high evolutionary potential due to its mode of reproduction (McDonald & Linde 2002).

It would be very beneficial to gain more sequence information of *P. brassicae* for the development of state-of-the-art markers, such as SSR markers, and to be able to screen for effector genes and other additional information of the pathogen (refer to Chapter 0).

Determination of the *Pyrenopeziza brassicae* population structure in field and *in planta*

Introduction

Information on population structure of plant pathogens can be gained according to their interactions with plant cultivars carrying different *R* genes. Frequencies of avirulent and virulent strains can be determined, respectively. The use of differential sets of host species is a useful tool to describe pathogen race composition of geographical regions where the commercial host species are cultivated and it can, therefore, be concluded on effectiveness of *R* genes in cultivars in the field. The information can be used for the rotation of *R* genes so that durability of these can be prolonged. Furthermore, the identification of pathogen races enables the screening of breeding material for major *R* genes that are recognising the effector genes of the pathogen. Therefore, this type of screening method is a valuable approach for the development and exploitation of resistance against pathogens (Peever et al. 2000).

The International Seed Federation has compiled a list of published differential cultivar sets for horticultural crops and corresponding pathogen races (http://www.worldseed.org/isf/differential_hosts.html). First described on the list is the interaction between *Phaseolus vulgaris* and *Colletotrichum lindemuthianum* distinguishing 11 major *R* genes and 19 pathogen races. However, not all described interactions are as complex such as the differential set of three cabbage cultivars (*B. oleracea*) for *Fusarium oxysporum* f. sp. *conglutinans* with two pathogen races to distinguish.

As an example for oilseed rape, a differential set of cultivars has been developed, with cultivars harbouring different *R* genes effective against strains of *L. maculans* (Balesdent et al. 2001). Extensive work has been undertaken to study the race structure of *L. maculans* and evaluate effectiveness of cultivars in the field and even rotate *R* genes (Balesdent et al. 2001, Balesdent et al. 2005).

Knowledge about the race structure of *P. brassicae* would be crucial for the better management of light leaf spot. The existence of at least one *R* gene and the rapid breakdown of cultivar resistance let suggest the presence of races of *P. brassicae.*

34

Information of cultivar resistance in field can indicate differences of the race structure of a pathogen at different locations.

Therefore, this chapter aims:

- To evaluate progress of the light leaf spot disease for oilseed rape cultivars from leaf to adult plant stage
- To identify differences of oilseed rape cultivars for *P. brassicae* resistance at different locations at leaf stage and before harvest
- To develop an inoculation method to test cultivar resistance in controlled conditions

Materials and Methods

Field sites

In cropping season 2013/14 field trials consisted of eight oilseed rape cultivars (not replicated) varying for light leaf spot resistance (AHDB Cereals & Oilseeds 2015a). The field trials in 2015/16 consisted of two additional cultivars and were replicated. Locations for each with co-ordinates year are listed in Table 2.

Field assessment

The field trials at the ADAS UK Ltd. sites were assessed for light leaf spot incidence (percent plants affected) and severity (percentage of plant leaf area affected) monthly from November to March with a main leaf assessment in March or April.

Assessments of stems and pods were done for the locations at ADAS Rosemaund, ADAS High Mowthorpe and Elsoms Seeds Ltd. at the end of the growing season in 2013/14 and 2014/15. An additional field site at Limagrain UK Ltd was included in 2014/15 but results are not shown because of very low disease severities. Severity scores were taken as percentage affected stem/pod area.

Controlled environment experiment

Cultivars "A", "E", "F" and "I" were inoculated with populations of *P. brassicae* spores from different locations (Herefordshire=Here2, Hertfordshire=Hert21, Aberdeen-shire=Scot46, Kincardineshire=Scot21 and Cambridgeshire=Camb13).

The experiment had 12 plants per treatment. Plants were randomized in a complete block design with 4 replicate blocks.

Table 2. Locations of field sites for cropping seasons 2013/14 and 2014/15

Cropping season	Company	Location	Grid Reference		Latitude	Longitude	X (Eastings)	Y (Northings)
2013/14	Trials Force	Potterton (Scotland)	NJ 15147	92668	57.226998	-2.123077	392668	815147
	ADAS High Mowthorpe	Settrington	N/A		N/A	N/A	N/A	N/A
	Elsoms Seeds Ltd	Deeping St. Nicholas	TF 16082	22153	52.728590	-0.192547	522153	316082
	ADAS Boxworth	Boxworth	TL 62060	34298	52.240351	-0.034578	534298	262060
	ADAS Rosemaund	Kings Caple	SO 29134	56186	51.958954	-2.6390286	356186	229134
2014/15	Trials Force	Potterton (Scotland)	NJ 15237	92406	57.227798	-2.127420	392406	815237
	ADAS High Mowthorpe	East Heslerton	SE 78087	93996	54.189947	-0.560963	493996	478087
	Limagrain UK Ltd	Caenby Corner	SK 89359	98071	53.391966	-0.526737	498071	389359
	Elsoms Seeds Ltd	Deeping St. Nicholas	TF 17015	21718	52.737079	-0.19865062	521718	317015

| ADAS Boxworth | Boxworth | TL 65536 | 34839 | 52.271448 | -0.025263620 | 534839 | 265536 |
| ADAS Rosemaund | Burley Gate | SO 47245 | 58763 | 52.121971 | -2.6037182 | 358763 | 247245 |

Results

Severities of light leaf spot on oilseed rape were different for the ADAS field sites with values below one percent at ADAS Boxworth and highest values ranging up to 9.5% at ADAS Rosemaund (Figure 11, Figure 13). Light leaf spot severities were lower in April compared to March at ADAS Boxworth, whereas severity from the first to the second time of assessment increased at the other two ADAS locations (Figure 11, Figure 12, Figure 13).

Differences between cultivars could be observed at ADAS High Mowthorpe and ADAS Rosemaund (Figure 12, Figure 13).
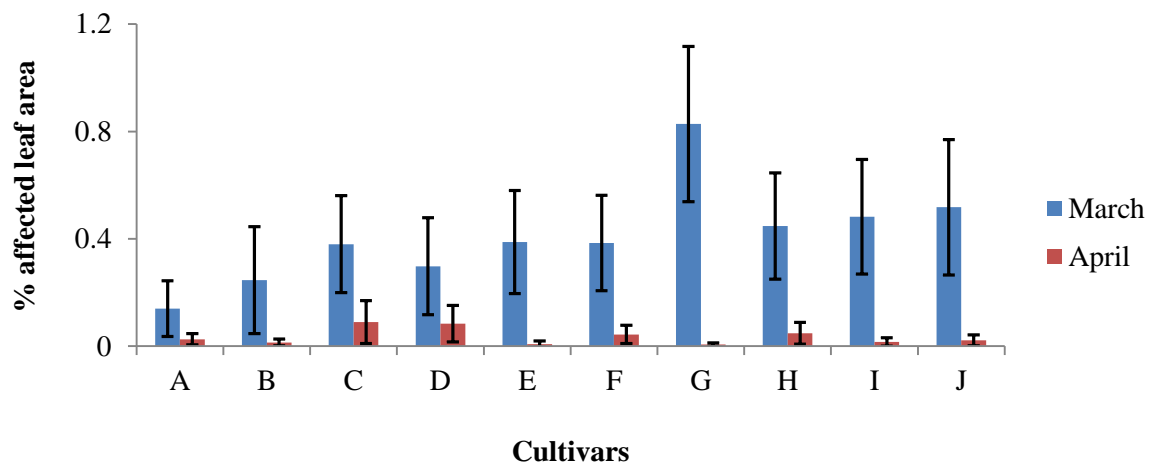
Figure 11. Severity of light leaf spot (in % affected leaf area) on cultivars (A to J) at ADAS Boxworth (Cambridgeshire) in March and April 2015

Bars show mean and error bars the standard error.
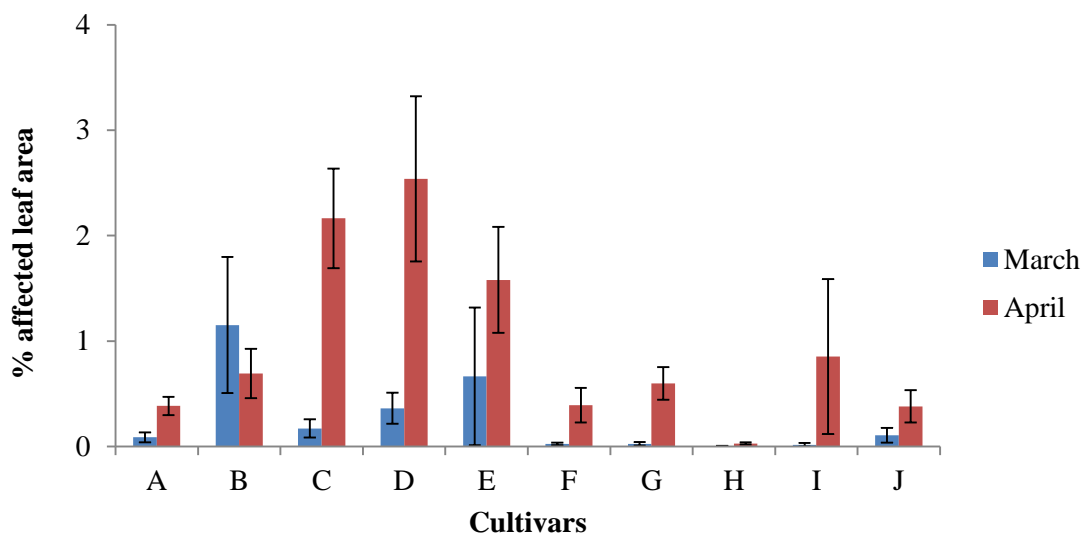
Figure 12. Severity of light leaf spot (in % affected leaf area) on cultivars (A to J) at ADAS High Mowthorpe (Yorkshire) in March and April 2015

Bars show mean and error bars the standard error.
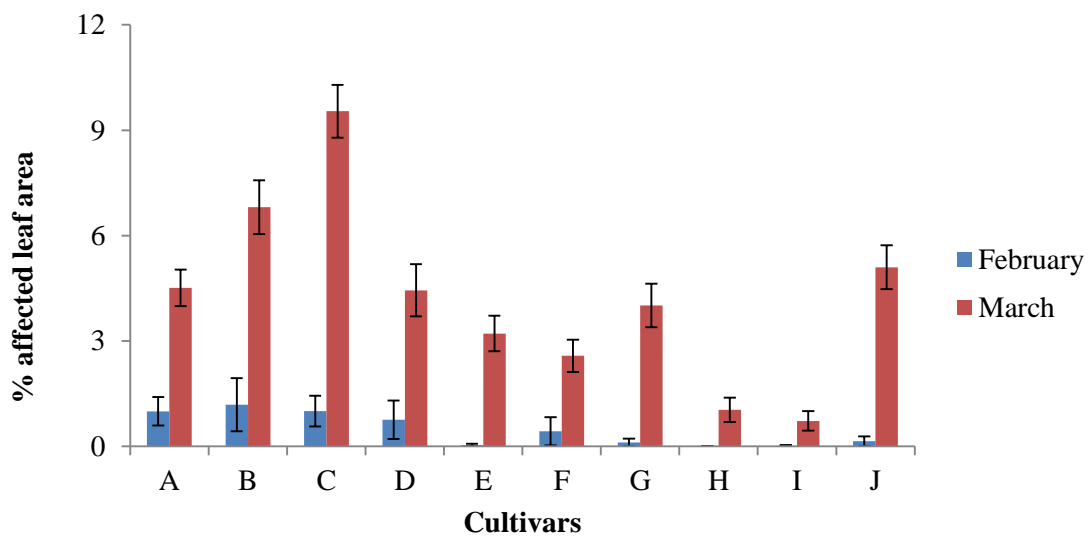
Figure 13 Severity of light leaf spot (in % affected leaf area) on cultivars (A to J) at ADAS Rosemaund (Herefordshire) in February and March 2015

Bars show mean and error bars the standard error.

Light leaf spot assessments for severity on oilseed stems were higher in 2014 compared to 2015 (Figure 14, Figure 15). Disease severity was highest at ADAS Rosemaund (Herefordshire) for both years.

Cultivar J has the lowest disease severity values at all locations for both years.
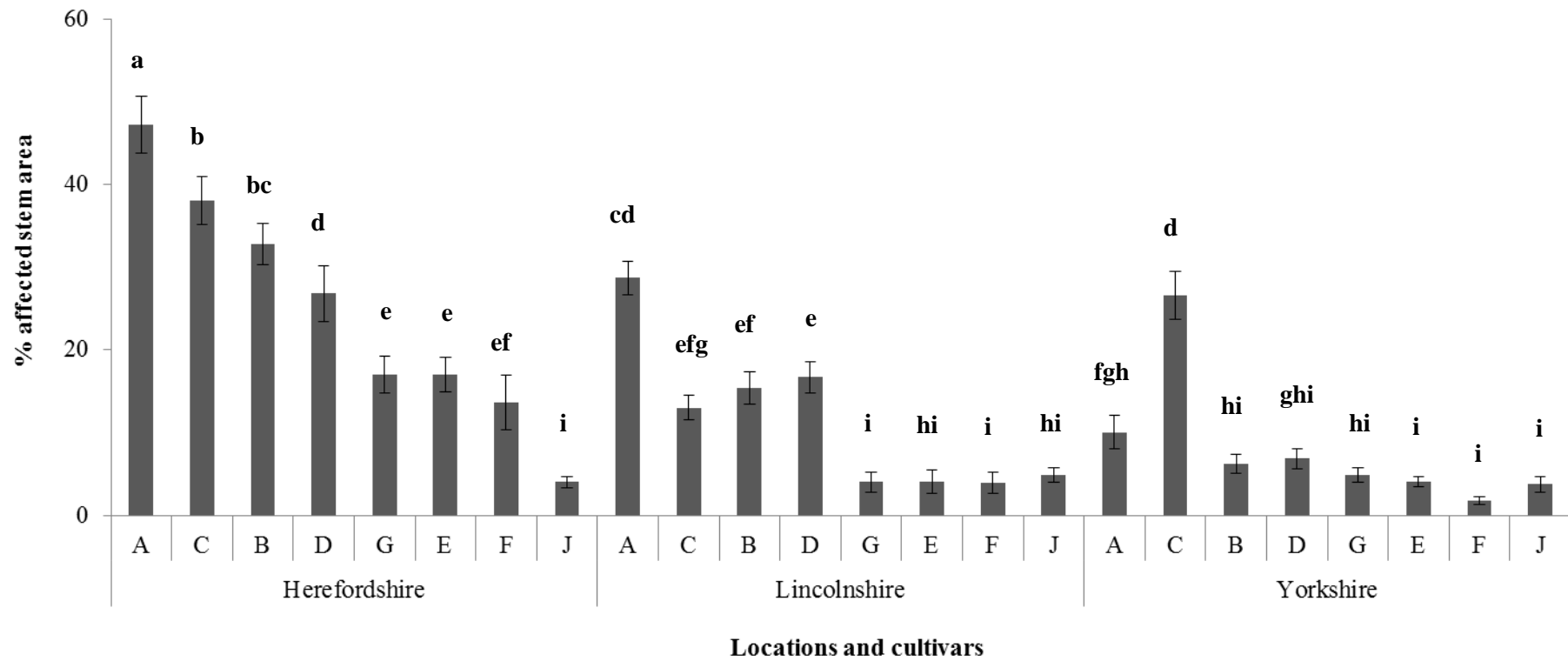
Figure 14. Light leaf spot disease severity (% affected stem area) of oilseed rape cultivars (A-G, J) before harvest at different locations in 2014

Location Herefordshire (ADAS Rosemaund), Lincolnshire (Elsoms Seeds Ltd.), Yorkshire (ADAS High Mowthorpe). For exact locations please refer to Table 2. Bars display mean values and error bars are the standard error. Different small letters show significant differences at $\alpha=0.05$
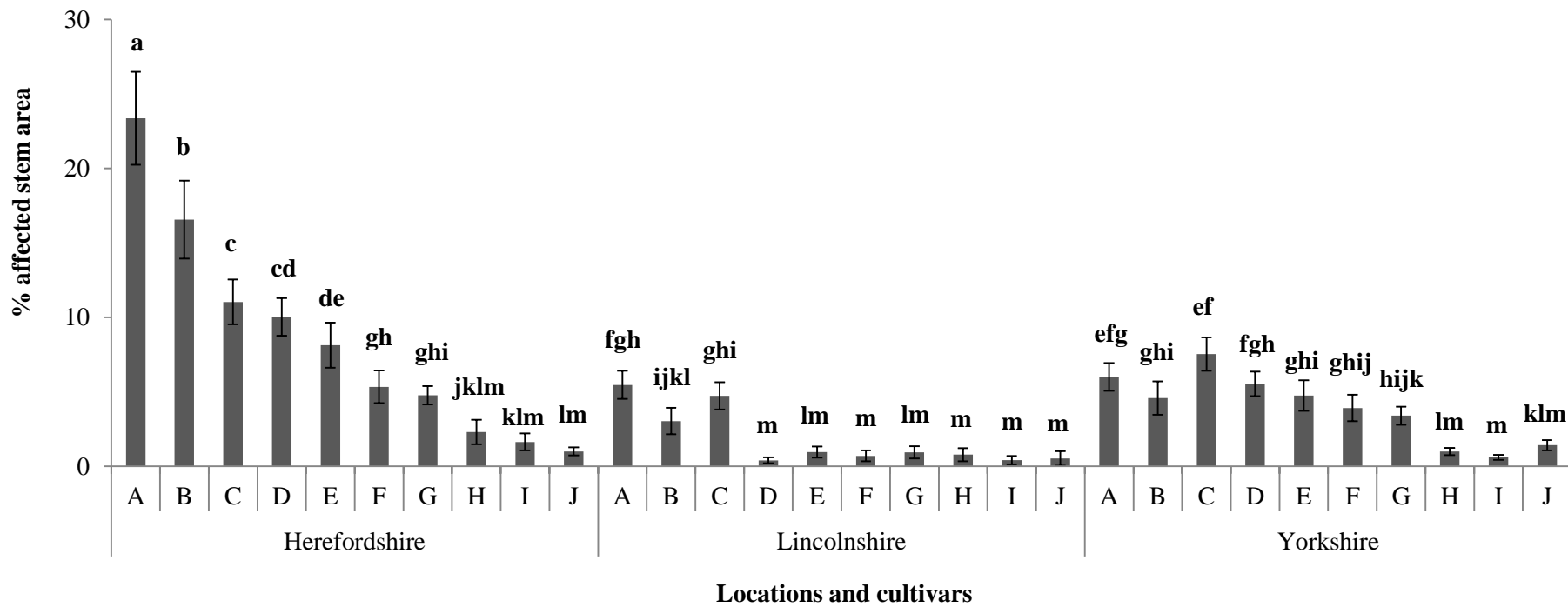
Figure 15. Light leaf spot disease severity (% affected stem area) of oilseed rape cultivars (A-G, J) before harvest at different locations in 2015

Location Herefordshire (ADAS Rosemaund), Lincolnshire (Elsoms Seeds Ltd.), Yorkshire (ADAS High Mowthorpe). For exact locations please refer to Table 2. Bars display mean values and error bars are the standard error. Different small letters show significant differences at α=0.05

For the controlled environment experiment differences between cultivars, isolates and the interaction between cultivars and isolates were significant (P<0.05; Figure 16)

The spore suspension from Hereford (Here2) was as virulent as the isolates originated from Scotland (Scot46, Scot14), whereas the isolate from Cambridgeshire caused lowest light leaf spot severity (Camb13).

Cultivar F shows clear differences between isolates, whereas cultivar E shows lower variation between the isolates.
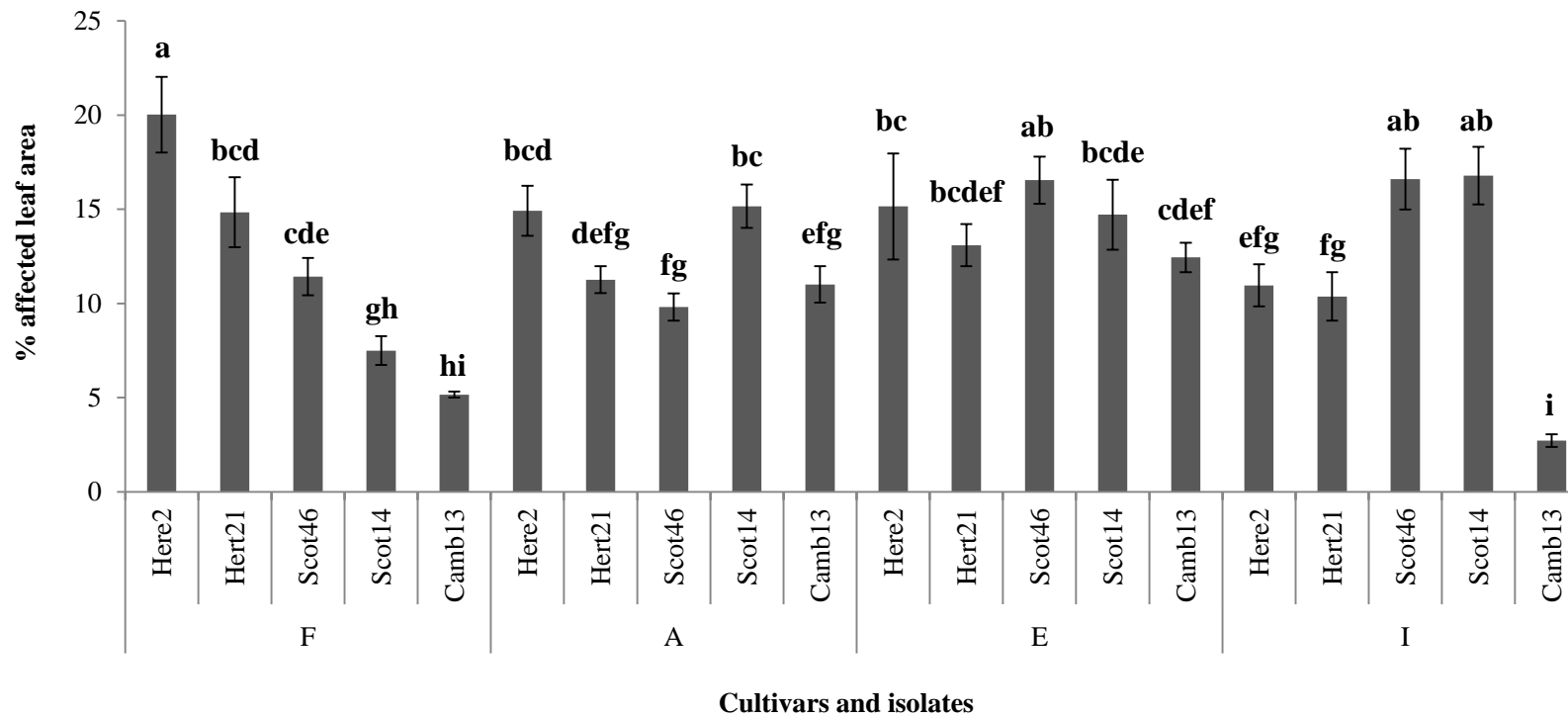
Figure 16. Light leaf spot severity (% affected leaf area) of oilseed rape cultivars (F, A, E, I) artificially inoculated with spore suspensions from leaf washings

Spore suspension originated from Herefordshire=Here2, Hertfordshire=Hert21, Aberdeenshire=Scot46, Kincardineshire=Scot21 and Cambridgeshire=Camb13. Bars show mean values and error bars are standard error. Different small letters indicate significant differences at α=0.05
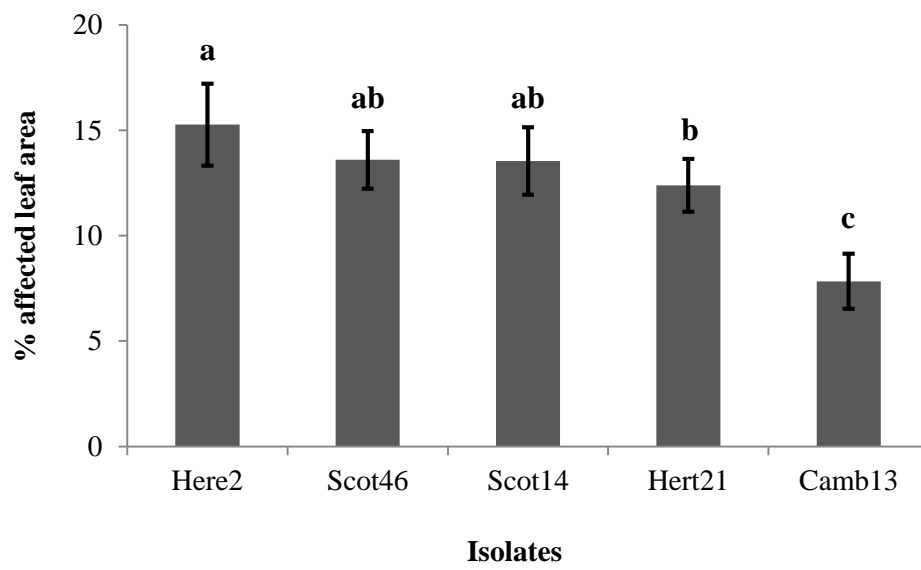
Figure 17. Light leaf spot severity (% affected leaf area) caused by *Pyrenopeziza brassicae* isolates across the

Discussion

AHDB Cereals & Oilseeds publish a list of oilseed rape cultivars evaluated for certain traits such as resistance against *P. brassicae* (AHDB Cereals & Oilseeds 2015a). The evaluation follows a protocol with assessments from leaf production up to end of flowering in a 28 day interval if disease is continuously progressing (AHDB Cereals & Oilseeds 2015b). For the assessment the amount of *P. brassicae* sporulation is taken into account. The occurrence of sporulation as disease assessment criterium alone could be unreliable as asexual conidia can be washed off by rain or even morning dew. That is the most likely explanation for lower disease severity scores at ADAS Boxworth in April compared to March 2015. Moreover, the disease pressure was very low and therefore, the assessment and differentiation between cultivars for their resistance against *P. brassicae* is difficult. An influence of sampling technique is unlikely, although light leaf spot occurs patchy and the assessment could be influenced by that, but the results would have been more inconsistent (Gilles et al. 2000, Evans et al. 2003). Incubating the leaves in polyethylene bags would be a solution to avoid underestimation of the amount of affected leaf area by *P. brassicae* when sporulation is absent (Fitt et al. 1998).

In contrast to the results at ADAS Boxworth, progression of the disease was recorded for ADAS High Mowthorpe and ADAS Rosemaund with more disease at the later assessment compared to the earlier. Although the disease pressure in spring at ADAS High Mowthorpe was only little higher than at ADAS Boxworth (Maximum Mean ± SE = 2.538 ±0.78, cultivar D in April 2015) differentiation of cultivars for resistance is possible. Cultivar "C" had high severity scores at ADAS High Mowthorpe and ADAS Rosemaund, whereas cultivar "H" was most resistant at both locations.

However, in terms of occurrence of pathogen races cultivar differences between locations are important to be taken into account. Cultivar "J" could give a hint towards varying pathogen population structure between locations with an intermediate disease severity score at ADAS Rosemaund in March 2015 compared to a low disease severity score at ADAS High Mowthorpe. This leads to the assumption that the *P. brassicae* population at the location in Herefordshire

might consist of higher abundance of isolates that can overcome the resistance of cultivar "J" at leaf stage than the location in Yorkshire. Interestingly, the stem data for disease severity of cultivar "J" shows very good resistance for both years and both locations (Figure 14, Figure 15). In 2014, light leaf spot severity on stems of cultivar "A" was higher than on "C" in Herefordshire but "C" was more susceptible than "A" which could also be an indicator for different populations. This was not seen end of the season in 2015 which can be due to different field sites between the two cropping seasons that differed by ten miles at ADAS Rosemaund (Herefordshire) and nine miles at ADAS High Mowthorpe. Similar to cultivar "J", leaf assessment for "A" is different to observations at stems. Cultivar "A" performs well at leaf stage but had highest disease severity scores on stems for both years, in particular at ADAS Rosemaund. This suggests that resistance in leaves could be different to resistance in stems for specific cultivars. This could be problematic for screening methods in controlled environments that are used to estimate cultivar resistance in the field.

Although, the evaluation of cultivars for resistance against a pathogen experiments in controlled environments (CE) can be done to gain information about the mode resistance of cultivars against and to identify gene-for-gene interactions between the pathogen and the cultivar, which can be used to describe the pathogen population. In this study cultivar "E" has the highest disease severity scores in the CE experiment compared to "A", "F" and "I" that are not significantly different from each other. This is not in accordance with the findings in the field where cultivar "E" is not particularly more susceptible to the pathogen.

Cultivar "I" is most susceptible to the Scottish populations which was expected as the resistance of that cultivar is suspected to be broken down in Scotland. The resistance of cultivar "I" seems effective against the population Camb13 from Cambridgeshire. A potential reason for this could be exploitation of the cultivar in the particular areas. Unfortunately no statistics are available for cropping areas of cultivars per region.

Virulence of population Camb13 is lowest in this experiment which can be related to the interactions with the cultivars "F" and "I". It could be suggested that there are

different pathogen populations at different locations due to varying proportions of virulent and avirulent isolates/races within the tested populations.

Especially cultivar "F" shows varying resistance for the *P. brassicae* populations from different locations. This again indicates varying pathogen population structure.

However, the interaction of cultivars with *P. brassicae* should be tested with defined isolates to allow clear assumptions on gene-for-gene interactions and the determination of pathogen races. At least, according to the described results, it is likely that different races of the pathogen are present. Moreover, it should be tested if resistance against *P. brassicae* is organ-specific for some cultivars as the ranking of cultivars were different for leaf and stem assessments.

Host range of *Pyrenopeziza brassica* in the *Brassica* genus

Introduction

Plants face a great variety of microbes in their environment but only a small number of microbes are actually able to infect the plant species to cause disease. This phenomenon is based on the concept of non-host resistance and host resistance of the plants, reciprocal recognition and subsequent plant defence responses involved.

Non-host resistance is a result of structural and biochemical mechanisms that can either be constitutive or induced and cannot be overcome by a non-adapted/non-host pathogen (Thordal-Christensen 2003, Lipka et al. 2008).

Firstly, the pathogen encounters the plant surface and a number of pathogens require specific plant signals to distinguish between a host and non-host plant [described for rust fungi (Hoch et al. 1987, Thordal-Christensen 2003)]. The composition of the cuticle varies greatly and can define the outcome of a penetration event by a fungal pathogen. Attachment and germination of some fungal spores requires a moist environment and cuticular waxes influence both the hydrophilicity of the plant surface and surface topology (Kerstiens 2000). The germination of the fungal pathogens causing stemphylium leaf spot in clover, *Stemphylium* spp., is enhanced with increasing free water availability (Bradley et al. 2003). Furthermore, leaf wax constituents can affect infection processes of fungal pathogens, e.g. some leaf wax constituents of rice (*Oryza sativa* L.) inhibit appressoria formation by *Magnaporthe grisea*, whereas others contribute to it (Uchiyama et al. 1979, Uchiyama & Okuyama 1990, Howard & Valent 1996). Both, these preformed structural defence mechanisms and constitutive biochemical compounds can influence determination of the host range of a pathogen. Plants constitutively produce compounds that have an antimicrobial effect, so-called phytoanticipins. They are low molecular weight constituents of the plant secondary metabolism, i.a. phenols, phenolic glycosides, saponins and glucosinolates (Osbourn 1996a). For example, phenolic compounds inhibit germination of spores of *Colletotrichum circinans* (cause of onion smudge) on yellow and red skinned onions, whereas colourless onions are susceptible to the pathogen (Link & Walker

52

1933). Another group of secondary metabolites is saponins, which can be found in many plant families; these show a broad range antifungal effect by interacting with membrane sterols leading to loss of membrane integrity (Osbourn 1996b). The saponin avenacin is an important determinant of the host range of the pathogen causing take-all disease in cereals, *Gaeumannomyces* (Bowyer et al. 1995). *Gaeumannomyces graminis* var. *tritici* is not able to infect common oats (*Avena sativa* L.) or bristle oats (*Avena strigosa* Schreb.) because of its susceptibility to avenacin (Osbourn et al. 1994). In contrast, *G. graminis* var. *avenae* is less sensitive to avenacin because it produces the corresponding detoxifying enzyme avenacinase (Osbourn et al. 1991). In Brassicaceae the most abundant phytoanticipins are glucosinolates. When cells are injured, glucosinolates are hydrolysed into diverse products, such as isothiocyanates, thiocyanates, nitriles and other indol-3-ylmethyl derivatives, by an enzyme β-thioglucosidase, also called myrosinase (Velasco et al. 2008, Redovniković et al. 2008). The resulting compounds are beneficial to the plant as constitutive defence mechanisms, due to their biological activity against herbivorous insects and some pathogens (Kliebenstein et al. 2005, Bednarek et al. 2009, Hopkins et al. 2009).

Moreover, structural changes and biochemical defence can be induced by pathogen activity. Abscission layers are formed on leaves of cherry laurel (*Prunus laurocerasus* L.) to prevent the colonisation of healthy plant material by *Clasterosporium carpophilum* (Samuel 1927). Thereby, cells surrounding the lesion swell and the middle lamella dissolves so that the affected area of the leaf is cut off with the loss of a few layers of healthy plant cells (Agrios, 1969). Restriction of pathogen growth can be initiated by modification of cell walls, e.g. formation of papillae. Papillae are cell wall thickenings containing callose and occur around the sites of infection by pathogens (Prats et al. 2005, Luna et al. 2011). Resistance of barley (*Hordeum vulgare* L.) against the powdery mildew pathogen *Blumeria graminis* f.sp. *hordei* is partially based on formation of papillae (Prats et al. 2005). In terms of biochemical defence responses antimicrobial compounds, phytoalexins, can be produced and accumulated after both biotic and abiotic stresses (Van Etten et al. 1994). An example of the importance of phytoalexins in pathosystems is the interaction of faba beans (*Vicia faba* L.) with *Botrytis cinearea* and *B. faba* with involvement of wyerone acid (Mansfield & Deverall 1974). The

rapid increase in concentration of wyerone acid after infection by *B. cinerea* led to an incompatible interaction, whereas the increase in concentration of this phytoalexin after infection by *B. fabae* led to a compatible interaction because *B. fabae* is less sensitive to the compound than *B. cinerea*. As well as phytoalexins, reactive oxygen species (ROS), pathogenesis related proteins (PR proteins) and rapid programmed cell death (hypersensitive response (HR)) play a role not only in non-host-microbe interactions but also in host incompatible interactions with a pathogen (Nürnberger & Lipka 2005, Hiruma et al. 2013). ROS such as superoxide, hydrogen peroxide and hydroxyl radicals, are components of the defence signalling chain and are released by some plants immediately after pathogen infection (Torres et al. 2006). Release of ROS results in the hyperoxidation of membrane phospholipids and the formation of lipid hydroperoxides followed by cell membrane damage (Gutterbridge 1995). Another induced biochemical response of the plant to inhibit pathogen infection and growth is the formation of pathogenesis related proteins (PR proteins). PR proteins have been detected in many plant species, for example PR2 coding for β-1,3 glucanases and PR3 coding chitinases are able to degrade cell wall components of fungi and therefore decrease colonisation by fungal pathogens, especially when both compounds are produced (Mauch et al. 1988). HR results in localized cell death around the site of infection to restrict pathogen growth, particularly for interactions with biotrophic pathogens (Heath 2000, van Doorn et al. 2011, Hiruma et al. 2013).

Induced plant defence involves recognition of the microbe or recognition of microbial activity, respectively (Thordal-Christensen 2003, Schulze-Lefert & Panstruga 2011). Pathogen-associated molecular patterns (PAMPs) are recognised by pattern-recognition receptors of the plant (PRRs) leading to resistance responses, called PAMP-triggered immunity (PTI) (Jones & Dangl 2006, Boller & Felix 2009, Dodds & Rathjen 2010, Thomma et al. 2011). Another mode of plant immune response involves effector protein recognition of pathogens that are recognised by plant receptors and is known as effector-triggered immunity (ETI) (Jones & Dangl 2006, Schulze-Lefert & Panstruga 2011). ETI is considered to interact with specific strains/isolates of an adapted pathogen and therefore describes the concept of gene-for-gene interactions (Schulze-Lefert & Panstruga

2011). Another term ETD, effector-triggered defence, was introduced by Stotz et al. (2014) distinguishing between extra- and intra-cellular pathogens that cause a rapid (ETI) or a delayed (ETD) defence response of the plant and the ability to restrict or slow pathogen colonisation, respectively. PTI and ETI can both contribute to non-host resistance (Lipka et al. 2008).

The host range of pathogens within the Brassicaceae family and *Brassica* genus may be restricted. An example is the oomycete *Candida albugo*, causing white blister in oilseed rape and vegetable *Brassicas*, that could cause disease on *Arabidposis thaliana* when isolated from *B. oleracea* (Borhan et al. 2008). Isolates that originated from *B. juncea* or *B. rapa* are adapted isolates of *A. thaliana* and have been named *Albugo laibachii* (Thines et al. 2009, Schulze-Lefert & Panstruga 2011).

This chapter aims:

- To determine if *P. brassicae* populations from oilseed rape are able to infect Brussels sprouts and vice versa
- To test the host range of *P. brassicae* populations from oilseed rape an Brussels sprouts on other vegetable brassicas

Materials and Methods

Two experiments were done: Experiment 1 focussed on cross-infection between oilseed rape and Brussels sprouts and Experiment 2 included other vegetable brassicas, such as broccoli, cabbage, cauliflower and romanesco.

Inoculum was derived from infected material of oilseed rape and Brussels sprouts from different locations in the UK (Table 3).

For Experiment 1, twenty-four plants were inoculated per treatment. The experiment was randomized in a complete block design with eight replicate blocks. The experiment was replicated.

Experiment 2 had twelve plants per treatment and was randomized in a complete block design with four replicate blocks.

Plants were assessed for percentage of affected leaf area. The main assessment was done 28 days past inoculation (dpi). The replicate experiment of Experiment 1 was also assessed at 21 and 35 dpi. Plants from the replicate of Experiment 1 were harvested at 35 dpi and incubated in polyethylene bags for 24 h for another assessment of affected leaf area.

Experiment 2 was assessed after plants were incubated 28 dpi.

Table 3. Origin of *Pyrenopeziza brassicae* populations (from leaf washings) used for experimental work

	Spore suspension	Species	Location
Experiment 1	BSpr1	Brussels sprouts	Spalding (Lincolnshire)
	BSpr2	Brussels sprouts	St Andrews (Scotland)
	OSR1	Oilseed rape	Aberdeen (Scotland)
	OSR2	Oilseed rape	Boxworth (Cambridgeshire)
Experiment 2	BSpr1*	Brussels sprouts	Spalding (Lincolnshire)
	OSR1*	Oilseed rape	Aberdeen (Scotland)
	OSR3	Oilseed rape	Kings Caple (Herefordshire)
	OSR4	Oilseed rape	Harpenden (Hertfordshire)

*same as Experiment 1

Results

In Experiment 1 both oilseed rape and Brussels sprouts showed leaf symptoms for spore suspensions BSpr 1, OSR 1 and OSR 2 but not for BSpr 2 (Figure 18). The oilseed rape cultivar was significantly more affected by light leaf spot than Brussels sprouts (P < 0.001).

In Experiment 2, all brassicas were infected and developed light leaf spot symptoms (Figure 19). There was a significant difference between the oilseed rape isolates and the Brussels sprouts isolate (P < 0.001) with the Brussels sprouts isolate being less virulent. The interaction between species and isolates was also highly significant (P < 0.001).
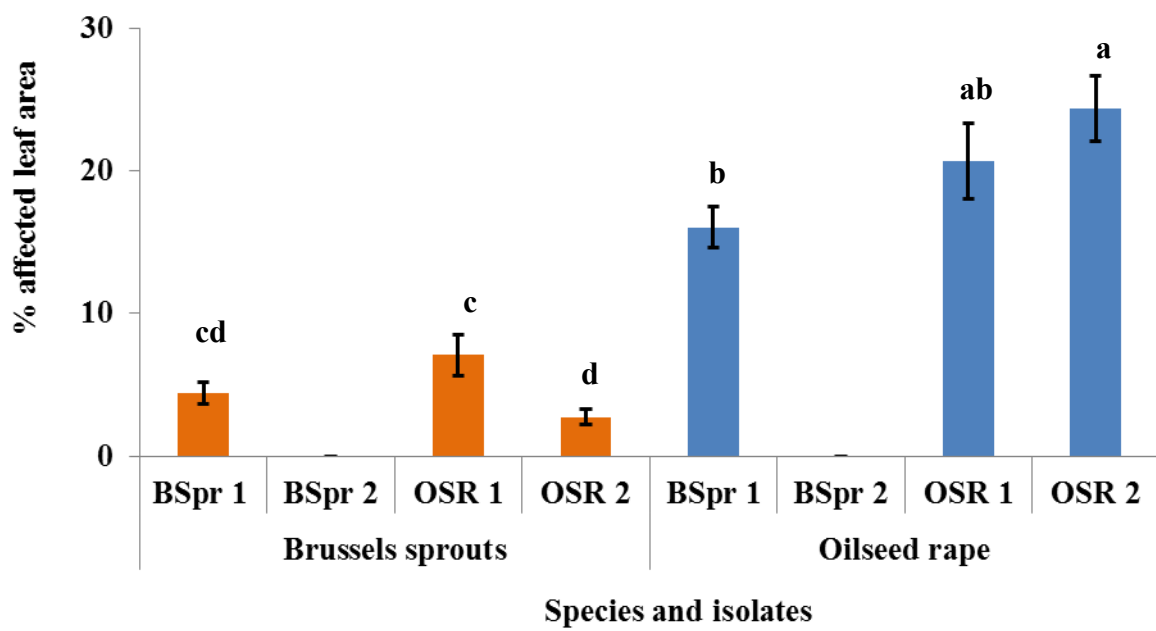
Figure 18. Light leaf spot severity (in % affected leaf area) of Brussels sprouts and oilseed rape with populations of *Pyrenopeziza brassicae*

Populations are originated from Brussels sprouts (BSpr 1, BSpr 2) and oilseed rape (OSR 1, OSR 2). Bars show mean and standard error. Different letters indicate significant differences at $\alpha=0.05$.
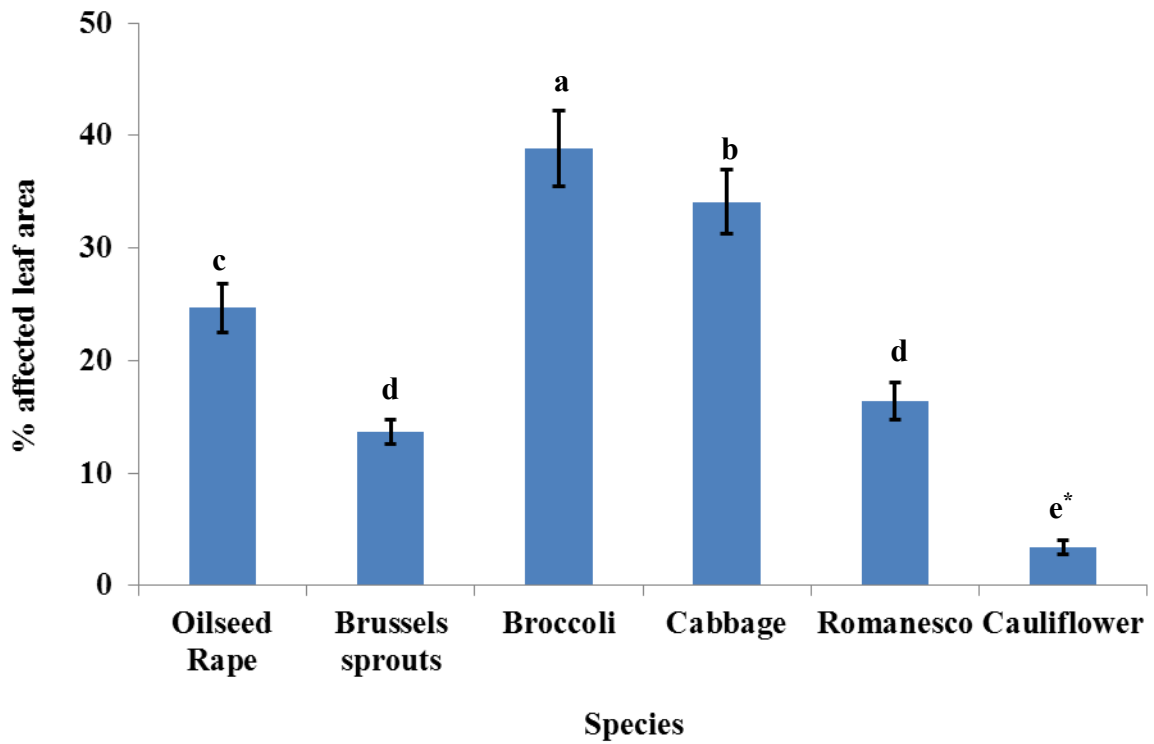
Figure 19. Light leaf spot severity (in % affected leaf area) of different Brassica species with populations of *Pyrenopeziza brassicae*

Bars show mean and standard error. Different letters indicate significant differences at α=0.05. *Result of cauliflower not comparable due to extensive loss of infected leaves

Discussion

Knowledge of the host range of a pathogen is vital when different species of the same family are grown in the same area. This can influence disease epidemics and more adapted cultural practices may need to be applied.

The results show that *P. brassicae* populations of oilseed rape can infect Brussels sprouts and Brussels sprouts populations also infect oilseed rape. Variation between the oilseed rape and the Brussels sprouts population was observed in the current study with the Brussels sprouts population being less virulent. Brussels sprouts as host was less susceptible compared to the other species which is in agreement with findings of Maddock et al. (1981). The absence of symptoms after inoculation with BSpr2 in Experiment 1 is most probably down to a processing error (causing damage to *P. brassicae* conidia). However, oilseed rape appeared to be more susceptible to *P. brassicae* than Brussels sprouts. The intrinsic properties of the wax layer can contribute to resistance against *P. brassicae* as here are hints in oilseed rape that cultivars with a thicker wax layer are more resistant (Boys et al. 2007). Comparison between thickness of wax layers and topology should be done to conclude clearly on the influence on penetration of *P. brassicae* and resistance performance. Furthermore, the influence of growth stage may play a role as the light leaf spot epidemic starts when Brussels sprouts are at a much later growth stage whereas, plants were only BBCH 14 when they were inoculated artificially. Another explanation could be the choice of the Brussels sprouts cultivar. In the vegetable industry there is no standardised recommended list for cultivars available that has information on resistance performance against pathogens. The cultivar was chosen by experience from field performance. More cultivars should be tested to judge variation of resistance in the Brussels sprouts material.

The other vegetable brassicas, such as broccoli, cabbage, Romanesco and cauliflower were able to be infected by both, oilseed rape and Brussels sprouts populations. The variation between the subspecies of *B. oleracea* can be caused by the same reasons mentioned above. Cross-infection between different brassicas is generally possible. Similar results were found by Simons & Skidmore (1988) for cabbage and Brussels sprouts F1 hybrids and their parental lines. The

tested hybrids and lines were inoculated with different single spore isolates from oilseed rape, a broccoli and a cabbage isolate. Speciation of isolates was not found but differential interactions were described. Maddock et al. (1981) were testing for host range of *P. brassicae* isolates in *Brassica* too. Variation between cultivars of different subspecies and between isolates was observed using a leaf disc method.

Low light leaf spot severity on cauliflower cannot be supported by the former study. This is probably due to loss of infected leaves during the time course of the experiment rather than resistance of the cauliflower against the tested *P. brassicae* populations. Cauliflower is considered to be rather susceptible in general (P. Gladders, personal communication).

However, the tested species are all containing the C genome (Triangle of U, Figure 3) and are closely related to each other. Experiments with representatives of the B genome could be conducted to identify non-host or host resistance. For example, *B. carinata* shows very good resistance to phoma stem canker, caused by *Leptosphaeria maculans* and *L. biglobosa* (Fredua‑Agyeman et al. 2014). More distantly related species of the Brassicaceae family could be tested for non-host resistance additionally. *Sinapis alba* was previously tested and could be infected by *P. brassicae* although *S. arvensae* did not develop symptoms of light leaf spot (Boys PhD thesis).

In conclusion, *P. brassicae* populations are not limited to specific hosts within the tested range of brassicas in this study. However, the presence of specified isolates cannot be excluded as experiments were conducted using populations of *P. brassicae* from leaf washings. Therefore, a number of cultivars of the species must be tested with a range of single spore isolates. Differential interaction could then be identified additionally.

Future Work

The work planned in this PhD project is shortly summarised below.

Molecular studies and population structure

Initial determination of population structure

To further distinguish isolates, molecular studies will be done. Therefore, DNA must be extracted from mycelium of the *in vitro* cultures of the isolates. The first differentiation of isolates will be determined on the basis of their mating types, using the *MAT-1* and *MAT-2* primers developed by Foster et al. (2002). Population structure of the pathogen population will initially be studied by RAPD markers because DNA sequence information is not needed for this marker type. However, repeatability can be problematic when using RAPD markers.

Sequencing of *Pyrenopeziza brassicae* isolate

An inoculation series will be done for the identification of a *P. brassicae* isolate that is virulent on the oilseed rape cultivar Bristol but avirulent on cultivar Imola. Purified DNA of that isolate will be sent for *de novo* genome sequencing (low depth sequence at 20X) and assembly at East Malling Research (to be confirmed).

Determination of *Pyrenopeziza brassicae* population structure with SSR markers

The sequenced *de novo* genome will be screened for 5-6 bp repeat units. Primers will be developed for the selected microsatellites. Selected isolates will be tested with the SSR markers for polymorphisms.

Morphological differentiation of *Pyrenopeziza brassicae* isolates

Morphological differences between *P. brassicae* isolates will be studied by distinguishing colony colour and growth rates on different culture media, for example PDA, MA, V8 medium and Cornmeal agar (CMA). Additionally, morphology of conidia will be studied (e.g. shape of conidia, length and diameter).

Fungicide sensitivity tests

Selected isolates will be tested for sensitivity to widely used fungicidal active ingredients according to the method used by Carter et al. (2013, 2014) to further describe the current *P. brassicae* population.

Pathogenicity tests on plant material

Plant material will be tested to distinguish differential interactions between cultivars and *P. brassicae* isolates. Six to eight oilseed rape cultivars will be inoculated with a range of *P. brassicae* isolates to identify gene-for-gene interactions and define races of the pathogen. The same will be done for Brussels sprouts cultivars to identify major genes in the plant material and differential interactions for the pathogen.

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Appendix

Table 4. ID numbers of *Pyrenopeziza brassicae* isolates collected from different oilseed rape cultivars (leaves) and different locations

Isolate ID	No. Isolates*	Season	Cultivar	Location
13OSR1	8	2013/14	Roxet	Banbury (Oxon)
13OSR2	4	2013/14	Roxet	Banbury (Oxon)
13OSR3	3	2013/14	Excel	Cowlinge (Suffolk)
13OSR4	1	2013/14	Excel	Cowlinge (Suffolk)
13OSR5	1	2013/14	Excel	Cowlinge (Suffolk)
13OSR6	1	2013/14	Bristol	Spalding (Lincolnshire)
13OSR7	2	2013/14	Bristol	Elsoms/Spalding (Lincolnshire)
13OSR8	1	2013/14	Bilbao	Cowlinge (Suffolk)
13OSR9	4	2013/14	Adriana	Cowlinge (Suffolk)
13OSR10	3	2013/14	PR46W21	Ryton (North Yorkshire)
13OSR11	3	2013/14	PR46W21	Ryton (North Yorkshire)
13OSR12	3	2013/14	PR46W21	Ryton (North Yorkshire)
13OSR13	3	2013/14	PR46W21	Ryton (North Yorkshire)
13OSR14	2	2013/14	PR46W21	Ryton (North Yorkshire)
13OSR15	3	2013/14	PR46W21	Ryton (North Yorkshire)
13OSR16	5	2013/14	PR46W21	Ryton (North Yorkshire)
13OSR17	3	2013/14	PR46W21	Ryton (North Yorkshire)
13OSR18	7	2013/14	Catana	Boxworth (Cambridgeshire)
13OSR20	1	2013/14	Catana	Boxworth (Cambridgeshire)
13OSR21	2	2013/14	Catana	Boxworth (Cambridgeshire)
13OSR22	1	2013/14	Catana	Boxworth (Cambridgeshire)
13OSR23	3	2013/14	Catana	Boxworth (Cambridgeshire)
13OSR24	4	2013/14	unknown	Kinkardine (Scotland)
13OSR25	2	2013/14	unknown	Kinkardine (Scotland)
13OSR26	5	2013/14	SWO24120	Elsoms/Spalding (Lincolnshire)
13OSR27	2	2013/14	Cuillin	Kings Caple (Herefordshire)
13OSR28	2	2013/14	Cuillin	Kings Caple (Herefordshire)

13OSR29	**3**	2013/14	Cuillin	Kings Caple (Herefordshire)
13OSR30	**5**	2013/14	Cuillin	Kings Caple (Herefordshire)
13OSR31	**5**	2013/14	Cuillin	Kings Caple (Herefordshire)
13OSR32	**1**	2013/14	Cuillin	Kings Caple (Herefordshire)
13OSR33	**3**	2013/14	Cuillin	Kings Caple (Herefordshire)
13OSR34	**3**	2013/14	Cuillin	Kings Caple (Herefordshire)
13OSR35	**2**	2013/14	Catana	Kings Caple (Herefordshire)
13OSR36	**3**	2013/14	Catana	Kings Caple (Herefordshire)
13OSR37	**2**	2013/14	Catana	Kings Caple (Herefordshire)
13OSR38	**3**	2013/14	Catana	Kings Caple (Herefordshire)
13OSR39	**1**	2013/14	Catana	Kings Caple (Herefordshire)
13OSR40	**3**	2013/14	Catana	Kings Caple (Herefordshire)
13OSR41	**1**	2013/14	Catana	Kings Caple (Herefordshire)
13OSR43	**2**	2013/14	Catana	Kings Caple (Herefordshire)
13OSR44	**4**	2013/14	Marathon	Spalding (Lincolnshire)
13OSR45	**4**	2013/14	Castille	Kings Caple (Herefordshire)
13OSR46	**3**	2013/14	Castille	Kings Caple (Herefordshire)
13OSR47	**3**	2013/14	Castille	Kings Caple (Herefordshire)
13OSR48	**4**	2013/14	unknown	Elsoms/Spalding (Lincolnshire)
13OSR49	**2**	2013/14	Castille	Kings Caple (Herefordshire)
13OSR50	**3**	2013/14	Castille	Kings Caple (Herefordshire)
13OSR51	**2**	2013/14	Castille	Kings Caple (Herefordshire)
13OSR52	**1**	2013/14	Catana	Elsoms/Spalding (Lincolnshire)
13OSR53	**1**	2013/14	unknown	Elsoms/Spalding (Lincolnshire)
13OSR54	**3**	2013/14	Catana	Boxworth (Cambridgeshire)
13OSR55	**3**	2013/14	Catana	Boxworth (Cambridgeshire)
13OSR56	**4**	2013/14	Catana	Boxworth (Cambridgeshire)
13OSR57	**3**	2013/14	Castille	Kings Caple (Herefordshire)
13OSR58	**4**	2013/14	Castille	Kings Caple (Herefordshire)
13OSR60	**1**	2013/14	Castille	Kings Caple (Herefordshire)

13OSR61	2	2013/14	Castille	Kings Caple (Herefordshire)
13OSR62	3	2013/14	Castille	Kings Caple (Herefordshire)
13OSR63	3	2013/14	Castille	Kings Caple (Herefordshire)
13OSR64	2	2013/14	SWO24120	Kings Caple (Herefordshire)
13OSR65	2	2013/14	SWO24120	Kings Caple (Herefordshire)
13OSR66	3	2013/14	Temple	Boxworth (Cambridgeshire)
13OSR67	3	2013/14	PR46W21	Boxworth (Cambridgeshire)
13OSR68	2	2013/14	Bristol	Boxworth (Cambridgeshire)
13OSR69	2	2013/14	Catana	Kings Caple (Herefordshire)
13OSR70	1	2013/14	Catana	Kings Caple (Herefordshire)
13OSR71	3	2013/14	SWO24120	Boxworth (Cambridgeshire)
13OSR72	3	2013/14	SWO24120	Boxworth (Cambridgeshire)
13OSR73	3	2013/14	Marathon	Boxworth (Cambridgeshire)
13OSR74	1	2013/14	Patron	Boxworth (Cambridgeshire)
13OSR75	4	2013/14	Castille	Kings Caple (Herefordshire)
13OSR76	2	2013/14	Bristol	Kings Caple (Herefordshire)
13OSR77	3	2013/14	Bristol	Kings Caple (Herefordshire)
13OSR78	3	2013/14	Bristol	Kings Caple (Herefordshire)
13OSR79	3	2013/14	Bristol	Kings Caple (Herefordshire)
13OSR80	3	2013/14	Bristol	Kings Caple (Herefordshire)
13OSR81	3	2013/14	Bristol	Kings Caple (Herefordshire)
13OSR82	1	2013/14	Bristol	Kings Caple (Herefordshire)
13OSR83	2	2013/14	Bristol	Kings Caple (Herefordshire)
13OSR84	3	2013/14	Bristol	Kings Caple (Herefordshire)
13OSR85	2	2013/14	Bristol	Kings Caple (Herefordshire)
13OSR86	4	2013/14	Marathon	High Mowth. (North Yorkshire)
13OSR87	3	2013/14	Marathon	High Mowth. (North Yorkshire)
13OSR88	4	2013/14	Marathon	High Mowth. (North Yorkshire)
13OSR89	5	2013/14	Marathon	High Mowth. (North Yorkshire)

13OSR90	**3**	2013/14	Temple	Kings Caple (Herefordshire)
13OSR91	**3**	2013/14	Temple	Kings Caple (Herefordshire)
13OSR92	**2**	2013/14	Temple	Kings Caple (Herefordshire)
13OSR93	**1**	2013/14	Temple	Kings Caple (Herefordshire)
13OSR94	**3**	2013/14	Marathon	Kings Caple (Herefordshire)
13OSR95	**3**	2013/14	Marathon	Kings Caple (Herefordshire)
13OSR96	**3**	2013/14	Marathon	Kings Caple (Herefordshire)
13OSR97	**2**	2013/14	Marathon	Kings Caple (Herefordshire)
13OSR98	**3**	2013/14	Marathon	Kings Caple (Herefordshire)
13OSR99	**1**	2013/14	Marathon	Kings Caple (Herefordshire)
13OSR100	**2**	2013/14	Marathon	Kings Caple (Herefordshire)
13OSR101	**3**	2013/14	Marathon	Kings Caple (Herefordshire)
13OSR102	**3**	2013/14	Marathon	Kings Caple (Herefordshire)
13OSR103	**2**	2013/14	Marathon	Kings Caple (Herefordshire)
13OSR104	**2**	2013/14	Recital	Kings Caple (Herefordshire)
13OSR105	**3**	2013/14	Recital	Kings Caple (Herefordshire)
13OSR106	**2**	2013/14	Recital	Kings Caple (Herefordshire)
13OSR107	**3**	2013/14	Recital	Kings Caple (Herefordshire)
13OSR108	**3**	2013/14	Recital	Kings Caple (Herefordshire)
13OSR109	**3**	2013/14	Recital	Kings Caple (Herefordshire)
13OSR110	**8**	2013/14	Cuillin	Kings Caple (Herefordshire)
13OSR111	**3**	2013/14	Temple	Kings Caple (Herefordshire)
13OSR112	**4**	2013/14	Temple	Kings Caple (Herefordshire)
13OSR113	**3**	2013/14	Temple	Kings Caple (Herefordshire)
13OSR114	**2**	2013/14	Temple	Kings Caple (Herefordshire)
13OSR115	**3**	2013/14	Temple	Kings Caple (Herefordshire)
13OSR116	**4**	2013/14	PR46W21	Boxworth (Cambridgeshire)
13OSR117	**2**	2013/14	PR46W21	Boxworth (Cambridgeshire)
13OSR118	**1**	2013/14	PR46W21	Boxworth (Cambridgeshire)
13OSR119	**4**	2013/14	PR46W21	Boxworth (Cambridgeshire)
13OSR120	**3**	2013/14	PR46W21	Boxworth (Cambridgeshire)
13OSR121	**1**	2013/14	Castille	Spalding (Lincolnshire)
13OSR122	**2**	2013/14	Catana	Boxworth (Cambridgeshire)

13OSR123	**3**	2013/14	Catana	Boxworth (Cambridgeshire)
13OSR124	**4**	2013/14	Catana	Boxworth (Cambridgeshire)
13OSR125	**2**	2013/14	Catana	Boxworth (Cambridgeshire)
13OSR126	**3**	2013/14	Temple	Boxworth (Cambridgeshire)
13OSR127	**1**	2013/14	Patron	Boxworth (Cambridgeshire)
13OSR128	**2**	2013/14	Marathon	Boxworth (Cambridgeshire)
13OSR129	**3**	2013/14	Temple	Kings Caple (Herefordshire)
13OSR130	**3**	2013/14	Temple	Kings Caple (Herefordshire)
13OSR131	**1**	2013/14	Temple	Kings Caple (Herefordshire)
13OSR132	**1**	2013/14	Temple	Kings Caple (Herefordshire)
13OSR133	**3**	2013/14	SWO24120	Kings Caple (Herefordshire)
13OSR134	**3**	2013/14	SWO24121	Kings Caple (Herefordshire)
13OSR135	**3**	2013/14	SWO24122	Kings Caple (Herefordshire)
13OSR136	**2**	2013/14	SWO24123	Kings Caple (Herefordshire)
13OSR137	**1**	2013/14	SWO24124	Kings Caple (Herefordshire)
13OSR138	**2**	2013/14	Anastasia	Kings Caple (Herefordshire)
13OSR139	**3**	2013/14	Anastasia	Kings Caple (Herefordshire)
13OSR140	**3**	2013/14	Anastasia	Kings Caple (Herefordshire)
13OSR141	**2**	2013/14	Recital	Kings Caple (Herefordshire)
13OSR142	**3**	2013/14	Recital	Kings Caple (Herefordshire)
13OSR143	**3**	2013/14	Recital	Kings Caple (Herefordshire)
13OSR144	**3**	2013/14	Recital	Kings Caple (Herefordshire)
13OSR145	**2**	2013/14	Recital	Kings Caple (Herefordshire)
13OSR146	**3**	2013/14	Recital	Kings Caple (Herefordshire)
13OSR147	**3**	2013/14	Recital	Kings Caple (Herefordshire)
13OSR148	**3**	2013/14	Recital	Kings Caple (Herefordshire)
13OSR149	**2**	2013/14	Recital	Kings Caple (Herefordshire)
13OSR150	**3**	2013/14	Recital	Kings Caple (Herefordshire)
13OSR151	**3**	2013/14	Recital	Kings Caple (Herefordshire)
13OSR152	**3**	2013/14	Catana	Boxworth (Cambridgeshire)
13OSR153	**3**	2013/14	Catana	Boxworth (Cambridgeshire)
13OSR154	**4**	2013/14	Catana	Boxworth (Cambridgeshire)
13OSR155	**3**	2013/14	Catana	Boxworth (Cambridgeshire)

13OSR156	**3**	2013/14	Catana	Boxworth (Cambridgeshire)
13OSR157	**4**	2013/14	Catana	Boxworth (Cambridgeshire)
13OSR158	**4**	2013/14	Catana	Boxworth (Cambridgeshire)
13OSR159	**5**	2013/14	SWO24120	Boxworth (Cambridgeshire)
13OSR160	**2**	2013/14	Bristol	Boxworth (Cambridgeshire)
13OSR161	**3**	2013/14	Bristol	Boxworth (Cambridgeshire)
13OSR162	**2**	2013/14	Catana	Boxworth (Cambridgeshire)
13OSR163	**4**	2013/14	Catana	Boxworth (Cambridgeshire)
13OSR164	**2**	2013/14	Catana	Boxworth (Cambridgeshire)
13OSR165	**3**	2013/14	Catana	Boxworth (Cambridgeshire)
13OSR166	**3**	2013/14	Bry x Fort	Harpenden (Hertfordshire)
13OSR167	**3**	2013/14	Bry x Fort	Harpenden (Hertfordshire)
13OSR168	**1**	2013/14	Catana	Boxworth (Cambridgeshire)
13OSR169	**3**	2013/14	Catana	Boxworth (Cambridgeshire)
13OSR170	**3**	2013/14	Harper	Boxworth (Cambridgeshire)
13OSR171	**3**	2013/14	SWO24120	Boxworth (Cambridgeshire)
13OSR172	**3**	2013/14	SWO24120	Boxworth (Cambridgeshire)
13OSR173	**3**	2013/14	Catana	Kings Caple (Herefordshire)

*No. Isolates indicates the number of isolates per leaf. The isolates from the same leaf have been obtained from different lesions.

Table 5. ID numbers of *Pyrenopeziza brassicae* isolates collected from different Brussles sprouts cultivars, plant parts and different locations

Isolate ID	No. Isolates*	Season	Cultivar	Plant Part	Location
13BSpr1	2	2013/14	Clodius	Bud	St. Andrews (Scotland)
13BSpr2	2	2013/14	Clodius	Bud	St. Andrews (Scotland)
13BSpr3	2	2013/14	Clodius	Bud	St. Andrews (Scotland)
13BSpr4	1	2013/14	Clodius	Bud	St. Andrews (Scotland)
13BSpr5	1	2013/14	Clodius	Bud	St. Andrews (Scotland)
13BSpr6	1	2013/14	Clodius	Bud	St. Andrews (Scotland)
13BSpr7	2	2013/14	Clodius	Bud	St. Andrews (Scotland)
13BSpr8	4	2013/14	Neptuno	Bud	Elsoms/Spalding (Lincolnshire)
13BSpr9	4	2013/14	Aurelius	Leaf	Kirton Holme (Lincolnshire)
13BSpr10	4	2013/14	NZ 16-682	Leaf	Kirton Holme (Lincolnshire)
13BSpr11	1	2013/14	NZ 16-682	Leaf	Kirton Holme (Lincolnshire)
13BSpr12	5	2013/14	1732	Leaf	Kirton Holme (Lincolnshire)
13BSpr13	3	2013/14	1732	Leaf	Kirton Holme (Lincolnshire)
13BSpr14	2	2013/14	Revenge	Leaf	Kirton Holme (Lincolnshire)
13BSpr15	2	2013/14	Cobus	Leaf	Kirton Holme (Lincolnshire)
13BSpr16	3	2013/14	Cobus	Leaf	Kirton Holme (Lincolnshire)
13BSpr17	1	2013/14	Neptuno	Bud	Kirton Holme (Lincolnshire)
13BSpr18	1	2013/14	Neptuno	Bud	Kirton Holme (Lincolnshire)
13BSpr19	1	2013/14	Neptuno	Leaf	Kirton Holme (Lincolnshire)
13BSpr20	2	2013/14	Bowintus	Leaf	Kirton Holme (Lincolnshire)
13BSpr21	2	2013/14	Bowintus	Leaf	Kirton Holme (Lincolnshire)
13BSpr22	1	2013/14	Bowintus	Leaf	Kirton Holme (Lincolnshire)
13BSpr23	3	2013/14	NZ 16-682	Bud	Kirton Holme (Lincolnshire)
13BSpr24	1	2013/14	NZ 16-682	Bud	Kirton Holme (Lincolnshire)
13BSpr25	4	2013/14	NZ 16-	Bud	Carnoustie (Scotland)

			628			
13BSpr26	**2**	2013/14	NZ 629	16-	Bud	Carnoustie (Scotland)
13BSpr27	**1**	2013/14	NZ 630	16-	Bud	Carnoustie (Scotland)
13BSpr28	**3**	2013/14	NZ 616	16-	Bud	Carnoustie (Scotland)
13BSpr29	**2**	2013/14	NZ 616	16-	Bud	Carnoustie (Scotland)
13BSpr30	**1**	2013/14	NZ 616	16-	Bud	Carnoustie (Scotland)
13BSpr31	**2**	2013/14	NZ 616	16-	Bud	Carnoustie (Scotland)
13BSpr32	**3**	2013/14	NZ 682	16-	Bud	Carnoustie (Scotland)
13BSpr33	**2**	2013/14	NZ 682	16-	Bud	Carnoustie (Scotland)
13BSpr34	**3**	2013/14	Neptuno		Bud	Kirton Holme (Lincolnshire)
13BSpr35	**1**	2013/14	Neptuno		Bud	Kirton Holme (Lincolnshire)
13BSpr36	**3**	2013/14	NZ 682	16-	Bud	Holbeach (Lincolnshire)
13BSpr37	**1**	2013/14	NZ 682	16-	Bud	Holbeach (Lincolnshire)
13BSpr38	**1**	2013/14	NZ 616	16-	Bud	Copath (Scotland)
13BSpr39	**2**	2013/14	NZ 616	16-	Bud	Copath (Scotland)
13BSpr40	**3**	2013/14	NZ 616	16-	Bud	Copath (Scotland)
13BSpr41	**3**	2013/14	NZ 616	16-	Bud	Copath (Scotland)
13BSpr42	**2**	2013/14	NZ 616	16-	Bud	Copath (Scotland)

13BSpr43	**3**	2013/14	NZ 616	16-	Bud	Copath (Scotland)
13BSpr44	**2**	2013/14	NZ 653	16-	Bud	Copath (Scotland)
13BSpr45	**1**	2013/14	NZ 653	16-	Bud	Copath (Scotland)
13BSpr46	**1**	2013/14	NZ 653	16-	Bud	Copath (Scotland)
13BSpr47	**1**	2013/14	NZ 653	16-	Bud	Copath (Scotland)
13BSpr48	**1**	2013/14	NZ 653	16-	Bud	Copath (Scotland)
13BSpr49	**2**	2013/14	Braemer		Bud	Copath (Scotland)
13BSpr50	**1**	2013/14	Braemer		Bud	Copath (Scotland)
13BSpr51	**2**	2013/14	Braemer		Bud	Copath (Scotland)
13BSpr52	**4**	2013/14	NZ 628	16-	Bud	Carnoustie (Scotland)
13BSpr53	**1**	2013/14	NZ 628	16-	Bud	Carnoustie (Scotland)
13BSpr54	**1**	2013/14	NZ 628	16-	Bud	Carnoustie (Scotland)
13BSpr55	**1**	2013/14	NZ 616	16-	Bud	Carnoustie (Scotland)
13BSpr56	**1**	2013/14	NZ 616	16-	Bud	Carnoustie (Scotland)
13BSpr57	**3**	2013/14	NZ 682	16-	Bud	Holbeach (Lincolnshire)
13BSpr58	**2**	2013/14	NZ 682	16-	Bud	Holbeach (Lincolnshire)
13BSpr59	**4**	2013/14	NZ 682	16-	Bud	Holbeach (Lincolnshire)
13BSpr60	**4**	2013/14	NZ 682	16-	Bud	Holbeach (Lincolnshire)

13BSpr61	**2**	2013/14	NZ 16-682	Bud	Holbeach (Lincolnshire)
13BSpr62	**3**	2013/14	pl. 5561	Leaf	Elsoms/Spalding (Lincolnshire)
13BSpr63	**3**	2013/14	NZ 16-682	Leaf	Kirten Holme (Lincolnshire)
13BSpr64	**2**	2013/14	NZ 16-682	Leaf	Kirten Holme (Lincolnshire)
13BSpr65	**2**	2013/14	NZ 16-682	Bud	Kirten Holme (Lincolnshire)
13BSpr66	**3**	2013/14	NZ 16-682	Bud	Holbeach (Lincolnshire)
13BSpr67	**3**	2013/14	NZ 16-682	Bud	Holbeach (Lincolnshire)
13BSpr68	**2**	2013/14	NZ 16-682	Bud	Holbeach (Lincolnshire)
13BSpr69	**2**	2013/14	NZ 16-681	Bud	Holbeach (Lincolnshire)
13BSpr70	**3**	2013/14	NZ 16-681	Bud	Holbeach (Lincolnshire)
13BSpr71	**3**	2013/14	pl. 5561	Leaf	Elsoms/Spalding (Lincolnshire)
13BSpr72	**2**	2013/14	pl. 5562	Leaf	Elsoms/Spalding (Lincolnshire)
13BSpr73	**4**	2013/14	Bejo	Bud	Copath (Scotland)
13BSpr74	**1**	2013/14	Bejo	Bud	Copath (Scotland)
13BSpr75	**3**	2013/14	pl.5561	Leaf	Spalding (Lincolnshire)
13BSpr76	**3**	2013/14	pl.5561	Bud	Spalding (Lincolnshire)
13BSpr77	**4**	2013/14	unknown	Leaf	Spalding (Lincolnshire)
13BSpr78	**3**	2013/14	unknown	Bud	Spalding (Lincolnshire)
13BSpr79	**2**	2013/14	NZ16-653	Bud	Copath (Scotland)
13BSpr80	**2**	2013/14	NZ16-	Bud	Copath (Scotland)

			653		
13BSpr81	**2**	2013/14	NZ16-653	Bud	Copath (Scotland)
13BSpr82	**1**	2013/14	NZ16-653	Bud	Copath (Scotland)
13BSpr83	**2**	2013/14	Neptuno	Leaf	Elsoms/Spalding (Lincolnshire)
13BSpr84	**5**	2013/14	Revenge	Leaf	Elsoms/Spalding (Lincolnshire)
13BSpr85	**4**	2013/14	Petrus	Bud	Elsoms/Spalding (Lincolnshire)
13BSpr86	**4**	2013/14	Petrus	Bud	Elsoms/Spalding (Lincolnshire)
13BSpr87	**1**	2013/14	pl. 5590	Bud	Elsoms/Spalding (Lincolnshire)
13BSpr88	**1**	2013/14	pl. 5590	Leaf	Elsoms/Spalding (Lincolnshire)
13BSpr89	**2**	2013/14	Sue's Line	Leaf	Elsoms/Spalding (Lincolnshire)
13BSpr90	**3**	2013/14	Sue's Line	Leaf	Elsoms/Spalding (Lincolnshire)
13BSpr91	**3**	2013/14	Sue's Line	Leaf	Elsoms/Spalding (Lincolnshire)
13BSpr92	**1**	2013/14	NZ16-681	Bud	Holbeach (Lincolnshire)
13BSpr93	**3**	2013/14	unknown	Leaf	Elsoms/Spalding (Lincolnshire)
13BSpr94	**4**	2013/14	unknown	Leaf	Elsoms/Spalding (Lincolnshire)

*No. Isolates indicates the number of isolates per leaf. The isolates from the same leaf have been obtained from different lesions.

Table 6. ID numbers of *Pyrenopeziza brassicae* isolates collected from different vegetable species and different locations

Isolate ID	Host Species	No. Isolates *	Season	Cultivar	Location
13Cbw1	White cabbage	8	2013/14	Caraflex	Spalding (Lincolnshire)
13Cbw2	White cabbage	3	2013/14	Caraflex	Spalding (Lincolnshire)
13Cbr1	Red cabbage	3	2013/14	unknown	Boxworth (Cambridgeshire)
13Cbr2	Red cabbage	1	2013/14	unknown	Boxworth (Cambridgeshire)
13Cbr3	Red cabbage	2	2013/14	unknown	Boxworth (Cambridgeshire)
13Cbr4	Red cabbage	3	2013/14	unknown	Boxworth (Cambridgeshire)
13Cbr5	Red cabbage	1	2013/14	unknown	Boxworth (Cambridgeshire)
13Rom1	Calabrese	1	2013/14	unknown	Willingham (Cambridgeshire)
13Rom2	Calabrese	1	2013/14	unknown	Willingham (Cambridgeshire)
13CFl1	Cauliflower	1	2013/14	Clemen	Kirten Holme (Lincolnshire)
13CFl2	Cauliflower	2	2013/14	Clemen	Kirten Holme (Lincolnshire)
13CFl3	Cauliflower	1	2013/14	Clemen	Kirten Holme (Lincolnshire)
13CFl4	Cauliflower	1	2013/14	Clemen	Kirten Holme (Lincolnshire)
13CFl5	Cauliflower	2	2013/14	Clemen	Kirten Holme (Lincolnshire)
13CFl6	Cauliflower	3	2013/14	Clemen	Kirten Holme (Lincolnshire)

13CFl7	Cauliflower	**2**	2013/14	Clemen	Kirten Holme (Lincolnshire)
13CFl8	Cauliflower	**3**	2013/14	Clemen	Kirten Holme (Lincolnshire)
13CFl9	Cauliflower	**1**	2013/14	Clemen	Kirten Holme (Lincolnshire)
13CFl10	Cauliflower	**3**	2013/14	Clemen	Kirten Holme (Lincolnshire)
13CFl11	Cauliflower	**2**	2013/14	Clemen	Kirten Holme (Lincolnshire)
13CFl12	Cauliflower	**2**	2013/14	Clemen	Kirten Holme (Lincolnshire)
13CFl13	Cauliflower	**3**	2013/14	Clemen	Kirten Holme (Lincolnshire)
13CFl14	Cauliflower	**3**	2013/14	Clemen	Kirten Holme (Lincolnshire)

*No. Isolates indicates the number of isolates per leaf. The isolates from the same leaf have been obtained from different lesions.