Infection biology and aggressiveness of *Puccinia striiformis* on resistant and susceptible wheat.

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Preface

This thesis has been submitted to the Graduate School of Science and Technology (GSST) at the faculty of Science and Technology, Arhus University in order to fulfill the requirements for obtaining the Ph.D. degree.

The project was mainly conducted at Research Center Flakkebjerg, unit of Plant Pathology and Entomology, Aarhus University during the period between September 2008 and November 2012. Experimental work was further carried out at INRA, Thiverval-Grignon, France during a six weeks stay in spring 2012 and parts of the microscopic work were performed at Danish Molecular Biomedical Imaging Center (DaMBIC) at University of Southern Denmark.

Professor Mogens Støvring Hovmøller was the main supervisor of the project and co-supervisor was associate professor Annemarie Fejer Justesen both from faculty of Science and Technology, Arhus University. The experimental work performed at INRA was further supervised by Dr. Claude de Vallavieille-Pope, INRA, Thiverval-Grignon, France.

The project work included design of experiments, experimental work in both greenhouse and field, statistical analysis of data and processing and analysis of images. In addition, Ph.D. courses covering a total of 30 ECTS was passed and public dissemination of results in forms of talks and a poster was undertaken.

This Ph.D. project resulted in three manuscripts. One manuscript on the development and structure of *Puccinia striiformis* haustoria in wheat seedlings has been accepted for publication in the American journal *Mycologia*. A second manuscript dealing with the fitness and cost of virulence for wild type and mutant isolates of *P. striiformis* has been submitted to *Plant Pathology*. The third manuscript addressing interaction between quantitative resistance in adult wheat plants and *P. striiformis* isolates of different evolutionary origin is in preparation for submission.

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Summary

Yellow rust on wheat caused by the fungal pathogen *Puccinia striiformis* is one of the most damaging diseases in cereal production worldwide. During this PhD project, we made new discoveries about *P. striiformis* haustoria development in infected wheat leaves and about the interaction between aggressive/non-aggressive pathogen strains and sources of qualitative and quantitative based resistance in the wheat host. *P. striiformis* is obligate biotrophic fungus, which during infection forms an intimate relationship with the host plant. This includes the formation of highly specialized structures, haustoria, which are formed between the cell wall and plasma membrane of host cells in leaves. Haustoria play an important role in acquisition of water and nutrient from the host and are further involved in signalling processes, including suppression and induction of plant defence. Due to its biotrophic nature *P. striiformis* cannot be grown in culture and infection structures can thus only be studied in leaf tissue. In this study, a classical staining technique was used in combination with advanced 2-photon microscopy to study haustoria in fixed whole leafs. This allowed 3D reconstruction of fungal structures and revealed several characteristics of haustoria not previously reported. The haustorial body gradually changed from small and spherical to highly irregular and apically branched, taking up significant space in the host cell. The haustorial neck area, which is thought to play an important role in the formation of haustoria, showed significant changes during haustoria development. These results indicate that haustorium formation is a highly dynamic process, which should be considered in future studies on the function and constitution of haustoria of *P. striiformis*.

*Puccinia striiformis* has shown a tremendous ability to adapt to resistance in the wheat host when the resistance has been based on resistance genes with major (qualitative) effects (*R*-genes). It has been hypothesized that the acquisition of virulence, i.e., the ability to compromise resistance in the host, could be associated with a fitness cost due to loss or change of gene function. In this thesis, we analysed data from an experiment where the fitness of wild type and virulence mutant had been measured by epidemiological parameters like latent period and lesion growth. No fitness cost was detected for the acquisition of virulence to the Yr2 resistance gene in wheat. In contrast, fitness differences were detected among isolates of different origin (genetic background). These results could indicate a potential selection based
on variability in fitness components independently of selection for virulence. Such a selection may influence the effects of quantitative resistance, which has often been considered to be based on several resistance genes with individual minor effects, resulting in host plants with incomplete resistance. However, recent genetic studies have indicated that pyramiding of genes for quantitative resistance can give complete and durable resistance in the sense of being long-term effective when used on a large scale in an epidemiological environment favourable for the disease. In this project, we studied whether different sources of quantitative resistance were equally effective against pathogen isolates of different evolutionary background. A typical *P. striiformis* isolate of NW-European origin and two isolates representing non-European (exotic) strains were considered on adult plants of nine recombinant inbred lines (RILs) from a cross between the French varieties Camp Rémy (resistant parent) and Récital (susceptible parent). The RILs were selected according to different combinations of QTLs for resistance to *P. striiformis*. One isolate representing an aggressive and high temperature adapted strain that has spread globally in recent years were not able to grow on any of the host genotypes. However, the NW-European isolate and a new isolate first detected in Europe in 2011 gave highly contrasting results. All QTLs effective against the NW-European isolate was fully or partly overcome by the new isolate. Furthermore, the parent Récital, which were susceptible to the NW-European isolate showed incomplete resistance to the new isolate. These results show that quantitative resistance can be isolate-specific, indicating that the incursion of exotic strains of *P. striiformis* may represent a potential threat to the durability of quantitative inherited resistance. The results emphasise the importance of maintaining high diversity for disease resistance and of using isolates of diverse origin in wheat breeding programs.

*Puccinia striiformis* har vist en enestående evne til at tilpasse sig resistens i hvede når denne resistens baserer sig resistensgener (R-gener) med stor (kvalitativ) effekt. Tidligere opstillede hypoteser, som antager at tilegnelsen af virulens, dvs. svampens evne til at etablere sygdomsangreb på en resistent plante er forbundet med et fitnesstab blev ikke bekræftet. Vi analyserede data fra et eksperiment, hvor fitness for vildtype- og virulensmutantisolater blev målt ved hjælp af epidemiologiske parametre som eks. latensperiod og læsionsvækst. Mutation fra avirulens (vildtype) til Yr2-virulent mutant var således ikke forbundet med tab af fitness. Samme resultat blev fundet i to uafhængige vildtype/mutant par. Derimod blev der fundet fitnessforskelle mellem de to isolatpar når sammenligningen var baseret forskelle i genetisk baggrund. Disse resultater kunne indikere en potential selektion baseret på forskelle i fitness uafhængigt af selektion for virulens. En sådan type selektion kan eventuelt influere på effekten.
af kvantitativ resistens, som ofte anses for at være baseret på mange resistensgener med lille, individuel effekt. Nyere genetiske studier har imidlertid indikeret, at man ved at samle gener for kvantitativ resistens i samme sort kan skabe en kompleks resistens med øget holdbarhed, dvs. øget evne til at forebygge sygdomsangreb i både tid og rum. I dette projekt undersøgte vi, om forskellig kvantitativ resistens havde samme effektiv overfor gulrustisolater med forskellig evolutionær baggrund. Et isolat af nordvest europæisk oprindelse og to isolater af ikke-europæisk oprindelse blev inokuleret på voksne planter af ni rekombinant indavlsliner (RIL) fra en krydsning mellem de franske sorter Camp Rémy (resistent forælder) og Récital (modtagelig forælder). De ni RIL’er var selekteret på basis af forskellige kombinationer af QTL’er for gulrust resistens. Et isolat, som repræsenterende en aggressiv og høj-temperatur tilpasset linie som har spredt sig på globalt plan i de senere år, var ude af stand til at vokse på nogen af de undersøgte hvedelinier. Sammenligningen af det nordvesteuropæiske isolat og et nyt isolat, der først blev detekteret i Europa i 2011, gav imidlertid meget forskellige resultater. Alle QTL’er som havde stor effekt mod det nordvesteuropæiske isolat var helt eller delvist uden effekt mod det nye isolat. Desuden var Récital, som var modtagelig overfor det nordvest europæiske isolat, delvis resistent overfor det nye isolat. Resultaterne viser, at kvantitativ resistens kan være isolat-specifik, samt at invasive, eksotiske isolater kan repræsentere en potentielt risiko for nedsat holdbarhed af kvantitative resistens. Resultaterne understreger betydningen af at udvikle og opretholde høj diversitet for værtplanteresistens i planteforædlingen, samt at resistensen i nye sorter af landbrugsplanter undersøges ved hjælp af patogenisolater med stor diversitet.
Table of content

Introduction

*Puccinia striiformis*

- Phylogeny and host specialisation
- Life cycle
- Epidemiology
- Infection process and infection structures
- Haustoria

Host resistance and pathogen adaptation

- Resistance against *P. striiformis* in wheat
- Assessment of host resistance
- Pathogen adaptation to qualitative resistance and cost of virulence
- Pathogen aggressiveness

Project aims

List of manuscripts

I. 3-D imaging of temporal and spatial development of *Puccinia striiformis* haustoria in wheat.
II. Fitness of wild type and virulence mutants of *Puccinia striiformis* on wheat.
III. Exotic strains of *Puccinia striiformis* reveal race-specificity of long-term effective adult plant resistance in wheat

General discussion and future perspectives

References

Appendix
Introduction

During evolution plants have evolved an extensive array of mechanisms to defend themselves against pathogen attacks and pathogens have developed tools to overcome host defence (Maor and Shirasu 2005; Skamnioti et al 2005). Throughout history plant diseases have made a significant impact on food supply and human welfare (Strange and Scott 2005) and the evolutionary relationship between plants and pathogen is an on-going battle that continues into modern agriculture.

Wheat is one of the world’s most produced cereal crops and in many countries it is a main source of daily calorie intake (FAO statistics). The task of preventing severe epidemics in wheat caused by plant pathogens are therefore of major concern for food security. Cereal disease management has mainly relied on deployment of resistant host varieties and fungicide application (Roelf et al. 1992; Chen 2005; Walter et al. 2012). The use of host resistance is the more environmental friendly and practical way to control disease (Hallwork 2009). It further has an economical advantage and in many developing countries the use of fungicides is not an option due to cost and availability. Development of cereal varieties that carries resistance against important diseases is thus a main focus for plant breeders and researchers (Johnson 1992; Singh et al. 2004; Pratt and Gordon 2006).

In wheat production the focus has been on integrating resistance against several diseases in the same varieties (Simmonds and Rajaram 1988; Johnson 1992). A strategy that was first initiated by Nobel Laureate Norman E. Borlaug (1914-2009) through the breeding of multiline varieties and which has contributed considerably to the dramatic increase in crop yield seen since the 1950 (Rajaram and Braun 2006; Joshi et al. 2007). Despite the success, use of host resistance involves several challenges and a thorough understanding of pathogen biology and host-pathogen interaction is needed in order to deal with these.

The most important diseases on wheat include fungal pathogens within the group of rust fungi. Wheat is a host for three rust fungi *Puccinia graminis, Puccinia triticina* and *Puccinia striiformis* causing the diseases stem rust, leaf rust and yellow rust, respectively (Roelfs and Bushnell 1985). Both historically and recently all three rust pathogens have caused severe epidemics. The success of these pathogens in an agricultural environment is due to their ability to spread by the wind over long distances and their ability to adapt to host resistance (Brown and Hovmøller 2002; Kolmer 2005). Many examples exist where these pathogens have overcome new resistant
wheat varieties only few years after they were first used at large scale in the field (e.g. Bayles et al. 2000; Kolmer et al. 2004; Jin et al. 2008).

Recently, international attention has been drawn to a new strain of stem rust that proved to be able to infect most of the commercial wheat varieties grown worldwide (Singh et al. 2006). The Borlaug global rust initiative (www.globalrust.org) was launched in order to take action against this serious threat to the world’s wheat production. The Initiative has been supported by several international institutions and foundations and has led to coordinated action at an international scale. While focusing on stem rust the initiative has also renewed focus on both leaf rust and yellow rust. Stem rust poses a serious threat due to its severe effect on crop yield but in fact the main epidemics on wheat in recent years have been caused by yellow rust (Wellings 2011). In a survey made among 25 internationally recognized plant pathologists with expertise in rust, yellow rust was estimated to be the most important disease on wheat currently (Wellings 2011). The consequences of significant attacks of yellow rust are reduced yield and grain quality (Roelfs et al. 1992). In case of epidemics yield losses have ranged from 10-70% but early infection on highly susceptible varieties can result in 100 % yield loss (Chen 2005). In epidemic years losses have been counted in million tons in some countries and resulted in great economic loss (Line 2002; Wan et al. 2004; Wellings 2007). In addition, millions have been spend on fungicide for protection of yield.

Historically, yellow rust has mainly been a problem in cool temperate regions and the earliest description of the disease comes from Europe (Eriksson and Henning 1896). The disease is found globally and is common in great wheat producing regions like China, US, Australia and the middle east (Yahyaoui et al. 2002; Wan et al. 2004; Chen 2005; Wellings 2007). Recent epidemics have been observed in new areas including the southern great plains of US and Western Australia (Wellings 2007). These areas have normally been considered too warm for epidemics but the emergence and global spread of new high temperature adapted strains has resulted in severe attacks (Hovmøller et al. 2008, Milus et al. 2009). In Europe a new multivirulent strain was observed for the first time in 2011 causing problems on previously resistant European wheat varieties (eurowheat.org). With these recent developments the presence of yellow rust seems to be ever-expanding and the threat from this disease always present. In order to take action we need to enhance our understanding of how this pathogen interacts with wheat as a host and to understand which factors determine the adaptation of this pathogen to host resistance.
**Puccinia striiformis**

**Phylogeny and host specialization**

The species was first described taxonomically under the name *Uredo glumarum* by Johann Carl Schmidt in 1827 (Stubbs 1985) but some very early disease reports are likely to describe *P. striiformis* as the cause of disease (Eriksson and Henning 1896). The current name was introduced by Cummins and Stevenson (1956) and was a reference to the original name *Puccinia striaeformis* proposed by the Dutch botanist Dr. Gérard Daniel Westendorp (1813-1868) in his taxonomical description of a specimen isolates from rye in 1854 (Westendorp 1854).

Due to the production of basidiospores during the life cycle *Puccinia striiformis* has been placed taxonomically in the phylum Basidiomycota (Kolmer et al. 2009). This relationship has been confirmed by phylogenetic studies (Grasso et al. 2006; Hibbett et al. 2007). The rust fungi include several genera, and molecular data indicates that they form a natural group of common origin (Maier et al. 2003; Maier et al. 2007). All rust fungi are placed in the order *Pucciniales* (formerly *Uredinales*) and *Puccinia* is by far the biggest genera including more than 4000 species (Cummins and Hiratsuka 2003).

All rust fungi are obligate biotrophs which means that they can only survive and reproduce on living host tissue. Otherwise they represent a highly variable group of fungi both in terms of morphology, host range and life cycle (Ellis and Ellis 1997; Cummins and Hiratsuka 2003). As for *P. striiformis* many rust fungi are the cause of some of the most devastating diseases on important crop species (Kolmer et al. 2009). The relationship between specific rust fungi and their host species is a very interesting area of research. Some species appear to have a very narrow host range whereas others are able to infect many different plant species (Ellis and Ellis 1997; Cummins and Hiratsuka 2003). The evolutionary connection between the pathogens and their hosts appear to have formed by a combination of co-evolution, host expansion and host jumps (van der Merwe et al. 2007; Schulze-Lefert and Panstruga 2011). The host range of *P. striiformis* has previously been described as very broad including species from up to 50 plant genera (Hassebrauk 1965), which includes the cereals wheat, barley and rye. However, as revealed by Liu and Hambleton (2010) the reliability of these early reports can be questioned and the host range may prove narrower. The host specialization of *P. striiformis* has been proposed to include the formation of formae speciales (f.sp.), which means that some varieties of the species are specialized to grow on specific host genera. A number of molecular and
taxonomic studies have supported the subdivision into formae speciales (e.g. Newton et al. 1985, Chen et al. 1995, Spackman et al. 2010) but as discussed by Hovmøller et al. (2011) the concept of formae speciales might not be useful for *P. striiformis* as genetic diversity between isolates from the same host but of different geographical origin can be larger than for isolates sampled from different host species. On the other hand Liu and Hambleton (2010) suggested that some of the previously described formae speciales of *P. striiformis* should be classified as new species. This suggests that host boundaries in some cases can be strong enough to drive speciation. Our knowledge about the evolutionary relationship between plants and plant pathogens are still limited. However, with the recent sequencing of genomes of several plant pathogens we are beginning to see indications that several factors such as horizontal gene transfer are important for understanding plant-pathogen co-evolution and host adaptation (Kemen and Jones 2012). A good understanding of such evolutionary relationships might assist the identification of effective resistance and in particular why some species can be hosts and others not is an important question.

Pathogen specialization on cereal hosts can further occur at the level of species where races of the pathogen are identified based on their ability to cause disease on particular host genotypes. For *P. striiformis* the concept of race was first introduced by Allison and Isenbeck (1930). Later Flor (1956) introduced the gene-for-gene hypothesis and the identification of races was found to be was tightly linked to this hypothesis. In the gene-for-gene relationship host resistance and susceptibility to the pathogen is determined by the presence or absence of matching pairs of host resistance genes and pathogen avirulence genes. In this context virulence, defined as the ability to cause disease, occurs when the pathogen is able to compromise host resistance (Hovmøller et al. 2011). Designation of pathogen races is based on reaction on differential sets of wheat varieties with known sources of resistance genes. Isolates of the pathogen are tested on seedlings on these varieties under controlled conditions and the disease level is evaluated on a scale ranging from full resistance to full susceptibility, indicative of the presence or absence of avirulence to specific resistance genes. Clearly the concept of race identification based on differential set is indefinite as one can only identify the races differentiated by the particular sets of host varieties. However when used consistently race identification has proven highly useful in many countries as an integrated part of early warning systems. In such systems the pathogen population is sampled for detection of races potentially dangerous to cereal production. Data
from such systems has also contributed significantly to our understanding of dynamics in pathogen populations (e.g. Hovmøller 2001; de Vallavieille-Pope et al. 2012).

**Life cycle**

Many rust fungi alternate between hosts during their life cycle and have separate hosts for asexual and sexual propagation. Until recently the sexual host of *P. striiformis* was unknown. Several unsuccessful attempts were made in the beginning of the 20th century to identify the alternate host of *P. striiformis* (Stubbs 1985) but now we know that *P. striiformis* is a macrocyclic rust with five spores types in the life cycle and heteroaecious with alternation between a graminaceous host for asexual reproduction and Barberry for sexual reproduction (figur1) (Jin et al. 2010).
During the growing season of the graminaceous host polycyclic infections occur by asexual urediniospores and later when the nutrient supply from the plant start to decline the production of telia is initiated resulting in the production of teliospores. In most rusts teliospores overwinter on residual plant material and germinate the following spring to produce basidiospores which can infect the host for sexual reproduction. Basidiospores are very prone to desiccation and therefore they cannot be dispersed for long distances without loss of viability (Gold and Mendgen 1991). When the alternate host has been infected pycnia with pyniospores form on the upper leaf surface. Evidence exists that at least in some rust species mating of
Pycniospores is mediated by insects, which are attracted by the production of nectar (Naef et al. 2002). Once fertilization has taken place new mycelium is formed that grows through the leaf to form aecia with aciospores on the lower side of the leaf. Teliospores of *P. striiformis* seem only to have a short dormancy and have no need for cold treatment to germinate (Rapilly 1979; Chen et al. 2012). It was further hypothesized by Chen et al. (2012) that in US Pacific Northwest where yellow rust is a common disease, teliospores would not be able to survive the winter and infect new Barberry leaves the following spring. Thus, despite that Barberry has been proven as a host under controlled condition our knowledge about the role played by Barberry and maybe other alternate host species in nature is still very scarce.

**Epidemiology**

During epidemics on the gramineous host asexual urediniospores are produced in huge quantities. Spores are mainly wind spread and most disperse to the neighbouring plants (Aylor 1987). A very small fraction of spores have been shown to be able to travel very long distance of up to several hundred kilometres when conditions are favourable (Hovmøller et al. 2002, Zeng and Luo 2006). Annual surveys conducted in e.g. the US have shown that long distance spread patterns of the pathogen can be explained by prevailing winds (Nagarajan and Singh 1990; Chen 2005). When susceptible host plants and inoculum are present weather conditions are the most important factor for infection (Zadoks et al. 1961, Gladders et al. 2007). Leaf moisture is essential for initiation of infection and a continuous period of minimum 3 hours of dew is required (de Vallavieille-Pope et al. 1995). The optimum temperature for spore germination is around 10-12° C (Rapilly 1979, Line 2002). Optimal conditions are most likely found at during the night and therefore night time conditions are more for determination of epidemics (Stubbs 1985). Latent period, the time from infection to spores are first formed in uredinia (pustules) on the leaf surface, has been found to be shortest at 12-19° C in susceptible cultivars, with a length of app. 10-15 days (McGregor and Manners 1985; Brown et al. 2001). The disease cycle can be repeated several times during the growing season of wheat. Because of the temperature and moist requirements yellow rust has mainly been a problem in temperate areas with a relatively cool environment and with regular precipitation and dew formation during the cropping season. In areas with cold winters the pathogen may not survive from season to season or year to year but can be reintroduced by long distance dispersal (Hovmøller...
et al. 2002; Brown and Hovmøller 2002). Cold winters negatively effects the survival of the pathogen and temperature during winter is of great importance for the severity of infection the following spring and summer (Hovmøller 2001). During mild winters *Puccinia striiformis* may survive as dormant mycelium on e.g autumn-sown wheat, creating a green bridge between seasons. In other areas disease development is halted due to high temperatures during the period of plant maturation. Temperatures above 30° C inhibit the systemic development of the pathogen and eventually spore production stops (Rapilly 1979).

**Infection process and infection structures**

As describe previously, wheat can be infected by both aeciospores produced by sexual reproduction and by asexual urediniospores. For *P. striiformis* on wheat no published information is yet available for infection initiated by aeciospores but in a study of *Uromyces vignae* on cowpea leaves the infection by aeciospores infection was found to be similar to infection by urediniospores (Stark-Urnau and Mendgen 1993). The general characteristics of how rust fungi infect their host(s) are well described and comprehensive reviews have been published by e.g. Perfect and Green (2001) and O’Connell et al. (2006).

The first histological examinations of how *P. striiformis* infects wheat were published in the 1970ties (Russell 1977; Mares and Cousen 1977, Mares 1979a and 1979b). Due to some limitation of the staining and microscopy techniques used in these first studies the best early description was given in a publication by Cartwright and Russell (1981). Cartwright and Russell (1981) used fluorescence microscopy to do observations on fungal structures in whole leaves stained with the fluorescent dye Uvitex2B. With the use of this technique Cartwright and Russell were able to do reliable observation of intact infection structures in non-disrupted leaf tissue from both seedlings and adult plants. They showed that like most other rust fungi, urediniospores of *P. striiformis* infect wheat by entering the leaf through stomata. Spores germinate on the leaf surface and form a germ tube that grows across the leaf in search for a stoma to enter. For several rust fungi evidence of directional growth of the germ tube has been found something that was also indicated in Cartwright and Russells (1981) observations. This type of growth optimizes the chance of encountering a stoma (Read et al. 1992) and is induced by the structure of the leaf surface (Allen et al. 1991). It seems that in most rusts an appressorium is formed upon contact with a stoma but it is now generally recognised that *P. striiformis* do not form an appressorium for penetration of stomata (Moldenhauer et al. 2006).
When the germ tube has entered the leaf through the stoma (Figure 2a) a substomatal vesicle is formed in the stomatal cavity and from this structure the formation of primary infection hyphae is initiated. When the primary infection hyphae reach contact with a mesophyll or an epidermal cell a haustorial mother cell (hmc) is formed (figure 2b). From the hmc an infection peg develops and penetrates the plant cell wall to establish a haustorium between the cell wall and the plasma membrane of the host cell (Ma et al. 2009). The haustorium probably represents the single most important structure for understanding host-pathogen interaction (Voegele et al. 2011; Cantanzariti et al. 2007) and its function and development will be described in more details later. Once the primary infection hyphae and the first haustoria have been established secondary infection hyphae will start to develop from the primary infection hyphae (figure 2c). These hyphae grow systemically in the intracellular space between mesophyll cells and ramify the mesophyll layer to form an extensive hyphal network (figure 2d). Approximately one week after infection the first pustule beds will start to appear and chlorotic spots will become visible at the leaf surface. Approximately 10-14 days after infection, spore bearing pustules will erupt through the leaf epidermis and the characteristic yellow spores will appear on the leaf. *P. striiformis* can infect both seedlings and adult wheat plants but whereas growth is unpolarized in seedlings transverse growth is restricted in adult plants due to strong vascular bundles in leaves (Cartwright and Russell 1981). Therefore, once pustules become visible on the leaf surface of adult plants they will appear in characteristic stripes. If the infected plant possesses some level of effective resistance various amounts of chlorosis or necrosis will become visible in the infected area.

During this thesis observation of infection structures and infection processes was performed on both adult plant and seedlings. Some of the staining and tissue processing techniques introduced in the 1970ties were used in slightly modified versions in combination with conventional epifluorescence and 2-photon microscopy. 2-photon microscopy is considered the most important advance in biological imaging since the introduction of confocal microscopy in the 1980ties (Feijo et al. 2004). Like confocal microscopy, 2-photon microscopy leaves out out-of-focus light and allows optical sectioning of thick specimens. The collection of layers of optical sections gives the opportunity to do 3D reconstruction of structures. In relation to the study of fungal structures in fixed whole leaf, 2-photon further has the advantage of allowing visualization of structures deeper into the tissue than confocal microscopy and in
minimizing photo bleaching in areas out of focus (Feijo & Moreno, 2004; Hepler & Gunning, 1998).

Figure 2. (a) A germinated urediniospore stained with the dye aniline blue and visualised by bright field microscopy. The germ tube of the germinated spore ends at a leaf stoma. Sp=spore; gt=germ tube; st=stoma; scale bar=50µm (b) Early colony inside a wheat leaf 44 hours after infection. Structures were stained with Uvitex2B and visualised by 2-photon microscopy. The substomatal vesicle (ssv) is the structure that forms right after the germ tube has entered the leaf through a stoma. From the ssv the primary infection hyphae (ph) develops and on these haustorial mother cells (hmc) are formed. Scale bar=20µm (c) From the primary infection hyphae secondary infection hyphae (sh) are developed which ramifies through the space between mesophyll cells and eventually the leaf is covered by an elaborate hyphae network. Scale bar=100 µm (d) Once the hyphael network is established pustule beds (p) will start to show up and app 10-14 days after infection the first spores appear on the leaf surface.

Clearly, basic microscopic observations on infection processes and structures have been and still are essential in setting up and testing hypothesis on how pathogens interact with their host. The development of a diverse array of molecular techniques has given us new insight and will be the main methods in the future. However, such molecular studies most often need to be assisted by microscopic observations to understand the spatial components of the interaction. Due to the biotrophic nature of rust fungi living colonies can only be observed in fresh host tissue and the next step in microscopy is to establish good systems for live-cell imaging. Such
observations have already been performed on biotropic plants pathogens which mainly infect the epidermal cells (e.g. Micali et al. 2011) but the deep layer growth of rusts represents a further challenge.

**Haustoria**

As obligate biotrophs, rust fungi has adapted to a life in close connection to their host. Obligate biotrophs are often referred to as `friendly´ parasites as in contrast to many other pathogens, they do not kill their host. Recent results also indicate that the life style of these pathogens share common features with mychorrhizal fungi which are involved in mutualistic relationships with their host plants (Rafiqi et al. 2012). Obligate biotrophs colonize their host in several different ways but a common characteristic is that successful establishment of a long-lasting intimate relationship with their host seems to be dependent on the formation of highly specialised structures that serve as a main interface between the pathogen and its host (Eichmann and Hückelhoven 2008; Gan et al. 2012). Depending on the species and type of mycelium these structures can take different shapes but during the uredinial stage of the life cycle rust fungi form structures that follows the definition of haustoria in sensu Bushnell (1972). Haustorial structures were first described more than 150 years ago and were hypothesized to be involved in nutrient and water uptake from the host (Voegele and Mendgen 2011). With the use of biochemical and molecular techniques supporting evidence for this hypothesis now exists. To sustain growth and reproduction rust fungi derive energy from living host cells and haustoria seem to play the lead role (Voegele et al. 2003; Voegele and Mendgen 2011). Haustoria have also been found to mediate the delivery of small proteins to host cells (Kemen et al. 2012). These proteins have been suggested to act as effectors for suppression and induction of host defence and for redirection of host metabolism (Heath 1997; O’Connell and Panstruga 2006; Catanzariti et al. 2007).

The structure and constitution of haustoria of rust fungi have been heavily studied in the past and extensive reviews of early observations are given by Littlefield and Heat (1971) and Harder and Chong (1991). Haustoria develop from haustorial mother cells (hmc) and terminate in living host cells (figure3). For *P. striiformis* haustoria on the primary infection hyphae start to develop with in the first day of infection (Sørensen et al. 2012). When the hmc reach contact with a host mesophyll cell a penetration peg form to penetrate the host cell wall after which the haustorium starts to develop between the cell wall and a membrane separating the haustorium
from the host cytoplasm (Ma et al. 2009; Mims et al. 2003). The membrane separating the haustorium from the host cytoplasm is called the extrahaustorial membrane (EHM) and is probably newly synthesised along with the formation of the haustorium (Koh et al. 2005). There is evidence that EHM is different from the plant plasma membrane. Most noticeable they seem to lack the ATPase activity of normal plasma membranes (O’Connell et al. 2006) and further they are deficient of proteins specific for normal plant cell plasma membranes (Koh et al. 2005; Micali et al. 2011). EHM is separated from the cell wall of the haustorium by an extra haustorial matrix (EHMx).

![Figure 3](image)

Figure 3. Generalized illustration of early infection structures of rust fungi. The figure illustrates where the haustorium (h) is placed in relation to other fungal infection structures and structures of the host leaf. The haustorium develops from a haustorial mother cell (hmc) and is formed between the cell wall and a plasma membrane separating it from the host cytoplasm. Fungal structures: U=urediniospore; GT=germ tube, A=appresorium; SV= substomatal vesicle; IH=infection hyphae; HMC=haustorial mother cell; N= haustorial neck; H=haustorial body; EHMx= interfacial matrix. Plant structures: G=guard Cell; E=epidermis cell; M=mesophyll cell. (Modified from Perfect and Green 2001).

Most evidence point towards that this matrix consists of partly fungal and partly plant derived material (Harder and Chong 1991). The matrix appears amorph and contains mostly carbohydrates and glycoproteins (Mendgen and Hahn 2002). Not much is known about the processes in this compartment but it probably serves as a space for exchange of compounds between the pathogen and the host. One of the most interesting observations was done by Kemen et al. (2005) who showed that a protein from the haustoria of *Uromyces fabae* passed through the EHMx before ending up in the host cytoplasm. Few studies have also revealed electron dense deposits in the matrix in close connection to tubular extensions ranging from the EHM and into host cytoplasm which could indicate involvement in secretory pathways (Mendgen 1991; Mims et al. 2002).
The haustorium is connected to the fungal mycelium by a narrow junction called the haustorial neck. A common feature surrounding the neck is a structure often referred to as the “neck ring” or “neck band”. This structure is thought to serve a role in sealing of the EHM from the bulk apoplast and the plant plasma membrane (Heath and Skalamera 1997; Panstruga 2003) which probably facilitate e.g. nutrient uptake from the host (Mendgen and Hahn 2002). In the study by Koh et al. (2005) it was found that the neck region marked the site where the extrahaustorial membrane became different form the host plasma membrane and they suggested models involving the neck band as a key component in formation of the extrahaustorial membrane.

In most case electron dense deposits are seen in connection to the neck area. These deposits form structures often referred to as collars (Harder and Chong 1984). Not much is known about their function and constitution but Mims et al. (2001) hypothesised that they could resemble secondary wall ingrowths found in plant transfer cells. Transfer cells are special plant cells which facilitate solute transfer over short distances (Offler et al., 2003) and they are common at sites where elevated nutrient flow is necessary for plant development (McCurdy et al., 2008). Therefore cell wall ingrowth might serve as a host cell response to compensate for solutes lost from the cell to the haustorium. A different hypothesis could be that they are the result of an inhibited defence response as for incompatible interactions the haustorial body is sometimes seen to be fully encased in such structures (Bushnell 1972). The shape of the haustorial body is very diverse across species. Some rust species form round and very regular shaped haustoria whereas others form haustoria with various degree of branching. In this thesis the shape and size of *P. striiformis* haustoria was found to change significantly during the time of infection. Newly formed haustoria were small and spherical whereas mature haustoria were apically branched and significantly larger (Figure4) (Sørensen et al. 2012).

Despite the general findings outlined above our understanding of haustorial function is still in its infancy and in particular very little is known about how haustoria develop and is accommodated by their host cell. Due to the unculturable nature of rust fungi the study of the function of haustoria still represent a significant challenge despite the development of sophisticated molecular methods. However, an integrated approach combining advanced microscopy with modern molecular and biochemical methods have resulted in some very interesting findings in recent years.
Host resistance and pathogen adaptation

How plants defend themselves against pathogens is a very active field of research and comprehensive reviews of the main concepts and hypothesis are given by e.g. Schulze-Lefert and Panstruga (2011) and Bernoux et al. (2011).

During coevolution with pathogens, plants have developed different layers of defence. Individual plant species are resistant to the large majority of pathogens based on either avoidance or a general surveillance system set up for detection of so-called pathogen associated molecular patterns (abbreviated PAMP). The leading hypothesis is that pathogens have developed effector proteins for suppression of this broad resistance system to gain access to the rich source of energy, which the living plant material represents (Bernoux et al. 2011). Once basic compatibility has been obtained, pathogens become involved in a new battle where the detection of pathogen effectors initiates a second layer of plant defence (Roussel and Balesdent 2010).

Investigation of the underlying mechanisms of resistance, susceptibility and how pathogens adapt to their host is an important basis for identification of ‘durable’ resistance, i.e., resistance
that remains long-term effective when used on a large scale in an epidemiological environment favourable for the disease Johnson (1984). Plant breeders have typically identified genetic sources of resistance against prevalent and important pathogens based on disease screenings under field conditions. However, often such resistances have failed to provide long-term protection against the considered disease.

**Resistance against *Puccinia striiformis* in wheat**

Resistance against *P. striiformis* in wheat basically comes in to forms. One that is qualitative inherited and relies on single genes with major effects and a second that is quantitatively inherited. The inheritance of yellow rust resistance in wheat was first studied by Sir Rowland Biffen (1874-1949). He demonstrated that resistance could be inherited as single-genes in Mendelian way and that a dominant allele resulted in full resistance (Biffen 1905). Single genes conferring such type of qualitatively inherited resistance is often named R-genes (*Yr*-genes in the case of resistance to yellow rust in wheat). Deployment of R-genes has been widely used to control yellow rust. Many such genes have been included in wheat breeding programs, some transferred to cultivated wheat from related cereal species and wild relatives (Wellings 2011). Potentially wild relatives represent a rich source of resistance (Anikster et al. 2005) but introgression of genetic material from wild species is not unproblematic and often results in undesired linkage drag (Randhawa et al. 2009; Niu et al. 2011). In most case R-genes are expressed in seedlings but a few genes for qualitative resistance are only expressed at the adult plant stage. Both molecular and histological investigations have revealed remarkable similarities between the effects of these genes, despite their seemingly widespread distribution across most plant-pathogen systems (Schulze-Lefert and Panstruga 2011). Qualitative resistance is the most studied type of resistance and phenotypically it follows the gene-for-gene interaction proposed by Flor (1956). With the invention of sophisticated molecular and genomic methods evidence supporting the hypothesis of gene-for-gene interaction at the molecular level is now compiling (Rouxle and Balesdent 2011). The recognition of the product of the avirulence gene (protein effector) either directly or indirectly by the product of the R-gene results in the initiation of pathways that eventually leads to a localized and special type of programmed cell death called hypersensitive response (HR) (Dodds and Rathjen 2010). Onset of HR seems in most cases dependent on the initiation of haustorium formation from which the avirulence product are hypothesized to be secreted (Kemen et al. 2005; Cantanzariti 2007). Despite the similarities
between function of R-genes their effect may vary, depending on plant development stage, the R-gene considered and genetic background of the host (Calonnec et al. 1997, McIntosh et al. 1995; Bozkurt et al. 2010). In this thesis, preliminary observation on haustorium development during incompatible interaction in seedlings between wheat varieties carrying the yellow rust resistance gene Yr2 and avirulent pathogen isolates, confirmed the association between the formation of haustorium and HR. The initiation and formation of haustoria on primary infection hyphae was observed on host cell prior to any sign of hypersensitive response (figure 5a). Later, most host cells showed clear sign of HR, and the development of haustoria was restricted (figure 5b). However, in some plant cells in such an interaction, the haustorium development appeared normal, and for these cells no hypersensitive response could be recorded (figure 5c). To the best of my knowledge, such diverging responses during an incompatible interaction have not previously been reported for *P. striiformis*. It could be hypothesised that such a response is an effect of a critical step in the recognition, where the haustorium, if by-passing this step without recognition, is able to develop normally.

Figure 5. Colonies and haustoria during incompatible interaction based on the Yr2 resistance gene in wheat seedlings and an avirulent *P. striiformis* isolate. Fungal structures were stained with Uvitex2B and visualised with 2-photon microscopy. Hypersensitive responses were visualised by autofluorescence. (a) Fungal colony 24 hours after inoculation. A single immature haustorium can be seen on two primary infection hyphae. Scale bar= 10µm. (b) haustoria on a primary infection hyphae 72 hours after infection. The size of the haustorium is clearly reduced and the host cell show signs of hypersensitive response (HR). Scale bar=10µm. (c) Fungal colony 120 hours after inoculation. Host cell with hypersensitive response and a small haustorium is present to the right, and another host cell with no hypersensitive response and a haustorium of normal size and morphology is present to the left. Scale bar=20. Ssv= substomatal vesicle, h=haustorium, HR=hypersensitive response.
Quantitatively inherited resistance is often expressed mainly at the adult plant growth stages tending to become increasingly effective as the plants matures (e.g. Park and Rees 1989; Ma and singh 1996). A number of terms have been used to describe quantitative resistance and a unifying concept, which will make comparisons between studies easier, is missing. Quantitatively inherited resistance relies on genes with mainly minor individual effects on disease development, often reported to have additive effects, and in some cases an epistatic effect (Navabi et al. 2004; Dedryver et al. 2009; Vazquez et al. 2012). The environment can have a large influence on the effect of this type of resistance, and the disease level on the same wheat variety may vary greatly in different environments (Boyd 2005, Vazquez et al. 2012). The effect of environment is particularly evident for a type of resistance designated ‘high temperature adult plant resistance’ (HTAP), where the resistance become increasingly effective with increasing temperatures (Chen 2005). Histological investigations have demonstrated that quantitative resistance against yellow rust in wheat can interact with the pathogen through different mechanism and during different stages of the infection process depending on the specific gene(s) (e.g. Parlevliet and Kievit 1986; Moldenhauer et al. 2006 and 2008; Feng et al. 2008). Moldenhauer et al. (2008) studied the function and effectiveness of four QTL’s (Quantitative trait loci) present in the variety Kariega. Each QTL and combinations of QTLs resulted in different effectiveness and phenotypic expression of the interaction. Furthermore, each QTL seemed to mediate different defence mechanisms. The resistance was most effective when all four OTL’s were combined. Similar results were obtained by Feng et al (2008) who studied quantitative resistance based upon additive effects of single genes. Different genes were involved in restricting P. striiformis growth during different stages of the infection process. In a histological study by Broers and Lopez-Atilano (1996) it was suggested that quantitative resistance could reduce the infection frequency by restricting the penetration through stomata. Disintegration of the substomatal vesicles and reduction in the growth of the infection hyphae was also observed. In another study no effect was found prior to haustoria formation, but the number of haustoria was reduced in resistant plants and necrosis of host cells resembling HR was also seen (Mares 1979b). Jagger et al. (2011) also found similarities to HR for quantitative resistance in the variety Alcedo. On the other hand Melichar et al. (2008) found no sign of cell death in the variety Guardian from which they had identified three QTLs for quantitative resistance. Despite these observations, histological evidence and characterisation of quantitative resistance against yellow rust in wheat is quite limited. As suggested by Jagger et al. (2011),
detailed phenotyping at the histological level of the effect of individual QTLs might assist in identifying genes with increased ‘durability’. In recent years several investigations have been performed on the genetics of quantitative resistance and some wheat varieties that have remained resistant in the field for several years have been found to possess specific combinations of QTL for quantitative resistance (e.g. Mallard et al. 2005, Dedryver et al. 2009, Agenbag et al. 2012). Singh et al. (2010) concluded that four-five of such genes could provide a durable resistance in wheat against rusts. Quantitative resistance genes are more difficult to use in breeding than genes for qualitative resistance. However with the increasing knowledge on marker assisted selection the future for routine incorporation of effective quantitative resistance into commercial wheat lines might seem promising (Lowe et al. 2010; St. Clair 2010).

Quantitative resistance in general has been proposed to share characteristics with the non-host resistance that gives broad protection against most potential pathogens (Niks and Marcel 2009). However, several very different hypotheses have been proposed to explain the nature of quantitative resistance and no clear unified concept has yet been established (Poland et al. 2008).

**Assessment of host-pathogen interactions**

Postulation and identification of R-genes in wheat varieties and accessions is often done by challenging seedlings with combinations of pathogen isolates with virulence phenotypes suitable for discriminating between individual R-genes (e.g. Hovmøller 2007, Pathan et al. 2008). Typing of the disease reaction of individual combinations of pathogen isolates and wheat genotypes have traditionally been performed on two different scales (McNeal et al. 1971; McIntosh et al. 1995). Both scales are divided into distinct steps ranging from full resistance with no visible disease symptoms to full susceptibility (figure 6a) where there appear to be no restriction on pathogen growth. Resistance levels are related to amount of visible chlorosis and necrosis and density of spore bearing pustules. In case of resistance, individual R-genes gives characteristic disease expression but the phenotype may vary e.g due the genetic background of the host genotype (McIntosh et al. 1995; Calonnec et al. 1997). Interpretation of disease reaction serves as the basis for division into the two categories of compatible and incompatible interaction.

In field trails the effect of quantitative resistance in adult plants are often assessed by registration of disease severity and area under disease progress curve (AUDPC). Disease
severity can be estimated by visual assessment of percentage leaf area affected by disease, most often as an average of the upper three leaves. Measurement of disease severity is more useful for quantitative resistance than determination of infection type because disease severity is correlated with effect on grain yield (e.g. Ali et al. 2009). Judgment of disease leaf area by eye can be unreliable but is often assisted by assessment keys (Brown and Keane 1997). The effect of quantitative resistance during polycyclic infections in the field have been estimated by performing a number of disease area assessments during the growing season and then calculating the area under disease progress curve (AUDPC). Both assessment of disease severity and AUDPC has in many studies formed the basis for genetic analysis of quantitative resistance.

The effect of quantitative resistance can be further detailed by performing assessment of epidemiological parameters. Components that have often been addressed in relation to quantitative resistance include infection frequency, latent period, lesion growth rate and spore production (e.g. Sandoval Islas 2007; Broers 1997; Kloppers and Pretorius 1995). Such studies are performed as monocyclic experiments under greenhouse condition. Two different definitions of latent period have been used in rust research. This first, designated “LP$_{50}$”, is defined as the time after inoculation where 50% of the final number of pustules has formed (de Vallavieille-Pope et al. 2000). Due to the systemically growth of *P. striiformis* it can be difficult to get a precise estimation of LP$_{50}$. Instead latent period has been defined as the time from inoculation to the first appearance of spores in new uredinia breaking the leaf surface (Hovmøller et al. 2011) (Figure6b). Such a definition involves an element of subjectivity where it depends on the person performing the assessment and all assessments in an experiment should be done by the same person or ideally independently by to different people. The same holds true for estimation of lesion growth, which is often measured as lesion expansion in mm/day. This can be done by marking the edge of the lesion at two or more different time points and then divide the length of the area with the time between markings. Other parameters that have been used in rust research includes infection frequency/efficiency, pustule density, pustule size and spore production. However, the practical value of these is sometimes limited due to being highly time consuming and/or associated with low precision. Nevertheless, such studies have added valuable information for interpretation of mechanisms behind resistance (e.g. Rubiales and Niks 1992; Klopper and Pretorius 1995).
For all types of interactions, image acquisition is an obvious but not always implemented method for documentation and analysis of phenotypes and effects. An example could be that images of the resistance phenotypes induced by specific R-genes can assist e.g. race-analysis. In this thesis scanned leaves were used for determination of lesion length and area affected by disease (figure 6c). The edge of lesions expanding from a point inoculation was marked consecutively with 3 days interval. At the end of the experiment leaves were scanned and lesion growth could be estimated by measuring the distances between markings. On the same images disease area was determined by the use of a colour threshold for separating the diseased area from healthy areas. Furthermore, image z-stacks obtained during microscopic visualization of haustoria were analysed for estimation of the volume of haustoria in wheat seedlings (Sørensen et al. 2012)

Figure 6. Illustration of some of the methods used for estimation of epidemiological parameters in this study. (a) Photo documentation of infection type. The leaf to the left display an incompatible interaction in seedlings of Heines Peko (Yr2) inoculated with pathogen isolate carrying Yr2 avirulence. The leaf to the right was inoculated with an isolate carrying Yr2 virulence. (b) Estimation of latent period was done by observing inoculated leaves at 24 hours interval with a 10X hand lens. (c) Lesion length at different times after latent period was estimated by measuring the distance between consecutive markings. For the same leaf estimation of the size of the diseased area (black) was done by separating it from the healthy parts of the leaf by use of a colour threshold.
Pathogen adaptation to qualitative resistance and cost of virulence

_**P. striiformis** has shown a tremendous ability to adapt to host varieties with new genes for qualitative resistance. Only for three out of about 70 R-genes virulence has yet to be found in the US (Lowe et al. 2010). Mutation is the ultimate source of variation, and new virulence is thought to arise from mutations in avirulence genes which lead to impairment of or change in the gene function (Ridout et al. 2006; Hovmøller and Justesen 2007b). The recent focus on sequencing of rust genomes is likely to answer some of the question of the rapid evolution of virulence. However, studies of the functionality of potential avirulence genes have been hampered due to the biotrophic nature of rusts, which constrains e.g. genetic transformation (Cantu et al. 2011; Hovmøller et al. 2011).

Farmers within a region often use varieties with the same sources of resistance which gives pathogen isolates with virulence matching new resistance a clear fitness advantage. Wide distribution of few resistance genes result in strong directional selection for virulence (Leonard 1987; Hovmøller et al. 1993). Epidemics where spores are produced in huge numbers could represent a window of opportunity for generation of beneficial virulence mutant. In a study with the bacterial species _Escherisia coli_ de Visser and Rozen (2005) showed that increased population size could increase adaptation through rapid generation of mutants with large fitness effects. However, in fungal populations the probability that a single new mutant will survive is often extremely small (Gale 1987). Most spores fail to infect and reproduce (de Vallavieille-Pope et al. 1995) which limits the establishments of new mutants. Further, bottlenecks as a result of introduction of new effective resistance or lack of winter survival may decrease the population to a size where genetic drift can reduce genetic variation (Salathé et al. 2005). Some _P. striiformis_ populations display low level of genetic variation indicative of clonal reproduction (Hovmøller et al. 2002; Enjalbert et al. 2005). Data from Australian surveys suggest that in cases of low variation a sequential use of resistance genes may lead to rapid stepwise evolution of virulence in a single lineage. _P. striiformis_ was first seen in Australia in 1979 probably due to an incursion from UK (Steele et al. 2001; Wellings 2007) and 15 new virulence phenotypes were collected during a ten year period from 1979 until 1988. Wellings and McIntosh (1990) suggested a stepwise mutational model to describe the process of generating genetic variation in a system derived from a single novel incursion. AFLP-data from Steele et al. (2001) and Keiper et al. (2004) supported the hypothesis that these new virulence phenotypes were the result of mutations in a single lineage. In a different study Hovmøller and Justesen
(2007b) used a combination of data for virulence phenotype and AFLP to clarify the connection between isolates of distinct virulence phenotype in the North-western European population. These data supported the hypothesis of sequential clonal evolution for pathogen avirulence and virulence in the North-western European population. Their study also showed that new virulence genes was established in the pathogen populations at a rate three magnitudes higher than the phenotypic AFLP mutation rate. This could be explained as an effect of selection but it could also indicate that the mutation rate in avirulence loci is higher than in the rest of the genome (Hovmøller and Justesen 2007b). Nevertheless, these results gave a good illustration of the high potential for host adaptation in populations of *P. striiformis*. It has often been hypothesized that virulence will be associated with a fitness cost in the absence of the matching R-gene (Sacristán et al. 2008). As touched previously avirulence genes have been proposed to be involved in the establishment of biotrophy (Catanzariti 2007) and in pathogenicity in general (Webb and Fellers 2006; Stergiopoulos et al. 2009). Across pathogenic viruses, bacteria and fungi avirulence genes have been found to code for a very diverse array of proteins (Skamnioti and Ridout 2006) but still very little is known about their exact function, in particular for fungal pathogens (Leach et al. 2001; Stergiopoulos et al. 2009). Thus, knowledge about their effect on fitness is still limited. Models for coevolution between plants and pathogens have often included a fitness cost of virulence as the most obvious factor for explaining maintenance of genetic variation for virulence in pathogen populations (Brown 2003). Cost of virulence together with cost of resistance ensures that virulence genes do not become fixed in the pathogen population (Brown and Tellier 2011). However, models including other evolutionary important factors like e.g. natural drift and variation in spatial population structure (metapopulations) have revealed that polymorphism for characters of virulence and resistance can be maintained without cost of virulence and resistance (Thrall and Burdon 2002; Salathé et al. 2005).

Prediction of cost of virulence has in some cases been based on survey data for dynamics of virulence frequencies. However, data for *P. striiformis* in Europe show that such dynamics may be largely unpredictable especially in clonal pathogen populations (Bayles et al. 2000; Bahri et al. 2009). In clonal populations other factors might be highly important for the faith of virulence genes. In agricultural systems the virulence gene frequencies in the aerial pathogen population to a large extend could be predicted by selection from host cultivars with resistance genes (Hovmøller et al. 1993). Brown (1995) showed in a theoretical model that during large selection
pressure by the use of same sources of resistance over large areas a virulence gene might become fixed in the pathogen population even if it is associated with a cost. Brown (1995) showed that unnecessary virulence might persist in populations of asexual pathogens as a consequence of genetic hitchhiking even if there is a cost of virulence depending on the level of the cost and the frequency of the host that selects for another virulence gene present in the same pathotype. Dynamics of unnecessary virulence genes mainly depend on whether they are in negative or positive gametic disequilibrium with virulence genes selected for (Østergård & Hovmøller 1991). In a situation with sequential use of pathotype-specific resistance new virulence arise at random in pathotypes present in the pathogen population and therefore the sign of the disequilibrium between a necessary and an unnecessary virulence gene cannot be predicted (Brown 1995; Hovmøller et al. 1997). Dynamics of virulence genes are likely to be different in sexual recombining populations (Groth and Roelfs et al. 1982; Liu and Kolmer 1998) and cost of virulence might be better reflected in such cases. However, in China where high level of variation has been found in some areas (Mboup et al. 2009; Duan et al. 2010) highly complex lines have dominated the populations in recent years (Chen et al. 2009) indicating no loss of unnecessary virulence. A tendency towards increase in the number of virulence carried by isolates has also been observed for populations of P. striiformis in other parts of the world (Yahyaoui et al. 2002; Enjalbert 2005; Chen 2005; Ochoa et al. 2007). This indicates that even if there is a cost of virulence in might only have a limited influence on the constitution of pathogen population in agricultural settings.

Direct experimental evidence of trade-off associated with loss of specific avirulences genes comes mainly from bacteria and viruses whereas only very few studies have been done for fungi (Leach et al. 2001; Sacristán 2009). In a study of near isogenic lines of Leptosphaeria maculans (phoma stem cranker of oilseed rape) loss of the function of the avirulence gene AvrLm4 was indicated to be associated with a loss of fitness (Huang et al. 2006). Bronson and Ellingboe (1986) found that fitness of isolates of Erysiphe graminis f. sp. tritici (=Blumeria graminis f. sp. tritici) was controlled by other loci than those for unnecessary virulence. Avirulence genes in Blumeria graminis f. sp. hordei was shown to contribute to pathogenicity but it was suggested that due to avirulence paralogues that could substitute each other, function of avirulence genes could be lost without a fitness penalty or loss of compatibility (Ridout et al. 2006). In this present thesis there was found to be no association between fitness and the gain of virulence against the Yr2
resistance gene (Manuscript II). On the other hand, Bahri et al. (2009) saw indication of a possible fitness cost of virulence to \( Yr4 \) and \( Yr6 \).

The above examples illustrate the complexity of addressing questions about cost of virulence, and there seems to be no general conclusion about the function of avirulence genes in relation to fitness. These effects probably have to be judged from gene to gene.

**Pathogen aggressiveness**

The term aggressiveness has most often been used to describe the relative ability of a virulent isolate to cause disease on a susceptible host plant (Hovmøller et al. 2011) but several definitions have been used in plant pathology (Pariaud et al. 2009). Components used for assessment of aggressiveness of rust fungi include some of the same parameters used for evaluation of effectiveness of quantitative resistance. For *P. striiformis* parameters like infection efficiency, latent period, lesion growth and spore production have been considered components of aggressiveness (Rapilly 1979; Broers 1997; Milus et al. 2009). Only very few studies have been carried out to investigate for differences in aggressiveness between isolates of *P. striiformis*. However, developments within the last decade have emphasised the importance of aggressiveness in shaping populations of *P. striiformis*. In year 2000 severe disease was observed in the South Central and Eastern United States, areas which traditionally have been largely unaffected by yellow rust because of high temperatures during summer (Chen et al. 2002; Chen 2005). Markell and Milus (2008) showed that these severe attacks were caused by pathogen isolates that were distinctively different from the old population in terms of AFLP and virulence phenotype. In a study of the aggressiveness of these new isolates Milus et al. (2009) found that they grew faster and produced more spore than the old isolate both at optimal temperatures and at temperatures considered to be relatively high for growth of *P. striiformis*. The difference between old and new isolates was more pronounced at high temperatures and it was concluded that the new isolates were adapted to high temperature. Within the last decade isolates of these new strains have spread on a global scale together with another closely related strain, causing severe attacks in e.g. Australia and Eastern Africa (Hovmøller et al. 2008; Wellings 2011).

Traits of aggressiveness are considered to be components of fitness (Brasier 1999; Pringle and Taylor 2002) and when heritable variation exist in pathogen populations for such traits selection is likely to occur. We have no information from *P. striiformis* on the genetics of
aggressiveness. In other pathogens aggressiveness has been found to be polygenic inherited with both additive and dominant effects (Burnett 2003). In populations of both *P. graminis* and *P. triticina* (=*Puccina recondita* f.sp. *tritici*) heritable variation for aggressiveness has been found (Lehman and Shaner 1996 and 1997; Liu et al. 1996). In a study by Lehman and Shaner (1997) where they selected for short latent on four wheat varieties with quantitative resistance, it was found that after five asexual generations latent period was shorter on all four varieties, although the change was only statistically significant on one variety. For *P. graminis* it was shown that isolates with higher infection rate and spore production i.e. a higher aggressiveness could outcompete less fit isolates (Liu et al. 1996). In other studies evidence of adaptation to both environment and individual host varieties have been observed. Isolates of the pathogen *Phytophtora infectans* from France and Morocco, respectively, were cross-inoculated onto the most common potato varieties in France (Bintje) and in Morocco (Desiree) (Andrivon et al. 2007). Isolates from France were most aggressive on Bintje and isolates from Morocco on Desiree. In *P. striiformis* evidence of local adaptation has been found within the French population (Mboup et al. 2012). Despite frequent migration of spores between northern and southern France a highly divergent populations have been found between these areas (Enjalbert et al. 2005; Mboup et al. 2012). The persistence of the southern population seemed related to adaptation to warmer Mediterranean climate whereas population structure in the north was related to adaptation to host resistance (Mboup et al. 2012). It has been discussed by e.g. Andrivon et al. (2007) and Lehman and Shaner (1997) that results on adaptation and selection for traits of aggressiveness could be highly important in relation to durability of quantitative resistance. For few pathogens indications of adaptation to quantitative resistance has been observed (Marcel et al. 2008; Pariaud et al. 2009). With the increased focus on use of quantitative resistance for control of cereal rusts (Boyd 2005; Lowe et al 2011) one important question could be whether the new aggressive isolates of *P. striiformis* represents a potential for adaptation to quantitative resistance and to what extent variation for aggressiveness on wheat varieties with quantitative resistance exist in pathogen populations.
Project aims

The main objectives of this Ph.D study were to investigate *Puccinia striiformis* infecting wheat with respect to 1) pathogen infection biology with special emphasis on the spatial and temporal development of haustoria in compatible interactions of *P. striiformis* and wheat and 2) interactions between pathogen aggressiveness and quantitative and qualitative based host resistance.

1) Preliminary microscopic observations had indicated that the structure and development of *Puccinia striiformis* haustoria in wheat leaves were different from what had previously been reported in the literature. Due to the fact that haustoria are primarily formed in mesophyll cells they can be hard to visualize in intact leaf tissue. It was therefore decided to investigate if a classical staining technique could be used in combination with advanced microscopy for visualization of haustoria. This techniques was used to gain new insight into the development and 3-dimensional structure of haustoria.

2) In the isolate bank at GRRC at Flakkebjerg Research Center we have access to unique pairs of wild type and virulence mutant isolates only differing with respect to virulence for the *Yr2* resistance gene. In a previous project data on the growth of these isolates on seedlings of both susceptible and resistant wheat varieties had been obtained. In the present study these raw data formed the basis for comprehensive analyses of possible trade-off effects of independent acquisition of a specific virulence gene in two different genetic backgrounds.

3) A third study addressed the interactions of pathogen aggressiveness and host resistance. It was hypothesised that the new high temperature adapted and aggressive isolates could be a potential threat for the break-down of disease control in wheat varieties with quantitative resistance. An experiment was set up to evaluate the aggressiveness of these isolates on wheat lines with known sources of quantitative resistance. Due to emergence of highly atypical and possibly aggressive *P. striiformis* isolates at multiple locations in NW-Europe in 2011 and 2012, it was decided to include such an isolate in the experiments.
List of manuscripts

I. 3-D imaging of temporal and spatial development of *Puccinia striiformis* haustoria in wheat.

II. Fitness of Wild-type and virulence mutants of *Puccinia striiformis* on wheat.

III. Exotic strains of *Puccinia striiformis* reveal race-specificity of long-term effective adult plant resistance in wheat
Manuscript I

3-D imaging of temporal and spatial development of *Puccinia striiformis* haustoria in wheat

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Abstract

Differentiation of haustoria on primary infection hyphae of the fungal pathogen *Puccinia striiformis* was studied in wheat seedlings by using 2-photon microscopy in combination with a classical staining technique. Our results showed a significant increase in the average haustorium size between 22, 44, 68, 92 and 116 hours after inoculation (hai). After 116 hai no significant change was observed until 336 hai. Haustorium morphology also changed significantly during the time course of infection. Initially, small spherical haustoria were seen but as they increased in size, the haustoria gradually became apically branched. At 22 hai all observed haustoria were spherical but at 44 hai most haustoria had an irregular structure and at 92 hai all observed haustoria appeared branched. Along with the changes of the haustorial body the haustorial neck changed from being narrow and slender to having an expanded appearance with a rough and invaginated structure. The structural changes were similar in two susceptible wheat varieties, 514W and Cartago, although the mean haustorium size was larger in 514W than in Cartago at all time points.

**Key words:** Fluorescence microscopy, Fungi, Plant pathogen, *Triticum aestivum*, Yellow rust,
Introduction

Yellow rust caused by the fungal pathogen *Puccinia striiformis* is one of the most important diseases on wheat, *Triticum aestivum* (Hovmøller et al. 2010). Like all rust fungi, *P. striiformis* is an obligate biotroph, depending on living host tissue for growth and reproduction. One of the characteristics of obligate biotrophs is the formation of haustoria, which are highly specialized structures that function as the main interface between the pathogen and its host. Haustoria play an important role in water and nutrient acquisition and in signaling between the pathogen and the host, including suppression and induction of plant defense (Catanzariti et al. 2007, Voegele and Mendgen 2003). Detailed knowledge of haustorium development and morphology therefore form an important basis for our understanding of plant-pathogen interaction.

Many rust species have complex life cycles with up to five different spore types that are either mono- or dikaryotic (Cummins and Hiratsuka 2003). Heteroecious rust species like *P. striiformis* show alternation between hosts for sexual and asexual reproduction (Bushnell 1972, Jin et al. 2010). The asexual host is infected by dikaryotic spores either aciddiospores produced on the sexual host or asexually produced urediniospores. In the case of *P. striiformis* urediniospores can be produced in great numbers on susceptible wheat varieties during the main cropping season. Mycelia originating from dikaryotic spores form specialized D-haustoria, which are clearly differentiated into specialized regions, including the haustorial neck and the haustorial body. In general, the host for the sexually reproducing stage is infected by monokaryotic basidiospores and the mycelia originating from these produce M-haustoria that resemble intracellular hyphae with less clear differentiation than D-haustoria (Chong et al. 1981). Both M- and D-haustoria are formed between the cell wall and plasma membrane of affected host cells, and for both types an extrahaustorial matrix is formed. Most structural and cytological studies of haustoria have examined D-haustoria by means of electron and bright field microscopy (Harder and Chong 1991, Littlefield and Heath 1979).

Previously, haustoria of *P. striiformis* in wheat leaves have been visualized by fluorescence microscopy (Cartwright and Russell 1981) and transmission electron microscopy (e.g. Kang et al. 2002, Ma and Shang 2004, 2009). In recent years, microscopic techniques like confocal and 2-photon microscopy, which allow optical sectioning and 3-D reconstruction of structures, have become increasingly popular in biological sciences. In two studies focusing on the effect of host resistance on *P. striiformis* development Moldenhauer et al. (2006) and Bozkurt et al. (
2010) used confocal microscopy to visualize infection structures and host cellular responses. However neither study reported observations of haustoria. In the present study, 2-photon microscopy was used in combination with a classical staining technique for visualization of D-haustoria of *P. striiformis* in fixed whole leaves of seedlings of two susceptible wheat varieties. The results represent the first detailed study of the spatial and temporal development of haustoria during compatible interaction between *P. striiformis* and wheat.

**Materials and Methods**

**Pathogen and plant material**

DK 24/95, a typical isolate of the NW-European lineage (Justesen et al. 2002), was used in this study together with two wheat varieties, Cartago and 514W (Milus et al. 2009), which have no identified yellow rust resistance genes. Twelve seeds of each variety were sown in 7.5 cm square pots filled with Pindstrup substrate, a standard peat-based mix with slow release nutrients (Pindstrup Mosebrug A/S, Ryomgaard, Denmark). Pots were placed in trays covered with plastic lids to ensure high humidity during germination and then moved to spore-proof greenhouse cabins. Artificial light of 50–100 µEm² s⁻¹ was applied when daylight was less than 10 000 lux. Alternating periods of 16 h light (17 C) and 8 hours darkness (12 C) were used before and after inoculation. Plants were trimmed to 10 uniform plants per pot prior to inoculation.

**Inoculation**

Spores were harvested from infected seedlings of Cartago which had been shaken 48 hours prior to spore harvest. The fresh spores were mixed 1:5 (w/w) with talcum and immediately used to inoculate the second green leaf of 16 days old seedlings. Spores were applied to a 2 cm long area of the adaxial, central part of the leaf by using a camel hair brush size 1. This method resulted in a colony density sufficient to give an appropriate number of non-overlapping infection sites at the early infection stages which allowed robust estimates of the observed parameters. Five pots of seedlings were inoculated for each variety. After inoculation plants were placed in trays, sprayed with water and covered to keep 100% r. h. during incubation at 10
C for 22 hours. After incubation pots were transferred to the greenhouse and randomized in a spore-proof cabin with light and temperature conditions as described above.

**Sampling and staining**

One inoculated leaf segment per pot was sampled at 22, 44, 68, 92, 116, 140, 164 and 336 hours after inoculation, resulting in five leaf samples per host variety per sampling point. Fixation and staining was done according to Moldenhauer et al. (2006) with a few modifications. Leaf segments were fixed and cleared in ethanol:chloroform (3:1, v/v) + 0.15 % (w/v) trichloroacetic acid for at least 24 h. After being washed twice in 50% ethanol they were incubated in 0.05 M NaOH for 30 min. Specimens were then rinsed in water before being submerged in 0.1 M Tris-HCl buffer (pH 5.8) for 30 min. Afterwards they were stained for 5 min in 0.1 % (w/v) Uvitex 2B (polysciences inc.). Following staining, specimens were washed four times in deionized water (DI) and stored overnight in DI. Before mounting on microscope slides specimens were dehydrated through a series of glycerol concentrations of 5, 15, 25 and 50% (w/w). Specimens were left in each concentration for 45 min. 75% glycerol were used for mounting and slides were sealed with VALAP (Vaseline:Lanolin:Paraffin (1:1:1) w/w).

**Microscopy and imaging**

Fungal structures were visualized by an inverted Zeiss LSM 510 confocal microscope (Zeiss, Jena, Germany) equipped with a Plan-Apochromat 63X/1.4 oil immersion objective. Excitation of Uvitex-2B stained structures was done with a Mai tai 2-photon laser at wavelengths of 720 nm. Emitted light was scanned with filters settings for short passing of 685 nm. The sizes of the haustoria were determined by focusing on the optical layer where the haustorium appeared largest. The haustoria on two primary infection hyphae originating from the substomatal vesicle were measured in five colonies per leaf segment. With five leaf segments for each combination of host variety and time point, this resulted in up to 50 haustoria observations per combination. Length and width of the haustoria were determined using measurement functions in the LSM 510 commercial software from Zeiss (version 3.2).

For 3-D projection, z-stacks were collected with 1 µm separation of images. 3D image projection was performed with Zeiss LSM image browser (version 4.2.0.121). Further image editing of 3D projections like size adjustments and sharpening was done with Adobe Photoshop CS5 (Adobe Systems Inc. San Jose, CA). Volume and surface area of selected
haustoria were determined by 3-D object counter (Bolte and Cordelières 2006) in Fiji (http://fiji.sc/wiki/index.php/Fiji). Images for determination of volume and surface area were pre-processed in Fiji by manual threshold and morphology operations. Varying intensities due to uneven staining were manually filled.

**Statistics**

The size of the haustorium was calculated as: length x width x π/4 (Baart et al. 1991) and the larger of the two observed haustoria per colony were used in the final statistical analysis. Data for haustorium size were square root transformed. The data were analyzed in a mixed model using variety, time and their interaction as fixed effects. Pot was treated as a random effect with leaf as the residual effect. Due to unequal variances for some time points separate residual variances were estimated for recording made early (at 22 hai) and late (after 22 hai). The different possible models were compared using Akaike information criteria (AIC) (Akaike 1974). A model with residual variance depending on the time of recording (without random pot effect) was chosen for the final analysis. The parameters were estimated using residual maximum likelihood (REML) and based of the estimates the marginal means were calculated. The means were compared using Tukey-Kramer adjustments for multiple comparisons (Kramer 1956, Tukey 1953). Statistical analysis of haustorium size was carried out using the procedure mixed in SAS (version 9.2, SAS institute Inc., Cary, NC, USA).

Pearson’s chi-square tests of independence were used to test the effect of time and variety on haustorium morphology. Fischer’s exact tests were used to analyze relevant parings of data for variety and morphology. Statistical analysis of haustorium morphology was carried out using the procedure frequency in SAS (version 9.2, SAS institute Inc., Cary, NC, USA).

**Results**

**2-photon microscopy combined with Uvitex 2B staining**

High-resolution 3-D images based on 2-photon microscopy and Uvitex 2B staining revealed the structure of *P. striiformis* haustoria in fixed whole leaves of infected wheat seedlings. Haustoria and other fungal infection structures are presented in Figure 1 together with structures of plant leaf tissue. Visualized fungal structures included the substomatal vesicle (ssv)
from which primary infection hyphae were formed (ph). Haustorial mother cells (hmc) were clearly separated from the rest of the mycelium by septa (s). The haustorium (h) originated from the hmc. Visualized plant structures include epidermal cell walls (e), mesophyll cell walls (m) and cell walls of guard cells (g), which make up the stoma (only one of the guard cells is fully visible in Figure 1). In the hollow substomatal cavity (ssc) proximal to the stoma no other structures than the ssv were visible. Chloroplasts (c) inside leaf mesophyll cells were also clearly visible.

Figure1. 2-photon microscopy combined with Uvitex 2B staining was used to visualize both fungal structures and plant structures in wheat leaves infected with *Puccinia striiformis*. Fungal structures: ssv = substomatal vesicle; ph=primary infection hyphae; hmc=haustorial mother cells; s=septa; h=haustorium. Plant structures: c=cell wall; m=mesophyll cell; g=guard cells; ssc=substomatal cavity; c=chloroplasts. Scale bar = 20 µm

**Temporal development**

Haustorial development was assessed by measuring the sizes of haustoria produced on the primary infection hyphae of colonies in two host varieties. Haustorium size was significantly affected by host variety and time without interactions between the two (Table I). Significant increase in mean haustorium size was observed between all sampling points up to 116 hai in both host varieties (Figure 2). After 116 hai no significant increase was observed until the latest
observation point at 336 hai. At this time spore production had been initiated, which was not observed for any of the previous time points. Also, a highly elaborate hyphal network and loss of leaf structure were observed at time point 336 hai. Mean haustorium size was larger in the host genotype 514W than in Cartago at all time points (Figure 2). Overall the effect of host genotype on mean haustorium size was highly significant (p<0.001) (Table I).

Table I. Statistical analysis of the fixed effect of host genotype, time and their interaction on the average size of haustoria formed by the primary infection hyphae

<table>
<thead>
<tr>
<th>Effect</th>
<th>Df</th>
<th>F value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Host genotype</td>
<td>1</td>
<td>27.03</td>
<td>&gt;.0001</td>
</tr>
<tr>
<td>Time</td>
<td>7</td>
<td>619.39</td>
<td>&gt;.0001</td>
</tr>
<tr>
<td>Variety*Time</td>
<td>7</td>
<td>1.63</td>
<td>0.1257</td>
</tr>
</tbody>
</table>

Figure 2. Mean haustorium size in 2nd leaf of wheat genotypes “514W” and ‘Cartago’ at different time points after inoculation. The examined haustoria were produced by haustorial mother cells on the primary infection hyphae. Columns with the same letter(s) are not significantly different (α=0.05), error bars indicate standard deviation.
Haustorial morphology

Haustorial morphology showed clear changes during the time course of infection. At 22 hai all observed colonies had a substomatal vesicle with one to three primary infection hyphae (Figure 3A). The haustoria at 22 hai had a spherical body with a slender neck that connected them to a haustorial mother cell clearly defined by a septum (Figure 3B). In order to register morphological differentiation as an effect of time this morphological type was referred to as regular. Later, at 44 hai, haustoria with a more irregular and allantoid morphology were seen. These haustoria showed clear signs of protrusion and the neck area had become more visible (Figure 3C). This morphological type was referred to as irregular. At 68 hai both the haustorium and neck area had developed further (Figure 3D). Most observed haustoria had become apically branched and the neck area had expanded further, now appearing rough and invaginated. This type was referred to as branched and seemed to represent the final morphological stage. The branched morphology was intact at 140 hai but the neck area had become even more pronounced than at 68 hai (Figure 3E). Two weeks after inoculation, 336 hai., the morphology of the haustoria were similar to structures seen at 116 and 140 hai but the central part of the haustorial body appeared rough and grooved (Figure 3F). Volume and surface area was estimated by image processing and analysis for all haustoria presented in Figure 3 (Table II).
Figure 3. Haustoria and colonies of *Puccinia striiformis* in seedling leaves of susceptible wheat from 22 to 336 hours after inoculation. Fungal structures were stained with Uvitex 2B and visualized by 2-photon microscopy. All images are made by 3D projection of acquired z-stacks. (A) A substomatal vesicle with two primary infection hyphae at 22 hai growing around a host mesophyll cell. A zone of contact appears to have formed. (B) Haustorial mother cell, haustorium and haustorial neck visible at 22 hai. (C) A haustorium with an allantoid structure and clear signs of protrusion observed at 44 hai. (D) Most of the observed haustoria were close to the final architecture at 68 hai with an apically branched structure. The neck area had expanded compared to 44 hai. (E) The structure observed at 68 hai was intact at 140 hai, but the neck area was more pronounced. (F) Haustorium at 336 hai. The surface of the central part of the haustorial body looks rough and groove. ssv=substomatal vesicle; p=primary infection hyphae; c=contact zone; h=haustorium; n=neck area; hmc=haustorial mother cell. All scale bars = 10 μm.

Table II. Estimates of volume, surface areas and surface area to volume ratio of the haustoria presented in figure 3 using 3D object counter plug-in for Fiji imaging software.

<table>
<thead>
<tr>
<th>Time point</th>
<th>Volume (µm³)</th>
<th>Surface area (µm²)</th>
<th>SA/V</th>
</tr>
</thead>
<tbody>
<tr>
<td>22</td>
<td>120</td>
<td>204</td>
<td>1.7</td>
</tr>
<tr>
<td>44</td>
<td>840</td>
<td>940</td>
<td>1.1</td>
</tr>
<tr>
<td>68</td>
<td>2079</td>
<td>2336</td>
<td>1.1</td>
</tr>
<tr>
<td>140</td>
<td>2182</td>
<td>2195</td>
<td>1.0</td>
</tr>
<tr>
<td>336</td>
<td>2511</td>
<td>2317</td>
<td>0.9</td>
</tr>
</tbody>
</table>

**Spatial development and distribution of haustoria**

The relative distribution of the three morphological types of haustoria presented in the previous section was recorded at all time points (Table III). A highly significant association between time and haustorium morphology was observed (Pearson’s Chi-square test: \( P<0.0001 \)), and likewise, variety had a significant effect (\( P=0.0258 \)). The variety 514W had a higher ratio of colonies with a haustorium at 22 hai (Fischer’s exact test; \( P=0.0378 \)) and a higher ratio of branched haustoria at 68 hai (Fischer’s exact test; \( P=0.0559 \)) compared to Cartago. In both varieties all observed haustoria on the primary infection hyphae were branched after 92 hai. As the colonies developed, haustoria at different developmental stage could be observed on other
hyphae close to the infection site and most haustorial mother cells close to the infection site had formed a haustorium (Figure 4). In the periphery of colonies where the haustoria on the primary infection hyphae had reached the branched stage, young and spherically shaped haustoria were often observed (Figure 5). In some colonies, haustoria on the primary infection hyphae were associated with host epidermal cells (Figure 6). Overall, 14 and 15 percent of the observed haustoria in 514W and Cartago, respectively, appeared to have developed in epidermal cells (data not shown).

Table III. Number of haustoria of each morphological type on primary infection hyphae in seedling leaves of two wheat genotypes “514W” and “Cartago”. At the time points 22, 44, 68 and 92 hours after inoculation haustoria were registered for up to five colonies in each of five leaves for both varieties.

<table>
<thead>
<tr>
<th>h.a.i.</th>
<th>514W</th>
<th>Cartago</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td>Globose</td>
</tr>
<tr>
<td>22</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>44</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>68</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>92</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 4. For colonies at 116 hai several haustoria were observed close to the infection site. ssv=substomatal vesicle; h=haustorium. Scale bar = 20µm.

Figure 5. Young haustoria (spherically shaped) were present in the periphery of many colonies at 116 hai. ih = infection hyphae; hmc=haustorial mother cell; h=haustorium. Scale bar = 20 µm.
Discussion

2-photon microscopy combined with a classical staining technique was used to study the development of haustoria in compatible interactions between *Puccinia striiformis* and wheat (*Triticum aestivum*). In contrast to previous studies, this allowed us to make high-resolution 3-D image reconstructions of haustoria and other infection structures in fixed whole leaves. Previous studies of *P. striiformis* haustoria have been based on electron microscopy (e.g. Kang et al. 2002, Ma and Shang 2004, 2009; Mares 1979a, 1979b) and standard widefield fluorescence microscopy (Cartwright and Russell 1981) which has much more limited options for 3D reconstructions and imaging of structures in whole leaves. Further, the present study provides the first detailed analysis of the spatial and temporal development of D-haustoria of *P. striiformis*, highlighting several characteristics in unprecedented detail and image quality. Our results showed significant structural changes of the haustorial body and the region around the haustorial neck during the time course of infection. The haustorial body gradually changed from a spherical and highly regular shape in young haustoria to an irregular and apically
branched appearance in older mature haustoria. The neck region changed from a slender and hardly visible structure at the beginning to an elaborate and invaginated structure later in the infection process.

It should be noted that fixation and clearing of the leaf tissue is necessary for in-depth imaging due to refractive and light scattering properties of living leaf material (Moreno et al. 2006). The methods used in the present study have been widely applied in plant pathology (e.g. Jagger et al. 2011, Moldenhauer et al. 2006). Methods involving the same chemicals have also proven highly useful for the study of plant tissue architecture in combination with multiphoton microscopy (Wuyts et al. 2010). Although these techniques may result in some level of shrinkage and distortion of the tissue due to dehydration, the fungal structures detected in our study generally appear non-distorted.

For other rust fungi, the body of D-haustoria has been reported to range from spherical forms to highly branched structures depending on species and age (Berndt 1999, Kemen et al. 2005, Mims et al. 2001). The branched structure of mature haustoria observed here is not uncommon in rust fungi, e.g. Hahn and Mendgen (1992) reported branching of haustoria from maize leaves heavily infected with Puccinia sorghi. Other studies of more distantly related species also reported branching of mature haustoria e.g. Frommeïla mexicana which is a pathogen on Duchesnea indica (Mims et al. 2001). The function of branching is most likely to increase the contact zone between the pathogen and the host, which may create a surface large enough to ensure sufficient nutrient fluxes for colony growth and to secure a strong host-pathogen interaction. Estimation of volume and surface area is a challenging task but in our study measurements were made for a number of selected haustoria. The results suggest that the surface area may increase more than tenfold from small spherical haustoria to branched mature haustoria and that the branched haustoria observed at 68 hai were close to a final volume and surface area. However, due to a possible dehydration of samples, these volume and surface estimates may be smaller than for haustoria in living plants. Nevertheless, mature haustoria seem to take up significant space in affected host cells. Observations for Blumeria graminis on barley indicates that processes affecting the cytoskeleton of host cells are involved in accommodation of haustoria (Hoefle et al. 2011, Opalski et al. 2005). Several studies have found strong correlation between pathogen attack and rearrangement of plant cytoskeleton. In general, the actine cytoskeleton serves an important function for plant defense both against adapted and non-adapted pathogens (Schmidt and Panstruga 2007). Based on the significant
changes in haustorium size and morphology reported here, one important question is how the interaction with the plant cytoskeleton changes during the time course of haustorium development, and to what extent this interaction is under fungal control. The plant cytoskeleton may serve a function in shaping and supporting haustorium formation.

A general characteristic of D-haustoria is a clear subdivision of the haustoria into specialized regions (Perfect and Green 2001). One region is the haustorial neck which is thought to serve a function in sealing off the extrahaustorial matrix from the plant apoplast and thereby facilitating nutrient uptake (Voegele and Mendgen 2003). Koh et al. (2005) also showed that the neck region marks the site where the extrahaustorial membrane becomes clearly different from the host plasma membrane in cells of Arabidopsis infected with Erysiphe cichoracearum. Based on transmission electron microscopy different structural features, connected to the haustorial neck area, have been reported for rust fungi. The formation of a so called collar at the site where the haustoria enter the host cell seems to be a general feature (Hu and Rijkenberg 1998, Kang et al. 2002, Mares 1979b). Collars have been defined as depositions of plant material between the host plasma membrane and the host cell wall (Harder and Chong 1984), either in direct contact with the haustorial neck or separated from the neck by the host cell plasma membrane. Our observation of P. striiformis haustoria showed that the collars gradually increased in size and shape, and in the oldest observed haustoria they seemed to cover most of the central body of the haustoria. The rough and invaginated appearance in mature haustoria are in accordance with previous observations based on electron microscopy (Baka 1992, Mims et al. 2001) and thus most likely not an effect of sample treatment. The function and effect of these changes are not yet known but Mims et al. (2001) discussed labyrinth structures surrounding the haustorial neck of haustoria of Frommeëla mexicana var. indicae as ingrowth of the host cell wall resembling secondary wall ingrowths found in plant transfer cells. Transfer cells facilitate solute transfer over short distances and are common at sites where elevated nutrient flow is necessary for plant development. Cell wall ingrowth might serve as a host cell response to compensate for solutes lost from the cell to the haustorium. The deposition of callose seems to be common characteristics of collars (Kang et al. 2002; Micali et al. 2011) a finding that has also been reported for cell wall ingrowths (McCurdy et al. 2008).

Average haustorium size increased significantly between sampling points up to 4–5 days after inoculation in both wheat genotypes after which no significant increase in size was observed between 116 and 164 hai. At 336 hai, when spore production had been initiated, the haustoria
were significantly larger than at previous time points. This significant change in size may be a result of spore production which probably increases the energy demand of the fungus. Haustorial morphology was identical in the two host genotypes 514W and Cartago although they differed in size at all time points. Data on morphology also indicated that haustorium formation was initiated earlier in 514W than in Cartago and that the branched stage was reached faster. As both hosts facilitate compatible interaction with *P. striiformis*, difference in size may reflect histological differences in tissue structure or cell morphology. In both varieties older pathogen colonies possessed haustoria of continuous morphology, ranging from young spherical haustoria in the periphery of colonies to older haustoria with varying degree of branching closer to the substomatal vesicle. Approximately 15% of the haustoria on primary infection hyphae were formed in epidermal cells in both host genotypes. Haustorium formation in epidermal cells has also been reported for two other wheat infecting rust fungi, *P. graminis* and *P. triticina* (Harder et al. 1979, Hu and Rijkenberg 1998).

In recent years several studies have dealt with proteome and gene expression analysis of haustoria formed by rust fungi (e.g. Godfrey et al. 2009, Puthoff et al. 2008, Song et al. 2011). Such studies have revealed several new and important insights about the role of haustoria in host-pathogen interactions. Most of these studies have focused on one particular stage of infection, but based on the results of haustorium formation reported here, it should be important to address the question of how the interaction in a particular host cell changes as the haustorium develops. With the significant changes seen for haustoria of *Puccinia striiformis* in compatible interaction with wheat it is most likely that the character of the interaction changes during the time course of infection. Thus, a detailed knowledge of haustorial development both in time and space serves as an important basis for a complete understanding of both the cytology and genetics of plant-pathogen interaction.

**Acknowledgements**

We want to thank Center for Biomembrane Physics (MEMPHYS) at University of Southern Denmark (SDU) for providing access to their microscope facilities, which made this study possible and Aarhus University for funding this research. We also thank Dr. Stephanie Walter for highly useful comments on the manuscript and Kristian Kristensen for statistical advice.
Literature cited


Manuscript II

Fitness of wild-type and virulence mutants of *Puccinia striiformis* on wheat

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Abstract

Differences in fitness between individuals of a plant pathogen may have a large impact on pathogen population dynamics. Here, we studied differences in fitness between two pairs of wild type/mutant isolates of *Puccinia striiformis*. Spontaneous virulence mutants, which were derived from a specially designed field trial, differed only from their respective wild types in virulence corresponding to the *Yr2* resistance gene in wheat. The wild type/mutant pairs represented different virulence phenotype groups and genetic lineages. Colony size, latent period, lesion growth rate and uredinia density and size were estimated on both susceptible and *Yr2* resistant wheat varieties. No significant differences for any of the evaluated parameters were observed within wild type/mutant pairs, i.e., the results did not support a hypothesis of a fitness cost in *P. striiformis* for acquiring virulence corresponding to *Yr2* resistance in wheat. In contrast, significant differences were found for most of the parameters when isolates were compared according to genetic lineage. Wild type/mutant isolates of one lineage had a significantly shorter latent period, a higher growth rate and larger colonies on the two susceptible host varieties than isolates of the other lineage, supporting a hypothesis that fitness of different virulence phenotypes is influenced mainly by their genetic background.

**Keywords:** Epidemiology, Pathogen evolution, Cost of virulence, Plant resistance, Yellow rust
Introduction

The gene-for-gene model has since its introduction by Flor (1955) been one of the most used models for understanding host-pathogen interactions. In the gene-for-gene model host resistance is controlled by resistance genes in the host and matching avirulence genes (effector) in the pathogen. In this context virulence is determined as the ability of the pathogen to compromise the matching resistance gene in the host (Hovmøller et al., 2011). Virulence is believed to arise as a result of loss or inactivation of an avirulence gene so that its product is no longer recognized in the host by the product of the matching resistance gene (Sacristán & García-Arenal, 2008).

Since the gene-for-gene model was put forward one of the major tasks has been to understand the fate of virulence genes in populations of fungal plant pathogens (Brown, 1995, Hovmøller et al., 1997). Based on observations for flax rust (*Melampsora lini*) (Flor, 1953) found that pathotypes with the least number of virulence genes necessary for survival dominated populations of this pathogen. Later, (van der Plank, 1968) introduced the concept of “stabilizing selection” and suggested that in general the fittest pathotypes in plant-pathogen systems were those with no unnecessary virulence genes. His hypothesis was that in a gene-for-gene system there will be a cost of virulence in the absence of the corresponding resistance gene (R-gene) which will lead to selection against the specific virulence gene when the R-gene is not present. The idea behind the concept of cost of virulence is that mutations in avirulence genes leading to virulence may impair the function of the gene and therefore result in a fitness penalty (Leach et al. 2001).

One of the best examples of a cost of virulence has been found in a natural population of the pathogen *Melampsora lini* (flax rust). For *M. lini* Thrall and Burdon (2003) found a negative correlation between number of virulence genes and spore production, which explained that highly avirulent pathotypes dominated populations of susceptible hosts. However, other studies of virulence frequencies in pathogen populations have revealed that the generalization made by van der Plank is inadequate (Bahri et al., 2009a, Bayles et al., 1997, Leach et al., 2001). In agricultural systems observed changes of pathotype frequencies have given no clear answer to the question of cost of virulence. In asexual populations of pathogen species like the fungal pathogen *Puccinia striiformis* aspects such as genetic hitch hiking and host specificity seem to be highly important for the fate of virulence genes, even when there might be a cost of virulence.
(Brown, 1995, Kirchner & Roy, 2002). Understanding how different factors affect pathotype frequencies in agricultural systems is of great importance for disease control. For *P. striiformis* it is well described how strong selection from host varieties possessing genes for specific resistance can lead to a great increase in the frequencies of pathotypes with virulence matching the resistance (Bayles et al., 2000, Hovmøller, 2001). But other factors such as difference in fitness between lineages and cost of virulence, might be equally important for understanding the genotype/pathotype dynamics in populations of this pathogen (Brown, 1995, Bahri et al., 2009b, Mboup et al., 2009).

For fungal pathogens several components in the life cycle add to the overall fitness of a genotype, but looking at only one or a few of these factors can be highly valuable for studying fitness differences (Pringle & Taylor, 2002). In plant pathology different aspects of pathogen fitness, often addressed under the term aggressiveness, have been used to define the relative ability of different genotypes to cause disease (Pariaud et al., 2009). Most often epidemiological parameters like infection frequency, latent period, lesion growth rate, and spore production have been used as components of aggressiveness. Variability for aggressiveness between genotypes has often been demonstrated (Pariaud et al., 2009), but in most cases no link has been made to virulence phenotype. In this study we tested two hypotheses of the relation between fitness and virulence phenotype in *P. striiformis*: 1) the acquisition of virulence corresponding to the *Yr2* resistance gene in wheat is associated with a fitness cost 2) fitness of different virulence phenotypes is influenced mainly by genetic background.

Two *Yr2* avirulent wild type isolates, each from a different lineage, and their corresponding virulence mutant, were inoculated on susceptible and *Yr2* resistant host varieties. Fitness was assessed at the macroscopic level by latent period, lesion growth and uredinium size and density. At a microscopic level, the sizes of colonies in infected leaves were assessed at different time points after inoculation.

**Material and methods**

**Pathogen isolates and hosts varieties**
Two pairs of wild type/mutant isolates of *P. striiformis* were used. In 2006 mutants were derived from experimental field plots, aiming to detect virulence mutants for *Yr2* resistance in wheat. Different rows of the susceptible wheat variety Anja were inoculated with wild type isolates of
Northwest European origin, DK24/95 and UK75/30 (Hovmøller & Justesen, 2007a). The rows of Anja were flanked by rows of variety Skater (Yr2, Yr32) (Hovmøller, 2007), which were resistant to the Danish population of *P. striiformis* at that time. In addition to differences in five virulence genes (Table 1), the two wild type isolates differed by 13 AFLP fragments among a total of 30 AFLP polymorphisms detected in the Northwest European *P. striiformis* population at that time. To detect whether lesions emerging on Skater were virulence mutants from the wild types, or exotic immigrants in the experiment, they were taken to the laboratory for pathotyping and AFLP fingerprinting according to Justesen et al. (2002). Based on these tests, Yr2 virulence mutant isolates were derived from both wild type isolates (Table 1). No polymorphism was detected within wild type/mutant pairs when screened by 20 AFLP primer combinations producing approximately 1400 AFLP fragments.

Fitness parameters of wild type and mutant isolates were evaluated on four host varieties: Two varieties with no known *P. striiformis* resistance genes: Avocet S and Cartago (susceptible), and two varieties carrying Yr2 resistance: Skater (Yr2, Yr32) and Heines VII (Yr2, Yr25, +). Seeds were sown in 7.5 cm pots filled with Pindstrup substrate, a standard peat-based mix with slow release nutrients (Pindstrup Mosebrug A/S, Ryomgaard, Denmark). Pots were placed in trays covered with plastic lids to ensure high humidity during germination in spore-proof greenhouse cabins. Artificial light of 50-100 µE m⁻² s⁻¹ was applied when daylight was less than 10000 lux outside. Light was applied 18 hours a day and with 17°C day/12°C night.

Table 1. Virulence phenotype of wild type (W) and virulence mutant (M) isolates of lineage 1 and 2.

<table>
<thead>
<tr>
<th>Isolate designation</th>
<th>Genotype</th>
<th>Lineage</th>
<th>Virulence phenotype&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>ID</td>
<td>Genotype</td>
<td>Lineage</td>
<td>1 2 3 4 5 6 7 8 9 10 15 17 25 27 32 Sd Sp</td>
</tr>
<tr>
<td>UK 75/30</td>
<td>Wild type (W1)</td>
<td>1</td>
<td>- - - - - - - - - - - 25 - 32 Sd Sp</td>
</tr>
<tr>
<td>Mut 15/05</td>
<td>Mutant (M1)</td>
<td>1</td>
<td>- 2 - - - - - - - - - 25 - 32 Sd Sp</td>
</tr>
<tr>
<td>DK 24/95</td>
<td>Wild type (W2)</td>
<td>2</td>
<td>- - 3 4 - 6 - - - - - - 25 - 32 Sd -</td>
</tr>
<tr>
<td>Mut 21/06</td>
<td>Mutant (M2)</td>
<td>2</td>
<td>- 2 3 4 - 6 - - - - - - 25 - 32 Sd -</td>
</tr>
</tbody>
</table>

<sup>a</sup> Figures and symbols designate virulence corresponding to specific yellow rust resistance genes. – avirulence, Sd: Strubes dickoff; Sp: Spalding prolific.
Experimental procedures

Three separate experiments were conducted for assessment of 1) latent period and lesion growth rate 2) uredinium size and density 3) colony size, respectively. For assessment of latent period and lesion growth rate a 0,5 cm long area on the adaxial side of the second leaf was inoculated approximately 11 cm below the leaf tip. 14 pots (replicates) each with one plant were used per isolate-variety combination (treatment). Latent period was defined as number of hours from inoculation until the first spores were visible in uredinia on the leaf surface. Plants were observed with a 10x hand lens at a 12 hours interval starting 8 days after inoculation. Four days after the first spores were observed on each leaf the first measurement of lesion length was done with a ruler to the closest mm. Any plants with infections outside the inoculated area were excluded from the experiment at this time point. The second lesion measurement was done four days later. The lesion growth rate was calculated as the difference between the two measurements divided by number of days between measurements (four).

In the set up for determination of uredinium size and uredinium density ten pots each with two plants were used per treatment and an adaxial area of 2 cm approximately 11 cm below the tip of the second leaf was inoculated. For assessment of uredinium size and uredinium density one inoculated leaf segment was cut off per pot 16 days after inoculation. Prior to microscopic observation leaf samples were stained according to Moldenhauer et al. (2006). Leaf segments were fixed and cleared in ethanol:chloroform (3:1, v/v) + 0,15 % (v/w) trichloroacetic acid for at least 24 hours. After being washed twice in 50% ethanol they were left in 0,05 M NaOH for 30 min. Specimens were then rinsed in water before being submerged in 0,1 M Tris-HCl buffer (pH 5,8) for 30 min. They were then stained for 5 min in 0,1 % (w/v) Uvitex 2B (polysciences inc.) in Tris-HCl buffer (pH 5,8). Following staining specimens were washed four times in DI, one time in 25% Glycerol and left overnight in DI. They were stored in 50% glycerol for later observation.

For estimation of colony size ten seeds were sown per pot and before inoculation the number was reduced to five uniform plants per pot. Ten pots were used per treatment and plants were inoculated in the same way as in the experiment for estimation of uredinium size and density. One leaf segment per pot was sampled at 3, 5, 7 and 16 days post inoculation (dpi). The inoculated part of the leaves were cut off and were prior to microscopic observation stained in the same way as leaf segments in the second experiment.
In all three experiments wheat seedlings were inoculated when they were 16 days old and the second leaf fully expanded. Urediniospores for inoculation were multiplied on the susceptible variety Cartago. Plants were shaken 48 hours prior to harvest of spores for inoculation to remove old spores. Freshly harvested spores were mixed with talc (1:19 w/w) and the mixture was applied to the second leaf with a camelhair paintbrush (size 1). The inoculated plants were incubated in a dark cold chamber (10°C) for 20 hours. Following incubation, plants were transferred to spore proof chambers and pots were randomized within and between chambers. All three experiments were conducted simultaneously and replicated twice.

Microscopy
For determination of uredinium size, uredinium density and colony size, whole mounts were prepared and microscopy was done with a Leica DMR equipped with optics for epifluorescence. Structures were visualized with UV-1D filter (excitation filter 355-425, barrier filter 455). On the leaf segment for determination of uredinium size and density the size of five randomly chosen uredinia were measured and the density was measured by counting the number of uredinia with in three random areas of 2x2 mm. On leaf samples for determination of colony size between two and five randomly selected colonies were measured per leaf segment depending on the number of infections. Some leaves had no visible infections. Dimensions of both colonies and uredinia were measured by recording the largest length and largest width with a calibrated eyepiece micrometer. Colony and uredinium size was calculated as: largest length x largest width x π/4 (Baart et al. 1996)

Statistics
Data for all variables were analyzed using mixed models (West 2006). Results for latent period and colony size were log-transformed before analysis and back transformed for presentation. For uredinium size, uredinium density and colony size, leaf average was used in the analysis. The fixed effect in the model was the combinations of isolate and variety. Random effects included in the model where the effect of experiment and the combinations of experiment, isolate and variety. Based on this model contrasts were formulated in order to test fixed effects of isolate, variety and their interaction. Likewise selected pairwise comparisons between isolates or pair of isolates were estimated based on the effects for the combinations of isolate and variety. No adjustments of P values for multiple comparisons were applied and the significance
level were set at $\alpha = 0.05$. The analyses were carried out using the MIXED procedure in SAS statistical software (version 9.2, SAS Institute Inc., Cary, NC, USA).

The effect of experimental replication was analysed using a general linear model. Experiment, isolate, variety and combinations of these were included as fixed effects. Results for latent period and colony size were log-transformed before analysis. Analysis was carried out with the GLM procedure in SAS (version 9.2, SAS Institute Inc., Cary, NC, USA).

**Results**

Our results showed significant main effects of both variety and isolate for all parameters and no significant isolate×variety interactions. Statistical results for the four epidemiological parameters: latent period, lesion growth rate, uredinium size and uredinium density are presented in table 2 and 3, and results for colony size are presented in table 4 and 5. Since only mutant isolates resulted in compatible interaction on Heines VII and Skater ($Yr2$ resistant) data for susceptible (Cartago and Avocet S) and resistant varieties were analysed separately.

**Latent period**

Latent period was significantly affected by replication of experiment ($P<0.0001$) but no interaction between experiment and main effects were found (data not presented). The significant effect of experiment was due to a longer latent period for all treatments in the second experiment.

For both the susceptible and $Yr2$ resistant varieties, the latent period was influenced by both isolate and variety (Table 2). Contrasts comparing wild type and corresponding mutant isolates on susceptible varieties revealed no significant effects, whereas contrasts comparing the two wild type/mutant pairs (lineages) showed a significant effect of genetic background. Lineage 2 had a generally shorter latent period than lineage 1. Similarly, on the $Yr2$ resistant varieties, Mutant 2 had a significantly shorter latent period than Mutant 1 (Table 3; Figure 1).

Significant differences were observed between varieties, with significant shorter latent periods for Cartago than for Avocet S. With respect to the two $Yr2$ resistant varieties, the virulence mutant isolates had significantly shorter latent periods on Skater compared to Heines VII. Even
for the compatible interactions, chlorosis and some level of necrosis was observed on Heines VII whereas only chlorosis was visible on Skater.

Table 2. Main effect of isolate and variety on the four fitness parameters latent period, lesion growth rate, uredinium density and uredinium size. Results for the susceptible varieties (Cartago and Avocet S - susceptible to all isolates) and the Yr2 resistant varieties (Heines VII and Skater – susceptible only to the mutant isolates) were analysed separately.

<table>
<thead>
<tr>
<th>Fixed effect</th>
<th>Latent period</th>
<th>Lesion growth rate</th>
<th>Uredinium size</th>
<th>Uredinium density</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F value</td>
<td>P value</td>
<td>F value</td>
<td>P value</td>
</tr>
<tr>
<td>All isolates on Cartago and Avocet S</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isolate</td>
<td>3</td>
<td>0.69</td>
<td>0.0241</td>
<td>5.96</td>
</tr>
<tr>
<td>Variety</td>
<td>1</td>
<td>14.87</td>
<td>0.0027</td>
<td>16.0</td>
</tr>
<tr>
<td>Isolate x variety</td>
<td>3</td>
<td>0.82</td>
<td>0.5087</td>
<td>0.51</td>
</tr>
<tr>
<td>Mutants on Heines VII and Skater</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isolate</td>
<td>1</td>
<td>37.60</td>
<td>&lt;.0001</td>
<td>6.6</td>
</tr>
<tr>
<td>Variety</td>
<td>1</td>
<td>175.13</td>
<td>&lt;.0001</td>
<td>13.6</td>
</tr>
<tr>
<td>Isolate x variety</td>
<td>1</td>
<td>0.12</td>
<td>0.7325</td>
<td>0.00</td>
</tr>
</tbody>
</table>

a. Analysis performed on log-transformed data.
Table 3. Estimates and probabilities of individual and pairwise comparisons of wild type and mutant isolates on susceptible varieties (Avocet S and Cartago).

<table>
<thead>
<tr>
<th>Contrasts</th>
<th>df</th>
<th>Latent period</th>
<th>Lesion growth rate</th>
<th>Uredinium size</th>
<th>Uredinium density</th>
</tr>
</thead>
<tbody>
<tr>
<td>W1 vs. M1</td>
<td>11</td>
<td>-0.0160</td>
<td>0.0582</td>
<td>-0.2028</td>
<td>0.1415</td>
</tr>
<tr>
<td>W2 vs. M2</td>
<td>11</td>
<td>0.0099</td>
<td>0.2052</td>
<td>-0.0845</td>
<td>0.5221</td>
</tr>
<tr>
<td>Lineage 1 vs. lineage 2 ( \text{a} )</td>
<td>11</td>
<td>0.0154</td>
<td>0.0140</td>
<td>-0.3365</td>
<td>0.0034</td>
</tr>
<tr>
<td>M1 vs. M2</td>
<td>11</td>
<td>0.0283</td>
<td>0.0034</td>
<td>-0.2773</td>
<td>0.0580</td>
</tr>
<tr>
<td>W1 vs. W2</td>
<td>11</td>
<td>0.0025</td>
<td>0.7429</td>
<td>-0.3956</td>
<td>0.0089</td>
</tr>
<tr>
<td>Wild types vs. Mutants</td>
<td>11</td>
<td>-0.0030</td>
<td>0.5773</td>
<td>-0.1437</td>
<td>0.1406</td>
</tr>
</tbody>
</table>

\( \text{a} \) Lineage 1 represents the combination of W1 and M1 whereas lineage 2 is the combination of W2 and M2.

\( \text{b} \) A positive estimate shows that the first mentioned isolate or pair of isolates represents the larger value.

Figure 1. Least square means of latent period (hours) for wild type (W1, W2) and mutant isolates (M1, M2) on two susceptible (Avocet S, Cartago) and two Yr2 resistant wheat varieties (Skater, Heines VII).
**Lesion growth rate**

As for latent period lesion growth rate was significantly affected by replication of experiment (P<0.0001) with the longer lesion growth rates in the second replication of the experiment (data not shown).

Lesion growth rate was significantly affected by variety and isolate. Lesion growth rate was higher on Cartago than on Avocet S. When comparing the lesion growth rates of wild type and corresponding virulence mutant isolates, no significant differences were found on susceptible varieties. However, as for latent period, isolates of different lineages revealed significant differences (table 3). Isolates of lineage 2 had a significantly higher lesion growth rate than isolates of lineage 1 on Cartago and Avocet S, and similarly, on the Yr2 resistant varieties, mutant of lineage 2 had a significantly higher lesion growth rate than the mutant of lineage 1 (Table 3 and Figure2). The lesion growth rate for virulence mutants was significantly higher on Heines VII than on Skater. Least square means of lesion growth rate for all treatments are presented in figure 2.

![Figure2. Least square means of lesion growth rate (mm/day) of wild type (W1, W2) and mutant isolates (M1, M2) on two susceptible (Avocet S, Cartago) and two Yr2 resistant wheat varieties (Skater, Heines VII).](image)

**Uredinium size**

Uredinium size was significantly affected by the replication of experiment with uredinia being biggest in the first replication (P<0.0001). The few interactions between experiment, variety
and isolate occasionally found were not considered important for the interpretation of the results.

On the susceptible varieties uredinium size was not significantly affected by either isolate or variety but the uredinia of isolates from lineage 2 were generally larger than uredinia of isolates from lineage 1 (Table 2 and 3). The mutant isolate of lineage 2 produced significantly larger uredinia on Skater and Heines VII compared to the mutant of lineage 1. Further, uredinia were larger on Skater compared to Heines VII. Least square means for all treatments are presented in figure 3.

Figure 3. Least square means of uredinium size (mm²) of wild type (W1, W2) and mutant isolates (M1, M2) on two susceptible (Avocet S, Cartago) and two Yr2 resistant wheat varieties (Skater, Heines VII).

**Uredinium density**

Uredinium density was significantly higher on the first replication of the experiment (P<0.0001) but no interaction was seen between experiment and other factors.

On susceptible varieties no differences between the wild types and their respective mutant but the wildtype/mutant par of lineage 1 had a significantly higher uredinium density than pair of lineage 2 (Table 3). Uredinium density was highest on Avocet S.
On the Yr2 resistant varieties the mutant of lineage 2 had a higher uredinium density than the mutant of lineage 1. Further, the uredinium density was higher on Skater than on Heines VII. Least square means of all combinations are presented in figure 4.

Figure 4. Least square means of uredinium density (uredinia/mm²) of wild type (W1, W2) and mutant isolates (M1, M2) on two susceptible (Avocet S, Cartago) and two Yr2 resistant wheat varieties (Skater, Heines VII).

**Colony size**

A significant effect of experiment replication was found for colony size, with significantly larger colonies in the first experiment at all time points (P<0.0001). Further, interactions between experiment, variety and isolate were found in few cases, but these were not considered important for the interpretation of the results.

On susceptible varieties, no significant effect of isolate was observed until 7 dpi (Table 4), where the wild type/mutant pair of lineage 2 had significantly larger colonies than the pair of lineage 1 (Table 5).

On the Yr2 resistant varieties, a significant effect of isolate was found at both 5 and 7 dpi for the two mutant isolates. At these time points the mutant of lineage 2 had significantly larger colonies than the mutant of lineage 1. For the incompatible interactions (wild types on Yr2 resistant varieties) there was no significant effect of isolate on colony size. The colonies of the incompatible interactions were associated with autofluorescence and at 5 and 7 dpi they were much smaller than for the compatible interactions (Figure 5).
Table 4. Effects of isolate, variety and their interaction on the size of *Puccinia striiformis* colonies in wheat leaves at four different times after inoculation. Results for the susceptible varieties (Cartago and Avocet S - susceptible to all isolates) and the *Yr2* resistant varieties (Heines VII and Skater – compatible only with mutant isolates) were analysed separately.

<table>
<thead>
<tr>
<th>Fixed effect</th>
<th>Colony size</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 dpi.</td>
</tr>
<tr>
<td></td>
<td>df</td>
</tr>
<tr>
<td>All isolates on Cartago and Avocet S</td>
<td></td>
</tr>
<tr>
<td>Isolate</td>
<td>3</td>
</tr>
<tr>
<td>Variety</td>
<td>1</td>
</tr>
<tr>
<td>Isolate x Variety</td>
<td>3</td>
</tr>
<tr>
<td>Mutants on Heines VII and Skater</td>
<td></td>
</tr>
<tr>
<td>Isolate</td>
<td>1</td>
</tr>
<tr>
<td>Variety</td>
<td>1</td>
</tr>
<tr>
<td>Isolate x Variety</td>
<td>1</td>
</tr>
<tr>
<td>Wild types on Heines VII and Skater</td>
<td></td>
</tr>
<tr>
<td>Isolate</td>
<td>1</td>
</tr>
<tr>
<td>Variety</td>
<td>1</td>
</tr>
<tr>
<td>Isolate x Variety</td>
<td>1</td>
</tr>
</tbody>
</table>

a. Due to expansive hyphal networks in leaves of compatible interaction colony size could only be measured for the incompatible interactions at 16 dpi.
<table>
<thead>
<tr>
<th>Estimates</th>
<th>3 dpi</th>
<th>5 dpi</th>
<th>7 dpi</th>
</tr>
</thead>
<tbody>
<tr>
<td>W1 vs. M1</td>
<td>15</td>
<td>0.0005</td>
<td>0.5950</td>
</tr>
<tr>
<td>W2 vs. M2</td>
<td>15</td>
<td>0.0003</td>
<td>0.7705</td>
</tr>
<tr>
<td>Lineage 1 vs. lineage 2</td>
<td>15</td>
<td>0.0059</td>
<td>0.6699</td>
</tr>
<tr>
<td>M1 vs. M2</td>
<td>15</td>
<td>0.0002</td>
<td>0.8590</td>
</tr>
<tr>
<td>W1 vs. W2</td>
<td>15</td>
<td>0.0004</td>
<td>0.6685</td>
</tr>
<tr>
<td>Wild types vs. Mutants</td>
<td>15</td>
<td>0.0008</td>
<td>0.5608</td>
</tr>
</tbody>
</table>

a. Lineage 1 represents the combination of W1 and M1 whereas lineage 2 is the combination of W2 and M2.

b. A positive estimate shows that the first mentioned isolate or pair of isolates represents the larger value.
Figure 5. Colony size least square means at three different time points for wild type (W1, W2) and mutant isolates (M1, M2) on two susceptible (Avocet S, Cartago) and two Yr2 resistant wheat varieties (Skater, Heines VII). (a) Colony sizes at 3 days post inoculation. (b) Colony size at 5 days post inoculation. (c) Colony size at 7 days post inoculation. Note the different scales on the y-axis.
Discussion

In this study we assessed the influence of virulence and genetic background on the fitness of *Puccinia striiformis*. In contrast to many previous studies of cost of virulence in fungal pathogens (Pariaud et al. 2009) we had access to unique pathogen stocks of wild type and mutant isolates. The mutant isolates were derived from a field experiment specifically designed for screening against spontaneous virulence mutants. The field experiments were based on the availability of unique and atypical wild type isolates in combination with two wheat host varieties, Skater and Robigus possessing the *Yr2* and *Yr32* resistance genes (Hovmøller 2007). All wild type isolates used in the field experiment had been absent for 10 years or more in the population of *P. striiformis* in Denmark and they were unable to infect Skater and Robigus due to avirulence for *Yr2*. Further, Skater and Robigus were resistant to all *P. striiformis* pathotypes detected in Denmark in the field experimental years, and in at least eight preceding years (Hovmøller & Henriksen, 2008). During the two experimental growing seasons, putative virulence mutants were collected on Skater and Robigus and they were subsequently assayed for virulence in the greenhouse according to Hovmøller and Justesen (2007a). Additional DNA fingerprinting for confirmation of identity was made by AFLP primer pairs selected to reveal maximum variability in the Northwest European *P. striiformis* population (Hovmøller & Justesen, 2007b). The two virulence mutant isolates in the present study shared both AFLP fingerprint and virulence phenotype with their respective wild type isolate, except for *Yr2* specificity.

The identification of the same phenotypic shift in virulence in two different genetic lineages gave an opportunity to study a possible trade-off between virulence and fitness. Eliminating the effect of genetic background for assessment of a possible fitness penalty of individual virulence genes has previously been considered highly important for such studies (e.g. Østergård 1987). However, this is difficult to achieve, especially for species with no experimental system for sexual reproduction (Pariaud et al. 2009).

None of the fitness parameters evaluated in the present study suggested a cost of virulence in *P. striiformis* against *Yr2* resistance in wheat. In contrast, significant differences were observed for several fitness components when comparing isolates of different lineages, suggesting a significant effect of genetic background. The differences were mainly expressed by a shorter latent period and a higher lesion growth rate. The lineages also differed for uredinia production, which was assessed by two parameters, uredinium size and density. On susceptible varieties,
isolates of lineage 1 produced relatively small uredinia at a high density whereas isolates of lineage 2 produced larger uredinia at a significantly lower density. However, on the Yr2 resistant varieties, the virulence mutant of lineage 2 produced both larger uredinia and the density was higher than for the mutant of lineage 1. These results demonstrate that the interactions between host and pathogen can be highly complex. Therefore, robust analyses of pathogen fitness must include several parameters.

Significant differences in interactions between pathogen and host varieties have previously been reported for varieties carrying Yr2. Calonnec et al. (1997) showed that Heines VII in addition to Yr2 had second resistance gene, probably Yr25 (Hovmøller, 2007) and also additional resistance genes with minor effects. Virulence for Yr25 is close to fixation in the European yellow rust population (Hovmøller, 2007) and Calonnec et al. (1997) stated that the additional resistance was only expressed against non-European pathotypes of P. striiformis. However, our study demonstrated that also P. striiformis isolates of European origin could detect an additional resistance in Heines VII. Four out of the five fitness parameters showed that the growth of both virulence mutants (sharing virulence to Yr2, Yr25 and Yr32) were significantly restricted on Heines VII (Yr2, Yr25, +) compared to Skater (Yr2, Yr32). Based on results for colony size our study suggests that the additional resistance in Heines VII was expressed relatively late in the infection process, from 7 dpi and onwards.

The parameters applied here have often been used to evaluate fitness of fungal plant pathogens (Milus et al. 2009; Pariaud et al. 2009) and to study the effect of resistance in host varieties (Broers, 1997, Cromey, 1992). Simulations studies using models for prediction of epidemic development have demonstrated that these parameters are highly important factors for disease severity (e.g. Luo & Zeng, 1995). It is important to notice that most of the evaluated fitness components are highly influenced by environment (McGregor & Manners, 1985, Chen, 2005). The effect of environment was also evident in our study where the effects of experimental replication were highly significant. However, an interaction between experiment and other factors were only observed in some few cases in this study, and these did not affect the overall conclusions.

In this study, the two virulence mutants had independently acquired virulence to Yr2, and in none of the cases fitness was affected. However, the generality of this result may be difficult to predict since different changes in an avirulence gene can result in the same virulence phenotype (Schürch et al., 2004, Rouxel & Balesdent, 2010). The exact nature of the genetic
changes from wild type to mutant, in this study is not known, i.e., other changes resulting in the acquisitions of Yr2 virulence may have a different effect on fitness. Nonetheless, the lack of a fitness penalty in pathogens acquiring a new virulence is not uncommon in plant pathogens (Leach et al 2001). In fact, the pair of isolates with the highest number of considered virulence genes had the highest fitness in our study, suggesting that the genetic background has larger impact on pathogen fitness than the detected number of virulence genes. Bronson and Ellingboe (1986), showed that fitness of *Erysiphe graminis* f.sp. *tritici* segregated independently of virulence genes.

The significance of increased fitness of specific pathogen strains is emphasised by the recent emergence and worldwide spread of two aggressive, and high-temperature tolerant strains of *P. striiformis* (Milus et al. 2009). Isolates of these strains have caused serious yellow rust epidemics in many wheat growing areas (Hovmøller et al., 2011). The emergence of isolates with enhanced fitness may thereby pose a serious threat to wheat production. As shown in our study fitness difference even exist between closely related lineages of the same geographical origin, and the effect of such differences on pathogen population dynamics is something that needs further evaluation.

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**References**


Manuscript III

Exotic strains of *Puccinia striiformis* reveal race-specificity of long-term effective adult plant resistance in wheat

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(Manuscript in preparation)
Abstract

Resistance to *Puccinia striiformis* (*Pst*) was examined in nine French recombinant inbred lines (RILs) from a cross between Camp Rémy (resistant parent) and Réctial (susceptible parent) using a typical *Pst* isolate of NW-European origin and two isolates representing non-European (exotic) strains. The RILs were selected according to different combinations of QTLs for resistance to *Pst*. Exotic strains were represented by an isolate of *Pst* S2, which is aggressive and tolerant to warm temperatures, and an isolate of a new, multivirulent strain, which was detected for the first time in NW-Europe in 2011. Isolates of the old NW-European strain and the new strain gave highly contrasting results. Four host genotypes including Camp Rémy were resistant to the old strain, but susceptible or moderate susceptible to the new strain, and the opposite was the case for five genotypes, which were susceptible to the old strain. Two RIL were resistant to isolates of both strains and all host genotypes were resistant to *Pst* S2. These large shifts in susceptibility were generally confirmed under field conditions, except for Camp Rémy and the two resistant RILs, which were resistant and susceptible under field conditions, respectively. The results stress the importance of maintaining high diversity for disease resistance and of using isolates of diverse origin in wheat breeding programs.

**Keywords:** Quantitative resistance, *Triticum aestivum*, Stripe (yellow) rust, durable resistance, race-specificity
Introduction

The severity of disease caused by plant pathogens on cereal crops depends on several factors including the virulence and aggressiveness of the pathogen, the resistance in the host and the environmental conditions (Chen 2005; Fabré et al. 2012). In modern agriculture, resistant host cultivars have often been introduced to control plant disease. However, the level of disease control may vary in time and space in case the pathogen is able to adapt to the resistant cultivars. Insights in the ability of the pathogen to adapt to host resistance which may have severe consequences in terms of yield loss and food security (Murray 1995; Chen 2005) are therefore highly important.

Stripe (yellow) rust of wheat, caused by *Puccinia striiformis* (*Pst*), is one of the most important cereal diseases worldwide (Hovmøller et al. 2010). Historically, *Pst* has mainly been a problem in cool climates but in recent years, the pathogen has become an increasing problem in areas normally considered too warm for yellow rust epidemics (Chen 2002; Wellings 2007). The global spread of *Pst*S1 and *Pst*S2, two aggressive and high temperature tolerant strains, has likely played a significant role for the changing epidemiology of yellow rust (Hovmøller et al. 2008; Milus et al. 2009). Isolates of these new strains have almost replaced the old populations in North America and Australia (Wellings 2007; Markell and Milus 2008). *Pst*S2 has also been found in Europe but generally in low frequencies (Hovmøller and Justesen 2007a), probably because many wheat cultivars in European agriculture have been resistant to these two aggressive strains (Hovmøller 2007).

In general resistance in wheat to stripe rust has been categorized as either qualitative or quantitatively inherited (Chen 2005). Qualitative resistance can in most cases be detected at the seedling stage based on host-pathogen interaction consistent with the gene-for-gene hypothesis (McIntosh et al. 1995). This type of resistance is very common in European wheat cultivars, possibly due to its simple inheritance and an effective disease control in the absence of virulent races. Quantitative resistance to stripe rust is mainly expressed at the post-seedling stage, where it tends to become more and more effective as the host plant matures (Ma and Singh 1996). It is therefore often referred to as adult plant resistance (APR), delaying pathogen growth, e.g., through an increased latent period and/or reduction of lesion growth (e.g., Park and Rees 1989; Cromey 1992; Broers 1997). In many cases, APR in wheat have remained effective for yellow rust control in many years even when used over large acreage for several years (i.e.,
'durable' in sensu Johnson 1984,) (Boyd 2005). Recent genetic studies of APR resistance to stripe rust have revealed a number of quantitative trait loci (QTLs) with additive, and in some cases epistatic effects of either minor or major importance (Ramburan et al. 2004; Agenbag et al. 2012).

A good example of durable stripe rust resistance has been observed in a number of French bread wheat cultivars with combinations of genes for qualitative and quantitative resistance, remaining effective for more than ten years (Mallard et al. 2005; Dedryver et al. 2009; Paillard et al. 2012) even when used on large areas. In most cases the alleged durability of quantitative resistance has been predicted in a particular epidemiological environment. An interesting question is therefore if the emergence of exotic pathogen strains of an evolutionary origin different from the native pathogen population can lead to erosion of previously effective quantitative resistance?

Exotic and atypical Pst strains have appeared quite often in the North West European population in recent years (Hovmøller and Justesen 2007a (eurowheat.org)). In this study, we examined the resistance to stripe rust in recombinant inbreed lines derived from a cross between the French cultivars Camp Rémy (resistant to stripe rust since 1980) and Récital (susceptible). The wheat lines were inoculated by three isolates which had all been sampled in France, i.e., a typical Pst race of NW-European origin and isolates of two exotic strains. The two latter were represented by an isolate of PstS2 (Hovmøller et al. 2010), which is aggressive and tolerant to warm temperatures, and clearly of non-European origin (Ali et al 2010), and an isolate of a multivirulent race of undetermined origin, which was detected for the first time and at multiple locations in NW-Europe in 2011. The resistance in Camp Rémy is known to be based on a combination of two genes for qualitative resistance and seven QTLs for adult plant resistance whereas the susceptible parent, Récital, carries the Yr6 resistance gene (Mallard et al. 2005). The inbreed lines possessed different combinations of resistance genes and QTLs derived from the parental cultivars. Based on the evaluation of a range of epidemiological traits assayed in field and greenhouse, race-specificity was demonstrated for both seedling resistance genes (typical Yr-genes) and QTLs. The implications of the results for wheat resistance breeding and disease management strategies are discussed.
Material and methods

Pathogen isolates and wheat genotypes.

Three pathogen isolates were selected based on evolutionary origin, virulence phenotype and aggressiveness. All three isolates had been collected in France and are a part of stripe rust collection at UR Bioger, INRA Grignon. Isolates were inoculated on to 27 differential host genotypes including 15 differential cultivars from the World and European set (Johnson et al. 1972) for test of virulence and avirulence to the resistance genes Yr1, Yr2, Yr3, Yr4, Yr5, Yr6, Yr7, Yr8, Yr9, Yr10, Yr17, Yr25, Yr27, Yr32, Sd, Sp, and Su (Table 1). Infection type was evaluated on a 0-9 scale (McNeal et al. 1971) and infection type 5 and 6 were considered intermediate and 7 and above was considered susceptible. Isolate J99198 (denoted ‘old’) was first detected in France 1999 and became common in 2002 (de Vallavieille-Pope et al. 2012). The isolate J11019 (denoted ‘new’) is a multivirulent isolate, which was first detected in North-Western Europe in 2011, and sampled in high frequencies in France in 2011 and 2012 (eurowheat.org). The third isolate J04003 (denoted ‘PstS2’), which was detected in France in 2004 (de Vallavieille-Pope et al. 2012), belongs to an aggressive and high-temperature tolerant strain, which was first detected in Europe year 2000 (Hovmøller et al. 2008).

The wheat genotypes used in the experiment included nine recombinant inbreed lines (RILs) obtained by single seed descent (SSD) derived from a cross between the two French bread wheat cultivars Camp Rémy and Récital. The lines were developed at UMR-IGEPP INRA, Le Rheu, France as a part of a project for genetic analysis of durable resistance in Camp Rémy (Mallard et al. 2005). Camp Rémy possessed a unique combination of genes for qualitative and quantitative resistance, whereas only Yr6 was detected in Récital. The five recombinant lines were expected to carry different combinations of QTLs for resistance derived from Camp Rémy (Table 2). Two additional pairs of isolines, which had been selected in F6 where approximately 3% heterozygocity is expected, were also considered. One pair, CRR2178R and CRR2178S, differed with respect to QYr.inra.2BL.2 and QYr.inra.2BS, and another pair, CRR2182R and CRR2182S, differed for QYr.inra.2BL.2 and QYr.inra.2DS. The seedling resistance genes in the nine RILs and their parents were confirmed by differential tests at the seedling stage, and all QTLs were detected by presence of marker alleles of Camp Rémy and inoculated field experiments. The viability of spores and the assessment of isolate
aggressiveness under the considered experimental conditions were tested by inoculating seedlings of two cultivars, Cartago and Victo, which were susceptible to all considered isolates.

Table 1. Virulence phenotype of *Puccinia striiformis* isolates used in the experiment. Numbers designate virulence corresponding to specific stripe rust resistance genes, Sd, Sp, Su refer to resistance in Strubes Dickoff, Spaldings Prolific, and Suwon 92 x Omar, respectively. — indicates avirulence

<table>
<thead>
<tr>
<th>Isolate ID</th>
<th>Common Name</th>
<th>Virulence for Yr genes</th>
<th>Race</th>
</tr>
</thead>
<tbody>
<tr>
<td>J04003</td>
<td><em>PsS2</em></td>
<td>1 2 - - - 6 7 8 9 - - 25 27 - (Sd)</td>
<td>6E16V9</td>
</tr>
<tr>
<td>J99198</td>
<td>Old</td>
<td>1 2 3 4 - 6 - - 9 - 17 25 - - Sd - Su</td>
<td>237E141V17</td>
</tr>
<tr>
<td>J11019</td>
<td>New</td>
<td>1 2 3 4 - 6 7 - 9 - 17 25 - 32 Sd (Sp) Su</td>
<td>239E171V17</td>
</tr>
</tbody>
</table>

b. Designation according to (Johnson et al. 1972)
c. Brackets indicate a high intermediate infection type of 5-6 on a 0-9 scale (McNeal et al. 1971).

**Field experiment**

Field trials were conducted at INRA Versailles (Ile de France) in 2010, 2011 and 2012. Percentages of leaf areas with sporulating lesions were assessed by visual assessment of the RILs and the parent cultivars inoculated with the isolate J99198 (old) in three years from 2010 to 2012. In 2010 and 2012 all lines and both parents were evaluated, whereas in 2011, only the four lines, CRR2010, CRR2020, CRR2088 CRR2178R, and the two parents were tested. In 2012, all genotypes were additionally tested to the multivirulent isolate J11019 (new). By the end of October, 30 seeds of each genotype were planted separately in 1.2 m rows in a complete randomized block design in two replicates. The two blocks inoculated with identical isolates were situated next to each other, and blocks inoculated with different isolates were located at least 700 m apart. The isolate J99198 (old) was multiplied on spreader rows of cultivar Victo, and the isolate J11019 (new) was multiplied on Toisondor (Yr32 resistant). Spreader rows were sown at both ends of the blocks, perpendicular to and flanking the rows of the investigated genotypes. Inoculation of spreader plants was done in March with infected seedlings of the cultivar Victo. Seedlings of Victo had been raised in growth chambers and inoculated at the two-leaf seedling stage. Just before sporulation, these seedlings were planted in the field between plants in the spreader row, one pot per two meter row.
The scorings of average percent infected leaf area (sporulating lesions) on the upper three leaves were done three times during the growing season using the modified Cobb scale (0-100%) (40).

Greenhouse experiment

Prior to the adult plant experiment, compatibility between pathogen isolates and the wheat genotypes were confirmed using sixteen-day-old seedlings. Isolate J04003 (Pst S2), which showed incompatibility with Camp Rémy and four of the RILs at the seedling stage, was only assessed on adult plants of Récital and the five lines CRR2019, CRR2020, CRR2178S, CRR2083 and CRR2283. The two other isolates, J99198 (old) and J11019 (new), were used on all 11 genotypes at the adult plant stage. Prior to the adult plant experiments, isolates were multiplied on seedlings of Victo under identical environmental conditions for three generations in order to standardize the viability of the inoculums. The seedlings were grown in pots filled with standard peat soil and incubated in climate-controlled cabinets at max 20°C day/14°C night temperature and 16h/8h photoperiod from natural and supplemental of light at 300 µE/m²/s PAR. When they were 1-cm high seedlings were treated with 20 ml of a maleic hydrazide solution (0.25 g/l) to slow the emergence of the secondary leaves and to increase spore production. The seedlings were inoculated when primary leaves were fully expanded by dusting spores from a pot of previously infected seedlings. Inoculated plants were incubated for 24h in dark in a dew chamber at 8°C and 100% relative humidity, after which they were placed in cabinets with conditions as described above. Before the onset of spore production, the infected plants were covered with cellophane bags to prevent cross-contamination between isolates. Spores for experimental use were collected approximately 14 days after inoculation, following partial desiccation at 5°C for 4 days and stored additional 3-4 days in liquid nitrogen prior to adult plant inoculation.

Adult plants were raised in the greenhouse in one liter pots filled with standard peat soil, following a vernalization period of 8 weeks at 6°C. Greenhouse temperature was maintained at 14°C at night and at a maximum of 20°C during daytime. A 16 h photoperiod from natural and supplemental light at 300 µE/m²/s PAR was kept by the use of sodium vapour lamps. Each pot contained one plant and the number of tillers was reduced to two before inoculation. Adult plants were inoculated when the flag leaf was fully expanded, growth stage 40-48 (Zadoks et al. 1974). Seedlings of Victo and Cartago were grown in 7x7 cm pots by sowing six seeds per
pot, reduced to five uniform plants prior to inoculation. Seedlings were grown under the same conditions as adult plants and inoculated when they were two-week-old and the second leaf fully expanded.

Prior to inoculation, adult plants were kept in high light intensity for at least 4 h to favor infection (de Vallavieille-Pope 2002). Spores were taken out of the liquid nitrogen and immediately heat shocked at 40°C for 10 min before use. Inoculum was prepared by mixing 5 mg of urediniospores with 25 mg of talcum powder. Inoculum was applied to the leaves on the adaxial side by gently pressing the edge of a plastic label (thickness app. 1mm) onto the central part of the leaves to form a narrow band of spores across the leaf. This technique allowed precise measurement of latent period and lesion growth rate.

For adult plants, ten pots each with two tillers were used for per treatment. For each tiller both the flag leaf and flag leaf minus one were inoculated. For seedlings of Victo and Cartago ten pots were used per treatment. All plants within a pot were inoculated with the same isolate. After inoculation plants were incubated in a dark dew chamber at 8°C and 100% relative humidity for 24 h to ensure infection. Following incubation adult plants were set in trays and placed at random in the same greenhouse section. Seedling pots were placed similar to adult plants but in a different greenhouse section.

**Sampling and observation**

Assessment of latent period started 8 days after inoculation on both adult plants and seedlings by examining inoculation sites using a 10x hand lens (Eschenbach, Germany). The observations were repeated at 24 h intervals for 17 days. The latent period was defined as the time interval (hours) from inoculation until the first appearance of spores in uredinia breaking the leaf epidermis. Assessment of lesion growth started three days after the latent period for individual leaves. Assessment was done by marking the growing edge of the spore producing area (lesion) with a waterproof tusch (Staedtler, Germany, Art.nr. 318-3). This marking was carried out at a three-day- interval on four consecutive dates. After the final marking all leaves were detached, placed on sheets of blue paper (A4) and digitally scanned using a flatbed scanner.

The image analysis software Fiji (http://fiji.sc/wiki/index.php/Fiji) was used for determination of lesion length and size of the spore producing area for each leaf. Measurement of lesion
length was done using Fiji. The length between consecutive markings was determined as well as the total length of the lesion. Disease areas defined as sporulation area as well as associated chlorotic and necrotic area, were determined by a color threshold using the HSB color space. Separation of the diseased area from the background and healthy parts of the leaf was done for individual leaves by adjustment of hue, saturation and brightness. The threshold method was set to default.

**Statistical analysis**

Field data for percentage of sporulating leaf area were analyzed by a general linear model (GLM) based on log-transformed data to obtain variance homogeneity and normal distribution of data. Variance structure and normal distribution of data were confirmed by plot of model residuals. One model, which was used to analyze data for isolate J99198 (old), included year, host genotype and the combination of these as fixed effect together with block nested in year. Two separate analysis were made, one including results from 2010 and 2012 and another including results for the 6 wheat genotypes, which were included in all three years, 2010, 2011 and 2012. A second model, which was used for analysis of field data from 2012, included isolate, host genotype and the combination of these together with block nested within isolate. Models were constructed using the Proc GLM procedure of SAS statistical software (version 9.2, SAS Institute Inc., Cary, NC, USA). Data obtained from the greenhouse experiment were analysed using a linear mixed model. Isolate, host genotype and leaf were included as fixed effects along with two and three way interactions. The effect of pot, the interaction of pot with fixed effects and the residual effects were treated as random effects. Data for latent period and disease area were log-transformed prior to the analysis and back transformed for presentation. Due to variance inhomogeneity of lesion length data, separate residual variances were recorded for host-pathogen interactions characterized by low, intermediate, and high infection types, respectively. For each variable the possible models were compared using Akaike’s Information Criteria (AIC) (Aikaike 1974) and the model with the lowest AIC value was chosen for final analysis. Test was performed using $F$ test with the denominator according to the theory of mixed models and degrees of freedom were calculated for the denominator using the principles of Kenwood-Rodgers. Model parameters were estimated with residual maximum likelihood based on which least square means were calculated. Comparisons of treatment were based on least square means for each
variable using \( t \) test with no adjustments for multiple comparisons. All analyses were carried out using the MIXED procedure in SAS statistical software (version 9.2, SAS Institute Inc., Cary, NC, USA).

For greenhouse data the relation between variables was studied separately for each isolates using Pearson’s linear correlation coefficients. For this analysis pot means were calculated and used as experimental unit. Comparisons of coefficients between isolates were performed by Fischer’s \( z \) test after Fischer’s \( z \) transformation of correlation coefficients. Analysis was performed using the procedure CORR of SAS statistical software (version 9.2, SAS Institute Inc., Cary, NC, USA).

Principal component analysis (PCA) was done for visualization of the interactions between isolate and host genotype. Pot means were used as experimental unit and data for latent period and disease area were log-transformed. Since latent period could not be recorded for incompatible interactions, input values for these missing data in the PCA were calculated using the equation for the linear relationship between latent period and infection type of treatments for which experimental data for latent period was obtained. The four variables infection type, latent period (log-transformed), lesion length and disease area (log-transformed) were reduced to two principal components (PC1 and PC2) explaining 89.7 % and 5.8% of the variation, respectively. For each treatment, means ± SE of the two components were plotted in a coordinate system with PC1 as the x-axis and PC2 as the y-axis. PCA was done with XLSTAT (XLSTAT version 2012.6.01).
Table 2. Identified seedling and adult plant resistance in the two parental cultivars Camp Rémy and Récital, and the recombinant inbred lines used in this experiment.

<table>
<thead>
<tr>
<th>Host genotype</th>
<th>Identity&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Yr6</th>
<th>Yr7</th>
<th>Yr&lt;sub&gt;sp&lt;/sub&gt;</th>
<th>Q&lt;sub&gt;Yr.inra-2BL.2&lt;/sub&gt;</th>
<th>Q&lt;sub&gt;Yr.inra-2BL.1&lt;/sub&gt;</th>
<th>Q&lt;sub&gt;Yr.inra-2BS&lt;/sub&gt;</th>
<th>Q&lt;sub&gt;Yr.inra-2AL.1&lt;/sub&gt;</th>
<th>Q&lt;sub&gt;Yr.inra-2AL.2&lt;/sub&gt;</th>
<th>Q&lt;sub&gt;Yr.inra-2DS&lt;/sub&gt;</th>
<th>Q&lt;sub&gt;Yr.inra-5BL.1&lt;/sub&gt;</th>
<th>Q&lt;sub&gt;Yr.inra-5BL.2&lt;/sub&gt;</th>
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</thead>
<tbody>
<tr>
<td>Camp Rémy</td>
<td>Parent</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Récal</td>
<td>Parent</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CRR2083</td>
<td>RIL</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<tr>
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<td>+</td>
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<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>CRR2019</td>
<td>RIL</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>CRR2020</td>
<td>RIL</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>CRR2088</td>
<td>RIL</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
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<tr>
<td>CRR2178R</td>
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<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
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<td>NIL</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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<td>+</td>
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</tr>
<tr>
<td>CRR2182R</td>
<td>NIL</td>
<td>-</td>
<td>-</td>
<td>+</td>
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<td>-</td>
<td>-</td>
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<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

<sup>a</sup> + indicates presence and – indicates absence of seedling resistance genes and QTLs. Designation of Q<sub>Yr.inra</sub> QTLs is based on (Mallard et al. 2005) and (Mallard 2005).

<sup>b</sup> RIL=Recombinant Inbreed Line; NIL=Near Isogenic Line (Mallard et al. 2005 and 2008)
Results

Field experiments

Disease severities on Camp Rémy and Récital (parental lines) and the nine RILs were generally consistent in years 2010-2012 when inoculated by the old French isolate (J99198) (Fig 1A). Largest variability was observed for host genotypes showing disease severities between 2 and 40% (intermediate), whereas resistant and susceptible genotypes were highly consistent. Results for all eleven host genotypes assessed in 2010 and 2012 revealed an interaction between year and host genotype (P=0.027), which was mainly due to a low level of disease on line CRR2182S in 2010. For the six host genotypes assessed in all three experimental years (2010, 2011 and 2012) similar results were shown with a weak interaction between year and host genotype (P=0.044).

In contrast, comparisons in 2012 involving the old (J99198) and the new isolate (J11019) revealed highly significant interactions (P<0.001) between host genotype and isolate (Fig 1B). Seven out of the 11 host genotypes had significant shifts in disease severity depending on the isolate. The old isolate induced significantly more disease on Récital and CRR2283 than the new isolate, and the opposite was the case for CRR2020, CRR2088, CRR2182R, CRR2178R, and CRR2083. The lines CRR2178R, CRR2182R and CRR2083, which were highly resistant to the old isolate, were moderately susceptible to the new isolate with disease severities from 25-50% leaf area covered by sporulating lesions.
Figure 1. Means of percentage sporulating leaf area for 9 recombinant inbred wheat lines (RILs) and two parental cultivars, Camp Rémy and Récital, inoculated with *Puccinia striiformis* isolates J99198 (old) and J11019 (new). Assessments were done on the upper three leaves (growth stage DC79) under field conditions. A) Results based on the old isolate in 2010, 2011 and 2012; note that CRR2182R, CRR2083R, CRR2182S, CRR2178S and CRR2283 were absent in 2011. B) Results based on the old and the new isolate in 2012. Stars above bars indicate the level of significance for comparison of isolates within host genotype, * <.01; **<.001, ***<.0001, NS=non-significant at $\alpha=0.05$. 
Greenhouse experiments

Seedling trials
All host genotypes were susceptible to the new isolate at the seedling stage (Table 6). In contrast, Camp Rémy and three of the RILs were resistant to the old isolate, indicating the presence of seedling resistance. Récital and five of the RILs had infection type 5 or above (0-9 scale) when inoculated with PstS2 and only three lines showed full susceptibility (infection type 8-9). The low infection type on CRR2088 suggests the presence of additional seedling resistance in the material.

Spore viability was confirmed on the susceptible controls, Cartago and Victo. In addition, results for latent period verified the aggressiveness of PstS2 which had a significantly shorter latent period than the old isolate on both cultivars (Fig.2). Moreover, a significant interaction between isolate and host genotype (P<.0001) was observed for latent period. The new isolate had a similar short latent period as PstS2 on Victo, but a longer latent period than the two other isolates on Cartago (Fig2). This extended latent period was associated with the emergence of chlorosis, indicating previously undetected resistance in Cartago.

Table 6. Infection types (0-9 scale) on seedlings of the nine RILs and two parental cultivars when inoculated by three isolates of Puccinia striiformis

<table>
<thead>
<tr>
<th>Host genotype</th>
<th>PstS2</th>
<th>Old</th>
<th>New</th>
</tr>
</thead>
<tbody>
<tr>
<td>Camp Rémy</td>
<td>3</td>
<td>2-3</td>
<td>8-9</td>
</tr>
<tr>
<td>Récital</td>
<td>5-7</td>
<td>8-9</td>
<td>8-9</td>
</tr>
<tr>
<td>CRR2083</td>
<td>5</td>
<td>2-3</td>
<td>8-9</td>
</tr>
<tr>
<td>CRR2283</td>
<td>6-7</td>
<td>8-9</td>
<td>8-9</td>
</tr>
<tr>
<td>CRR2019</td>
<td>8-9</td>
<td>8-9</td>
<td>8-9</td>
</tr>
<tr>
<td>CRR2020</td>
<td>8-9</td>
<td>8-9</td>
<td>8-9</td>
</tr>
<tr>
<td>CRR2088</td>
<td>2-3</td>
<td>8-9</td>
<td>8-9</td>
</tr>
<tr>
<td>CRR2178R</td>
<td>3</td>
<td>2-3</td>
<td>8-9</td>
</tr>
<tr>
<td>CRR2178S</td>
<td>8-9</td>
<td>8-9</td>
<td>8-9</td>
</tr>
<tr>
<td>CRR2182R</td>
<td>2-3</td>
<td>2-3</td>
<td>8-9</td>
</tr>
<tr>
<td>CRR2182S</td>
<td>6</td>
<td>8-9</td>
<td>8-9</td>
</tr>
</tbody>
</table>
Figure 2. Least square means of latent period in hours of three isolates of *Puccinia striiformis* on seedlings of Victo and Cartago. Letters indicate significant differences at $\alpha=0.05$ after Tukey –Kramer adjustment for multiple comparisons.

**Adult plant trials**

All combinations of host genotype and isolate, resulting in infection type 5 or above at the seedlings stage, were included in the adult plant trials. Overall, 80% of the inoculations on adult plants were successful and resulted in macroscopic symptoms, i.e., infection type of 1 or above. Line CRR2178S differed from this general trend by an infection efficiency of only 43%. All host genotypes were confirmed resistant to *PstS2*, resulting in infection type 0-1 for all combinations. Reliable assessment of infection type on CRR2178R and CRR2083 was restricted due to the appearance of leaf spots across the entire leaf, and corresponding results are therefore absent (Fig.3A). All five host genotypes susceptible to the old isolate (infection type of 5 or above) were less susceptible to the new isolate (infection types below 5), and the opposite was the case for the six most resistant host genotypes (infection type 3 and below). CRR2083 and CRR2178R remained resistant to the new isolate at the adult plant stage (infection types below 2), Camp Rémy and CRR2182R became moderately susceptible (infection type 4-5) whereas CRR2020 and CRR2088 became susceptible (infection type 6-7). Latent period (LP) was much influenced by both isolate and host genotype with contrasting LPs for the two isolates (Fig.3B). LP of the old isolate was related to the number of QTLs...
previously detected in the host lines (Table 2). LP was shortest for CRR2019 and CRR2178S, with only one identified QTL and longest for CRR2020 with six identified QTLs. No spore bearing pustules were observed on the four host genotypes with identified seedling resistance. For the new isolate, lack of spore production was only observed for CRR2083 and CRR2178R.

Lesion length was assessed every three days after the first appearance of spores on the individual leaf surface. The final lesion length 12 days after the end of the LP differed significantly between the old and the new isolates, on all host genotypes, generally following the pattern of the infection type data (Fig.3C).

The disease areas, which were assessed 25 days after the inoculation reflected the final outcome of the host-pathogen interactions (Fig.3D). The two isolates resulted in significantly different disease areas for 8 of the 11 host genotypes, confirming the huge impact of isolate on the susceptibility of the considered wheat genotypes.

Isolate-host genotype interaction was highly significant for all variables assessed in the greenhouse (Table 7). Only disease area showed an effect of leaf position but the reactions of flag leaf and flag leaf minus-one reaction were dependent on the interaction between isolate and host genotype. Further, high correlation coefficients were found between all variables for both isolates (Table 8) and all correlations were highly significant (P<.0001). However, the correlation between infection type and disease area, and between lesion length and disease area was significantly higher for the old isolate than for the new isolate.
Figure 3. Interactions between three isolates of *Puccinia striiformis* and 9 recombinant inbreed lines and two parental cultivars with different components of seedling and adult plant resistance, measured by infection type (A), latent period (B), lesion length (C) and disease area (D). Note that data for latent period, lesion length and disease area are absent for incompatible interactions: The *PstS2* isolate showed incompatibility with six host genotypes; the old isolate showed incompatibility with Camp Rémy, CRR2178R, CRR2182R, CRR2983R, and the new isolate was incompatible with CRR2178R and CRR2083. Stars above bars indicate the level of significance for comparison of isolates within line/cultivar, * <.01; **<.001, ***<.0001, NS=non significant at α=0.05.

Table 7. Main effects and interaction of *Puccinia striiformis* isolate, host genotype and leaf position on infection type, latent period, lesion length and disease area for greenhouse tests.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>F value</th>
<th>P value</th>
<th>F value</th>
<th>P value</th>
<th>F value</th>
<th>P value</th>
<th>F value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolate</td>
<td>1</td>
<td>0.36</td>
<td>0.5476</td>
<td>45.55</td>
<td>&lt;.0001</td>
<td>22.46</td>
<td>&lt;.0001</td>
<td>7.28</td>
<td>0.0078</td>
</tr>
<tr>
<td>Host</td>
<td>11</td>
<td>378.97</td>
<td>&lt;.0001</td>
<td>50.03</td>
<td>&lt;.0001</td>
<td>17.15</td>
<td>&lt;.0001</td>
<td>11.07</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Isolate*Host</td>
<td>11</td>
<td>265.62</td>
<td>&lt;.0001</td>
<td>51.95</td>
<td>&lt;.0001</td>
<td>77.36</td>
<td>&lt;.0001</td>
<td>27.55</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Leaf</td>
<td>1</td>
<td>0.64</td>
<td>0.4227</td>
<td>2.16</td>
<td>0.1435</td>
<td>2.48</td>
<td>0.1164</td>
<td>12.31</td>
<td>0.0005</td>
</tr>
<tr>
<td>Isolate*Leaf</td>
<td>1</td>
<td>0.92</td>
<td>0.3390</td>
<td>0.19</td>
<td>0.6661</td>
<td>0.01</td>
<td>0.9213</td>
<td>0.18</td>
<td>0.6684</td>
</tr>
<tr>
<td>Host*Leaf</td>
<td>11</td>
<td>6.31</td>
<td>&lt;.0001</td>
<td>4.66</td>
<td>&lt;.0001</td>
<td>3.73</td>
<td>0.0008</td>
<td>5.75</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Isolate<em>Host</em>Leaf</td>
<td>11</td>
<td>3.83</td>
<td>&lt;.0001</td>
<td>0.68</td>
<td>0.6893</td>
<td>2.58</td>
<td>0.0146</td>
<td>4.20</td>
<td>0.0002</td>
</tr>
</tbody>
</table>

* Data were log-transformed before analysis.

Table 8. Pearson’s correlation coefficients between four disease variables measured in the greenhouse for two *Puccinia striiformis* isolates on adult plants of 11 wheat genotypes. The table is divided in two where lower left corner shows correlation coefficients for the old isolate and the upper right corner shows coefficients for the new isolate.

<table>
<thead>
<tr>
<th>Old isolate</th>
<th>New isolate</th>
<th>Infection type</th>
<th>Latent period</th>
<th>Lesion length</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infection type</td>
<td>-</td>
<td>-0.80</td>
<td>0.84</td>
<td>0.62</td>
<td></td>
</tr>
<tr>
<td>Latent period</td>
<td>-0.82</td>
<td>-</td>
<td>-0.74</td>
<td>-0.61</td>
<td></td>
</tr>
<tr>
<td>Lesion length</td>
<td>0.89</td>
<td>-0.67</td>
<td>-</td>
<td>0.69</td>
<td></td>
</tr>
<tr>
<td>Disease</td>
<td>0.79</td>
<td>-0.67</td>
<td>0.90**</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

* Data for latent period and disease area were log-transformed.

* P<0.05, ** P<0.001 for comparison of correlations coefficients for each isolate.
A principal component analysis (PCA), provided a good overall summary of results for the individual epidemiological parameters. The first two components of the PCA accounted for 95.5% of the original variation. The first component explained 89.7% of the variation and the four original variables contributed almost equally to this component (Table 9). Latent period was inversely correlated with the first component. The second component was mainly correlated with latent period and disease area and explained 5.8% of the original variation. The second component primarily explained the variation caused by some isolate-host interactions having a relatively short or long latent period together with relatively low or high level of disease.

Table 9. Eigen vectors of principal component analysis for the four variables infection type, latent period, lesion length and disease area.

<table>
<thead>
<tr>
<th></th>
<th>PC1</th>
<th>PC2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infection type</td>
<td>0.512</td>
<td>-0.215</td>
</tr>
<tr>
<td>Latent period</td>
<td>-0.492</td>
<td>0.654</td>
</tr>
<tr>
<td>Lesion length</td>
<td>0.508</td>
<td>0.173</td>
</tr>
<tr>
<td>Disease area</td>
<td>0.488</td>
<td>0.704</td>
</tr>
<tr>
<td>Percentage of total variation</td>
<td>89.7</td>
<td>5.8</td>
</tr>
<tr>
<td>Eigenvalue</td>
<td>3.587</td>
<td>0.231</td>
</tr>
</tbody>
</table>

Most of the QTLs derived from Camp Rémy became ineffective by the new isolate. CRR2020 and CRR2088, which had the highest level of quantitative resistance to the old isolate, were highly susceptible to the new isolate (Gray circles, Figure 4). In contrast, some host genotypes with none or few identified QTLs were more resistant to the new isolate, i.e., CRR2019 and CRR217S both changed from highly susceptible to moderately susceptible and grouped close to Camp Rémy and CRR2182R, which had changed from resistant to moderately susceptible. Récital and CRR2283, which were highly susceptible to the old isolate, became moderately resistant to the new isolate (broken circles). Two lines (CRR2178R and CRR2083) were more resistant to the new isolate than both Camp Rémy and Récital, indicating the presence of resistance components derived from both parents.

The disease level on individual host genotypes when inoculated with the old isolate corresponded with the number of QTLs previously detected, and the host genotypes with
effective seedling resistance grouped resistant. The seedling resistance thus masked the effect of QTLs in these lines. The two lines CRR2020 and CRR2088, with 6 and 4 QTLs respectively, had the highest level of quantitative resistance to the old isolate. CRR2182S with three QTLs were moderately susceptible whereas the last three lines CRR2019 (one QTL), CRR2178S (one QTL) and CRR2283 (none) showed slightly reduced susceptibility compared to Récital which was found to be highly susceptible to the old isolate. In contrast, the level of resistance to the new isolate did not correspond to the number of previously identified QTLs in individual host genotypes.
Discrepancies between results of field and greenhouse experiments were observed for some host-pathogen combinations, when comparing with respect to disease area in the greenhouse (Fig3D) and percentage sporulating leaf area in the field (Fig1B). Camp Rémy changed from
resistant in the field to moderately susceptible in the greenhouse. The two lines CRR2178R and CRR2083 which were moderately susceptible and moderately resistant to the new isolate in the field, respectively, show full resistance to this isolate in the greenhouse. Further, the disease level for the new isolate was relatively low on Récital, CRR2020, CRR2182S and CRR2178S in the greenhouse compared to the field. On line CRR2019 disease level was relatively high in the greenhouse when inoculated with the old isolate whereas disease level on CRR2178S was relatively low in the greenhouse with this isolate. The near isogenic lines CRR2178R and CRR2178S differed for the QTL $QYr.inra.2BS$ which was overcome by the new isolate. Both greenhouse and field results indicated an effect of $QYr.inra.2BS$ against the new isolate with a higher effect seen in the greenhouse. The other pair of isogenic lines, CRR2182R and CRR2182S, differed for $QYr.inra.2DS$ which were absent in CRR2182S. CRR2182R was more susceptible to the new isolate under both field and greenhouse conditions which indicates that the presence of $QYr.inra.2DS$ lead to higher susceptibility towards the new isolate.

**Discussion**

Much effort has been invested in identifying new sources of resistance to *Puccinia striiformis* (*Pst*), and within recent years several components of adult plant resistance providing high levels of disease control have been identified in European wheat cultivars (e.g. Mallard et al. 2005; Melichar et al. 2008; Dedryver et al. 2009; Jagger et al. 2011). In contrast to effect of seedling genes for resistance to *Pst*, which may break down after few years deployment of the resistant wheat cultivars (e.g Bayles et al. 2000; Hovmøller 2002), adult plant resistance based on several genes with individual minor effects are often considered to maintain an effective disease control longer (Johnson 1984; Singh et al. 2010). This study addressed the impact of exotic incursions of *Pst* on the susceptibility of wheat genotypes diverging with respect to the number of QTLs for resistance expressed mainly at the adult plant growth stages. The results of the present study revealed highly significant interactions between *Pst* isolates of diverse evolutionary origin (‘exotic’) and quantitatively expressed QTLs for resistance to *Pst* derived from the French cultivars Camp Rémy and Récital.

None of the evaluated host genotypes carried seedling resistance effective against the new strain but the $QYr.inra.2BL.2$ present in Camp Rémy, CRR2178R and CRR2182R was
effective against both the old French isolates and the isolate from PstS2. Further, Camp Rémy and CRR2083 carried Yr7 effective against the old isolate. As result the effect of QTL against the old isolate and the isolate from PstS2 were masked by the presence of qualitative resistance in these host genotypes.

At the adult plant growth stages all host genotypes were resistant to the isolate of strain PstS2 (Hovmøller et al. 2008), which has increased aggressiveness on susceptible cultivars compared to isolates common to Europe (Milus et al. 2009). The aggressiveness was confirmed on susceptible seedlings in the present study, where it had a significantly shorter latent period than the isolate representing the previous Pst population in France and NW-Europe in general (de Vallavieille-Pope et al. 2012). However, the PstS2 strain does not carry virulence for any of Yr1, Yr3, Yr4, Yr17, and Yr32, which are common in North West European cultivars (e.g., Hovmoller 2007; Pope et al. 2012). Our study suggest the presence of additional adult plant resistance to this isolate in European wheat cultivars, and together these are likely reasons why this strain only appear in low frequencies in Europe (Hovmøller and Justesen 2007a) despite being widespread in other parts of the world (Wellings 2007; Hovmoller et al 2008; Markell and Milus 2008; Ali et al. 2012 (in prep.)).

Based on three years field experiments, the resistance components derived from Camp Rémy and Récital showed stable interactions with the old isolate of North-west European origin. The effects of the QTLs from Camp Rémy were additive to this isolate, i.e., additional numbers of QTLs leading to higher levels of resistance both under field and greenhouse conditions. Together with other isolates of similar genetic background, this isolate was used in the study by Mallard et al. (2005) where the resistances in Camp Rémy were mapped. The new isolate detected for the first time in France in 2011 (Vallavieille-Pope et al. 2012) produced highly contrasting results compared to isolates of the two other lineages for most host genotypes, both in the field and in the greenhouse. The general picture was that the more complex lines in terms of number and effect of known QTLs against the old isolate had a higher level of disease with the new isolate. On the other hand host genotypes susceptible or moderately susceptible to the old isolate where more resistant to the new isolate. The moderate susceptibility of CRR2020 indicated that all 6 QTLs in this line had been fully or partly overcome by the new isolate. Only CRR2088 with 4 QTLs were more susceptible than CRR2020 and thus these 4 QTLs had no effect to the new isolate.
The existence of at least one major QTL in the susceptible parent Récital, detected by isolates of the new strain, was confirmed under both greenhouse and field conditions. A previous study of resistance in the French cultivar Renan also identified a QTL in Récital, but with very limited effect (Dedryver et al. 2009). Two lines, CRR2178R and CRR2083, were more resistant in the greenhouse to the new isolate than both the parents, Camp Rémy and Récital. This may be explained by components of resistance from both parents. These two lines share \( QYr.inra.5BL1 \) and a hypothesis could be that combining this QTLs with resistance from Récital leads to full resistance under greenhouse conditions. The line CRR2178S which only carry \( QYr.inra.5BL1 \) was only moderately susceptible to the new isolate supporting the hypothesis of an effect of this QTL.

Few discrepancies were seen between results recorded in the field and results recorded in the greenhouse. Most importantly Camp Rémy showed full resistance to the new isolate in the field while being moderately susceptible in the greenhouse. A similar effect was found in a study with leaf (brown) rust by Goyeau and Lannou (2011), where the cultivar Renan was resistant in the field despite being susceptible in the greenhouse (H. Goyeau, personal communication). Furthermore, cultivar Soissons, which has been grown in large areas in France since 1989, is still resistant to stripe rust despite being moderately susceptibility in greenhouse test before registration (anonymous 1989).

The unidentified resistance in Recital appeared to be influenced by environment. Lines grouping with Récital in the principal component analysis all seemed more susceptible in the field than in the greenhouse. In addition, the two lines with full resistance to the new isolate in the greenhouse were moderately susceptible and moderately resistant in the field, respectively. The influence of environment on adult plant resistance has often been reported and several studies reveal an effect of light levels and temperature on the expression of Adult plant resistance (e.g. Ash and Rees 1994; Agenbag et al. 2012). The low disease level of CRR2178S which only carried \( QYr.inra.5BL1 \) was a result of low infection rate in the greenhouse. The OTL \( QYr.inra.5BL1 \) thus appeared to confer al low infection rate which may be alleviated in the field by polycyclic infections. The two pairs of isogenic lines revealed an environmental effect on expression of \( QYr.inra.2DS \) and \( QYr.inra.2BS \). \( QYr.inra.2DS \) seemed to confer elevated susceptibility under greenhouse conditions while \( QYr.inra.2BS \) seemed to enhance resistance to the new isolate under greenhouse conditions. The effect of \( QYr.inra.2BS \) was
supported by the fact that CRR2020 had similar disease level as CRR2088 in the field but lower disease in the greenhouse. Elucidation of the differences between greenhouse and field and of the interaction between sources of resistance from Camp Rémy and Récital will require new genetic studies involving the new pathogen isolate.

Race-specificity for quantitative resistance to leaf rust in barley (Puccinia hordei) has been demonstrated in a study where pathogen isolates were selected to optimize identification of race-specificity (Marcel et al. 2008). In a study by Paillard et al. (2012) a QTL for adult plant resistance was found to be effective only against one out of three isolates. However it is possible that this QTL represent a gene for qualitative resistance as some genes for qualitative resistance are mainly expressed in adult plants (Boyd 2005). Our results are in agreement with the hypothesis that all true (full) resistance genes interact in a gene-for-gene manner of either major or minor effects (Parlevliet and Zadoks 1977). Our results are also supported by the fact that quantitative resistance in cultivars related to Camp Rémy seems to have been partly overcome under field conditions in England (Agenbag et al. 2012). In addition, Dolores Vazquez et al. (2012) reported that QTL identification was heavily dependent on the constitution of pathogen population to which the mapping population was exposed.

Isolates of the new strain used in this study is both genetically and in terms of virulence phenotype different from any isolate previously detected in Europe (Hovmoller et al., unpublished, eurowheat.org). It readily produces telia both in the field and in the greenhouse, and at both seedlings and adult plants (data not shown), which may suggest a sexual origin in the near-past according to Ali et al. (2010). The cultivar Renan (Yr3, Yr17) has been shown to be resistant to isolate of the new strain (C. de Vallavieille-Pope, unpublished data) despite that it carries virulence for both Yr3 and Yr17. Identification of previously undetected resistance using an exotic isolate was also shown by Calonnec et al. (1997), where Yr25 was identified in the three differential lines, Heines VII, Heines Peko and Strubes Dickkopf.

Breeding for durable resistance based on genes for quantitative resistance has been a main focus for CYMMIT breeding station for more than 25 years (Singh et al. 2010) and several studies and field observations have reported stability of quantitative resistance across years and locations when artificially inoculated with a particular isolate or during natural infection (e.g. Broers, 1989, Broers et al. 1996). The results we present here should encourage the identification and maintenance of a diverse array of genes for quantitative resistance to Pst in
wheat germplasm. Our study also suggests cultivars to be tested in the epidemiological environments where use is intended. Using the same cultivar across different regions might lead to unexpected results as they may encounter a genetically different pathogen population. The acclaimed race-nonspecific nature of quantitative resistance is most likely an effect of combining several genes with different effect in the same cultivar which makes it unlikely that the pathogen can accumulate the required virulence within a short timespan (Mallard et al. 2008; Coram et al. 2008). However, the nature of quantitative resistance is still poorly understood (Poland et al. 2008). The high correlation seen between epidemiological traits confirmed the quantitative effect of the evaluated QTLs against the old isolate and probably reflected that all these traits is a measure of the ability of the pathogen to grow on and extract nutrient from a particular host genotype. High correlation was also seen for the new isolate, although slightly lower than for the old isolate, which suggested a quantitative effect of previously unidentified resistance acting on this isolate. This discovery of isolate-specificity (or ‘race specificity’) of quantitatively inherited APR resistance to Pst stress the difficulties in predicting the durability of disease control based on the nature of the inheritance, or the phenotypic expression of resistance to Pst, and the necessity to use pathogen isolates of diverse origin in breeding programs. Further, the increasing knowledge on resistance in European wheat has made it clear that intensive and long-term selection by European cereal breeders have led to accumulation of diverse resistance genes not all identified in commercial cultivars. Epidemics caused by invasive strains in other parts of the world might be a result of a general lower selection for resistance against Pst.

**Acknowledgements**

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References


General discussion and perspectives

The present thesis presents a range of new discoveries on development of haustoria of *Puccinia striiformis* in infected wheat leaves and new insights in the interaction between aggressive/non-aggressive pathogen strains and sources of qualitative and quantitative based resistance in the wheat host.

Haustoria

With a combination of a classical staining technique and advanced 2-photon microscopy it was possible to visualize the structure and formation of haustoria at different time points during the infection process of *P. striiformis* on wheat seedlings. The staining technique used here was adapted from Moldenhauer et al. (2006) but was originally developed by Rohringer et al. (1977) and later modified by Kuck et al. (1981). It has the advantage of giving a deeper staining of fungal material than plant material which makes it easier to differentiate between fungal and plant structure during visualization and processing of images. In opposition to many other staining procedures, it also provides a good staining of fungal structures in the deeper leaf layers.

The main disadvantage of the applied technique relates to the harsh treatments used for fixation and clearing of leaves. In order to keep the leaf structure as intact as possible the protocol from Moldenhauer et al. (2006) was slightly modified to be used for seedling leaves. The modification included a lower concentration of sodium hydroxide (0.05M) and clearing of samples at 80°C. This sometimes resulted in samples that were not suitable for microscopy due to insufficient clearing and staining, but in general it had a positive influence on the integrity of the samples. The method was further modified by taking the specimens through a glycerol series before mounting. Placement of specimens directly into glycerol of high concentration may cause serious shrinkage of tissue structures due to the hydrophilic properties of glycerol. Plant cells in samples treated according to the modified protocol appeared less distorted based on direct assessment by eye.

Most recent microscopic studies of interaction between *P. striiformis* and wheat have involved confocal microscopy. Like 2-photon microscopy, confocal microscopy is a laser based method where a focused spot is scanned across the sample. 2-photon microscopy has several
advantages compared to confocal microscopy (Feijo and Moreno 2004). In relation to the present study the most noticeably advantages includes a deeper penetration of the tissue and a higher resolution, in particular along the z-axis. None of the previous confocal studies have reported a branched appearance of haustoria. Confocal microscopy was also evaluated during this Ph.D project, but it failed to produce consistent and reliable visualization of haustoria in fixed whole leaves. In conclusion, the present thesis have demonstrated that 2-photon microscopy can be a valuable tool to provide further insights in the interactions between *P. striiformis* and its wheat host.

A large number of fluorescent dyes and probes are available that could potentially be used for differential staining of fungal structures and plant structures (Johnson and Spence 2010). A methodology development in this direction was outside of the scope of this Ph.D study. However, based on my current knowledge it seems possible that refinement of the staining methods could give an even more detailed visualization on e.g. how host cells may change in response to accommodation of the developing haustoria. Infection by pathogen has been found to lead to rearrangement of plant cytoskeleton and changes in the arrangement of cell organelles (Schmelzr et al. 2002; Takemoto et al. 2003) but how host cells accommodate haustoria are still poorly understood. The significant secondary growth of haustoria observed in this thesis is likely to impose several constrains and changes on the host cells and indicates that the formation of haustoria as a highly dynamic process. It is truly an exciting relationship where the plant cell accommodates an organ that takes up significant space without disrupting cell function.

The detail report of the structural development of haustoria could represent a framework for doing studies on the function and constitution of haustoria. One example could be in relation to expressed sequences tags (ESTs), which have been identified in isolated haustoria of *Puccinia striiformis* (Yin et al. 2009). If technical possible, sorting of haustoria according to developmental stage before DNA extraction might reveal aspects of the dynamics of gene expression in haustoria. It is for instance likely that many effectors will be produced during the first steps of hauotorium development.
“Cost of virulence” and pathogen aggressiveness

A good understanding of cost of pathogen virulence and aggressiveness can add to our knowledge about which factors that are important for pathogen population dynamics and the durability of host resistance in terms of time and area where different sources of resistance maintain sufficient disease control. Not much is known about cost of virulence in fungal pathogens, in particular for *P. striiformis*, in part due to the lack of appropriate near-isogenic fungal isolates diverging with respect to the virulence considered. Moreover, as discussed in the introduction other factors might by more important for understanding dynamics in virulence gene(s) and pathogen genotypes under agricultural conditions, especially in pathogen populations with a clonal structure as populations of *P. striiformis* in many parts of the world (Hovmøller et al. 2002; Enjalber et al. 2005, Keiper et al. 2004). The results in this thesis showed no fitness cost of the acquisition of virulence to the *Yr2* resistance gene in wheat, based on two wild-type mutant pairs of isolates diverging only for the virulence phenotype corresponding to *Yr2*, but in two different genetic backgrounds. This result support the fact that virulence to *Yr2* is almost fixed in the European population (eurowheat.org) despite that the *Yr2* resistance gene has only been detected in relatively few commercial wheat varieties (e.g., Hovmøller 2007).

The strength of this experiment was the access to unique pairs of wild type and mutant isolates, where a possible effect of genetic background was eliminated within each pair. In many previous studies of cost of virulence, similar pathogen resources have not been available (Pariaud et al. 2009). The importance of genetic background was also evident from this study as isolates between pairs of different genetic background showed variation for aggressiveness. The two pairs of isolates came from closely related lineages (Hovmøller and Justesen 2007b) and results were in accordance with findings for *Puccinia triticina*, where variation for aggressiveness was observed between closely related isolates (Pariaud et al. 2012). It has previously been found that *P. striiformis* isolates of different evolutionary origin can differ significantly for aggressiveness (Milus et al. 2006; Milus et al. 2009). The importance of this has been highlighted by the recent spread of aggressive high-temperature adapted strain causing epidemics on a global scale. The importance of differences in aggressiveness between closely related lineages are less well understood. As suggested by results from e.g. Hovmøller and Justesen (2007b), the same virulence phenotype can arise independently in different lineages.
within or between regions with in a relatively short time span, which is further supported by the fact that the two mutants used in this study had been sampled from field experiments as discussed in manuscript II. In agricultural systems pathogens are often under strong directional selection for virulence and it can be hypothesized that the virulence mutant with the more aggressive background will out compete those less fit. Thus results presented here indicate a potential for selection of components of aggressiveness independently of selection for virulence even in populations with a clonal structure. Such variation for aggressiveness both within and between populations should be considered in relation to large scale use of quantitative resistance. It is possible that variation for aggressiveness represents a potential for adaptation to incomplete quantitative resistance.

**Pathogen interactions with host plants with different levels of quantitative resistance**

The study comprising wheat lines diverging with respect to quantitative resistance illustrated that incursion of exotic pathogen strains can pose a serious threat to large scale deployment of sources of quantitative resistance for control of yellow rust. Two recently emerged and atypical isolates in the North-west European *P. striiformis* population interacted significantly different with sources of qualitative and quantitative resistance derived from two French bread wheat varieties compared to a typical Northwest European isolate.

During the set up and planning of this experiments some general problems related to the study of exotic isolates and quantitative resistance were experienced. Before the final set up of the experiments reported in Manuscript III, using wheat lines from a cross between the varieties Camp Rémy and Récital, other lines and varieties were tried. A number of unforeseen problems hampered these preliminary experiments, e.g., by the presence of previously undetected resistance genes in some of the lines coupled with extensive physiological leaf spot reaction, which were not related to rust infection. Experiments equivalent to several months of work and greenhouse space had to be discarded due to such technical problems. Such complications make both planning and execution of such experiments difficult, but on the other hand they also stress the importance of doing experiments with atypical isolates, as such studies might lead to results that reveal new important aspects of plant-pathogen interactions.
Isolate-specificity of QTLs for quantitative resistance has only been reported in very few plant-pathogen systems (e.g. Marcel et al. 2008). In the present study it was demonstrated that 6 QTLs previously effective against *P. striiformis* isolates of typical Northwest European origin had been fully or partly overcome by a new multivirulent isolate that emerged at multiple sites in Europe in 2011. However, equally important the isolate representing the high-temperature adapted strains (Milus et al. 2009) could not grow on adult plant of any of the evaluated host genotypes. As discussed in manuscript III, this result together with other factors may explain the aggressive *PstS2* strain has not been established in Northwest Europe (Hovmøller et al. 2008).

If new invasions have a clear fitness advantage either due to an appropriate virulence phenotype and/or increased aggressiveness, they may have a high probability of replacing the native population, especially in populations with clonal reproduction. Several examples of such invasions in *P. striiformis* populations have been documented, and in some cases they have had devastating effects by initiating large-scale yellow rust epidemics, either due to regional breakdown of qualitative resistance or due to increased pathogen aggressiveness and climatic adaptation (Hovmøller et al. 2011). In Europe there has been an apparent increase in the frequency by which atypical isolates are recovered (Hovmøller and Justesen 2007a) and this change may be related to an increase in human travel and commerce (Hovmøller et al. 2011). Furthermore, Long distance wind dispersal of yellow rust spores are well documented and have been indirectly shown to have a large influence on regional populations structures and on the emergence of exotic strains (Brown and Hovmøller 2002; Hovmøller et al. 2002). Based on results in this thesis, new incursion of *P. striiformis* should not only be evaluated in relation to occurrence of new virulence but also in relation to the interaction between immigrants and wheat varieties or breeding material with quantitative resistance. In this respect the recent efforts in coordination of regional and global survey systems are highly important. In Europe a centralized reporting system based on results from national surveillance systems has been established through several projects and networks funded by the European commission, e.g., COST817 about population studies of airborne pathogens on cereals, and later initiatives as ENDURE and eurowheat.org (Bayles et al. 1998; Jørgensen et al. 2010). Furthermore the Global rust reference center (GRRC) was launched at Aarhus University in 2008 (Hovmøller et al. 2011). At GRRC rust samples from all over the world are virulence phenotyped under
standardized, quarantine conditions, which insures that results can be compared across countries and regions. GRRC thus serves an essential role in tracking down new virulent and aggressive races and in bringing the information to the relevant communities. Moreover it can provide breeders with isolates of e.g. high diversity in order to evaluate possible sources of resistance. This discovery of isolate-specificity (or ‘race specificity’) of quantitatively inherited resistance stress the difficulties in predicting the durability of disease control based on the nature of the inheritance, or the phenotypic expression of resistance, and the necessity to use pathogen isolates of diverse origin in breeding programs.

References


Appendix

List of public disseminations and other contributions that have not been included in the thesis but were carried out during the Ph.D. period.


