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Supervisor:
Professor Aarne Kurppa
Plant Production Research
MTT Agrifood Research Finland
Jokioinen, Finland

Pre-reviewers:
Associate Professor Lisa Munk
Department of Plant Biology
University of Copenhagen
Copenhagen, Denmark

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Plant Pathology Laboratory
Department of Agricultural Sciences
University of Helsinki, Finland

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Marja Jalli

MTT, Plant Production Research, Jokioinen, FI-31600 Jokioinen
marja.jalli@mtt.fi

Abstract

In Finland, barley, *Hordeum vulgare* L., covers 50 % of the total acreage devoted to cereal cultivation. The most common disease of barley in Finland is net blotch, a foliar disease caused by the ascomycete *Pyrenophora teres* Drechsler. Disease resistance based on plant genes is an environmentally friendly and economical way to manage plant diseases caused by biotic stresses. Development of a disease resistance breeding programme is dependent on knowledge of the pathogen. In addition to information on the epidemiology and virulence of a pathogen, knowledge on how the pathogen evolves and the nature of the risks that might arise in the future are essential issues that need to be taken into account to achieve the final breeding aims.

The main objectives of this study were to establish reliable and efficient testing methods for *Pyrenophora teres* f. *teres* virulence screening, and to understand the role of virulence of *Pyrenophora teres* f. *teres* in Finland from a disease resistance breeding point of view. The virulence of *P. teres* was studied by testing 239 Finnish *P. teres* f. *teres* isolates collected between 1994 – 2007 originating from 19 locations, and 200 *P. teres* progeny isolates originating from artificially produced *P. teres* matings.

According to the results of this study, screening for *P. teres* f. *teres* isolates on barley seedlings under greenhouse conditions is a feasible and cost efficient method to describe the virulence spectrum of the pathogen. However, the environmental conditions of temperature, light and humidity, need to be stable to achieve reliable and comparable results between different studies. Inoculum concentration and the seedling leaf used to gauge virulence had significant effects. Barley grain size, morphological traits of *P. teres* isolates, spore production and growth rate on agar did not affect the expression of virulence. A common barley differential set to characterize the *P. teres* virulence was developed and is recommended to be used globally: c-8755, c-20019, CI 5791, CI 9825, Canadian Lakeshore, Harbin, Prior, Skiff, and Harrington.

The virulence spectrum of Finnish *P. teres* f. *teres* isolates collected in 1994-2007 was constant both within and between the years. The results indicated differences in the pathogen's aggressiveness and in barley genotypes resistance. However, differences in virulence were rarely significant. No virulent reactions were recorded on barley genotypes CI 5791 and CI 9819. Unlike in laboratory conditions, no indications of changes in virulence caused by the sexual reproduction have been observed in Finnish barley fields.

In Finland, durable net blotch resistance has been achieved by introducing resistance from other barley varieties using traditional crossing methods, including wide crossing, and testing the breeding material at early generations at several sites under natural infection pressure. Novel resistance is available, which is recommended to minimize the risk of selection of virulent isolates and breakdown of currently deployed resistance. Barley genotypes c-8755, CI 9825 and CI 5791 are potential resistance sources to be used in Finnish barley. They differed in their reaction to the *P. teres* f. *teres* isolates collected globally and all of them showed excellent resistance to Finnish *P. teres* f. *teres* isolates.

Keywords:
Hordeum vulgare, barley, Drechslera teres, net blotch, net form of net blotch, virulence testing, differential set, sexual reproduction, evolution, tillage method, disease resistance

Suomalaisten *Pyrenophora teres* f. *teres* - isolaattien virulenssi ja sen huomioiminen taudinkestävyysjalostuksessa

Marja Jalli

MTT, Kasvintuotannon tutkimus, 31600 Jokioinen
marja.jalli@mtt.fi

Tiivistelmä

Puolella Suomen viljanviljelyalasta kasvaa ohraa. Yleisin ohran kasvitauti on lehtiä vioittava verkkolaikku, jonka aiheuttaja on kotelosieniin kuuluva *Pyrenophora teres* Drechsler. Taudinkestävyys on ympäristöystävällinen ja taloudellinen keino hallita pieneliöiden aiheuttamia kasvitauteja. Taudinaiheuttajan tuntemus on taudinkestävyysjalostuksen perusta. Taudinaiheuttajan yleisyyden lisäksi on huomioitava taudinaiheuttajan virulenssi (taudinaiheuttamiskyky) ja sen muuntelun todennäköisyys. Taudinaiheuttajan kyky muuntua tuo mukanaan riskejä, joiden ennakoiminen on oleellinen osa onnistunutta taudinkestävyysjalostusta.

Tutkimuksen tavoitteena oli kehittää luotettavat ja tehokkaat menetelmät *Pyrenophora teres* f. *teres* – virulenssin testaamiseksi ja selvittää, mikä on suomalaisten *P. teres* f. *teres* –isolaattien virulenssi ja miten se huomioidaan taudinkestävyysjalostusohjelmassa. Aineisto koostui vuosina 1994–2007 Suomesta 19 paikkakunnalta kerätyistä 239 *P. teres* f. *teres* –isolaatista sekä 200 laboratoriossa tuotetusta suvullisten *P. teres* –risteytysten jälkeläisisolaatista.

Tutkimus osoitti, että kasvihuoneessa toteutettava virulenssitesti on luotettava ja taloudellinen menetelmä *P. teres* f. *teres* -sienen virulenssin kuvaamiseksi. Ympäristöolosuhteiden, lämpötilan, valon ja ilmankosteuden tulee olla testeissä vakioidut, jotta eri testien tulokset ovat keskenään vertailukelpoiset. Tartukkeen itiöpitoisuus sekä havainnoitavan ohralehden ikä vaikuttivat merkittävästi virulenssin ilmenemiseen. Sitä vastoin ohran siemenen koko, *P. teres* -isolaattien morfologiset ominaisuudet, itiöntuottokyky tai kasvunopeus agar-maljalla eivät vaikuttaneet virulenssiin. Tutkimuksessa määritettiin ohragenotyypit, joiden reaktiot ilmentävät *P. teres* -sienen virulenssia eri maissa ja joita suositellaan käytettäväksi kansainvälisesti. Virulenssia tehokkaasti mittaaviksi ohragenotyypeiksi osoittautuivat: c-8755, c-20019, CI 5791, CI 9825, Canadian Lakeshore, Harbin, Prior, Skiff, ja Harrington.

Suomalaisten *P. teres* f. *teres* -isolaattien virulenssi on pysynyt muuttumattomana vuosien 1994 ja 2007 välillä. Tulokset osoittivat eroja taudinaiheuttajan aggressiivisuudessa mutta erot isolaattien virulenssissa olivat harvoin merkittäviä. Yksikään testatuista *P. teres* f. *teres* -isolaateista ei ollut virulentti ohragenotyypeillä CI 5791 ja CI 9819. Toisin kuin laboratoriotesteissä, Suomen ohrapelloilla ei havaittu suvullisesta lisääntymisestä johtuvaa virulenssin muuntelua.

Tutkimustulokset osoittivat, että suomalaisissa ohralajikkeissa esiintyy pitkäkestoista verkkolaikunkestävyyttä. Tämä on saavutettu laajoilla perinteisiin menetel-

miin perustuvilla risteytyksillä, sekä testaamalla jalostusaineistoa jo ensimmäisten sukupolvien aikana eri ympäristöissä luonnon tartunnalle altistettuna. Tutkimuksessa valikoitui jalostustyössä aiemmin käyttämätöntä, hyvin verkkolaikun kestävää ohramateriaalia. Laaja-alaisuudeltaan ja tehokkuudeltaan poikkeavien taudinkestävyyslähteiden käyttö on suositeltavaa, jotta *P. teres* -isolaattien muuntumista sekä tästä aiheutuvaa taudinkestävyyden murtumisen riskiä voidaan hillitä. Ohragenotyypit c-8755, CI 9825 ja CI 5791 ovat suositeltavia uusia verkkolaikun kestävyyslähteitä käytettäväksi suomalaisessa ohranjalostuksessa. Näillä on toisistaan poikkeava yhdysvaikutus eri *P. teres* f. *teres* -isolaattien kanssa, ja kaikki genotyypit ovat erityisen kestäviä suomalaisia *P. teres* f. *teres* -isolaatteja vastaan.

Asiasanat:
Hordeum vulgare, ohra, Drechslera teres, verkkolaikku, verkkolaikun verkkotyyppi, virulenssin testaus, differentiaalisetti, suvullinen lisääntyminen, evoluutio, muokkausmenetelmä, taudinkestävyys

List of original publications

This thesis is based on the following original articles, which are referred to in the text by their Roman numerals (I-IV).

I Robinson, J. and Jalli, M. 1996. Diversity among Finnish net blotch isolates and resistance in barley. Euphytica 92: 81-87.

II Jalli, M. and Robinson, J. 2000. Stable resistance in barley to *Pyrenophora teres* f. *teres* isolates from the Nordic-Baltic region after increase on standard host genotypes. Euphytica 113: 71-77.

III Afanasenko, O. S., Jalli, M., Pinnschmidt, H.O., Filatova, O and Platz, G.J. 2009. Development of an international standard set of barley differential genotypes for *Pyrenophora teres* f. *teres*. Plant Pathology 58: 665–676.

IV Jalli, M. Sexual reproduction and soil tillage effects on virulence of *Pyrenophora teres* in Finland. (Manuscript, submitted).

Contents

1 Introduction

In 2007, barley, *Hordeum vulgare* L., was the fourth most important cereal crop both in terms of quantity produced and global area of cultivation (FAOSTAT 2009). In Finland, barley covered 50 % (601 000 ha) of the total acreage used for cultivation of cereals in 2009 (Matilda 2009). Plant pathogens can cause an average loss of 20 % of the average annual value of the barley crop (Murray and Brennan 2010).

The most common disease of barley in Finland is net blotch, a foliar disease caused by the ascomycete *Pyrenophora teres* Drechsler. The imperfect state of *Pyrenophora teres* is *Drechslera teres* (Sacc.) Shoemaker (syn. *Helminthosporium teres* Sacc.). Two forms of the pathogen exist: *Pyrenophora teres* Drechs. f. *teres* Smedeg., the causal agent of the net form of net blotch, and *Pyrenophora teres* f. *maculata* Smedeg., the causal agent of the spot form of net blotch. These two forms are morphologically indistinguishable in culture media but they produce different types of symptoms on barley leaves (Smedegård-Petersen 1971). Rau et al. (2007) studied the mating type genes in net and spot form isolates and found a long genetic isolation between the two forms.

In 1970 and 1971, net blotch was found in over 50 % (Mäkelä 1972) and in 1971-1973 nearly 60 % of the Finnish spring barley fields, and in nearly 90 % of the localities studied (Mäkelä 1975). In 1985, 84 % of the 234 inspected Finnish barley fields had net blotch symptoms as observed on the two uppermost leaves of plants (Avikainen and Isotupa 1986). The latest results from 2006-2007 showed that net blotch exists in 60 % of Finnish barley fields (Manninen et al. 2009).

1.1 Role of virulence in host-pathogen interactions

Infectious plant diseases result from an interaction between at least two organisms, a host plant and a pathogen. The outcome in each host-pathogen combination is determined by the genetic constitution of the host and the pathogen. Host plants vary in resistance and tolerance while pathogens are variable in their ability to infect a host (Barrett et al. 2009). Plant-pathogen interactions are due to continuous long-term co-evolution of plants and the microbes. Plants have evolved systems to recognize pathogens while pathogens have evolved effectors to turn off plant defences (Stukenbrock and McDonald 2009).

According to Ebert and Hamilton (1996), the impact that parasites have on the evolution and ecology of their hosts depends on their virulence, which is a product of the host-parasite interaction. Pathogens produce compounds termed pathogen associated molecular patterns (PAMPs) that are molecules unique to pathogens and conserved across many pathogen species (Navarro et al. 2004). PAMPs have a key role in the basal defence system of the host. Pathogenic species have developed specialized strategies to overcome the plant basal defence (Abramovitch and Martin 2004). Production of enzymes, toxins, and effector proteins can promote pathogen virulence by interacting with the host. Effectors do not usually have a function outside of the host. Pathogens may deliver various effectors by different mechanisms (Bent and Mackey 2007). Several of the fungal effectors are cloned and most of them are small proteins of unknown function (Chisholm et al. 2006). However, with novel and sophisticated biotechnological tools the function of most fungal effec-

tors can be investigated experimentally and it is expected that their role in the prevention of PAMPs triggered immunity is significant (De Wit et al. 2009).

In a gene-for-gene model, the term virulence is used for the pathogen genotype that is able to overcome a resistance factor (Sacristán and García-Arenal 2008). Gene-for-gene interaction was first described by Flor (1955) based on investigations on the host-parasite interaction between flax, *Linum usitatissimum* L., and rust, *Melampsora lini* var. *lini* (Ehrenb.) Lév. Flor's studies led to the gene-for-gene hypothesis, which applies to cases in which resistance is controlled by monogenic, dominant or semi-dominant resistance genes. Resistance response results when plant resistance proteins (R proteins) recognize corresponding proteins of the pathogen, named avirulence (Avr) factors. When a pathogen carrying an *Avr* gene attacks the host carrying the corresponding *R* gene, the *Avr* gene product can act as an elicitor, or it can direct the synthesis or modify a metabolite or protein that is a race-specific elicitor that can be recognized by a receptor of a resistant plant (Figure 1).

Contrary to non-specific resistance induced by PAMPs, the interaction between the products of *R* and *Avr* genes specify resistance between a particular host and a pathogen (Stukenbrock and McDonald 2009). The gene-for-gene model is commonly connected to biotrophic fungal pathogens as a result of long-term co-evolution (De Wit et al. 2009). However, several necrotrophic pathogens have been reported to produce proteins that may function as effectors in inducing necrosis in plants following a gene-for-gene model (Figure 1). Host specific toxins (HST) are defined as pathogen effectors that are critical for the virulence in necrotrophic pathogens. They facilitate the disease infection, for example, in species of *Alternaria*, *Cochliobolus* and *Pyrenophora* (Friesen et al. 2008).

Progress towards understanding the molecular basis of the gene-for-gene interaction has been made and numerous novel *Avr* genes and *R* genes have been identified (Bent and MacKey 2007; De Wit et al. 2009). The role of avirulence determinants has been questioned in pathogen populations: why should a microbe keep a mol-

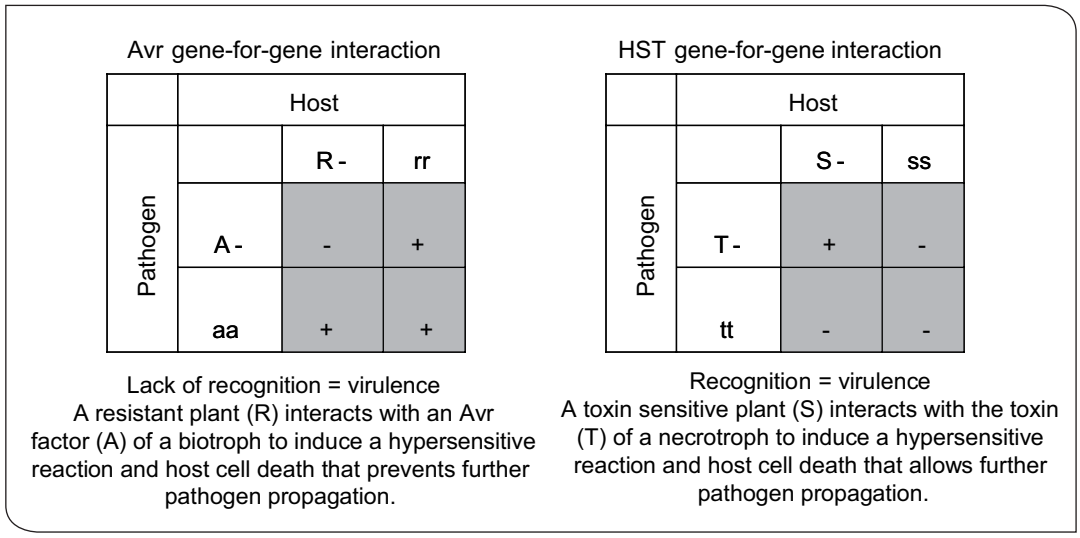

Figure 1. Genetic basis for gene-for-gene interactions based on avirulence proteins and host specific toxins (HST). Adapted from Stukenbrock and McDonald (2009).

ecule that allows it to be recognized? *Avr* genes may perform some essential functions for pathogen survival and must be maintained (Skamnioti and Ridout 2005). In a plant-pathogen system where evolution of a pathogen is directed towards avoiding recognition and induction of resistance, increasing evidence implicates *Avr* genes in roles other than race-specificity (Leach and White 1996). In some cases the products of diverse *Avr* genes are not only for *R*-gene recognition but have a functional role by encoding effectors that facilitate virulence (De Wit et al. 2009). In susceptible hosts *Avr* genes may enhance the pathogen infection (Stukenbrock and McDonald 2009).

Different *Avr* genes do not share common features (Sacristán and García-Arenal 2008). A number of avirulence genes have a measurable fitness function, and mutation of these genes might impose a cost on virulence. Huang et al. (2006) studied near-isogenic isolates of *Leptosphaeria maculans* (Desm.) Ces. & De Not. (phoma stem canker) differing at the *AvrLm4* avirulence locus in oilseed rape, *Brassica napus* L., cultivars that were lacking the resistance gene *Rlm4*, which corresponds to *AvrLm4*. They determined that the isolates without the *Avr* gene incurred a fitness cost compared with *AvrLm4* isolates. In contrast, Schürch et al. (2004) showed how deletion of an avirulence gene may allow pathogens to escape recognition by the corresponding resistance gene in the host. Virulence of a barley pathogen *Rhynchosporium secalis* (Oudem.) Davis isolate to a barley genotype carrying the *Rs1* resistance gene was achieved through deletion and point mutations of the *Avr* gene *NIP1* that produces a phytotoxic peptide which acts as an elicitor of the defence response. Plant pathogens have evolved *Avr* determinants in a complex environment. Although *Avr* genes function as effectors to promote virulence, not all have obvious function in pathogen-host interaction (Leach et al. 2001).

Plant pathogens are difficult to control because their populations are variable over time, space and genotype. Pathogen effector genes, including Avr elicitors and host specific toxins, are diverse and local selection pressure plays an important role in their evolution (Stukenbrock and McDonald 2009). Barrett et al. (2009) summarized that environmental heterogeneity increases the potential for variation in host-pathogen interactions. They also stated that host range and traits associated with fitness vary, making broad ecological and evolutionary predictions difficult. Selection on the pathogen population favours escape of host recognition and the pathogen is pressured to keep updating its virulence factors (Does and Rep 2007). Thrall and Burdon (2003) investigated the *Linum usitatissimum* - *Melampsora lini* interaction and demonstrated that virulent pathogens occurred more frequently in highly resistant host populations and avirulent pathogens in susceptible populations. Gene loss and mutation in a pathogen population may be compensated for by gene gain through horizontal gene transfer (HGT) (Van der Does and Rep 2007). New combinations of *Avr* genes generated by HGT may evolve in unpredictable situations (Skamnioti and Ridout 2005). Friesen et al. (2006) have provided strong evidence on HGT, where a gene encoding a virulence factor (*ToxA*) was transferred from a wheat, *Triticum aestivum* L., pathogen, *Stagonospora nodorum* (Berk.) E. Castell. & Germano, to another wheat pathogen, *Pyrenophora tritici-repentis* (Died.) Shoemaker.

The term virulence has a confused history and various usages for the term exist (Sacristán and García-Arenal 2008). In the current study I use the term virulence as it is defined by Sacristán and García-Arenal (2008): virulence is the degree of damage caused to a host by parasite infection. More specifically, in gene-for-gene interactions virulence is used for the pathogen genotype that is able to overcome a resistance factor.

1.2 Plant disease resistance

The majority of plants possess natural defence that enables them to avoid or resist infection by the majority of plant pathogens present in their environment. Recognition of pathogen results in a massive reprogramming of the plant cell to activate and deploy defence responses to interrupt pathogen growth (Bolton 2009). Plants have evolved two lines of defence. The first line is based on recognition of PAMPs, which provides basal defence against all pathogens. The second line is based on pathogens' effector recognition by plant R (resistance) proteins. Jones and Dangl (2006) introduced a four phased 'zigzag' model to illustrate the quantitative output of the plant innate immune system. In phase 1, PAMPs are recognized by pattern recognition receptors (PRRs) resulting in immunity that can halt further colonization. In phase 2, successful pathogens deploy effectors that contribute to pathogen virulence. Effectors can interfere with PAMP-triggered immunity (PTI) which results in effector-triggered susceptibility (ETS). In phase 3, a given effector is recognized by proteins resulting in effector-triggered immunity (ETI). ETI results in disease resistance and usually, a hypersensitive cell death response. In phase 4, natural selection drives pathogens to avoid ETI. Natural selection results in new *R* specificities and the ETI can be triggered again (Figure 2).

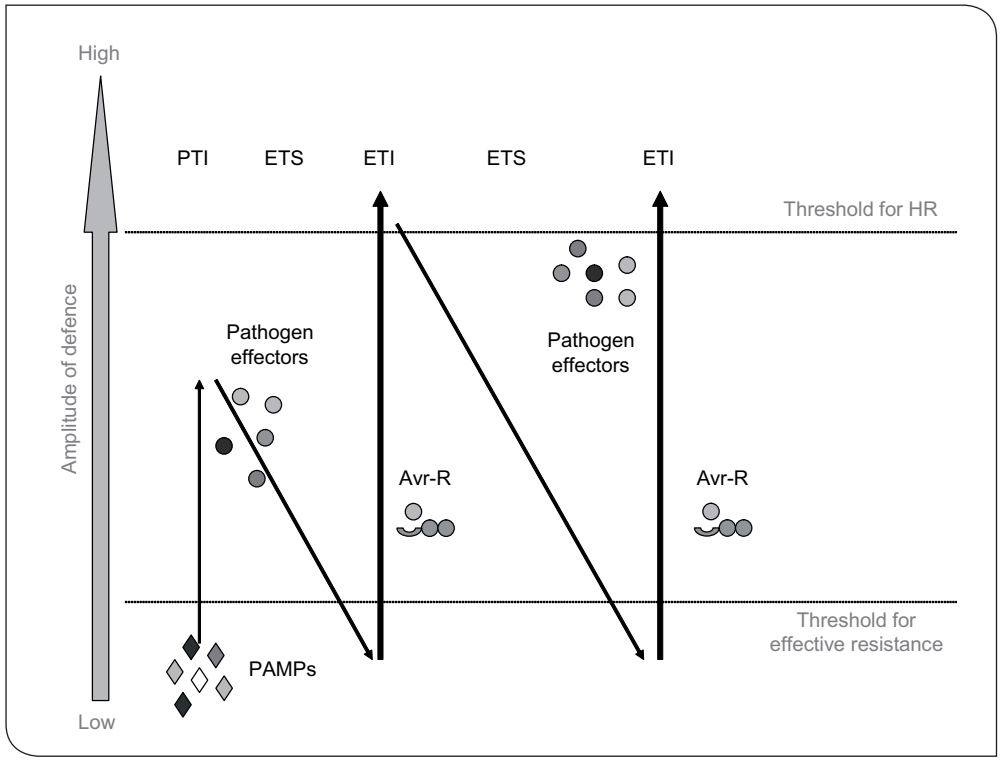

Figure 2. A zigzag model that illustrates the quantitative output of the plant immune system. PAMP: pathogen-associated molecular pattern. PTI: PAMP-triggered immunity. ETS: effector-triggered susceptibility. ETI: effector-triggered immunity. Adapted from Jones and Dangl (2006).

Plants are resistant to certain pathogens either because they belong to taxonomic groups that are outside the host range of these pathogens, or because they possess genes for resistance directed against the avirulence gene of the pathogen, or because they tolerate or escape infection by these pathogens. Van der Plank (1968) divided disease resistance into two types: vertical (race-specific) and horizontal (non-specific). According to Stuthman et al. (2007) Van der Plank's terms were more epidemiological than genetic. Vertical resistance reduces initial inoculum by screening out avirulent races but has no effect on the rate of increase of the virulent races. Horizontal resistance is at least partially effective against all races.

R-genes are important in many systems. They typically provide high levels of resistance and are easy to manipulate, but their utility varies among pathosystems. Several *R*-genes have been isolated and their functions have been studied in detail. The limitations of *R*-genes in crop protection are a lack of durability in some systems and a lack of availability in others (Poland et al. 2008). For highly specialized pathogens with narrow host ranges, monogenic race-specific resistance has generally been unreliable (Stuthman et al. 2007).

Resistance breakdown is considered to be less of a problem with multiple gene based quantitative (horizontal) disease resistance, which leads to lower selection pressure on the pathogen (Poland et al. 2008). Palloix et al. (2009) demonstrated that the frequency of breakdown of major gene resistance against Potato Virus Y was high when introgressed into a susceptible background and no breakdown occurred when the same gene was introgressed into a partially resistant genetic background. Even though quantitative disease resistance is generally considered being non-race-specific, race-specific quantitative disease resistances have also been identified. However, the molecular mechanism of the quantitative disease resistance is still poorly understood (Poland et al. 2008).

1.3 Breeding for disease resistance

Breeding crops for disease and pest resistance is one method used to protect them from damage due to biotic factors. Inherited resistance is valuable because it is easy for the grower and reduces the need for other methods of control. The breeder has to choose weather to select for resistance, or against high susceptibility. Surveys of disease incidence are useful in indicating the importance of diseases providing information for breeders on priorities (Johnson and Jellis 1992). Stuthman et al. (2007) identified three aspects of disease resistance relating to practical breeding strategies: inheritance, effectiveness and specificity of disease resistance.

Special and increased interest in disease resistance breeding has emphasised durable resistance that is cost effective, environmentally sound, and promotes the conservation of genetic resources. Durable resistance can be defined as the adequacy of the resistance throughout the useful lifetime expected from a variety. Keeping ahead of the changing pathogen populations is a continuous challenge for plant breeders. The capacity to predict the durability of resistance genes would be desirable when making investments in plant breeding (Leach et al. 2001).

The durability of disease resistance is highly influenced by the recognition method of R protein, by the capability of the pathogen to retain virulence after altering or eliminating the recognized effector, and by the ability of a pathogen to evolve. Using *R* genes in rotation, with monitoring the current races of pathogen, using *R* genes only when needed, removing them from use before they become widely ineffective, keeping more than one effective *R* gene present in plant, combining use of fungicides and

R genes, and coordinating the system carefully among pathologists, plant breeders, and growers, are examples of how to enhance the durability of genetic resistance (Bent and Mackey 2007). The challenge and main goal in plant breeding is to optimize the plant genotype by choosing the most promising resistance genes and gene combinations for durable disease resistance (Palloix et al. 2009).

Although disease resistance is a valuable tool to be used in plant breeding, the active defence processes in plants occur at the expense of the energy resources and may be a limiting factor in plant growth and yield. The use of energy for defence responses relies on several metabolic pathways (Bolton 2009). If resistance is costly and has a negative effect on yield, a breeder's most effective strategy may not be to select for excellent resistance but to select for moderate resistance and eliminate very susceptible lines. The gene content, instead of comprising single genes, determines a cultivar's value to end-users (Brown 2002).

1.4 *Pyrenophora teres* as a pathogen

The life cycle of *P. teres* f. *teres* involves both an asexual and a sexual stage. The asexual lifecycle includes over-wintering of the pathogen as mycelium in seed or in crop debris. The sexual fruiting bodies, pseudothecia, are formed on barley straw after harvest in autumn. Asci and ascospores are formed during the late autumn and the following spring and summer, depending on the climatic conditions (Smedegård-Petersen 1972, Shipton et al. 1973). Infection of barley seedlings is caused by *D. teres* conidia or *P. teres* ascospores, and is more efficient at cool temperatures (10-15 °C). The role of ascospores in the life cycle of *P. teres* is considered more as a source of novel variation than as an important source of primary inoculum (Shipton et al. 1973). The most severe damage caused by seed-borne infection of *P. teres* f. *teres* occurs in dry soils at temperatures of 12 °C (Youcef-Benkada et al. 1994). Infected volunteer crop species may also serve as sources of primary inoculum. Brown et al. (1993) demonstrated the successful net form net blotch infection on 65 gramineous species in California. One of the volunteer crop species for net blotch is couch grass, *Elymus repens* L. Gould, a common weed of cereal fields that exists in 66 % of the spring cereal fields in Finland (Salonen et al. 2001). Besides barley, Finnish *P. teres* f. *teres* isolates have shown pathogenicity on spring and winter wheat, winter rye and oats (Mäkelä 1972).

During the growing season, *D. teres* conidia produced on the surface of primary lesions caused by ascospores or conidia serve as secondary inoculum. Sporulation occurs when relative humidity is near 100 % for 10 – 30 hr and the temperature remains between 15 and 25 °C. Isolates of *P. teres* from areas where barley is grown over mild winter periods have lower temperature requirements for sporulation and infection (Shipton et al. 1973). In a successful penetration, a primary vesicle develops within the epidermal cell, followed by the formation of a secondary vesicle. Subsequently, infection hyphae grow from the secondary vesicle to penetrate the inner cell wall of the epidermal cell and the apoplastic space of the mesophyll. No hyphae penetrate the mesophyll cells during the early stages of infection. Cells within the developing lesions exhibit various degrees of disruption (Keon and Hargreaves 1983).

In infected barley tissue, *P. teres* induces long dark brown netted necrotic lesions (the net form) or dark brown circular or elliptical spots (the spot form) surrounded by chlorosis (Smedegård-Petersen 1971). Smedegård-Petersen (1977) isolated two toxins, toxin A and toxin B, from *P. teres* cultures that produced necrosis, chlorosis and water-soaking on barley leaves. A third aspergillomarasmine-derived toxin, toxin C, was later identified by Bach et

al. (1979). Weiergang et al. (2002) found a positive correlation between the reactions on detached barley leaves caused by the toxins A B, and C of both net and spot form *P. teres* strain cultures. Toxins A, B and C induce water soaking, chlorosis, and general necrosis but not well-defined necrotic net or spot lesions. Sarpeleh et al. (2007) isolated phytotoxic low molecular weight compounds and proteinaceous metabolites both from *P. teres* f. *teres* and *P. teres* f. *maculata* which were responsible for symptoms on susceptible barley cultivar. Later Sarpeleh et al. (2008) found that the activity of toxins on barley is both temperature and light dependant. Toxins were only active in the presence of light and no necrotic lesions were induced when treated plants were kept for 168 h at 4 °C.

The severity of net blotch is related to the climatic conditions, to the cultivation practice and to the susceptibility of cultivars used. The seed borne *P. teres* infection reduces leaf area, plant height and total mass compared to healthy plants (Deadman and Cooke 1988). The net blotch infection of the uppermost three leaves of a barley plant has the main effect on yield, which may be reduced by 30 %. However, the effect of the disease on barley yield is more than the pure effect of the destroyed leaf area (Jebbouj and Yousfi 2009). Kernel weight and size are the yield components most affected by *P. teres* f. *teres* infection (Steffenson 1991).

The studies on avirulence genes in *P. teres* f. *teres* provide information on the role of virulence in the *P. teres* – barley interaction. Weiland et al. (1999) reported that a single major gene controls virulence in *P. teres* f. *teres* on 'Harbin' barley. They found five random amplified polymorphic DNA (RAPD) markers associated with the low virulence on Harbin and designated the trait *avrHar* denoting avirulence to 'Harbin'. Lai et al. (2007) constructed an AFLP (amplified fragment length polymorphism) based genetic linkage map and identified three major genes in *P. teres* f. *teres* involved in avirulence/virulence of the fungus: *AvrHar* conferring avirulence to Tifang and Canadian Lake Shore, and two epistatic genes *AvrPra1* and *AvrPra2* conferring avirulence to Prato. Beattie et al. (2007) identified six AFLP markers linked to a *P. teres* f. *teres* avirulence gene *AvrHeartland*. Despite the identification of the *P. teres* f. *teres* avirulence genes their function in the virulence of *P. teres* is still unclear.

1.5 Net blotch resistance in Finnish barley

Studies on barley net blotch resistance were already conducted in 1948 in California when resistance of 4 526 barley varieties was tested in the field (Schaller and Wiebe 1952). Seventy-five of the tested varieties were highly resistant to net blotch and most of the resistant varieties originated from Manchuria. Later, Buchannon and McDonald (1965) studied the reaction of *P. teres* infection on 6 174 barley genotypes in Canada. Forty varieties, seventeen originating from Ethiopia, showed good resistance to a wide range of *P. teres* isolates. Already then it was concluded that *P. teres* isolates from different regions, and even within the same region, may differ in pathogenicity and the results of the disease screening tests are highly dependent on the methodology and isolates used. Later on, several experiments on screening for *P. teres* resistance have been conducted and a considerable amount of valuable information is available (e.g. Steffenson and Webster 1992b; Robinson and Jalli 1996; Jørgensen et al. 2000; Gupta et al. 2003; Bonman et al. 2005).

Research on the net blotch resistance genes indicates a complex host-pathogen interaction controlled by both quantitative and major resistance genes. The reports on the presence of qualitative net blotch resistance suggest the potential existence of a gene-for-gene interaction in the *P. teres* - barley pathosystem (Afansenko et al. 2007). Stef-

fenson et al. (1996) identified three QTL (quantitative trait loci) for seedling resistance and seven QTL for adult plant resistance. Also several other studies on the genetic background on the net blotch resistance show that the resistance is controlled by a single or several genes depending on source of resistance, plant development stage and *P. teres* isolate used (Manninen et al. 2000; Cakir et al. 2003; Manninen et al. 2006; Grewal et al. 2008). When further studying barley net blotch resistance, molecular marker technology provides potential tools both for mapping disease resistance genes and for genotypic selection and may promote considerable resource savings (Grewal et al. 2008).

The Finnish barley breeding programme is working systematically on improving barley net blotch resistance. Special input has been targeted to reduce the susceptibility of six-rowed barley cultivars by selection of crossing parents that inherit high levels of quantitative resistance, which mainly originates from two-rowed North American barley genotypes. The programme relies on a multi-locational testing system where the material is tested already in early generations under a high disease pressure (Nissilä 2009, personal communication).

The level of net blotch infection in barley varieties has been monitored in the official variety trials in Finland since 1989. The main purpose of the official variety trials is to assess the value of new genotypes and make recommendations on yield and quality parameters. Plant disease resistance is an increasingly important factor in the cultivation value of cereal varieties (Kangas et al. 2008). Robinson and Jalli (1997a) studied the severity of net blotch in Finnish official barley variety trials over six years and five sites. Net blotch occurred at all five sites. The disease severity differed between years but was similar across sites. The data indicated that differences in resistance to net blotch existed among the 19 genotypes studied.

The disease pressure by net blotch in Finnish official trials is based on natural infection whose average level varied between 5 and 12 % over all tested cultivars and breeding lines in 2000-2008. The data were analyzed using linear mixed models and the estimated means for the different varieties are comparable despite the different trial period. The general level of the resistance against barley net blotch in the barley material tested in the Finnish official variety trials has improved. When comparing the barley genotypes to the standard cultivar Scarlett, a malting barley originating from Germany, 55 % of the tested barley genotypes were more susceptible in 2002, and only 21 % in 2008. In neither of the years were any of the genotypes significantly more resistant than Scarlett (Kangas et al. 2002, Kangas et al. 2008). In 2001-2008, the average net blotch infection level in Scarlett was 1.5 %. Thirteen of the 16 Finnish barley cultivars that are on the official variety list in Finland in 2009 express resistance comparable to Scarlett. Nine of these cultivars are six-rowed barleys (Table 1). None of the cultivars is known to carry any specific resistance genes against net blotch.

Currently, plant breeding is business that has to respond rapidly to end-users' needs. At the same time it represents a long term project that has to look to the future. Building up a disease resistance breeding programme relies on knowledge of the pathogen. Besides information on the epidemiology of the pathogen, knowledge on how the pathogen evolves and which risks might arise in the future are key points that need to be taken into account to achieve the final breeding goals.

MTT Agrifood Research Finland, in close cooperation with the Finnish breeding company Boreal Plant Breeding Ltd., has concentrated on improving barley net blotch resistance in several research projects (Figure 3). The research has concentrated on understanding the function of the

Table 1. The net blotch infection % of the Finnish barley cultivars in the Finnish Official Variety Trials in 2001-2008 compared to the standard cultivar Scarlett (Kangas et al. 2008).

Variety	Head type	Net blotch infection % in Finnish Official Variety Trials 2001-2008	Breeder	Year of Approval on National List of Plant Varieties
Harbinger	two-rowed	0.6	Boreal Plant Breeding Ltd	2009
Elmeri	six-rowed	0.7	Boreal Plant Breeding Ltd	2009
Edvin	six-rowed	0.7	Boreal Plant Breeding Ltd	2008
Eerik	six-rowed	0.8	Boreal Plant Breeding Ltd	2009
Minttu	two-rowed	1.4	Boreal Plant Breeding Ltd	2005
Scarlett	two-rowed	1.5	Saatzucht Josef Breun GmbH & Co	1998
Einar	six-rowed	1.6	Boreal Plant Breeding Ltd	2008
Olavi	six-rowed	2.2	Boreal Plant Breeding Ltd	2006
Saana	two-rowed	2.4	Boreal Plant Breeding Ltd	1996
Jyvä	six-rowed	2.4	Boreal Plant Breeding Ltd	2000
Kunnari	six-rowed	2.5	Boreal Plant Breeding Ltd	2001
Erkki	six-rowed	2.7	Boreal Plant Breeding Ltd	1998
Polartop	six-rowed	3.0	Boreal Plant Breeding Ltd	2005
Pohto	six-rowed	4.9 * [1)]	Hankkija Plant Breeding	1994
Rambler	two-rowed	5.2	Boreal Plant Breeding Ltd	2009
Rolfi	six-rowed	5.4 ***	Boreal Plant Breeding Ltd	1997
Voitto	six-rowed	25.4 ***	Boreal Plant Breeding Ltd	2005

[1)] Significantly different from Scarlett: * at 5 % level, ** at 1 % level, *** at 0.1 % level

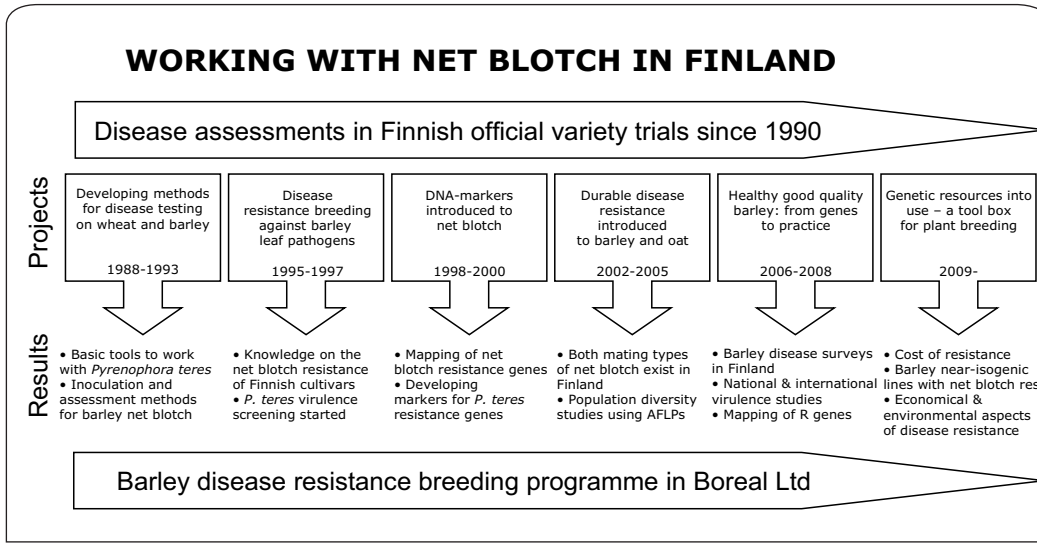

Figure 3. The background of the barley net blotch research in Finland at MTT Agrifood Research Finland.

disease triangle that illustrates interactions among the three components of net blotch: the pathogen, the environment and the host.

1.6 Objectives of the thesis

The main objectives were to establish reliable and efficient testing methods for *Pyrenophora teres* f. *teres* virulence screening, and to understand the role of virulence of the *Pyrenophora teres* f. *teres* pathogen in Finland from a disease resistance breeding point of view. The background material in this study includes four publications that are discussed together with related scientific papers. The papers included in my thesis were chosen based on the results of *P. teres* virulence screening in Finland using greenhouse test methods. Only the parts of the articles that support the objectives of this study are presented and discussed. In addition to the data already published in the individual papers I-IV, this work includes new results on combined data (Finnish *P. teres* f. *teres* isolates in collection 1-4), which made it possible to study the virulence over a longer time period. Those results are published only in this work.

The specific aims were:

- To establish stable virulence testing methods under greenhouse conditions (papers I, II, III, IV)
- To assess diversity of virulence among Finnish isolates of *Pyrenophora teres* f. *teres* (papers I, II, III, IV)
- To identify the impact of *P. teres* f. *teres* virulence on a net blotch resistance breeding programme (papers I, II, III, IV)

2 Materials and methods

2.1 *Pyrenophora teres* f. *teres* isolates (I-IV)

Virulence of 239 Finnish *P. teres* f. *teres* isolates was tested in 1994 - 2008. The isolates that were collected in 1994, 1997, and 2000-2007 across 19 locations originated from four *P. teres* collections: 1) 20 *P. teres* f. *teres* isolates collected in 1994 from 11 sites in Finland originating from two barley cultivars, a net blotch susceptible cultivar Arve and a field resistant cultivar Pohto (Kangas et al. 2008). Three Swedish and one Canadian isolate were also included in the studies. One of the Swedish isolates represented *P. teres* f. *maculata*. 2) 120 Nordic-Baltic and Irish *P. teres* f. *teres* isolates collected in 1997. The collection consisted of 19 Estonian, 29 Finnish, 11 Irish, 20 Latvian, 22 Norwegian, and 19 Swedish isolates. 3) 247 isolates originating from 13 countries from Europe, North America and Australia collected between 1984 and 2007. 153 of the isolates originated from Finland, collected in 2000-2007. 4) Six *P. teres* populations isolated from experimental fields in Jokioinen Finland in 2006-2007 (276 *P. teres* f. *maculata* and 37 *P. teres* f. teres isolates). The fields had a five to eight year history of barley monoculture. Three of the populations were collected from no-tillage and three from normal tillage cultivation systems.

Leaf samples with net blotch lesions were surface sterilized in 50 % ethanol for 15 s and in 2 % NaOCl for 30 s, rinsed in distilled water and placed on 2.3 % limabean (LB) or 20 % V8-agar. Plates were kept under near-UV light for 5-7 days. Single spores were collected with a needle and placed on LB- or V8-agar. For inoculum production, single spore cultures were grown under near-UV-light in a 12-h photoperiod at 16 ºC for 14 days. A spore suspension for inoculum was made by flooding Petri-dishes with sterile water. For long-term storage the isolates were deep-frozen as mycelium and conidia and stored in –80 ºC at MTT Agrifood Research Finland and Boreal Plant Breeding Ltd.

2.2 Mating studies with *Pyrenophora teres* isolates (IV)

Twenty-four *P. teres* isolates were used for mating studies. One of the isolates represented the spot form. Sterilized barley straw was placed on Sachs's agar (Smedegård-Petersen 1971) and inoculated at both ends with 100 µl of two *P. teres* suspensions. The plates were incubated in darkness for six months at 15 ºC. The success of crossings was studied under the microscope by assessing the mature pseudothecia. The ascospores were released from the pseudothecia by breaking the asci with a thin needle. Single ascospores were placed on 2.3 % LB-agar.

Morphological character measurements were made on one successful crossing population between a net and a spot form *P. teres* isolate. Colony growth, weight of mycelium, conidia production, conidia length, width and amount of septa were measured on six subcultures from one hundred single ascosporic cultures.

2.3 Set of differential barley genotypes used in *P. teres* f. *teres* virulence studies (I-IV)

The barley genotypes suggested by Steffenson and Webster (1992a) constituted the net blotch differential set in the first *P. teres* virulence study. In the later studies, that set formed the basis of barley genotypes used and was modified according to the aim of the study. Barley genotype CI 9819 was included in all test sets. Arve, Rolfi, or Pirkka was included in a test set

as a susceptible check (Table 2). In addition to the barley differential set, barley varieties cultivated in Finland were included in the virulence tests. The total amount of barley genotypes in the ten virulence tests was 46, including 16 varieties that have been cultivated in Finland.

2.4 Inoculation and scoring (I-IV)

The virulence studies were carried out in two greenhouses at MTT Agrifood Research Finland and at Boreal Plant Breeding Ltd. Barley seeds were sown in pots containing nutrient-supplemented peat and placed in the greenhouse at 18-22 °C with a 12-h photoperiod. The pots were arranged in a split plot design; isolates assigned to main plots and barley genotypes to sub plots with 2-4 replicates. At the two to three leaf stage (14 days after the sowing) relative humidity in the greenhouse was raised to 100 % and plants were inoculated with a conidial suspension at 0.1 – 0.2 ml / plant by using a pressurized sprayer. Lights were switched off and high humidity was maintained for 24 h after inoculation. The infection response was recorded 7 to 14 days after the inoculation on the first, second or third seedling leaf using the 10-point scale of Tekauz (1985) or the percentage of leaf area damaged. The infection scores < 5 were classified as avirulent reactions and ≥ 5 as virulent reactions (Figure 4).

2.5 Data analysis (I-IV)

The virulence data were analysed as split plot designs by using SAS PROC ANOVA, PROC GLM and PROC GLIMMIX. Significantly different means were separated with Tukey's HSDs. SAS PROC FREQ was used to study the general association of virulence of the *P. teres* isolates and tillage method. Kendall Correlation coefficients between the morphological characters and virulence data were analyzed with SAS PROC CORR (SAS Institute 2004). Hierarchical cluster analyses were carried out to examine similarities and dissimilarities in the reaction patterns of the accessions with respect to the net-blotch isolates. The clustering method of Ward (1963) and Simpson's diversity index was computed to characterize pathotype diversity of the isolates.

Table 2. The barley genotypes in barley differential sets used for screening virulence in *P. teres* f. *teres*.

Genotype	CI-number	Used in studies
Algerian	CI 1179	I
Atlas	CI 4118	I, II
Beecher	CI 6566	I
c-20019	c-20019	III
c-8755	c-8755	III
Canadian Lakeshore	CI 2750	I, II, III
Cape	CI 1026	I, II
CI 4922	CI 4922	I, II
CI 5791	CI 5791	I, II, III, IV
CI 5822	CI 5822	I, IV
CI 7584	CI 7584	I, II
CI 9214	CI 9214	III
CI 9819	CI 9819	I, II, III, IV
CI 9825	CI 9825	III
CI 11458	CI 11458	I, II
Coast	CI 2235	I, II
Corvette		III
Harbin	CI 4929	I, II, III
Harrington		III
Haruna Nijo		III
Kombar	CI 15694	I, II
Manchuria	CI 2330	I, II, IV
Manchurian	CI 739	I, II, III
Ming	CI 4797	I
Pirkka		III, IV
Prato	CI 15815	I
Prior		III
Rika	CI 8069	I, II
Rojo	CI 5401	I, II
Skiff		III
Tifang	CI 4407	I, II, III

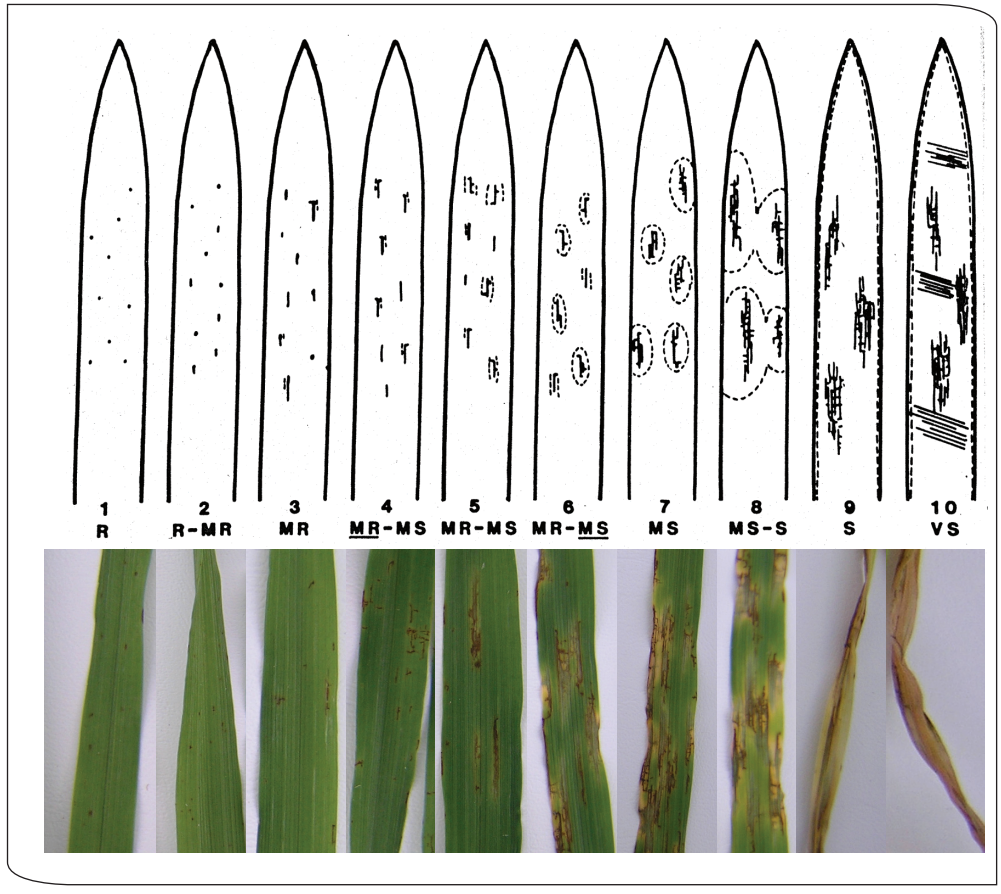

Figure 4. The 10-point scale of Tekauz (1985) and the corresponding infection response on living barley genotypes 10 days after inoculation on the second seedling. Photo Marja Jalli.

3 Results and discussion

3.1 *Pyrenophora teres* f. *teres* virulence testing methods

In the first virulence study (paper I) it was shown that the comparison and interpretation of results from the same laboratory from different times, and between laboratories is made difficult by the variable nature of the host-pathogen interaction. That was also observed in the second study (paper II) where there were significant differences in the results between the replicates that were located in different greenhouse rooms.

The environmental factors, temperature, light and humidity, were stable in all trials and their effect was not studied in this research. However, it has been shown that even the slight changes in environmental conditions can significantly affect the expression of plant infection. Khan and Boyd (1969) indicated the importance of stable testing methods based on their research on *P. teres* resistance in Manchurian varieties, which was enhanced by providing either pre-inoculation high temperature or high light intensity during the incubation period.

The virulence of *P. teres* has been studied by using barley genotypes with specific resistance as virulence markers (Tekauz 1990; Sato and Takeda 1993; Afanasenko et al. 1995). The lack of a universal barley differential set makes the comparison of separate virulence studies difficult. The aim in paper III was to develop an international standard set of barley differential genotypes to standardize the characterization of *P. teres* f. *teres* virulence globally. In the greenhouse tests, the virulence of 174 *P. teres* f. *teres* isolates (part of collection 3) was studied on 17 barley genotypes. Most of the *P. teres* isolates were virulent on Harrington, Pirkka and Haruna Nijo, while the lowest frequencies of virulent reactions was observed on CI 5791, CI 9819, CI 9214, Beecher, c- 8755 and CI 9825 (Table 3). Based on similarity analysis, the genotypes CI 5791 and CI 9819 were almost identical in the reaction to the isolates. Also, Skiff and Corvette resembled each other and were more similar to susceptible cultivars Pirkka, Harrington and Haruna Nijo than to other cultivars. Excluding the cultivars Beecher, CI 5791, CI 9819 and Harrington from the test set had a minor effect on the relative number of pathotypes and diversity

Table 3. Range and mean susceptible reactions of *P. teres* f. *teres* isolates tested on barley differential genotypes.

Barley differential	Range of susceptible reactions (%)	Mean of susceptible reactions (%)
CI 5791	0-14	1.7
CI 9819	0-25	2.9
CI 9214	0-25	7.2
Beecher	0-24	7.6
c-8755	0-25	9.7
CI 9825	0-50	10.2
c-20019	0-50	13.1
Manchurian	0-35	17.9
Tifang	0-75	22.4
Prior	0-50	31.3
Harbin	0-64	41.1
Canadian Lakeshore	0-75	41.5
Skiff	0-97	63.6
Corvette	13-86	71.4
Haruna Nijo	25-100	78.5
Pirkka	50-94	80.9
Harrington	50-100	89.4

index, whilst omission of cultivars Harbin and Skiff markedly affected both the relative number of pathotypes and the diversity index. The results of greenhouse tests carried out in Finland were incorporated with the detached-leaf studies made by the All Russian Research Institute of Plant Protection (VIZR), Russia. Based on the similarity analyses and the ability of individual barley genotypes to discriminate pathotypes and indicate pathotype diversity, barley genotypes c-8755, c-20019, CI 5791, CI 9825, Canadian Lakeshore, Harbin, Prior, Skiff and Harrington were identified to be the most appropriate to characterize *P. teres* f. *teres* virulence globally. A further aim was to study the genetic background of the resistance and to develop near-isogenic lines that carry different resistance genes in the same background, similarly as the Pallas-lines for powdery mildew (Kølster et al. 1986). If *P. teres* f. *teres* resistance that has not been identified earlier exists, it needs to be included in the test set. The composition of a barley differential set should be flexible when studying a pathogen-host system that evolves and might change rapidly.

The effect of barley seed size on the infection response via differences in growth speed was recorded for biotrophic pathogens (Jørgensen 1992). In our studies, the barley seed size correlated positively with plant growth. The seed size (sorting through sieves with mesh diameters 2.2 mm, 2.4 mm, 2.6 mm and 2.8 mm) used in the virulence test affected the plant height at seedling stage. Larger seeds produced larger plants for all tested genotypes. Seed size, and its consequent effect on plant height, did not significantly affect the infection response or percentage leaf area damaged. Even though no correlation between the seed size and the *P. teres* infection response was found, the seed may carry other elements that interact with the disease infection. Molinier et al. (2006) determined that stressed plants inherited the capacity for genomic change in *Arabidopsis*. The possible inheritance of a plant's expression to stress from one generation to another may cause unpredictable interactions when measuring the plant-pathogen interaction. Moreover, treating the seed with fungicides may affect infection response at the seedling stage. There is evidence that *triadimenol* is systemic and could be found even in the third wheat leaves 21 days after sowing (Thielert et al. 2007). *Triadimenol* is an active ingredient in fungicides Baytan I and Baytan Universal (Finnish Food Safety Authority 2010), which are commonly used seed dressings in Finland. Therefore, the seed used in virulence studies should have been grown in a completely stress-free environment to avoid the effect of seed treatment products and the possible inheritance of expression to stress factors.

Passaging the *P. teres* isolates through barley might be necessary after several cycles of sub culturing on artificial culture media, the effect of which varies on different isolates (McDonald 1967). A fall-off in conidia production capacity was also noticed in our studies, but was improved after passaging the isolate through a living barley plant. However, we found no evidence that passaging the *P. teres* f. *teres* isolates, which were used directly after the isolation from field passaging once through barley cultivars differing in resistance, were affected in terms of their virulence. As Lohan and Cooke (1986) reported, when comparing different agar media for *P. teres* spore production, we found the spore production to be most efficient in diurnal NUV light on V8-juice agar. In our studies, inoculum concentration (1 250 ml^{-1}, 2 500 ml^{-1}, 10^4 ml^{-1} and 2 x 10^4 ml^{-1}) had a significant effect on plant height, suppressing it at higher concentrations. Infection response and percentage leaf area damage were also influenced by inoculum concentration. A higher concentration led to higher infection response and proportion of leaf area damaged. The inoculum concentration used in papers III and IV was

fixed at 4 x 10^4 ml^{-1} and the amount of inoculum was adjusted to 0.2 ml/plant. This quantity of spores per plant was sufficient to get an even infection and to differentiate the infection response reactions of different barley genotypes.

Net blotch symptoms developed well in all our studies, lesions becoming apparent within two days post-inoculation. The differences in the degree of symptom expression on the inoculated first, second and third leaves were significant. The second seedling leaf was the most affected. Regression lines for the means infection response indicated that barley germplasm was more clearly differentiated into resistant and susceptible categories by scoring the second seedling leaf rather than the first. The information on the seedling leaf from which the virulence scorings are made is often missing in descriptions of materials and methods. Even if the second seedling leaf seems to be the most commonly used for disease scoring in seedling tests (e.g. Gupta 2001; Wu et al. 2003, Beattie et al. 2007), it is an essential detail to report when analysing and comparing *P. teres* virulence results.

The Tekauz 1-10 numerical scale for scoring the infection response is based on symptom expression as it appears 7-9 days after inoculation (Tekauz 1985). Tekauz's scale to classify barley reactions to *Pyrenophora teres* is used worldwide both for the *P. teres* virulence (e.g. Sato and Takeda1993; Jonsson et al. 1997; Gupta 2001; Wu et al. 2003) and for resistance studies (e.g. Sato and Takeda 1997; Cakir et al. 2003; Ma et al. 2004; Manninen et al. 2006; Grewal et al. 2008). Our results showed that the infection response values based on Tekauz scale were not affected by any of the fungal isolates' morphological characters (growth rate on agar, spore production ability, size of conidia or amount of septa per conidium). That supports the uses of Tekauz scale for pathogen-host interaction studies either to measure virulence or resistance that are not influenced by other characters of *P. teres* isolates.

The qualitative Tekauz scale provides a common understanding on infection reaction types. In our studies the differential genotypes ranked similarly to the infection rate (Tekauz scale) and the percentage of leaf area damaged. However, the scale based on the infection type is less sensitive to the microclimatic conditions on a barley leaf at the time of inoculation and incubation than the scale based on percentage leaf area damaged. When measuring infection type, a few successful symptoms on the second leaf are enough for proper investigations while for the scale based on percentage leaf area damaged it is assumed that all spores have standard conditions for germination. Both scales are based on visual observations, which might cause some error between different studies. The Tekauz scale has clear descriptions for all infection types and the risk for misscoring is relatively low.

When comparing the studies made at the seedling stage in a greenhouse using the Tekauz scale and the field adult plant test, Robinson and Jalli (1997b) found no correlation between the tests on six Nordic barley varieties carrying no known *P. teres* resistance genes. The contrast was explained by the higher infection rate in the greenhouse than in the field. Therefore, for quantitative disease resistance screening in the greenhouse it is necessary to optimize the test methods. In *P. teres* virulence screening studies, when a barley differential set is used, a high positive correlation between the reactions in greenhouse and in field is observed (Jalli 2009, unpublished data).

Tekauz (1985) rated lesions below 5 as resistant reactions and greater than 5 as susceptible reactions. In different studies a score of 5 is considered either as a virulent/susceptible reaction (e.g. Beattie et al. 2007) or avirulent/resistant reaction (e.g.

Jonsson et al. 1997; Wu et al. 2003). In our virulence studies, a value of 5 was considered to be a virulent reaction. However, the fixed limit between an avirulent and a virulent reaction over all tested barley genotypes might be misleading. For example, on barley genotypes CI 5791 and CI 9819, a score of five is clearly a sign of virulence, which may develop further in time. On genotypes Canadian Lakeshore and Manchuria it is more considered as an avirulent, stable reaction. The expression of defence differs slightly between different barley genotypes. On some genotypes, like CI 9819, no netting is observed and the symptoms are closer to symptoms caused by *P. teres* f. *maculata*. Therefore, when monitoring the *P. teres* f. *teres* virulence, to follow the possible evolution in pathogen population, defining the limit between avirulent and virulent classes individually for each barley genotypes instead of having a fixed limit over the entire barley differential set may be a more sensitive tool.

An important part of the research is the use of different statistical analyses and their effect on the final conclusions. In our studies the statistical methods were chosen based on the trial design, on the type of data (categorical or continuous variable), on the distribution of data, and on the statistical methods available at the time of analyses. Several different statistical methods are used when analysing the virulence or resistance data based on the Tekauz scale: e.g. general linear models (GLM) procedure, restricted maximum likelihood (REML) procedure, best linear unbiased predictors (BLUPS), and protected least significant difference test (PLSD) (Grewal et al. 2008; Gupta et al. 2003; Tuohy et al. 2006). Statistical methods evolve and give increased opportunities for better understanding the data. That demands optimal trial design, close cooperation between researchers and statisticians, and careful comparison of studies with different statistical methods used. Definitively, statistical methods are the part of the research that should be as carefully evaluated as other parts of the research and their value is often underestimated.

The screening of *P. teres* virulence with molecular tools instead of resistant barley plants might be possible in the future. There already exist molecular methods to detect *P. teres* avirulence genes *AvrHar, AvrPra1, AvrPra2* (Lai et al. 2007) and *AvrHeartland* (Beattie et al. 2007). However, the relatedness between avirulence genes and virulence is variable between different pathogens (Sacristán and García-Arenal 2008) and the role of the detected *P. teres* avirulence genes in virulence is still unclear (Beattie et al. 2007). Also, the research on phytotoxic compounds isolated from *P. teres* (Sarpeleh et al. 2007) and their role as pathogen effectors (Stukenbrock and McDonald 2009) may open novel possibilities to study virulence of *P. teres*. However, for routine virulence screening to monitor the changes of virulence in *P. teres* f. *teres* populations, greenhouse seedling tests still represent a valuable and efficient tool.

3.2 Diversity of *Pyrenophora teres* f. *teres* isolates in Finland

3.2.1 Diversity in virulence

The *P. teres* isolates used in this study were originally collected for several different purposes. Some of the isolates were collected systematically from selected locations, cultivars and fields with known cultivation histories, while others were collected randomly from experimental or farmers' fields. The testing conditions in different studies were close to each other and all the disease observations were made by the author, allowing the analyses and conclusions to be made over the separate trials.

In total, 239 Finnish *P. teres* f. *teres* isolates were tested in 1994 – 2008. None of the isolates was virulent on CI 5791.

On CI 9819, 1 % of the isolates scored 5, the lowest value considered to be a virulent reaction. All other reactions on CI 9819 were avirulent. The median of the infection score was above four (virulent reaction) on 11 of 31 barley differential genotypes: Cape, Corvette, Harbin, Harrington, Haruna Nijo, Kombar, Manchuria, Ming, Pirkka, Rika and Skiff (Figure 5). The average infection score of the tested isolates over the all tested barley genotypes varied from 1.9 to 7.4 (Figure 6).

The first virulence study on 20 *P. teres* f. *teres* isolates collected from 11 sites in 1994 in Finland provided the basis for this research (collection 1). The results were illustrated with regression plots and each isolate exhibited a unique pattern of response on the differential genotypes. The results indicated differences between the isolates and genotypes, but not always in slope of the regression line. All the isolates were avirulent only on genotypes CI 5791 and CI 9819 and virulent on the six Nordic barley genotypes that showed a range of symptoms in the field.

We investigated if the host plant from which the isolate originated significant-

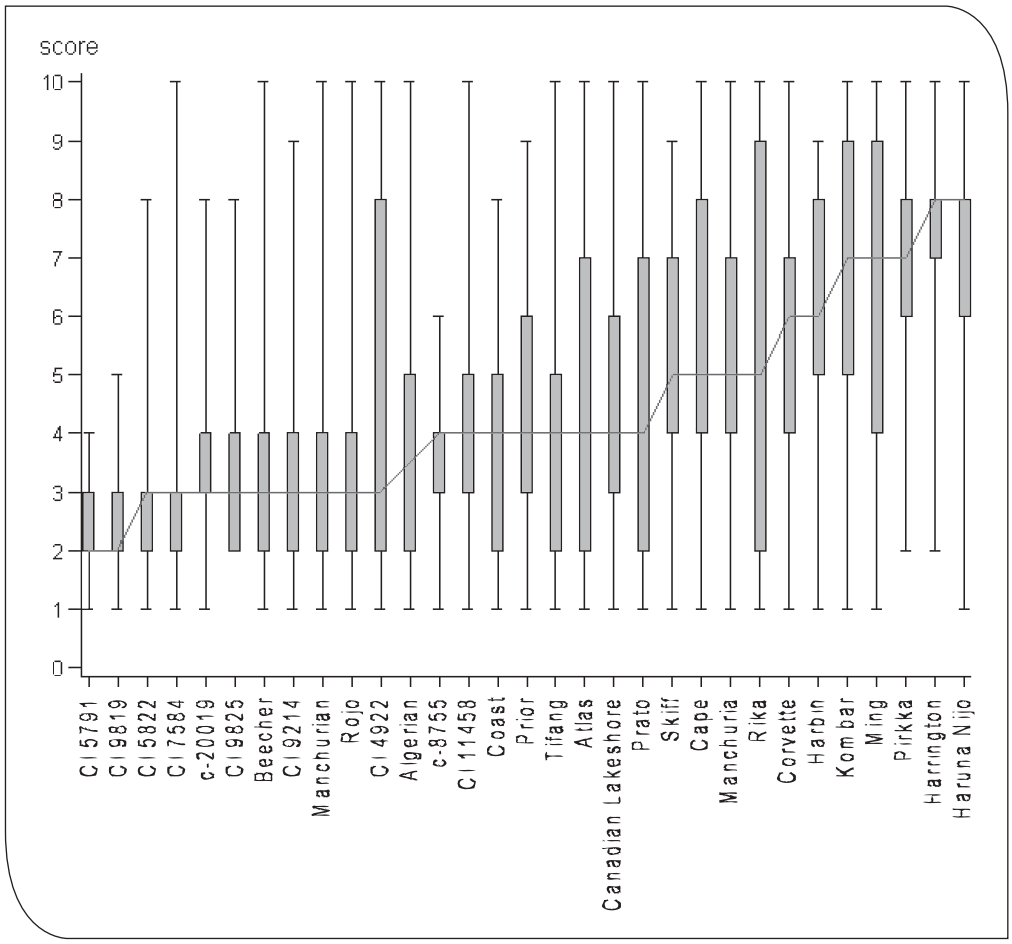

Figure 5. Box plot displaying the median, quartiles, and minimum and maximum observations for the symptom scores of the 239 Finnish *Pyrenophora teres* f. *teres* isolates on barley differential set genotypes. The medians are connected by a line.

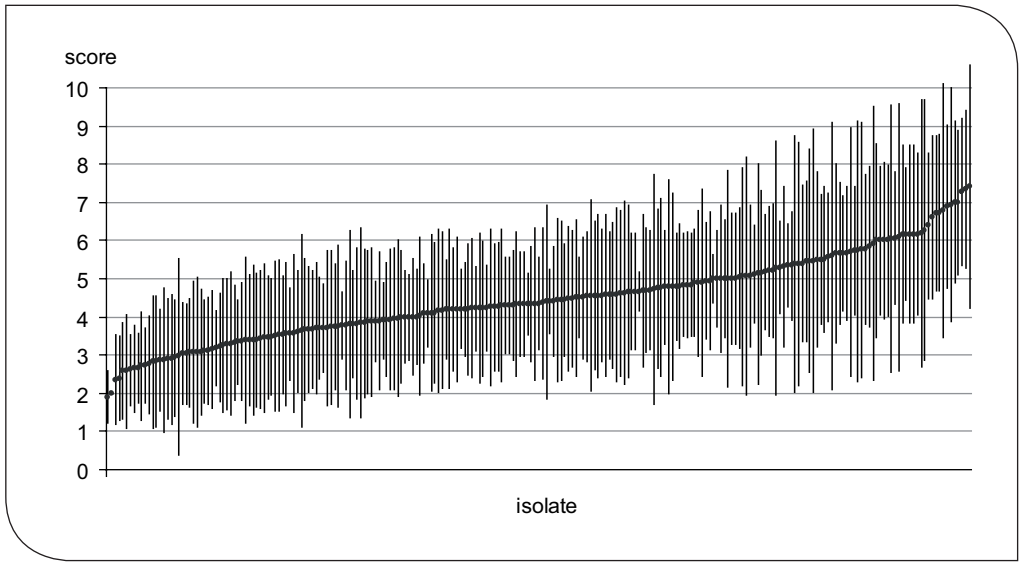

Figure 6. The average and standard deviation for the symptom scores of the 239 Finnish *Pyrenophora teres* f. *teres* isolates over the tested barley differential genotypes.

ly affected the virulence of the isolate. The isolates from susceptible barley cultivar Arve were more virulent than those from less susceptible Pohto. That was confirmed by Jalli & Robinson (1997) using 64 *P. teres* isolates originating from cultivars Arve and Pohto. However, as Thrall and Burdon (2003) reported in their studies on flax rust and flax, aggressiveness is favoured over virulence in susceptible host populations, whereas virulence is favoured in resistant populations, and it might be that instead of greater virulence the issue in our studies was one of greater aggressiveness. The differences in definitions between various terms are not always clear. Aggressiveness and virulence are even used as synonyms (Shaner et al. 1992). The same problem of nomenclature exists when discussing virulence on the barley genotypes that are quantitatively resistant or susceptible to the *P. teres*. The proper term to use in such cases would be aggressiveness (Pariaud et al. 2009).

In paper II, the virulences of 29 Finnish isolates collected in 1997 were compared to those of the isolates originating from Estonia, Latvia, Norway, Sweden and Ireland. The study identified large differences in response of barley genotypes to the *P. teres* f. *teres* isolates. Only little variation was detected among the *P. teres* isolates from Nordic-Baltic countries on the tested genotypes Cape, CI 9819, Coast, and Manchuria. Contrary to our studies, Tuohy et al. (2006) found significant variation in virulence among Northern European *P. teres* f. *teres* isolates, including isolates from Finland. However, the two studies included only one barley genotype in common (Coast), which partly explains the different conclusions from the two host-pathogen interaction studies.

In paper III, the greenhouse test virulence data on 174 *P. teres* f. *teres* isolates (including 80 Finnish isolates) were analysed together with the virulence data from detached leaf tests on 885 isolates (including 150 Finnish isolates). Based on Afanasenko's detached leaf studies on the Finnish isolates (Afanasenko 2009, personal communication), the most resistant

barley genotypes identified were Tifang, CI 9819, Canadian Lakeshore, c-8755, and CI 5791. The results correlate well with our greenhouse virulence data collected between 2000 and 2005. The highest frequency of avirulent reactions was found on CI 9819 and CI 5791. 86 % of the isolates were avirulent on c-8755, but only 47 % on Canadian Lakeshore. In the seedling tests Canadian Lakeshore was mainly considered to be a medium resistant genotype and some isolates were highly virulent on it. The slight differences between Afanasenko's and our studies may have resulted from the origin of the isolates and the testing methods. In Afanasenko's research the Finnish isolates originated from three fields while in our studies they were collected from 14 sites and several cultivars.

In paper IV, the virulence of *P. teres* f. *teres* isolates originating from a no-tillage and normal-tillage cultivation system was studied. Surprisingly, only 37 of the 313 single spore isolates represented the net form of *P. teres* and the rest represented *P. teres* f. *maculata*. The virulence reactions of net form isolates on barley differential genotypes CI 9819 and Manchuria were almost identical and no influence of the tillage system was observed. All the isolates were avirulent on CI 9819. Two of the 37 isolates were virulent on Manchuria, one originating from a no-tillage and the other from a normal tillage plot.

3.2.2 Change in virulence over years

The combined dataset (Finnish isolates in the collections 1–4) was used to study the change in *P. teres* virulence over time. Chi-Square values that illustrated the association between the year of collection of the isolate and the virulence of all tested 239 *P. teres* isolates on a susceptible barley genotype and on CI 9819, indicated no significant interaction (P= 0.97). The virulence of 98 *P. teres* f. *teres* isolates collected in 1994, 2003, 2004 and 2005 was tested in four trials and nine of the barley genotypes in different trials were common: Beecher, Canadian Lakeshore, CI 5791, CI 9819, CI 11458, Harbin, Manchuria, Manchurian, and Tifang. Based on the results of GLIMMIX analyses made on the combined data there were no significant average differences in the virulence of the *P. teres* isolates among the years (P=0.10). The pairwise analyses showed a slight difference in virulence of the studied *P. teres* isolates between 1994 and 2004 (P=0.04). The estimated frequencies of virulent isolates over the nine barley genotypes in different years were 1994: 29 %; 2003: 40 %; 2004: 20 %; and 2005: 35 %. The results of GLIMMIX analysis on 44 isolates collected in 2000, 2003 and 2007 that were tested on five common barley genotypes (Annabell, CI 9819, Kunnari, Manchuria, and Pirkka,) showed no significant interaction between the collection year and the virulence (P=0.52).

The virulence studies on the combined dataset provided novel possibilities for analyses of the specific and overall virulence among the isolates collected in 1994-2007. No clear virulence was found on CI 9819, which was the only barley genotype included in all trials. The collecting year had no significant effect on the infection response when using the avirulent/virulent classification. However, the expression of avirulence on CI 9819 varied while the infection response ranged between scores from one to five. Some of the isolates were highly aggressive, causing numerous leaf spots even when the spot size remained small and no chlorosis was observed. The same phenomenon was observed in field trials where CI 9819 seemed to be sensitive to physiological leaf spots. The physiological leaf spots appeared at later growth stages and no physiological spots were observed in seedling tests. No virulence on CI 9819 is recorded in *P. teres* virulence studies made in other barley growing areas (paper III Afanasenko's studies; Gupta et al. 2003; Tuohy et al. 2006).

Regarding the separate trials and the overall analyses, the virulence of Finnish *P. teres* f. *teres* isolates collected between 1994 and 2008 was fairly stable, both within and among years. In Finland, the pathogen has not been exposed to major resistance genes, and therefore it has not been under a selective pressure, making coevolution of the pathogen-host interaction to produce a more virulent pathogen unlikely. Similar results were reported by Gupta (2001). He concluded that the virulence among the isolates from Western Australia remained stable for 19 years due to the cultivars grown. More detailed knowledge on the evolution of Finnish *P. teres* f. *teres* populations could be achieved by designating pathotypes (Steffenson and Webster 1992a) or isolate groups (Gupta 2001) that illustrate the similarities and differences between isolates in a very comprehensive way. However, in our studies, the rationale has been to serve the practical barley resistance breeding programme and therefore emphasis has been put on following the evolution of virulence on specific barley genotypes instead of the general evolution of *P. teres* pathogen populations. To get a more comprehensive overview of the changes in pathogen populations, larger numbers of isolates rather than single isolates would have to be tested. Also the number of barley genotypes included in virulence tests increases knowledge on the population structure. The variability of a population of *P. teres* depends on the number of barley cultivars examined and the differences in their genetic background, the presence of both net and spot forms in studies, and the number of isolates examined (Tekauz 1990).

Not many comparable novel studies on *P. teres* virulence exist in the literature, which together with the different methodologies used weakens the comparison between the *P. teres* virulence situation in Finland and in other countries. Instead, the rapid development of molecular tools has made it possible to put more emphasis on population genetic studies of *P. teres*. Wu et al. (2003) combined the results of virulence and genetic variation studies based on restriction fragment length polymorphism (RFLP) analysis. The results indicated that *P. teres* possesses a high degree of diversity at species and subspecies level (*P. teres* f. *teres* and *P. teres* f. *maculata*). Serenius et al. (2005) studied the genetic variation of two *P. teres* populations originating from Finnish barley fields using AFLP analysis. The mean genetic similarity among all the isolates was 93 % and most of the variation was observed within field populations. They concluded that Finnish *P. teres* isolates are genetically more variable than expected based on virulence surveys in papers I and II. In later *P. teres* population studies Serenius et al. (2007) showed that most of the total variation within Finnish *P. teres* f. *teres* isolates was within the collection years rather among them and they were genetically more different than those collected from other Nordic areas. Virulence is only one indicator used to describe the population structure. The combination of systematic population genetic and virulence studies would be an ideal tool to study the factors affecting the *P. teres* – barley interaction and to predict the risk for changes in the *P. teres* virulence spectrum.

3.2.3 Effect of sexual reproduction on virulence

Mating studies with pathogen isolates are a valuable method for better understanding pathogen-host interactions. The effect of sexual reproduction on virulence in *P. teres* was studied in three artificially produced populations (paper IV): in one crossing between two net form isolates (isolates 14 x 15) and in two crossings between a net form and a spot form isolate (isolates 14 x 24 and 16 x 24). The virulence of the *P. teres* f. *teres* parental isolates 14, 15 and 16 were identical on five tested barley genotypes Arve, CI 5791, CI 5822, Manchuria, and Pohto: avirulent on CI 5791 and

CI 5822 and virulent on Arve, Manchuria and Pohto. The virulence reaction of the *P. teres* f. *maculata* parental isolate was opposite to the net form isolates on CI 5791, CI 5822, and Manchuria. None of the crossing progenies was entirely identical to its parents in terms of virulence. The average similarity of the progeny isolates to their parents was calculated by assessing the percentage of similar reactions between the progeny isolates and parental isolates over the five barley genotypes. The average similarity of the net by net form mating progeny isolates to its parents was 92 %. The average similarity of the net by spot form mating progenies isolates was 58 % to the net form parents and 73 % to the spot form parent.

The *P. teres* crossing studies done in the laboratory showed the possible risk for changes in virulence on genotypes CI 5791, CI 5822, and Manchuria if sexual mating exists in the field. In the crossing between the two net form isolates, the changes in virulence were smaller than in crossings between the net and spot form isolates because of the different virulence profiles of the net and spot form parents. All three progenies included isolates that were either avirulent or virulent on each barley genotype. Both net form parents were avirulent on CI 5791, but still 8 % of their progeny isolates were virulent on it. Both the parents scored 2 while in progeny isolates the maximum score was 7. The studies included two net and spot form matings, having the same spot form parental and two net form parental isolates sharing the same virulence profile. However, the inheritance of virulence was not identical among the two net x spot form matings. On CI 5822, 68 % of the 16x24 and 94 % of the 14x24 progeny isolates were virulent. The unpredictable results confirm that virulence of a pathogen is result of several factors (Bent and Mackey 2007) and in the studied host-pathogen interactions it seemed to be controlled by several genes. Our study is related to the specific isolates and host genotypes, and generalization of the results should be done with caution.

The sexual mating of heterothallic ascomycetes is controlled by fast evolving mating type genes (*MAT1* and *MAT2*) (Turgeon 1998) that determine the sexual compatibility between different haploid individuals. The heterothallism among ascomycetes supports the importance of generating new genetic combinations. A pathogen must choose between sexual and asexual development, and they use nutritional, temperature and light clues to make this decision (Nelson 1996). Serenius et al. (2005) reported that both mating types (*MAT1* and *MAT2*) exist in Finland at a ratio close to 1:1. Based on the AFLP studies of Finnish *P. teres* f. *teres* isolates Serenius (2006) concluded that sexual reproduction occurs in Finland. However, no visual evidence of the naturally existing pseudothecia has been reported. In contrast, pseudothecia were easily observed in Denmark and in southern Sweden (Smedegård-Petersen 1972; Jonsson 2001). The artificially inoculated straw samples that were stored at different soil depths over the winter 2006-2007 in Jokioinen, Finland had visible pseudothecia in spring but at the time of collection, just before sowing time in early May, the ascospores were already ejected (Jalli 2007, unpublished data). The equal amounts of mating types in Finnish populations (Serenius et al. 2005), the conclusions reached on AFLP studies of Finnish isolates (Serenius 2006), and the success of artificial mating in nature (Jalli 2007, unpublished data) indicate the high probability of the existence of sexual reproduction of *P. teres* f. *teres* in Finland. However, the possible effects of sexual reproduction on the virulence of *P. teres* have not yet been noted in Finnish barley fields.

Due to the different genetic structure (Rau et al. 2007) and virulence profile of net and spot forms of *P. teres* isolates (paper

IV) their recombination may lead to unpredictable virulence. Our crossing studies in the laboratory were successful in producing large amounts of viable ascospores. There are several examples of the success in mating the two forms under laboratory conditions (e.g. Smedegård-Petersen 1971; Campbell et al. 1999). Campbell and Crous (2003) investigated the hybrid progeny of a mating between net and spot form isolates, concluding that they retain their virulence and fertility over time and are genetically stable after two cycles of inoculation, mitosis and re-isolation. Evidence on the recombination between net and spot form isolates in the field in South Africa was reported (Campbell et al. 2002). In contrast, the results of Serenius et al. (2007) on the AFLP studies on *P. teres* populations, which also included Finnish isolates, indicated that recombination between the two forms hardly occurs in nature. Also the studies by Rau et al. (2007) confirm that the hybridization between the two forms is rare or absent under field conditions. In that regard our results on the net and spot form crossings should be considered more as an example of possible changes in virulence when two isolates of different virulence profile mate.

3.2.4 Effect of cultivation practices on virulence

Reproduction and mating systems affect how gene diversity is distributed within and among individuals in a pathogen population. Populations of sexual pathogens usually exhibit a high degree of genotype diversity and represent greater risks to break down of resistance (McDonald and Linde 2002). One phenomenon that may affect the occurrence of sexual reproduction is the cultivation system. Reduced and no-tillage cultivation systems are becoming popular among farmers around the world. Reduced tillage improves a soil's physical, hydro-physical and biological properties, especially in a good crop rotation system (Rusu et al. 2009). Due to the potential for enhanced productivity, cost and environmental savings, most agricultural institutions and governments are promoting reduced tillage. However, in a minimum or no-tillage system the risk for residue-borne plant diseases may increase (Joshi et al. 2007). There are a few studies on the influence of no-tillage and reduced tillage on the incidence and severity of net blotch (e.g. Jordan and Allen 1984; Duczek et al. 1999). There is also evidence on the effect of both the no-tillage and crop rotation on pathogen population structure. Almeida et al. (2008) showed that *Macrophomina phaseolina,* an important disease of soybean, is a genetically variable species and can be affected by crop rotation. Chulze et al. (2000) indicated that the *Fusarium* populations isolated from no-till maize are similar to those recovered from maize managed with conventional tillage. However, no research has been done on the effect of cultivation system on *P. teres* population structure and virulence.

In this study the effect of no-tillage cultivation practices on the virulence of *P. teres* populations was studied among 313 isolates that were obtained from three fields that had five to eight year histories of barley monoculture either under no-tillage or in normal tillage cultivation (paper IV). Of the isolates, 276 represented the spot form and only 37 the net form of *P. teres*. Based on Cochran-Mantel-Haenszel statistics, the tillage method (no-tillage or normal tillage) had no effect either on the virulence structure of the *P. teres* f. *teres* or *P. teres* f. *maculata* populations.

Collections 1-3 in our studies represent only the net form of *P. teres* and the low frequency of net form isolates in collection 4 was not predicted. The fungicide treated seed and the net blotch infections observed at the early tillering stage in no-tillage blocks confirms that the infection was mainly straw-borne. Besides the possible effect of the microclimatic conditions in the fields, the high proportion of spot form

isolates may be explained by long monoculture of barley cultivars Annabell and Saana in the fields from which the samples were isolated. Both cultivars are medium resistant against the net-form of the fungus (Kangas et al. 2008), which might have caused selection towards the spot form. Based on the virulence studies there were no signs of increased variation among the isolates originating from no-tillage compared to isolates originating from normal tillage plots. No recombinants between net and spot form isolates were observed either; all the isolates produced clear net or spot form symptoms. The AFLP analyses on some of the isolates support the results of virulence studies. No differences in the genetic variation were found among the populations originating from different cultivation systems (Serenius 2008, unpublished data). Even though natural drift of isolates between blocks with different cultivation systems is probable, there was no such variation in virulence either in the isolates originating from no-tillage or normal tillage field.

What was observed in the study on the effect of sexual mating on virulence, and the sexual recombination of *P. teres* isolates could not have been proved based on the virulence studies. The virulence of the net form isolates originating from no-tillage and normal tillage background was comparable to the results earlier presented in this work: all net form isolates were avirulent on CI 9819 and virulent on Pirkka. However, this research was a preliminary study on the possible effects of cultivation methods on *P. teres* related to specific environments, and to better understand the long-term effect of no-tillage on sexual recombination and on the virulence of *P. teres*. The research continues, considering also the effects of monoculture and crop rotation on the virulence and on population genetics.

3.3 Implications of *P. teres* f. *teres* virulence for net blotch resistance breeding

Besides the differential sets that include barley genotypes with different resistance phenotypes, our virulence studies included barley genotypes that are cultivated in the Nordic countries and whose resistance background is unknown. The virulence study results identified large differences in response of barley genotypes to *P. teres* f. *teres*. When infection scores from 1 to 4 were considered as resistant reactions, CI 5791 was the only genotype resistant to all tested isolates. Of the 46 barley genotypes, 23 were resistant to at least 50 % of the isolates. Those genotypes included two commonly grown malting barley cultivars in Finland: Scarlett (resistant to 80 % of the isolates) and Annabell (resistant to 72 % of the isolates), and a six-rowed cultivar Kunnari (resistant to 61 % of the isolates). The frequency of resistant reactions was less than 10 % on cultivars Tiril, Rolfi, Arve, WW 7977, Harrington and Agneta (Table 4).

Scarlett, Annabell and Kunnari have shown good net blotch resistance also in the Finnish official variety trials (Kangas et al. 2008). Robinson and Jalli (1997b) found adequate levels of quantitative resistance in genotype H 6221 ('Thule') and later (2001) in Bor 94007 ('Jyvä'). These genotypes are examples of widely adapted genotypes that possess adequate levels of resistance to make them useful parents in crossing programmes. Testing the breeding material at several sites over several seasons provides an opportunity to monitor and develop adapted genotypes that exhibit stable resistance to *P. teres*. Based on the analysis made on Finnish official variety tests, Robinson and Jalli (2001) concluded that there are no site-specific populations of net blotch that differ greatly in virulence

Table 4. Number of tested isolates, the minimum infection score, the maximum infection score, and the percentage of resistant reactions (score < 5) on barley genotypes tested with different Finnish *P. teres* f. *teres* isolates.

Genotype	Number of tested isolates	Min infection score	Max infection score	% of resistant reactions
CI 5791	142	1	4	100
CI 9819	238	1	5	98
CI 7584	53	1	10	93
CI 5822	53	1	8	89
CI 9825	51	2	8	88
CI 9214	50	1	9	87
c-8755	51	1	6	86
Rojo	53	1	10	86
c-20019	51	1	8	83
Beecher	110	1	10	83
Scarlett	33	2	6	80
Manchurian	110	1	10	77
Coast	82	1	8	74
Annabell	22	1	10	72
Algerian	20	1	10	72
CI 11458	110	1	10	71
Tifang	110	1	10	63
Prato	20	1	10	63
Canadian Lakeshore	110	1	10	62
Kunnari	66	2	8	61
Prior	51	1	9	60
CI 4922	20	1	10	60
Atlas	20	1	10	51
Rika	20	1	10	48
Olavi	44	2	9	42
Skiff	51	1	9	37
Cape	49	1	10	36
Manchuria	160	1	10	33
Ming	20	1	10	30
Harbin	110	1	10	27
Kombar	20	1	10	24
Edel	43	3	9	18
Corvette	51	1	9	18
Pohto	49	3	9	17
Artturi	20	3	6	17
Pirkka	156	1	10	15
H 6221	20	1	10	14
Pilvi	44	2	10	13
Voitto	44	2	9	10
HarunaNijo	32	1	10	10
Tiril	44	2	9	9
Rolfi	33	1	10	9
Arve	93	2	10	5
WW 7977	20	1	10	4
Harrington	51	2	10	3
Agneta	20	4	10	1

in Finland. That was confirmed by the virulence studies made during this research. Cultivar Kunnari was released in 2000 and Jyvä in 2001 (Table 1). Both cultivars are widely cultivated in Finland (Kunnari 30 000 ha and Jyvä 36 000 ha in 2009) but no breakdown of the resistance has been recorded (Kangas et al. 2008). The durability of resistance may be explained by the genetic background of the resistance and the stability of Finnish *P. teres* f. *teres* populations. In the Danish studies on net blotch infection on barley during several years and at several locations, the total variation in disease severity (61-81 %) was explained by plant genotype and environment, and only a minor part of the total variation was assumed to be caused by virulence characteristics of pathogen populations (Pinnschmidt and Hovmøller 2002).

The results of our experiments indicate that the barley genotypes used in the differential sets contain useful levels of resistance to *P. teres* f. *teres* that could be used in the Finnish barley disease resistance breeding programme. Several genotypes were more resistant than most of the cultivated barleys and could be used to strengthen the level of resistance that exists among barleys as a result of the regular breeding programme.

Barley genotypes CI 5791 and CI 9819 were resistant to all Finnish *P. teres* f. *teres* isolates. In paper III, where genotypes were tested against 1059 *P. teres* f. *teres* isolates from 14 countries, the lowest mean frequency of virulent isolates across populations and testing methods was observed on CI 5791, CI 9819, c-8755 and CI 9825, which indicates a high level of resistance in these cultivars. Genotypes c-8755 and CI 9825 showed good resistance also against Finnish isolates. Despite the good polygenic resistance of CI 5791 and CI 9819 against barley net blotch (Jonsson et al. 1999; Manninen et al. 2006; Afanasenko et al. 2007), it seems that they are fully efficient only against the net form of net blotch (Arabi et al. 1992; Tuohy et al. 2006; Grewal et al. 2008). According to the cluster analyses and the ability of individual genotypes to discriminate *P. teres* f. *teres* isolates, c-8755, CI 9825 and CI 5791 showed a different ability to illustrate virulence in *P. teres* isolates. Therefore, it is likely that these resistant genotypes carry at least partly different resistance genes. The genetic studies made on the host-pathogen interaction of these barley genotypes and *P. teres* f. *teres* isolates confirm that the resistance is mostly isolate-specific and controlled by one or two genes (Afanasenko et al. 2007).

Diverse sources of resistance must be deployed to achieve long-lasting resistance. Novel net blotch resistance sources are essential in environments that encourage the risks for changes in *P. teres* populations. Even though this study demonstrated the slow evolution of *P. teres* virulence between 1994 and 2007, there are examples on host-pathogen interactions where the situation has changed rapidly resulting from recombination and the selection imposed by resistance genes (McDonald et al. 1989).

Novel resistance sources and allelic variation could be found from landraces and wild genotypes originating from areas with high net blotch pressure. Robinson and Jalli (1996) showed that barley genotypes originating from Latin America carry efficient net blotch resistance against Finnish *P. teres* f. *teres* isolates. Bonman et al. (2005) demonstrated that disease resistance varies depending on geographic origin. Adult plant net blotch resistance was most common in accessions from Eastern Asia, China, South Korea and Japan. Accessions from Eastern Africa showed the highest levels of resistance at the seedling stage. Wild barley, *Hordeum vulgare* spp. *spontaneum*, may represent a totally novel input for improving barley net blotch resistance. However, the value of genetic resources of wild barley will only

be realised when their resistance genes are transferred into cultivated barley. Several *H. vulgare* spp. *spontaneum* and cultivated barley crosses have been developed and resistance was successfully transferred into adapted barley germplasm (Steffenson et al. 2007). Sato and Takeda (1997) found *H. vulgare* spp. *spontaneum* accessions from Afghanistan and Russia that showed high levels of net blotch resistance. Using wild barleys and landraces to increase biodiversity in barley breeding programmes affords the development of plant varieties with novel genetic combinations that will be required to meet the challenges arising from the changing environment, like drought and pests.

The net blotch disease infection level is highly influenced by the environment and the reliability of the resistance screening results may decrease if they only rely on non-inoculated survey data or on data from too few on non-representative environments (Pinnschmidt and Hovmøller 2002). The testing methods for screening virulence of *P. teres* f. *teres* on seedlings in the greenhouse with specific isolates seem to be efficient also in disease resistance testing. Gupta et al. (2003) and Grewal et al. (2008) concluded there was strong agreement between seedling and adult-plant reactions, which indicates that seedling screening could be useful in selecting for adult-plant resistance. Jonsson et al. (1998) reported that net blotch resistance in barley seedlings inoculated at the one to two leaf stage correlated with net blotch reactions on the fourth and flag leaves when barley was grown in growth chambers, as well as with disease levels recorded on the three uppermost leaves in a field experiment. However, based on our virulence studies on the barley differential set and the studies by Khan and Boyd (1969) on the sensitivity of resistance expression to temperature and light intensity, resistance screening only under the greenhouse conditions can lead to false conclusions. The value of expression of resistance in diverse climatic conditions is increasing due to climate warming. Climate change can also affect the durability of resistance. There is evidence that some forms of disease resistance might be overcome more rapidly following changes in levels of CO_2, ozone and UV-B (Chakraborty et al. 2000). The optimal way to get profitable and long-lasting results in improving net blotch resistance could be to find a balance between different breeding methods most appropriate to the needs and resources: molecular tools to map and screen specific resistance genes, greenhouse seedling tests to select against specific virulence, and multi-environmental field tests to follow the resistance-environment interactions and to secure durability.

4 Conclusions

Screening for *P. teres* f. *teres* virulence on barley seedlings under greenhouse conditions is a reliable and feasible method, especially in the absence of modern molecular tools to monitor the complex *P. teres* f. *teres* – barley interaction. Because the method is based on visual observations of disease symptoms on living plants it is also a comprehensive tool for studying the *P. teres* – barley pathosystem. Besides the genetic backgrounds of the host and the plant, disease expression is related to the environmental conditions, temperature, light and humidity, which need to be stable to achieve reliable and comparable results among different studies. Even slight changes in environmental conditions may change the disease expression of some barley genotypes. A common barley differential set to characterize the *P. teres* f. *teres* virulence was developed based on its ability to discriminate pathotypes and indicate pathotype diversity, and is recommended for use globally: c-8755, c-20019, CI 5791, CI 9825, Canadian Lakeshore, Harbin, Prior, and Skiff. Cultivar Harrington is recommended to be used as a susceptible check. These genotypes form the basis of the set that needs to be enlarged when novel resistance is introduced. The barley seed for virulence tests should be produced in an environment that minimizes the risks for biotic or abiotic stresses. Seed dressing treatment is not recommended. Barley seed size, *P. teres* isolates morphological characters, spore production or growth rate on agar have no effect on the expression of virulence. The Tekauz numerical scale based on infection type is more appropriate to describe the virulence of the isolate than the percentage of leaf area damaged. The second seedling leaf is best for differentiating the isolates.

The virulence spectrum of Finnish *P. teres* f. *teres* isolates collected in 1994-2007 is stable both within and between years. The results indicated differences between the isolates and genotypes but not always in their interaction. There is no virulence to barley genotypes CI 5791 and CI 9819. The virulence of Finnish *P. teres* f. *teres* isolates is highest on Cape, Corvette, Harbin, Kombar, Manchuria, Ming, Rika, and Skiff of the barley differential genotype set. In Finland, there is no evidence of any effect of sexual reproduction on virulence under natural conditions; not even in barley monoculture no-tillage fields, which should represent an optimal environment for sexual recombination. However, based on artificial mating studies the virulence of a pathogen is a result of several factors that may be influenced significantly by sexual recombination even when two isolates with similar virulence profiles mate. The mating and host-pathogen interactions studies related to specific isolates and host genotypes, and generalization of the results, needs to be done with caution.

Even though no changes in the Finnish *P. teres* f. *teres* virulence spectrum were observed, changes in climate and cultivation methods could change the situation rapidly and breeders need to be prepared. Therefore, virulence surveys, concentrating on areas with high risk for evolution, should be an essential and continuous part of a disease resistance breeding programme. Large differences in response of barley genotypes to *P. teres* f. *teres* exist. Finnish six-rowed cultivars Kunnari and Jyvä are examples of durable resistance achieved through traditional breeding methods. Novel resistance that has not been introduced earlier into Finnish barley is also available. Barley genotypes c-8755,

CI 9825 and CI 5791 have excellent resistance against Finnish *P. teres* f. *teres* isolates and it is assumed that their resistance background is not entirely identical. The greenhouse virulence screening methods are valuable for *P. teres* f. *teres* resistance monitoring, especially when screening against specific virulence. However, when screening for quantitative and durable resistance, testing breeding material at early generations in multi-environmental field tests is an efficient way to gauge the effect of different environments and *P. teres* populations on the resistance expression. It is obvious that the importance of plant pathogens in barley production will not decrease in the future. There is also evidence that besides *P. teres* f. *teres,* the spot form of the pathogen *P. teres* f. *maculata* will be an important pathogen of barley in the future, which needs to be taken into account in disease resistance breeding programmes. The barley disease resistance breeding represents a palette of several pathogens, resistance genes, positive and negative interactions, test methods, and also compromises but has a common aim to develop a plant that is valuable for farmers, end-users, the environment, and the breeder.

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MTT, FI-31600 Jokioinen, Finland.
Tel. +358 3 4188 2327, email julkaisut@mtt.fi