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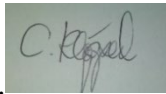
2 AUTHENTICATION

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 ....22/10/2014.................

3 CONTENTS

4 Grower summary

4.1 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.

4.2 Background

Light leaf spot is a very important disease in vegetables and oilseed rape. Over the last decade the importance of the disease in oilseed rape has increased dramatically increased in the UK (Figure 1).
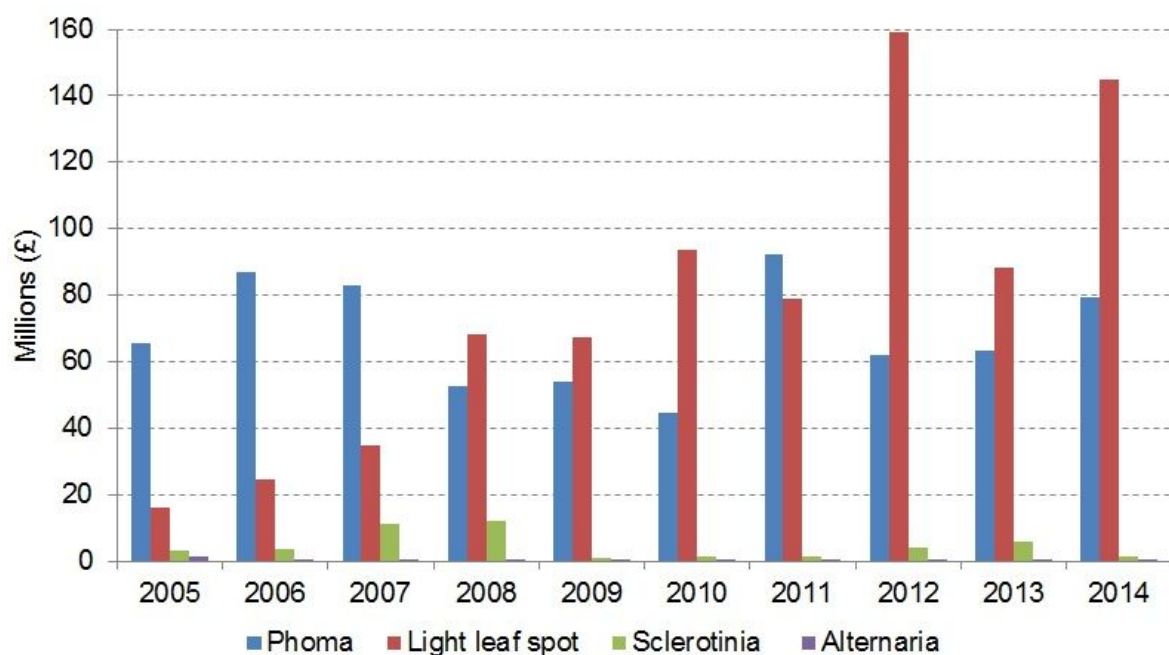
Figure 1: Estimated yield losses due to diseases in oilseed rape in England from 2005 until 2014 (CropMonitor, 2014)

Effective control of light leaf spot is difficult to achieve. Chemical control is challenging as fungicides must be applied during the period when the pathogen grows asymptomatically in plant tissue (Figueroa et al. 1994). The efficacy of fungicides may also be reduced as methyl benzimidazole carbamate (MBC's) and azole resistant *P. brassicae* strains have been identified (Carter et al. 2013, 2014). A forecasting system is available that assists farmers with their decisions on spray timings for light leaf spot on oilseed rape (http://www.rothamsted.ac.uk/light-leaf-spot-forecast/regional-light-leaf-spot-risk-forecast). This forecasting model is currently being updated. Furthermore, the exploitation of plant resistance against the pathogen could help control the disease but current commercial oilseed rape cultivars show poor light leaf spot resistance.

The disease is damaging in vegetables as well, Brussels sprouts in particular. It has been assumed that there is a potential spread between oilseed rape and vegetable brassicas but this has not been confirmed yet.

4.3 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. Furthermore, there could also be better advice on cultivar choice on a regional scale according to the population in a certain area (e.g. Recommended List for oilseed rape).

Therefore, isolates of *P. brassicae* are being collected from infected leaf (oilseed rape, vegetables) and bud tissue (Brussels sprouts). The isolates will be studied morphologically and molecularly using a combination of neutral markers to determine differences between the isolates. Certain isolates will be used for *in planta* screenings to discover differential interactions between isolates and potential host cultivars.

5 Introduction

5.1 Plant defence responses against pathogens

Plants face a great variety of microbes in their environment but only a small number of microbes are actually able to infect the plants 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. Defence responses of plants can be preformed/constitutive or induced for both non-host and host resistance. The host range of pathogens is determined by their ability to overcome resistance of plants.

5.1.1 Defence mechanisms of plants against pathogens

Defence mechanisms can generally be distinguished between structural and biochemical responses.

5.1.1.1 Structural defence mechanisms against pathogens

Structural characteristics of plants can be the first hurdle for a pathogen to face. Pathogen propagules encounter plant surfaces (e.g. plant cuticle) at first contact. The composition of the cuticle varies greatly and can define the outcome of a penetration event by a fungal pathogen. The basic components of the cuticle are cutin and waxes, which are cuticular lipids (Bargel et al. 2006). Attachment and germination of some fungal spores necessitates a moist environment and cuticular waxes influence both the hydrophilicity of the plant surface and surface topology (Kerstiens 2000). Therefore, water retention and hence leaf wetness duration may be influenced by the amount of waxes. 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 appressorium formation by *Magnaporthe grisea*, whereas others contribute to it (Uchiyama et al. 1979, Uchiyama & Okuyama 1990, Howard & Valent 1996). Nevertheless, the occurrence of natural openings such as stomata has a considerable effect on some plant-pathogen interactions (Melotto et al. 2006). Stomata are usually considered as passive openings for pathogen entry but active closure of stomata after bacterial action was found in *Arabidopsis thaliana* (Melotto et al. 2006, Melotto et al. 2008).

Moreover, structural changes 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 infected leaf area is cut off with the sacrifice of a few cell layers of healthy plant material (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). Other cell wall appositions, such as lignin, strengthen the cell wall against mechanical forces that are applied by directly penetrating fungi (Vance et al. 1980).

5.1.1.2 Biochemical defence against pathogens

Biochemical defence plays an important role in plant defence mechanisms against. Plants constitutively produce compounds that have an antimicrobial effect, so-called phytoanticipins. Phytoanticipins are constitutive low molecular weight constituents of the plant secondary metabolism, i.e. phenols, phenolic glycosides, saponins and glucosinolates (Osbourn 1996a). Phenolic compounds inhibited germination of *Colletotrichum circinans* (cause of onion smudge) in yellow and red skinned onions, whereas colourless onions are susceptible to the pathogen (Link & Walker 1933). Another group of secondary metabolites are 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 a constitutive defense mechanism due to their biological activity against herbivorous insects and some pathogens (Kliebenstein et al. 2005, Bednarek et al. 2009, Hopkins et al. 2009).

Another group of 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*.

Moreover, reactive oxygen species (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, e.g. PR2 coding for β-1,3 glucanases and PR3 coding chitinases, are able to degrade cell wall components of fungi and therefore decrease colonization by fungal pathogens, especially when both compounds are produced (Mauch et al. 1988).

These biochemical responses, accumulation of phytoalexins, ROS and PR proteins, are part of an important physiological event for incompatible interactions between plants and pathogens, the hypersensitive response (HR) (De Gara et al. 2003). 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). HR plays an important role in both host and non-host resistance reactions (Hiruma et al. 2013).

5.1.2   Concepts for interactions between plants and pathogens

In the interactions between plants and pathogens, there is a distinction between PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI) (Jones & Dangl 2006).

PTI is involved in non-host resistance responses where pathogen-associated molecular patterns (PAMPs) are recognized by plant receptor proteins also called pattern recognition receptors (PRRs) on the plant cell surface (Boller & Felix 2009, Dodds & Rathjen 2010, Thomma et al. 2011). ETI, on the other hand, involves the interaction of plant hosts with pathogen strains in a gene-for-gene manner, based on the concept of Flor (1955). When an *Avr* gene product of the pathogen, so-called effector, is recognized by a resistance (*R*) gene product of the plant, there is an incompatible reaction, which mostly involves a hypersensitive response and local cell death (Dodds & Rathjen 2010, de Jonge et al. 2011). Just recently Stotz et al. (2014) suggest another form of defence response to distinguish more clearly: effector-triggered defence (ETD). ETD describes defence responses against apoplastic pathogens, which do not lead to a rapid hypersensitive response and do also not restrict pathogen growth completely (Stotz et al. 2014).

5.2   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.

5.3   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 then 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 *Avr* genes (refer to Chapter 5.1.2). 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

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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 a great extent (Barrett et al. 2008). Therefore, 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 (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 in 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 then 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).

5.4   Determination of population structures

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

5.4.1   Determination of allele frequencies

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, respectively, can be determined to investigate effectiveness of *R* genes in cultivars on the field. As an example, a differential set of oilseed rape cultivars has been developed, with cultivars harbouring different *R* genes (Balesdent et al. 2001). With the help of this differential set, *L. maculans* strains can be distinguished whether they have avirulent (*Avr*) or virulent (*avr*) alleles at corresponding loci (Balesdent et al. 2001, Balesdent et al. 2005). The information can be used to guide strategies for the rotation of *R* genes so that durability of these *R* genes can be prolonged (http://www.cetiom.fr/). This type of screening method is a valuable approach for the development and exploitation of resistance against pathogens (Peever et al. 2000). However, plant screenings only

determine the virulence phenotype rather than the genotype of the fungal isolates (Kolmer 1992, Peever et al. 2000). Therefore, molecular approaches are necessary to study the population structure of plant pathogens.

5.4.2 Determination of population structures with molecular markers

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 about 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 or absence of an allele whereas co-dominant markers also distinguish if the individual is homo- or heterozygous at the given locus and, therefore, provide 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 lengths. 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 of 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 can 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*,

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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; their use involves digestion of DNA by restriction enzymes and is based on PCR (Mueller & Wolfenbarger 1999). AFLPs do not allow conclusions about 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 are characterised by tandem repeats of base pairs in the 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 therefore, 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*; they allow studies of 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 2).
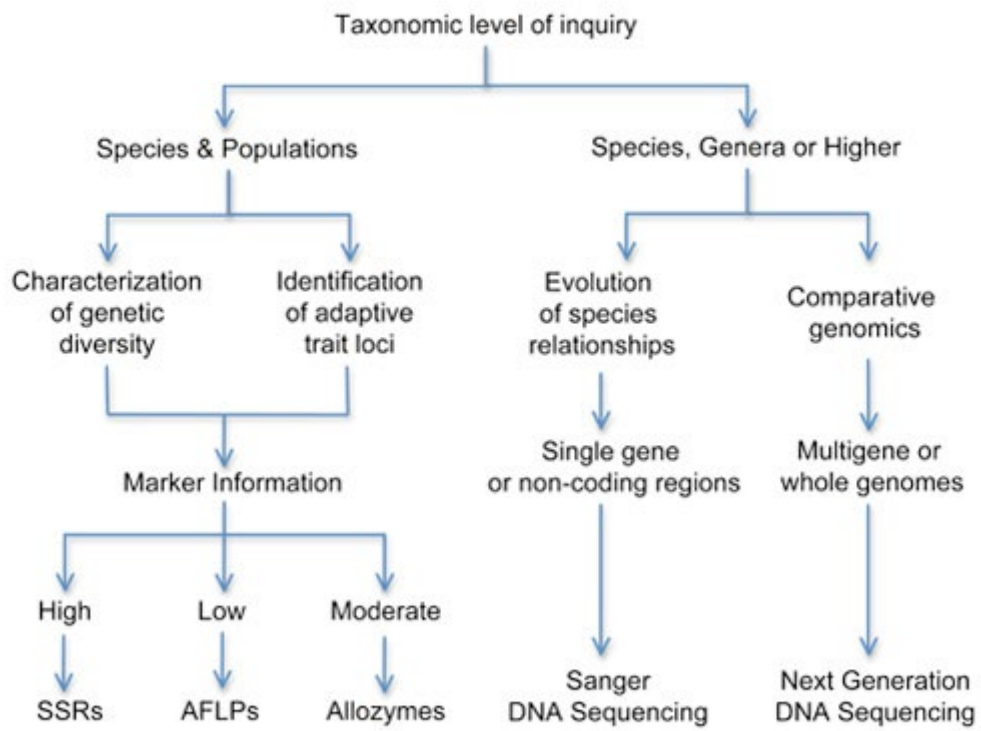
Figure 2: Decision tree for the use of molecular markers based on taxonomic level (Allan & Max 2010), Abbreviations: SSR = simple sequence repeats, AFLPs = amplified fragment length polymorphism.

5.5 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 lead 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).
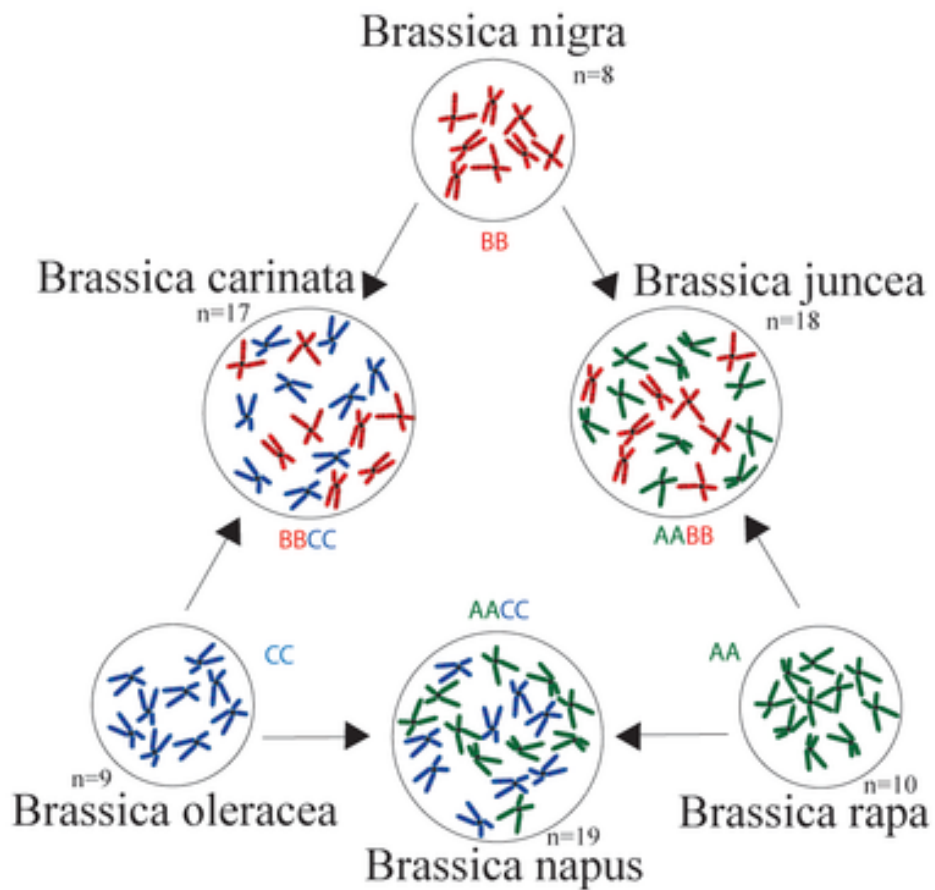
Figure 3: Triangle of U showing the relationship between *Brassica oleracea*, *B. rapa* and *B. nigra* forming allotetraploid species *B. napus*, *B. carinata* and *B. juncea* (U, 1935; picture: Anonymous 2014b)

Oilseed rape oil, also called rapeseed oil, is used as vegetable oil for human consumption. The rapeseed oil contains about 60% oleic acid (C18:1), 20% linoleic acid (C18:2) and 10% linolenic acid (C18:3) (Hu et al. 2006). This fatty acid composition is almost ideal for human consumption and health (Ackman 1990). After the process for extracting the oil, the remains (the 'meal') can be used as animal feed (Pustjens et al. 2014). Furthermore, rapeseed oil is also a renewable resource for the production of biofuel (McKendry 2002).
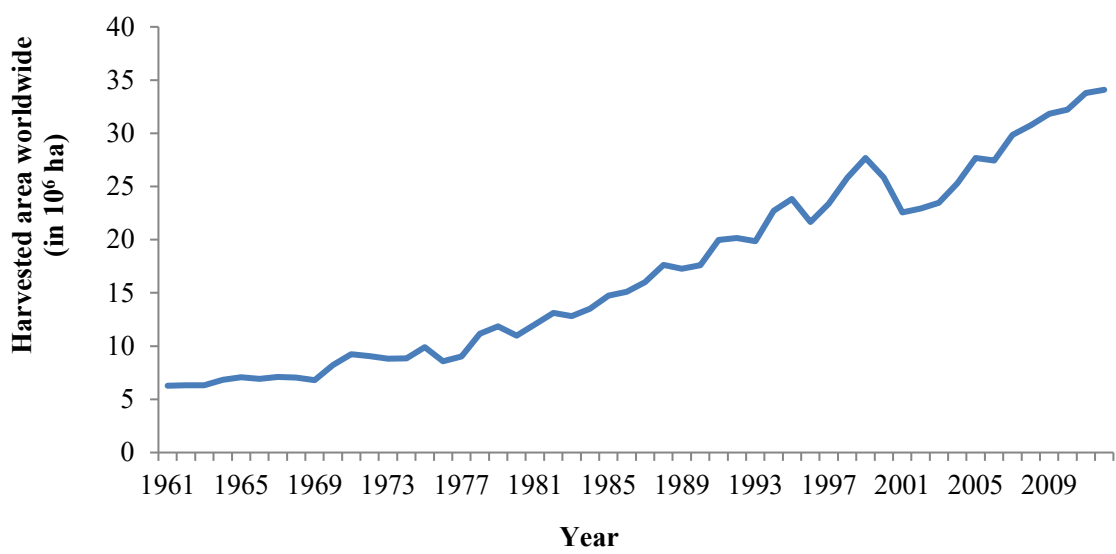
Figure 4: Harvested area of oilseed rape worldwide (in 10^6 ha) from 1961 until 2012 (FAOSTAT 2014).

5.6 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).

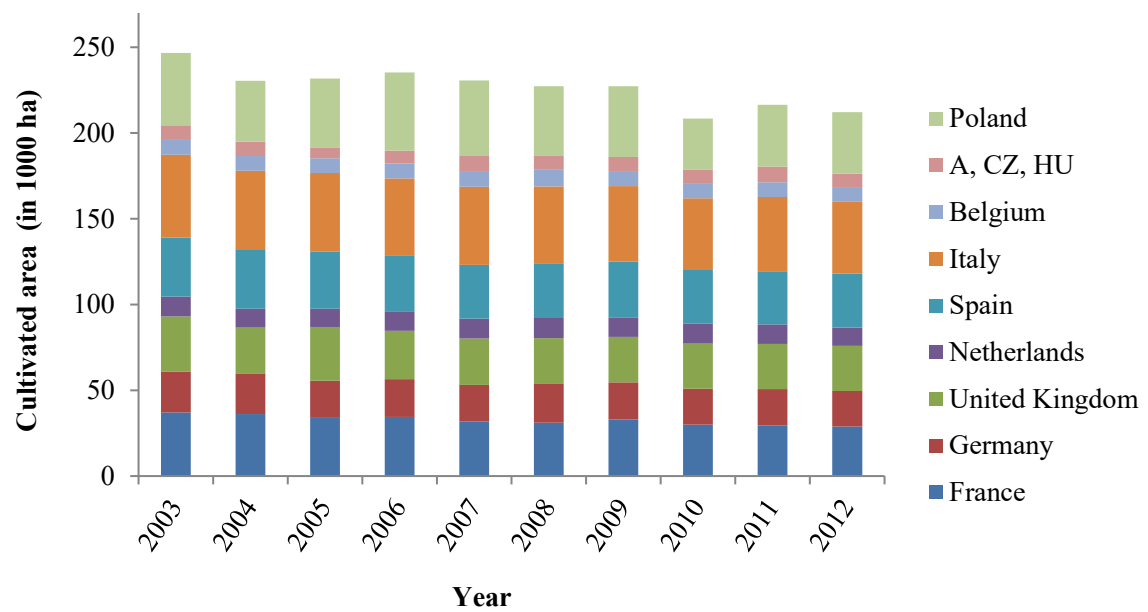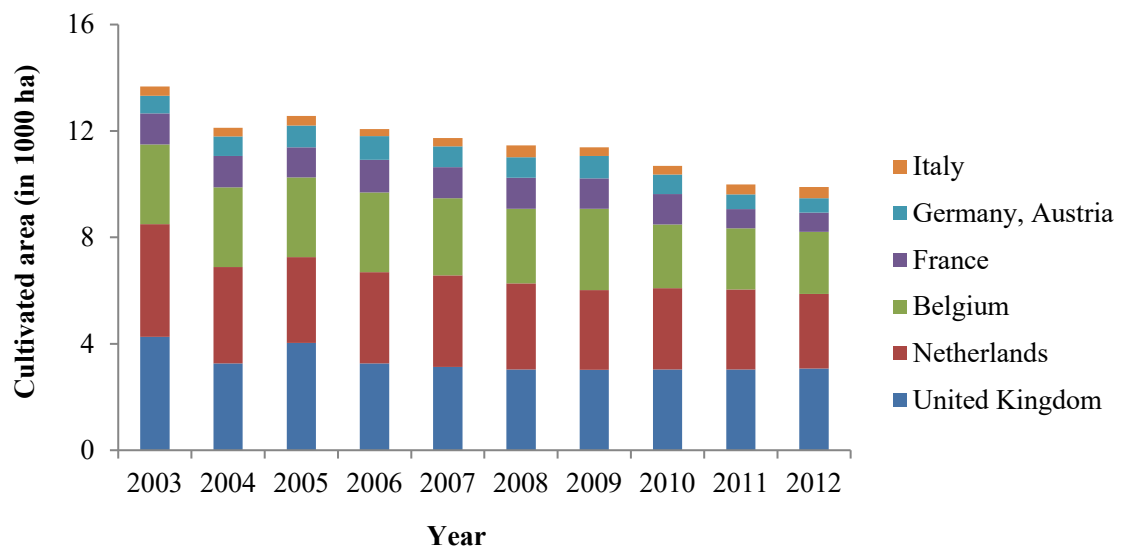### 5.7 *Pyrenopeziza brassicae* and light leaf spot

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 2010 it caused an annual yield loss of approximately £ 150 million (Neal Evans, CropMonitor, www.cropmonitor.co.uk). 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).

5.8 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 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 develop 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).
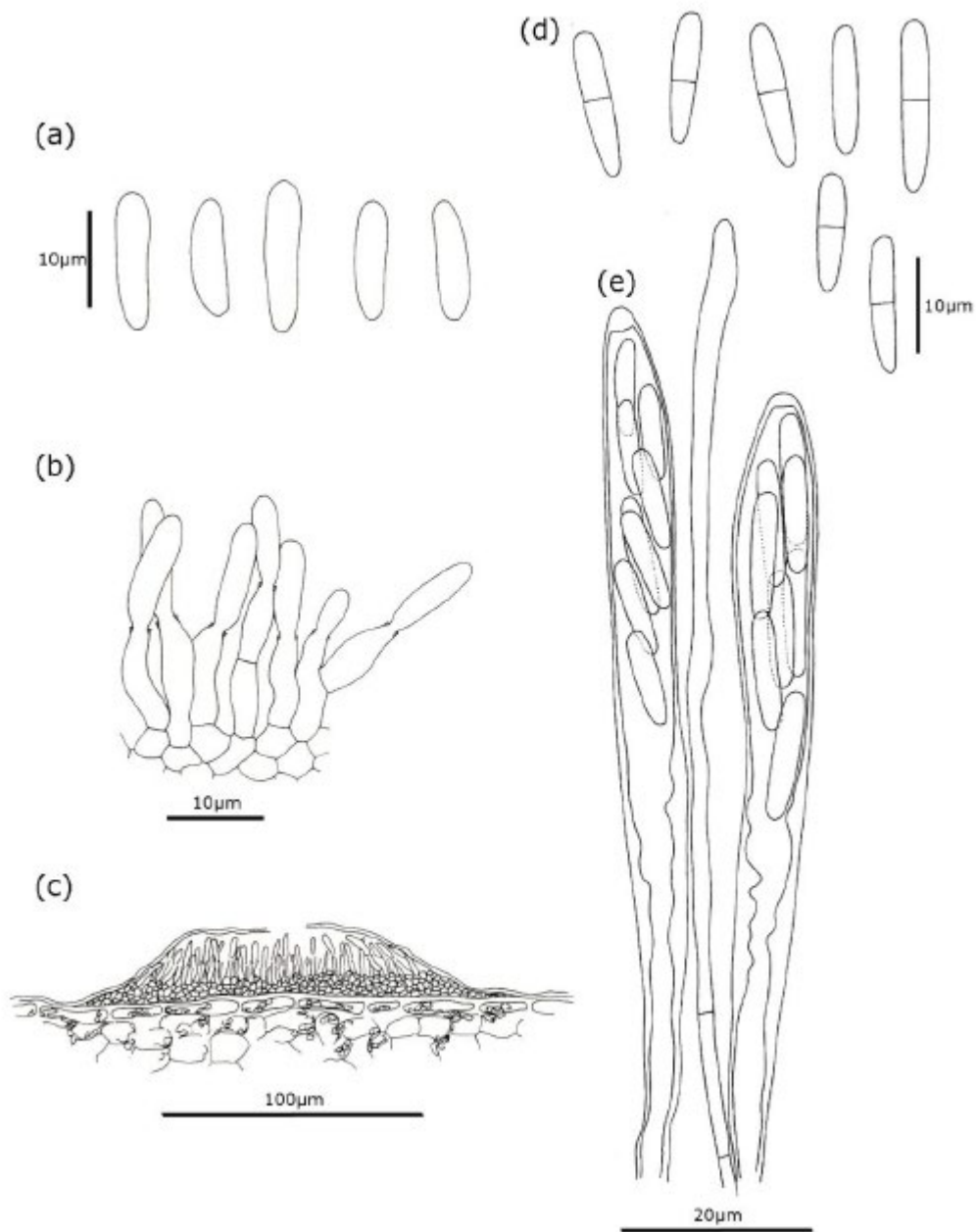
Figure 7: Spores of *Pyrenopeziza brassicae*: asexual forms: conidia (a), conidiophores (b) and acervuli (c); sexual forms ascospores (d) and asci (e) (CMI Descriptions of Pathogenic Fungi and Bacteria No. 536, Rawlinson et al. 1978, Boys 2009).
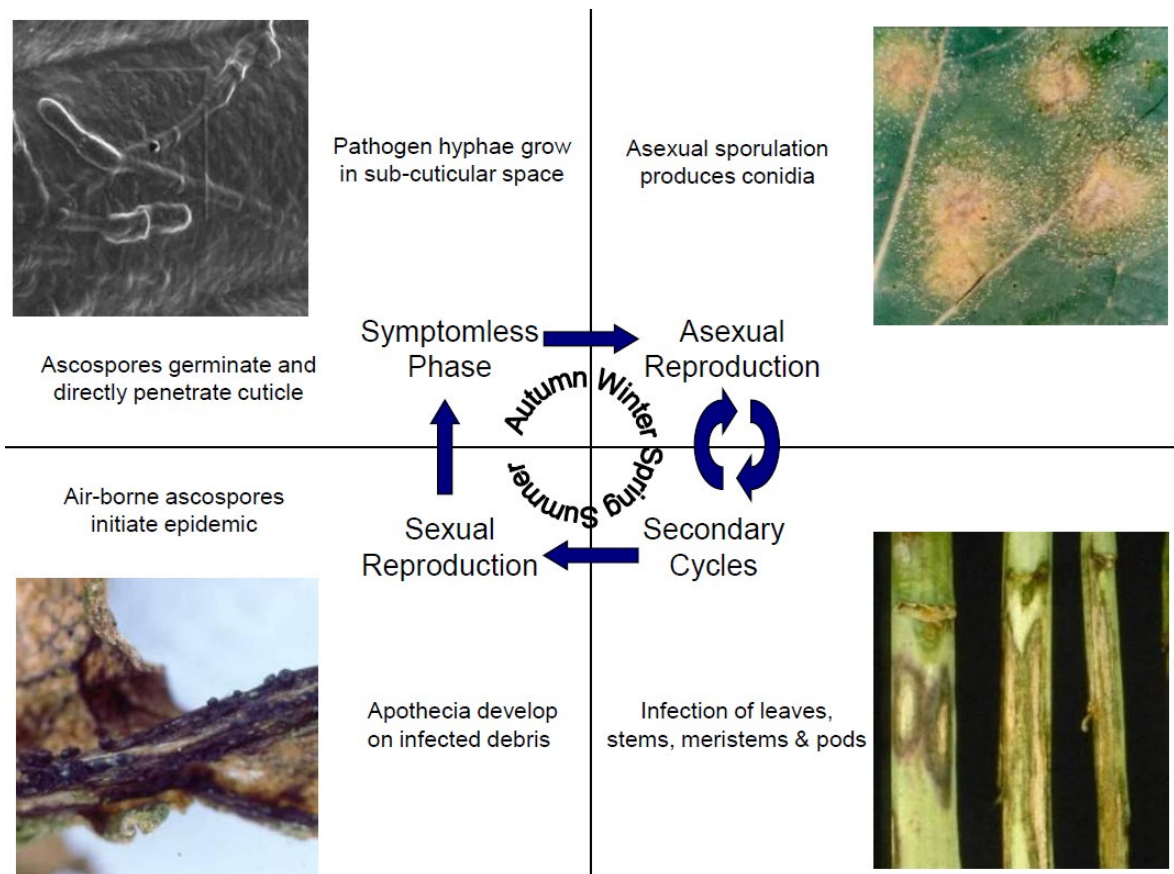
Figure 8: Life cycle of *Pyrenopeziza brassicae* on winter oilseed rape (graph: Bruce Fitt).

5.9 Diagnosis and symptom development on plants

5.9.1 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 extensively 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.

5.9.2 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.

5.9.3 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.
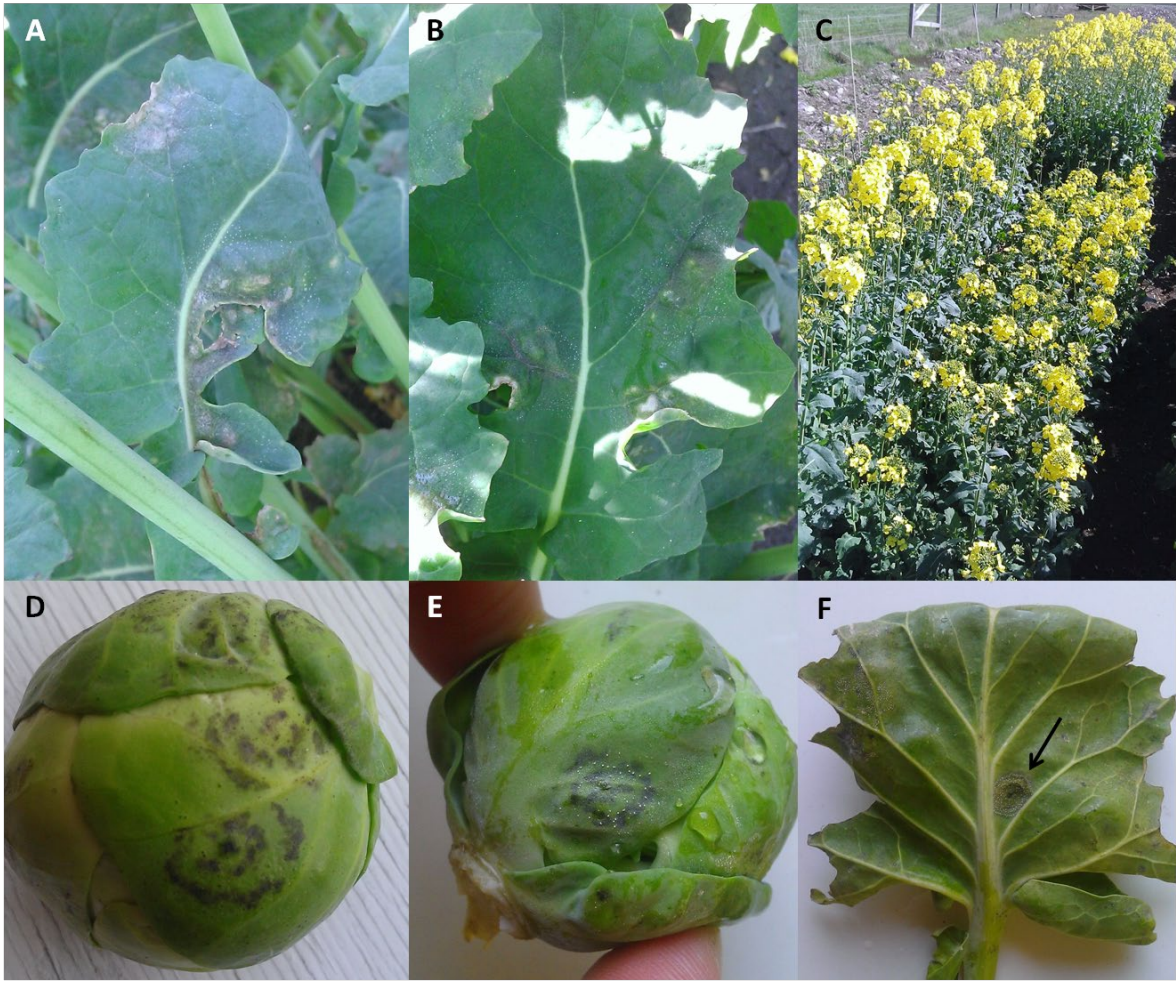
Figure 9: Light leaf spot symptoms on oilseed rape (A-C) and Brussels sprouts (D-F). A) distortion of a leaf and light leaf spot symptoms with sporulation, **B)** light leaf spot symptoms with sporulation, **C)** oilseed rape cultivar stunted (right hand side) due to light leaf spot compared with a more 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).

5.10 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.

5.10.1 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 remove 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 be 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 susceptible stage of the plant host or more likely the maximum 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.

5.10.2 Resistance against *Pyrenopeziza brassicae*

Furthermore, the cultivation of resistant cultivars is a very effective method for control a disease.

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 HGCA 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* (HGCA 2014). Disease resistance is indicated on a scale from 1 to 9 with the higher numbers indicating better resistance. On the recommended list for the current cropping season, the mean disease ratings for listed cultivars recommended for the East/West and North region are 5.7 (SD = 0.74) and 6.36 (SD = 0.87), respectively. Therefore, the resistance in cultivars is generally moderate. Only the cultivar "Cracker" (score = 8) and cultivar "Cuillin" (score = 8.7) show very good resistance scores. Nevertheless, it seems that resistance of "Cracker" has been rendered ineffective in Scotland this year (Mark Nightingale & Jim Anderson, personal communication). There is a need for improved resistance against *P. brassicae* in cultivars.

To date, they have not been many scientific studies on resistance against *P. brassicae*. 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 that Bradburne et al. (1999) had 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 *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 the 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 effector gene in *P. brassicae* strains. Simons & Skidmore (1988) seem to have found differential interactions for *P. brassicae* and brassica cultivars that indicates a gene-for-gene relationship. The interaction of *P. brassicae* with different brassica species and cultivars should be investigated more closely.

5.10.3 Fungicide application

Chemical control of pests and diseases is a common tool to reduce yield losses. The timing of fungicide 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 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 forecasting paper more references) 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 2014). This forecast includes regional mean rainfall and summer temperature data from 30 years as well as data for pod disease incidence from the previous cropping season. It is updated in spring for the deviation of winter temperature from the 30 year mean (Anonymous 2014).

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, due to mutations in the *CYP51* gene encoding for sterol 14α-demethylase has been reported (Carter et al. 2014).

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

5.11 Aims of the study

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

- to study the *P. brassicae* population structure morphologically
- to study the *P. brassicae* population structure molecularly with the use of neutral markers
- to identify differential interactions between *P. brassicae* strains and oilseed rape and vegetable brassicas

- to study if the same *P. brassicae* strains are able to infect both oilseed rape and vegetable brassicas
- to identify possible *R* genes in the Q population

6 Materials and Methods

6.1 Culture media

Culture media were prepared as described in the following.

Potato dextrose agar (PDA)

PDA   39 g

Distilled water   1000 ml

Streptomycin   50 ppm

Penicillin   50 ppm

Malt agar (MA)

Malt Extract   30 g

Agar Agar   15 g

Streptomycin   50 ppm

Penicillin   50 ppm

Germination medium (GM)

Murashige Skoog Basal Medium   4.4 g

Sucrose   30 g

Difco Bacto   0.8 g

Distilled Water   1000 ml

6.2   Collection of *Pyrenopeziza brassicae* isolates

Ten oilseed rape cultivars chosen had different light leaf spot disease ratings on the HGCA recommended list (Table 2). The cultivars were drilled at four different locations (Cambridgeshire, North Yorkshire and Herefordshire) by ADAS Ltd or Elsoms Seeds Ltd. Younger leaves showing clear light leaf spot symptoms were sampled and wrapped into absorbent paper. Additionally, the OREGIN field plots at Rothamsted Research were used as source for *P. brassicae* isolates (http://www.oregin.info/). Samples from other locations, especially vegetable samples, were kindly provided by various people.

6.3   Isolation and culturing of *Pyrenopeziza brassicae* isolates

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 on PDA plates including antibiotics. 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.

Table 2: List of cultivars drilled on field sites with information on HGCA recommended list ratings for light leaf spot (HGCA 2014). Brackets include information about the particular regional list and the cropping season.

Cultivar	Recommended List Rating
Anastasia	6.0 (East/West 2013/2014)
Bristol	Susceptible*
Castille	5 (East/West 2012/2013)
Catana	7.4 (North 2013/2014)
Cuillin	8.7 (North 2013/2014)
Marathon	5.1 (East/West 2013/2014)
Recital	5 (2007/2008)
SWO24120	Moderately resistant*
Temple	6.7 (North 2013/2014)

* Information not available in the archive of HGCA recommended lists. Categorisation according to personal communication with Peter Gladders and Mark Nightingale.

6.4 *Pyrenopeziza brassicae* inoculum production

6.4.1 Preparation of spore suspension from leaf material

If more than ten leaves with good sporulation were available, spore suspensions were produced from the infected leaf material. Leaves showing symptoms of any other disease were discarded. Leaves with sporulation light leaf spot symptoms were put in a polyethylene bag and 300 ml of distilled water was added. The polyethylene bag including leaves and water were shaken from time to time and incubated for one hour. Spore suspensions were counted for their concentration using a Bright-Line™ haemocytometer slide and subsequently stored at -20 °C.

6.4.2 Preparation of spore suspension from *in vitro* cultures

After *P. brassicae* isolates had grown to a diameter of ca. 4 to 5 cm, agar plugs were cut out for the mass production of asexual spores. Therefore, agar plaques with young mycelial growth were cut and transferred to Eppendorff tubes with 1 ml of distilled water. The agar plugs were ground with micro vial homogenizers and 200 µl of the resulting solutions were spread on each MA plate evenly with a Drigalski spatula. After 5 weeks, the plates overgrown with mycelium and spores were used for the production of spore suspensions.

To each culture plate, 10 ml of sterile tap water was added and the mycelium and spores were scraped off the MA plates with sterile microscope slides. The resulting solution was filtered with Miracloth tissue (Calbiochem) into Falcon tubes. The concentration of spore suspension was determined a Bright-Line™ haemocytometer slide.

6.5 Growth of plant material

Two days before sowing, seeds were pre-germinated in Petri dishes with wetted Whatman™ qualitative filter paper No. 1. Seeds were sown 2 cm deep into a 1:1 soil mixture of all-purpose compost (Miracle Gro) and John Innes No.3 soil based compost (J. Arthur Bower's). Vermiculite Fine (1 – 3 mm, Sinclair) was evenly distributed on the soil surface after sowing. Plants were grown under glasshouse conditions until further use.

6.6 Inoculation of true leaves

Plants that reached BBCH 14 – 15 (GS 1,4 – 1,5 Sylvester-Bradley et al. 1984) were used for inoculation experiments. Spore suspensions from natural inoculum (leaf material) were used for inoculation with a spore concentration of 1×10^5 spores per ml. Plants were sprayed with the help of a 50 ml spraying bottle (Boots Ltd.) until leaves were evenly covered with suspension. After inoculation plants were covered with polyethylene bags for 48 h to ensure high humidity. Plants were kept in controlled environment (CE) cabinets (Conviron Adaptis, Controlled Environments Ltd.) under a 12 h day/12 h night light regime at 16 °C day/14 °C night.

6.7 Light leaf spot assessment of OREGIN diversity set

The OREGIN diversity set consists of 100 accessions of brassica material of different origin. These accessions were drilled on West Barnfield at Rothamsted Research on the 31. August 2013. The experiment was randomized in a split block design with two different nitrogen treatments, low (0 kg N/ha) and high (30 kg N/ha) and two replicates per treatment.

The OREGIN diversity set was assessed for light leaf spot incidence and severity in mid-March 2014. Incidence was determined in % plants affected by assessing ten plants for presence or absence of disease. Severity was scored according to the scale shown in Table 3.

Table 3: Rating scale and description for light leaf spot assessment in the field (modified according to Boys 2009).

Score	Description
0	No disease observable
1	Trace of disease
2	Diseased leaves with one small lesion, plants with a few scattered lesions
3	Diseased leaves with a few small lesions, plants with several scattered lesions
4	Diseased leaves with a few large lesions or many small lesions, area quite heavily infected
5	Half green/half diseased plants, leaf curling and distortion
6	Plants heavily diseased, evidence of stunting
7	Plants heavily stunted or dead
8	All or most of the plants dead

7 Results

7.1 Collection of *Pyrenopeziza brassicae* isolates

Thus, so far 734 isolates have been collected from 15 locations, nine in England and six in Scotland. 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 isolates from cauliflower and 2 isolates from calabrese have been maintained.

7.2 Growth of fungal cultures, morphology and complications

The isolates collected show a great variation in their morphology varying from orange-pink pigmentation, grey-greenish to dark grey-black pigmentation (Figure 10).

Problems have occurred during maintenance of isolates. After the subculturing of isolates from PDA to MA agar plates a bacterial species was able to grow on the fungal hyphae (Figure 11).
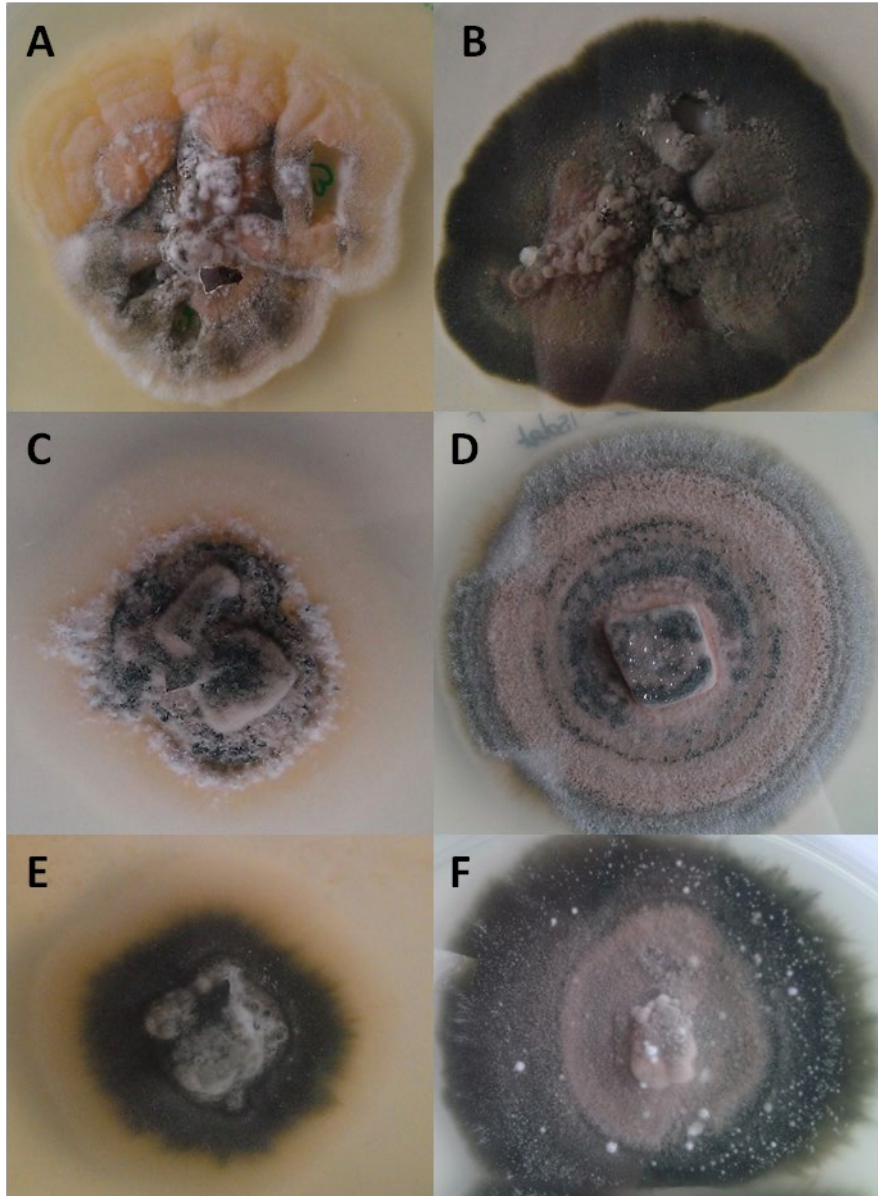
Figure 10: Morphology of *Pyrenopeziza brassicae* isolates A) PbUK001 (OREGIN), B) PbUK031 (OREGIN), C) 13BSpr78, D) 13Cb2.1, E) 13OSR102, F) 13OSR46 on PDA.
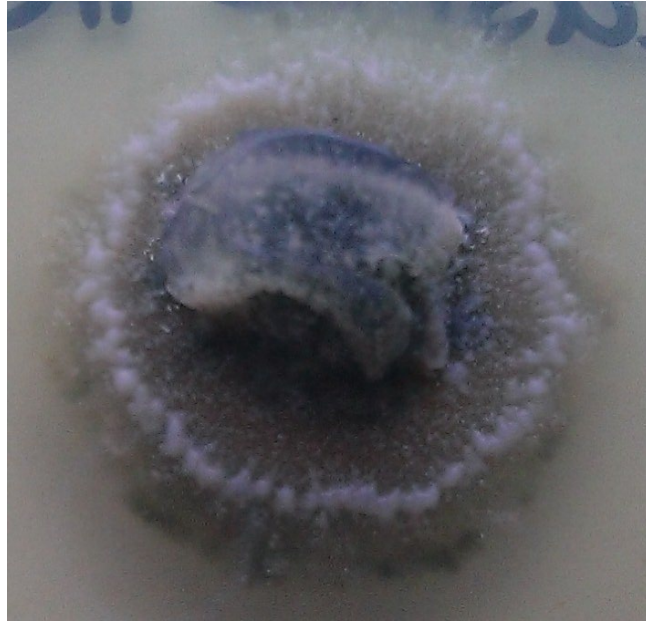
Figure 11: *Pyrenopeziza brassicae* **isolate 13OSR53 infested with bacteria that grow on fungal hyphae.**

7.3   Plant growth issues

Plants which have been grown for inoculation were firstly damaged in the controlled environment (CE) cabinet and therefore did not show light leaf spot symptoms (Figure 12). Furthermore severe infestation with green peach aphids (*Myzus persicae*) made a use of plants for inoculation impossible (Figure 12).

Figure 12: A and B) Leaves of oilseed rape leached due to complications in CE cabinets; C and D) heavy infestation of oilseed rape with *Myzus persicae*.

7.4   Light leaf spot assessment of the OREGIN diversity set

The incidence of light leaf spot was high in the field experiment, with 50% of the accessions had more than 80% of plants affected. For each crop type differences between accessions were observed in incidence and severity of light leaf spot (Figure 13, Figure 14).
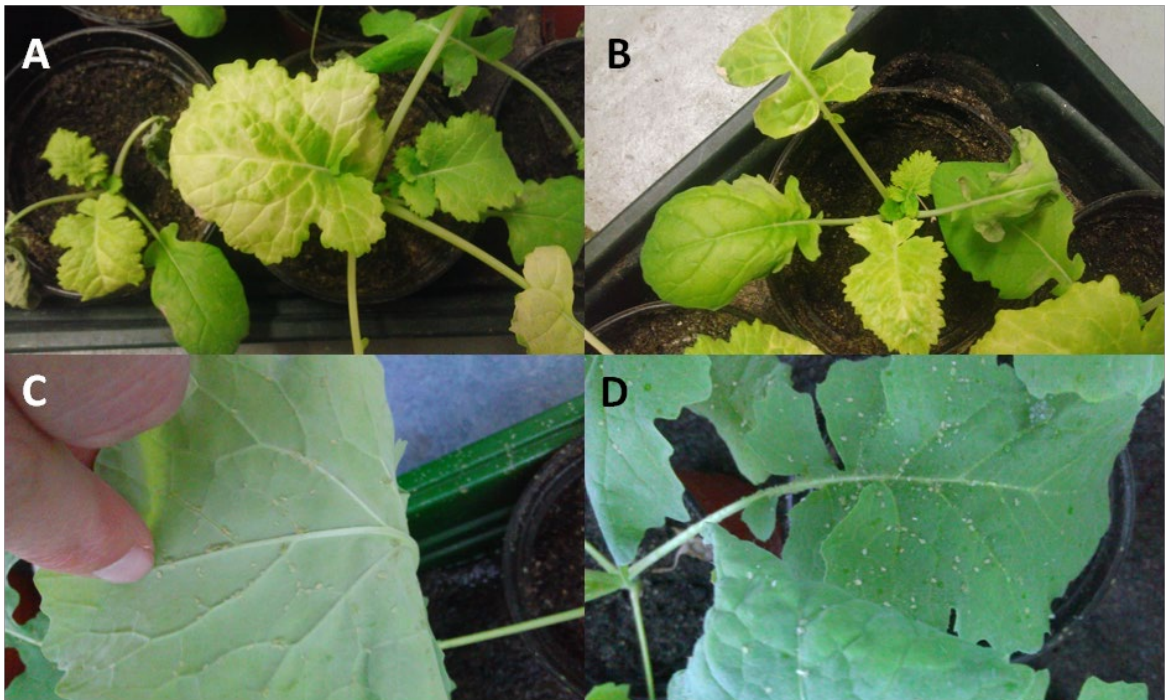
Twelve accessions, in particular, had a low incidence of light leaf spot (Figure 13), less than 30%, and a low severity of light leaf spot (Figure 14). This group included seven swede accessions (*B. napus* var. *napobrassica*), one forage rape, two spring oilseed rape cultivar and one winter oilseed rape cultivar.

For this assessment, light leaf spot incidence strongly correlated with the severity score (r = 0.92) in an exponential manner (Figure 15). Although for some accessions light leaf spot incidence was relatively high, light leaf spot severity was small. An example for that is the swede accession "Jaune" (Figure 13, Figure 14)

The different nitrogen treatments (high or low) did not influence light leaf spot incidence (t-test, P = 0.95) or severity (t-test, P = 0.18) significantly (Figure 16).
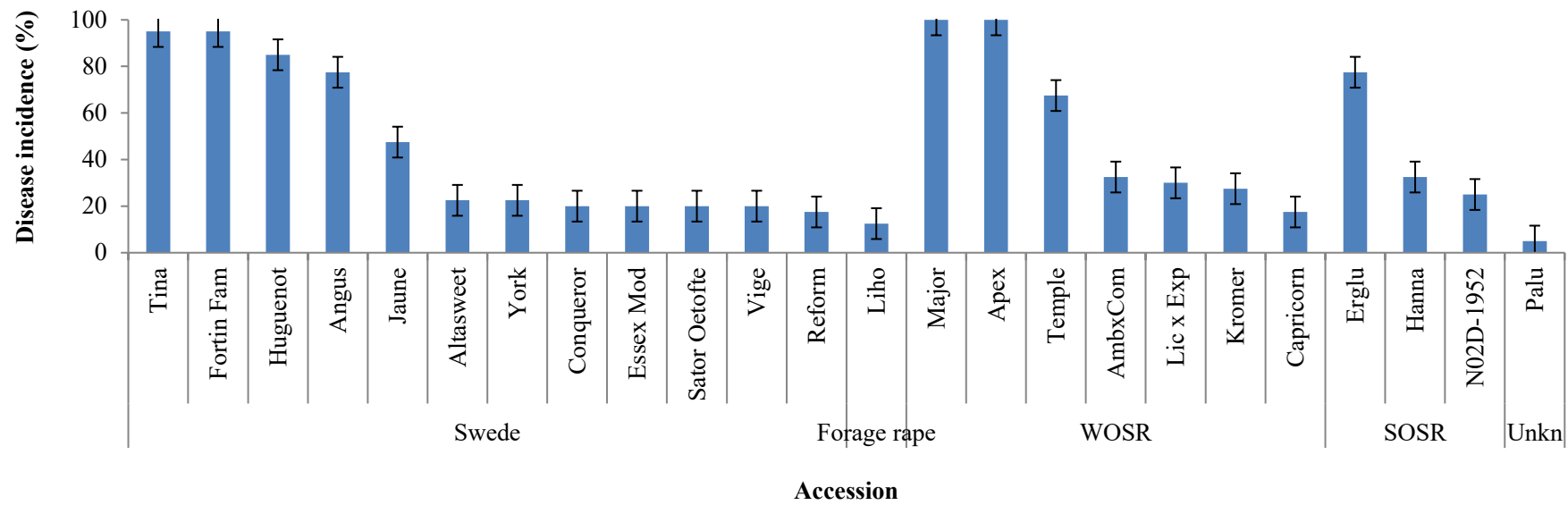
Figure 13: Light leaf spot incidence (% plants affected) of accessions of different brassica crop types (swede, forage rape, WOSR = winter oilseed rape, SOSR = spring oilseed rape and an Unkn = unknown, error bars are SE.
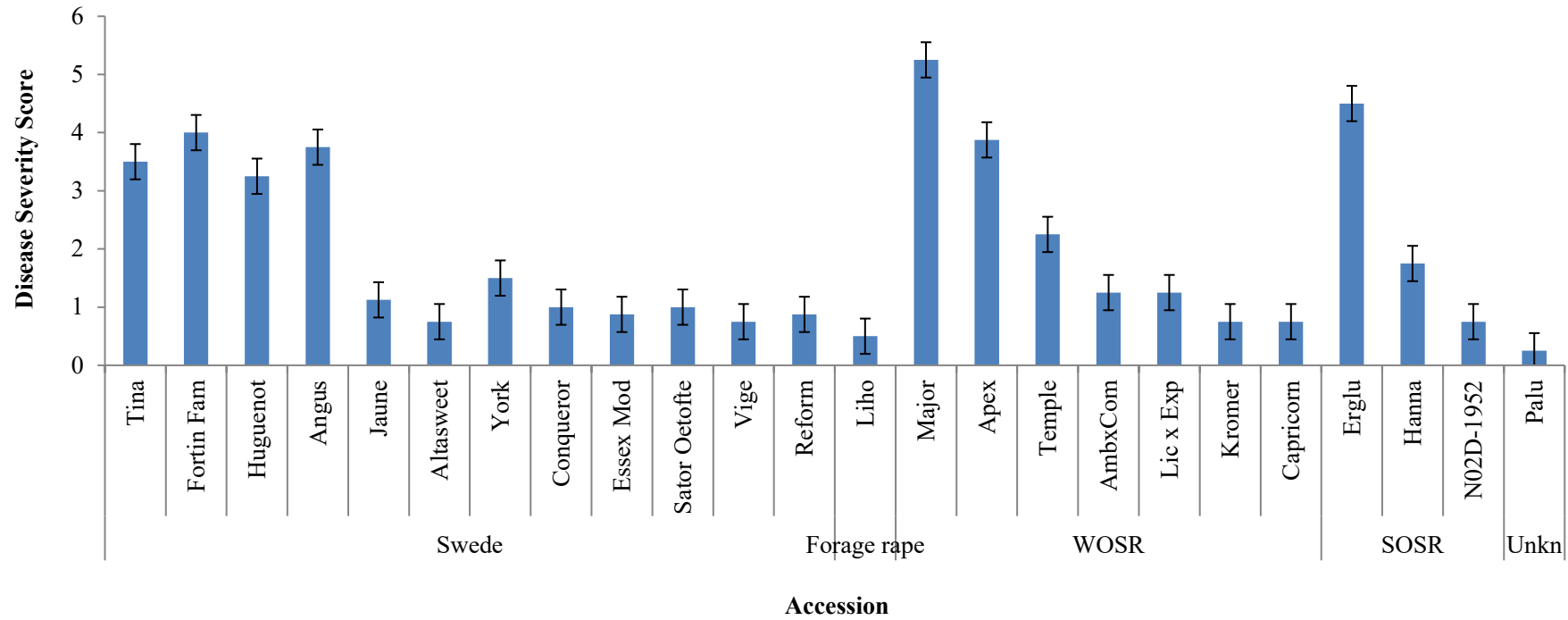
Figure 14: Light leaf spot severity (0 - 8 scale) of accessions of different brassica crop types (swede, forage rape, WOSR = winter oilseed rape, SOSR = spring oilseed rape and an Unkn = unknown, error bars are SE.
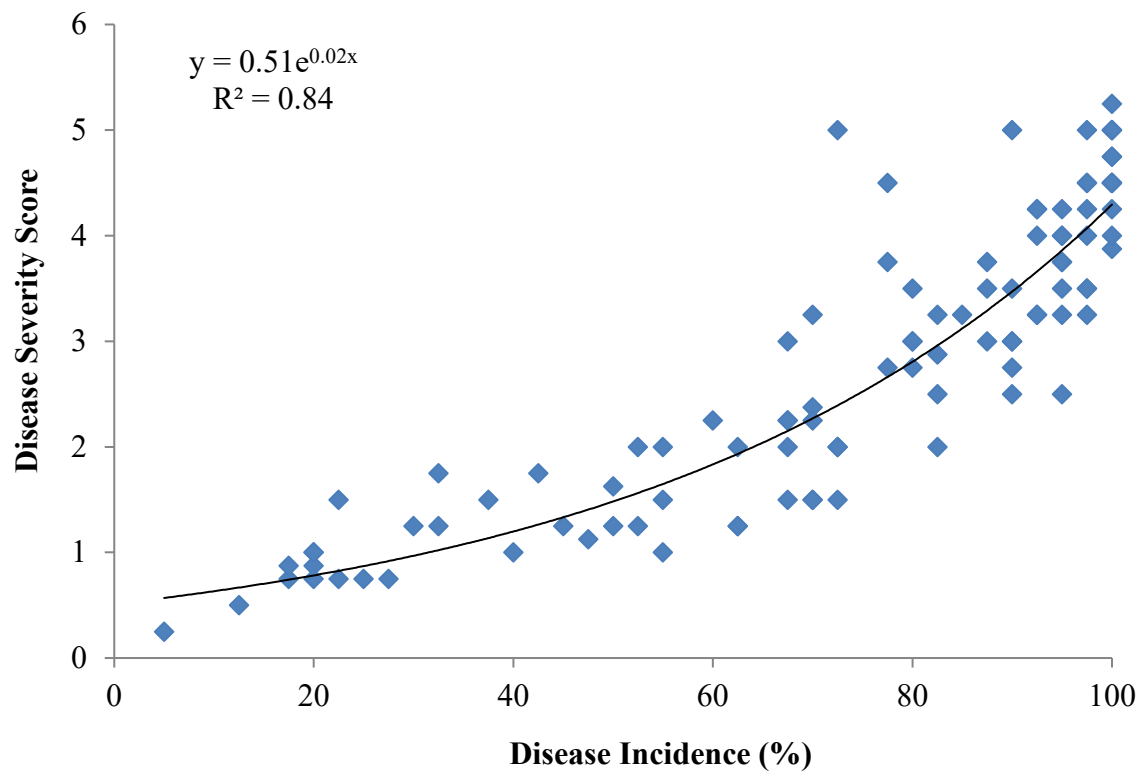
Figure 15: Relationship between light leaf spot incidence (% plants affected) and disease severity (0 – 8 scale) for the assessment of the OREGIN diversity set.
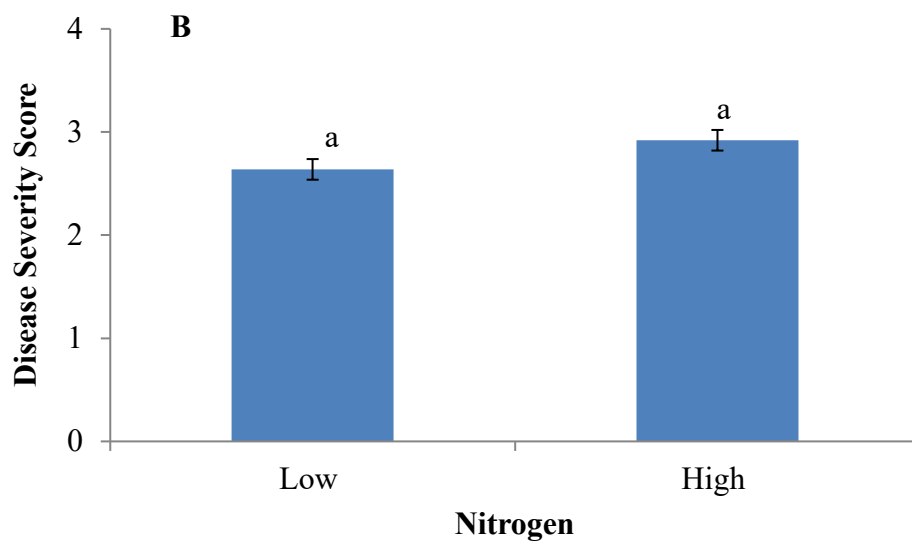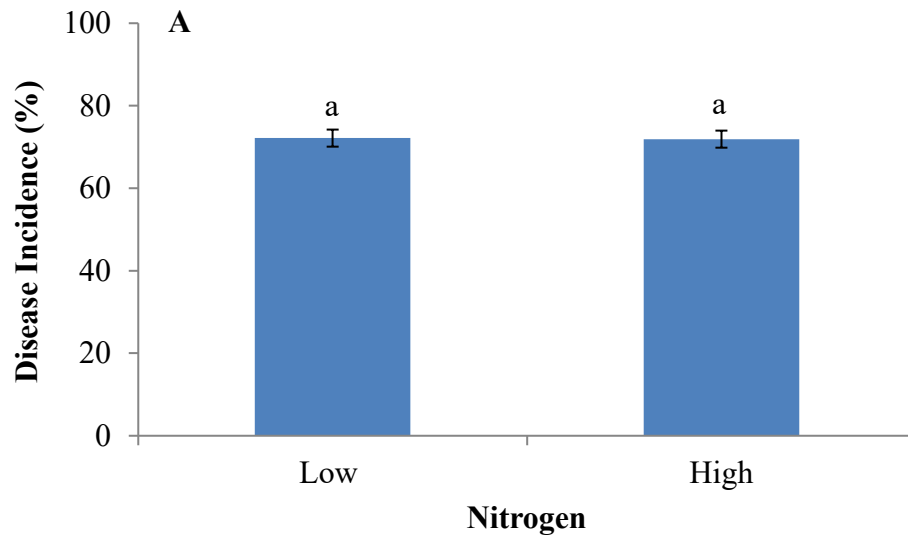
Figure 16: A) light leaf spot incidence (% plants affected) and B) light leaf spot severity score (0 – 8 scale) for different nitrogen treatments (low or high), error bars are SE.

8 Discussion

8.1 Morphological variation between *Pyrenopeziza brassicae* isolates

P. brassicae isolates show great variation in terms of colony colour ranging and colony colour also changed over time, which made it more difficult to distinguish isolates correctly. Other fungal growth media should be tested for their usefulness to distinguish colony colour more consistently. For example, *L. maculans* and *L. biglobosa* can be distinguished for their colour on PDA medium with *L. biglobosa* showing yellow pigmentation. Moreover, *L. biglobosa* shows higher growth rates than *L. maculans* (Williams & Fitt 1999).

Information on colony colour and growth rate may not be sufficient to distinguish between fungal isolates. Morphology of conidia, i.e. conidial length, diameter and shape, could be other indicators of differences between isolates. King et al. (2013) have identified a new subspecies of *Rhynchosporium*, *R. lolii*, which is closely related to *R. orthosporum* but showed significant differences in conidial length.

Morphological differentiation may not be sufficient to distinguish between possible subspecies. As an example, *Magnaporthe oryzae* and *M. grisea* cannot be differentiated morphologically and isolates were previously just characterized by the host they were isolated from, until Couch & Kohn (2002) were able to distinguish these pathogens by molecular methods.

Problems occurred after the transfer of *P. brassicae* isolates from PDA to MA medium. Bacterial growth on the fungal hyphae was observed. Bacteria seem to have escaped the contact with antibiotics contained in the medium. Therefore, agar plugs overgrown with mycelium and bacteria were dipped into streptomycin and penicillin to ensure that bacteria came into contact with the antibiotics before transferring agar plugs onto PDA plates. Tween, a detergent, should have been added to ensure that the bacteria come into contact with the antibiotics. However, cultures with bacterial infection should also be maintained because the bacteria could play a role in the interaction of the pathogen with the plant and possibly acts as pathogenicity factor (Bruce Fitt, personal communication).

8.2 Plant growth and inoculation

So far it has not been possible to infect plant material with *P. brassicae* artificially due to problems with growth facilities and aphid infestation. Plants are now grown in a different environment (CE rooms) to ensure better conditions for inoculation experiments.

8.3 Variation of resistance against *Pyrenopeziza brassicae* in the OREGIN diversity set

Accessions in the OREGIN diversity set showed differences in resistance against *P. brassicae.* Besides the spring oilseed rape N02D-1952 and Palu, a brassica of unknown crop type, swede accessions had the lowest light leaf spot incidence and severity score, whereas other swede accessions developed severe light leaf spot. Maddock et al. (1981) also found variation for resistance in swede varieties but most varieties were rather susceptible to *P. brassicae*. However, they used a detached leaf method for inoculation, which can have an influence on the results because resistance mechanisms may be circumvented by leaf detachment. Nevertheless, swede could easily be used as a resistance source for new oilseed rape cultivars because crosses between swede and oilseed rape are fertile (Bradshaw 2010). The disadvantage of crosses with related species is that undesirable agronomic factors are introgressed in the material and make time-consuming backcrossing programmes necessary (Von Korff et al. 2006).

Oilseed rape accessions also showed differences in severity of light leaf spot (Figure 13, Figure 14). The oilseed rape cultivars "Kromer" and "Capricorn" had the lowest light leaf spot incidence and severity and could possibly be sources of resistance against *P. brassicae.*

Light leaf spot disease incidence and severity showed a strong exponential relationship. Therefore, disease severity would not necessarily need to be assessed. According to Seem (1984), the observed relationship in the present data set is typical for a polycyclic disease. When few plants are affected and disease severity is low, secondary propagules are likely to land on uninfected plants; thus influences disease incidence. When disease has spread, disease incidence is less affected by severity because secondary propagules are more likely to land on plants that are already affected.

However, one light leaf spot assessment may be insufficient for judging resistance of plant material because some accessions might be severely affected and suffering from light leaf spot early in the growing season but at a later stage cope better with the disease (Mark Nightingale, personal communication). Therefore, it is recommended to assess resistance against *P. brassicae* at different time points during the cropping season.

Differences in nitrogen application did not have a significant effect on light leaf spot incidence and severity. In accordance with that, Sutherland et al. (2004) did not find differences between treatments with high (200 kg N/ha) and low nitrogen (100 kg N/ha).

9 Future Work

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

9.1 Morphological differentiation of *Pyrenopeziza brassicae* isolates

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

9.2 Molecular studies and population genetics

To further distinguish isolates, studies on molecular level will be conducted. 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 be studied using a combination of neutral genetic marker. Firstly, RAPD markers will be used because DNA sequence information is not needed for this marker type. However, repeatability can be problematic when using RAPD markers. Depending on the results of the RAPD marker screening, other techniques can be used to support the initial data, for example rep-PCR, BOX-PCR and ERIC-PCR, similar to the studies of King et al. 2013. Furthermore, comparative genomics could be used to develop more reliable types of markers. *Rhynchosporium* spp. is closely related to *P. brassicae* (Goodwin 2002) and data of the whole genome sequence is available.

9.3 Pathogenicity tests on plant material

Plant material will be tested to distinguish differential interactions between cultivars and *P. brassicae* isolates. Therefore, isolates from different origin, isolated from different cultivars and locations, will be chosen. Inocula will be prepared as described in chapter 6.4.2. If problems occur with inocula derived from *in* vitro cultures, experiments will be continued with natural inocula prepared from infested leaf material. True leaf and cotyledon inoculation will be conducted. It will be tested, if cotyledon tests give representative results compared to true leaf testing. Cotyledon experiments would save time and facility space.

Furthermore, it will be studied if isolates derived from vegetables are able to infect oilseed rape and vice versa. Oilseed rape will be inoculated with vegetable isolates and vegetables will be with isolates originated from oilseed rape.

9.4 Identification of *R* genes in the Q population

The Q Population has been produced from a synthetic cross *B. oleracea* var. *atlantica* and *B. rapa* and has been backcrossed with the *B. napus* cv. Tapidor. Material of the same cross had been studied by Bradburne et al. (1999) and two *R* genes operating against *P. brassicae* were described. The *B. napus* cv. Imola had been derived from the Bradburne material but just one *R* gene was identified (Boys et al. 2012). Therefore, it will be studied if *R* genes operating against *P. brassicae* can be mapped. Previous investigations on this doubled haploid (DH) population have found segregation for resistance between DH lines (Neal Evans). Currently material of the DH lines is being propagated for field testing by project partners (Elsoms Seeds Ltd., Limagrain UK Ltd.). Field assessments for light leaf spot resistance from two seasons will be combined with additional genetic marker analyses to be able to map possible *R* genes.

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