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Phenotyping of barley (*Hordeum vulgare*) responses to spot blotch caused by different isolates of the fungus *Cochliobolus sativus*

– Searching for resistance sources in barley germplasm

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Kvantifiering och karakterisering av bladfläcksymptom orsakade av olika isolat av svampen *Cochliobolus sativus* i korn (*Hordeum vulgare*) - Sökning efter resistenskällor i kornmaterial

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Summary

Barley is one of the major crops in the world, and spot blotch, caused by the fungus *Cochliobolus sativus*, is an important disease that affects it. Spot blotch can cause major yield losses and reduce the quality of the seeds, especially in wet, warm climates. The pathogen can also infect the roots of barley plants, causing common root rot. Due to climate change and the spread of the pathogen through modern transportation, the risk of yield losses due to spot blotch has been increasing. *Cochliobolus sativus* is a hemibiotrophic fungus that is usually found in nature in its asexual form (*Bipolaris sorokiniana*).

I used the tape method, a so far little used technique for testing resistance, to try to find good sources of resistance to *C. sativus* in barley. The tested lines were provided by Nordic breeders, who previously tested them under standard field and greenhouse conditions. Different fungal isolates that had been isolated from different locations in Sweden were used in this experiment. The lesions were later scored with a 1-9 scale, and the plants were classified as resistant, moderately resistant, moderately susceptible, or susceptible to a certain fungal isolate.

It was found that there is a significant barley genotype-fungal isolate interaction, which means that barley lines may respond differently to each fungal isolate. The barley lines suggested as resistance sources were resistant or moderately resistant to all three fungal isolates. The barley lines coded as PPP112, PPP201, PPP206, PPP207, PPP250, PPP252, PPP260, PPP265, PPP269, PPP272 and PPP274 are the genotypes thus identified as potential resistance sources for breeding. I found that there is a positive correlation between lesions in the leaves with diffuse necrotic reactions and gray spots and fungal aggressiveness, suggesting possible mechanisms of infection that could be studied further. In the end, I could not compare the results from the tape method with the results provided by breeders, since the fungal isolates used in these experiments were too different.

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1. Introduction

Barley (*Hordeum vulgare* L.) is a grass species (Poaceae), and one of the main cereal crops in the world, with 144,755,038 tonnes produced in 2013 (FAOSTAT, 2017). Barley is the fourth cereal grain in world production after maize, rice and wheat. However, it is the second cereal in production in Sweden, with 96,000 tonnes of winter barley and 1,576,000 tonnes of spring barley in 2016 (Jordbruksstatistik, 2016). Spring barley grows very well in temperate areas, as it is adapted to this type of climate, and it has a short growing season. Sweden, despite the small area of land used for agriculture, is number 18 in barley production in the world (FAOSTAT, 2017).

Barley is mainly used for beer production and livestock feed in northern climates, and a small amount is used for food in some traditional porridges. The economic value of barley makes it very important to breed plants resistant to the various barley diseases. The main barley foliar diseases are leaf blotch, rust and powdery mildew.

These diseases are caused by different pathogens; powdery mildew is caused by *Blumeria graminis* f. sp. *hordei*, barley leaf rust by *Puccinia hordei*, stem rust by *Puccinia graminis*, net blotch by *Pyrenophora teres* and spot blotch by *Cochliobolus sativus* (anamorph *Bipolaris sorokiniana*) (DAF, 2016).

Cochliobolus sativus (Ito & Kurib.) Drechsl. ex Dast. [anamorph: *Bipolaris sorokiniana* (Sacc. in Sorok.) Shoem.], is the causal agent of spot blotch in barley, as well as common root rot. Although spot blotch is not the main disease in barley, it still causes yield losses, and, because it is favored by warm, wet conditions, global warming can intensify the problem. Spot blotch is one of the major diseases in warm and humid regions (Kuldeep et al., 2008), indicating that an increase in temperature due to global waming could result in an increase of spot blotch ocurrence and severity. In fact, spot blotch can cause yield losses that range between 10-100% (Clark et al., 1979; Mathre, 1997), besides causing deterioration in grain quality.

In order to combat disease and maintain yield and quality, foliar fungicides can be used. However, fungicide use causes farmers to incur into additional costs, and fungicides may have adverse effects. Besides, as spot blotch has a tendency to survive on stubble, it can persist until the following season (Singh et al, 2014). This is why developing barley varieties resistant to spot blotch is crucial in order to maintain yield and lower costs for farmers. In order to find resistance sources, several phenotyping methods can be used, *e.g.* field trials with direct inoculations via infected straw.

Environmental conditions

As was mentioned earlier, spot blotch is a major disease in warm and humid environments (Kuldeep et al., 2008). Extended periods of high humidity are associated with the development of the disease, whereas the disease does not develop during dry periods (Clark et al., 1979).

Studies done on diseases of wheat similar to spot blotch of barley, such as the one caused by the pathogen *Pyrenophora trichostoma* (Fr.) Fckl., show that the duration of the wet period significantly affects the speed of disease development. In fact, when the exposure is long (30-48 h or more), both susceptible and resistant varieties suffer from major yield losses, whereas at exposure times of 6-30 h, only susceptible varieties were affected (Hosford and Busch, 1973).

Exposure to humidity is also a crucial factor for spot blotch. In a three year spanning study (1975-1977), the disease developed insignificantly when the weather was dry, during the year 1975 and the dry period of 1977, and became more infectious during the extended wet periods in 1976 and the wet period of 1977 (Clark, 1979). In fact, the exposure to humidity is so important, that in rainy years, both the inoculated and not inoculated plots reached high levels of infection, with the exception of the control plots treated with Maneb fungicide. Humidity also speeds up disease progression; a brief, 10 day humidity period in July of 1977 (during which the plants were exposed to 6 periods of humidity ranging from 21 to 34 h), decreased the yield by 16%, even though the plants did not appear to have a significant level of infection before this period (Clark, 1979).

Temperature is another factor that significantly affects the infectivity of wheat spot blotch, as long as the humidity is high enough. The temperature range for *C. sativus* is of 12-34ºC, with an ideal temperature range of 18-30ºC (Dosdall, 1922). This means that the disease is well adapted to the temperate regions in which barley is grown.

The duration of the infection is also critical. A spot blotch epidemic 1-2 weeks before maturity produces a yield loss of 10-20%, whereas a 3-4 week long infection period causes a 20-30% yield loss, together with a 10-15% reduction in kernel weight (Clark, 1979). Since the major factor for disease development is humidity, as long as the temperatures are not much higher or lower than the range provided by Dosdall (1922). This is important, because if climate change in barley growing regions brings more extended periods of humidity, this will increase the disease infection and reduce yield.

Cochliobolus

Cochliobolus is a genus of filamentous ascomycetes that consists of around 40 pathogenic species characterised by specificity to their hosts (Condon et al., 2013). *C. sativus* was recently reclassified as a hemibiotroph (Kumar et al., 2002). Other *Cochliobolus* species are still considered as necrotrophs, but their trophic behaviour has not been extensively studied. *Cochliobolus* pathogens often act by using HSTs (host selective toxins), hence, their specificity (Condon et al., 2013). Pathogens of the *Cochliobolus* genus use effectors to modify their host to their advantage and HSTs to trigger cell death (Condon et al., 2013).

The effectiveness of plant defense mechanisms depends on the type of pathogen that attacks it. For a biotroph, which needs the plant cells to be alive in order to obtain nutrients, a hypersensitive response (HR), with localized cell death, is an effective way to stop the pathogen, as a biotroph cannot grow on dead cells

(Heath, 2001). The HR can also work against hemibiotrophic pathogens, as they also need the cells to be alive during the initial stages of infection (Deller et al., 2011). It seems that both biotrophic and necrotrophic fungi activate the same genetic mechanisms, although in the first case it leads to resistance, and in the latter, to susceptibility (Deller et al., 2011).

The biotrophic phase of *C. sativus* is limited to the invasion of epidermal cells by infection hyphae with apressoria-like structures (Kumar et al., 2002). During the necrotrophic phase, mesophyll tissue is invaded and the host cells die (Rohringer et al., 1977). The host cell death is triggered by toxins, such as prehelminthosporol, that create necrotic spots on leaves (Kumar et al., 2002). The fungus also seems to secrete fungal hydrolases that destroy cell walls (Rohringer et al., 1977).

The necrotic phase of the disease development is characterised by the production of H_2O_2 in epidermal and mesophyll cells (Kumar et al., 2002). The production of hydrogen peroxide seems to benefit the necrotic stage, as shown by the positive association between H_2O_2 production and the development of the necrotrophs *Botrytis cinerea* and *Sclerotium sclerotiorum* (Govrin and Levine, 2000). Although H_2O_2 helps stop fungal infection during its biotrophic phase, it also leads to cell death, making it advantageous to the fungus if it is able to reach the necrotic phase (Kumar et al., 2002).

This shows that defense against hemibiotrophic pathogens is very complex, since a plant trait that may be beneficiary to the plant in the earlier infection stages can promote susceptibility in the later stages. In fact, the defense mechanisms of plants are the same for biotrophs and the early phases of hemibiotrophs, and include accumulation of H_2O_2, papillae formation, and the hypersensitive response (Görlach et al., 1996; Thordal-Christensen et al., 1997; Hückelhoven et al., 1999). Some of these mechanisms can be co-opted by the fungus to help infection development during its necrotic phase (Deller et al., 2011).

Sexual and asexual reproduction

Bipolaris sorokiniana is the spot blotch morph found in nature (Kumar, 2002). In fact, *C. sativus*, the teleomorph, has so far only been found in nature in Zambia (Raemaekers, 1988), and was first discovered in the lab (Dastur, 1942). The perfect stage was induced by cultivating the fungus in the presence of opposite mating types (Dastur, 1942).

B. sorokiniana has elliptical conidia with a thick cell wall. The morphology of the conidiophores and conidiospores is what distinguishes *B. sorokiniana* from other species of *Bipolaris* (Subramanian, 1971).

Although there is quite a lot of genetic variability in spot blotch, as shown by differences in virulence, the mechanisms of its appearance are not well understood. Sexual reproduction is uncommon, because the telemorph is so rare. In fact, a genetic analysis of the *C. sativus* population in wheat reveals the presence of linkage disequilibrium, which indicates that sexual reproduction plays

no significant role in the epidemiology of the fungus (Gurung et al., 2013). One source of variability for the *B. sorokiniana* anamorph is the fusion (anastomosis) of hyphae from different conidia (Kumar et al., 2002). The main sources of variation for *B. sorokiniana* are mutations, chromosomal rearrangements and gene flow (Ghazvini and Tekhauz, 2012).

In a study by Ghazvini and Tekhauz (2012) only isolates belonging to pathotypes with low and differential virulence had significant genetic differences from the other pathotypes in North Dakota. Hence, they hypothesized that more virulent pathotypes evolved from the low virulence pathotype, which means the oldest pathotype is the most diverse one. Molecular analyses did not find significant differences between the other pathotypes, proving that the classical method of pathotype classification is not robust enough for highly virulent (more recent) pathotypes of *C. sativus.*

However, even though genetic differences between the pathotypes were slight, there was a closer association between AFLP markers and virulence than between the markers and the geographical origin (Ghazvini and Tekhauz, 2012). A study by Fordyce and Meldrum (2012) confirmed the correlation between genetic markers and virulence. However, Zhong and Steffenson (2001) did not find a significant correlation between AFLP patterns and virulence. Other studies found strong associations between virulence and molecular markers in other asexually reproducing plant pathogens (Assigbetse et al., 1994; Kolmer 2001; Liu and Kolmer 1998).

The difference in markers between non-virulent and virulent (non-aggressive and aggressive in the terminology used in the present thesis) fungal pathotypes means that the virulent and non-virulent pathotypes of *C. sativus* likely form two different phases of the fungus' evolution (Knight et al., 2010). Zhong and Steffenson (2001) found that there is more allelic diversity in *C. sativus* pathotypes with low virulence (aggressiveness), suggesting that the pathotypes of low virulence (aggressiveness) existed for a longer time (Zhong and Steffenson, 2001; Ghazvini and Tekhauz, 2012). As the co-existence of the plant and pathogen will lead to a lower level of virulence (aggressiveness) and a higher level of resistance, this would make perfect sense. A study conducted on another species of *Cochliobolus* also showed greater genetic diversity in the least pathogenic pathotypes (Welz et al., 1994).

Although virulence is often used interchangeably with aggressiveness, because these concepts are poorly defined in agronomy textbooks, they do not have the same meaning in the plant biology research area. According to Van der Plank (1975) "The concepts of virulence and aggressiveness are entirely distinct. Virulence involves gene diversity, probably through mutation. Aggressiveness may well involve enzyme dose (as distinct from enzyme diversity) and the switching on and off of enzyme action." So virulence is the ability to cause disease, a qualitative trait of the pathogen that depends on the genetic makeup of the pathogen, and it is the counterpart of vertical resistance (specific strain resistance, often controlled by R genes) (Van der Plank, 1975). This is also the common use of the word in the literature, where virulence is used to refer to a

pathogen capable of causing a compatible interaction (Andrivon, 1993). The definition of virulence and avirulence as qualitative traits seems to be widely supported in the literature, as reviewed by Andrivon (1993).

Agressiveness refers to the degree of pathogenicity, and is linked to horizontal or broad spectrum resistance (Van der Plank, 1975). This definition is also widely used in the literature (Andrivon, 1993). The fact that aggressiveness is a quantitative trait means that aggressiveness depends on the fitness of the host plant and the environmental effects as well as by the genetic makeup of the pathogen (Andrivon, 1993). This makes it much harder to know whether a difference in aggressiveness is genetic or caused by environmental effects.

Spot blotch vs common root rot

Cochliobolus sativus is a fungal pathogen that can cause different barley diseases, depending on its infection mechanism. If it infects the seed or the root via contaminated soil, it causes common root rot, a highly lethal disease (Karov et al., 2009). It can also be airborne and spread by wind, infecting leaves and causing spot blotch. Infested grains, with black points are undesirable as the quality of the seeds is lowered and the resulting seedling will be infected (Kumar et al., 2002).

The *C. sativus* fungal complex has a wide host range, as it is able to invade many cereals (*Triticum aestivum, Secale cereale, Hordeum vulgare, Hordeum murinum* and *Avena sativa*) and many other grasses (*Agropyron pectinatum, Agropyron repens, Alopecurus pratensis, Beckmannia eruciformis, Bromus erectus, Bromus inermis, Dactylis glomerata, Festuca heterophylla, Festuca ovina, Lolium perenne, Pennisetum villosum, Poa pratensis* and *Setaria virdis*) (Bakonyi et al., 1998). But the other pathotypes are not of much interest, because, unlike the barley and wheat ones, they infect plants of a lower commercial value.

Spot blotch caused by *C. sativus* is a foliar disease that can be easily confused with net blotch caused by *Pyrenophora teres* f. sp. *maculata*, and in some cases, samples that superficially looked like spot-type *P. teres* actually contained spores of *B. sorokiniana* (Arabi and Jawhar, 2004). This is why it is critical to have pure fungal isolates for inoculation, as the fungus can only be properly identified under the microscope.

In common root rot, brown to black spots appear near the base of the hypocotyl, and, as the infection progresses, the seedling turns yellow and dies, before or after emergence. This leads to a reduction of the stand density. The plants may also face dwarfing, defective emergence (not being able to break the soil), or poor kernel filling (Karov et al., 2009).

But even if the plants avoid the more severe forms of seedling infection, the disease may still appear at later stages, in the form of spot blotch. At heading time, leaf lesions may appear on the lower leaves after warm, moist weather. These lesions, typically, are oblong or lens shaped, with a dark brown colour in the center that gradually becomes green towards the edges. These lesions may

also merge and become irregular. Severely infected leaves die prematurely. The older lesions are covered with a dark layer of fungal mycelium, with an abundant production of conidia (asexual spores). Normally, the disease will spread from older leaves to newer ones via spores (Karov et al., 2009).

The relationship between the resistance to root rot, black spots and leaf blotch seems to depend on the host plant species. Conner (1990) found a weak negative correlation between resistance to black point (seed disease) and spot blotch in wheat. He also found one universally susceptible cultivar in his sample, but there were no universally resistant ones. There were also large differences between the cultivars in the resistance to each disease.

Arabi and Jawhar (2007), though, found a strong correlation between the resistance to spot blotch and to common root rot in barley. There were no universally resistant cultivars in barley either, but there was one universally susceptible one. Their results suggest that similar mechanisms are involved for resistance in leaves and subcrown internodes. As it is harder to screen for resistance to root rot than for spot blotch, breeders can save time by screening for resistance to spot blotch and obtain plants resistant to both diseases.

Phenotyping methods

Other than scoring in the field, there are two main methods of phenotyping plants for leaf resistance to *C. sativus*, both used in the greenhouse: whole plant inoculation, executed by spraying the plants with the inoculum (Arabi and Jawhar, 2004; Bilgic et al., 2005; Arabi and Jawhar, 2007; Arabi et al., 2010; Afanasenko, 2015), or by inoculating individual leaves, which can be detached (Afanasenko, 2015; Arabi and Jawhar, 2007) or kept on the plant (Arabi and Jawhar, 2010).

Arabi and Jawhar (2007) found that the infection responses were correlated between *in vitro* detached leaves and whole plant seedling or adult plant tests. Other studies also show a high correlation between *in vitro* and whole plant results (Mikhailova and Afanasenko, 2005; Tuohy et al., 2006; Afanasenko et al., 2009).

Both methods have their advantages and disadvantages; *e.g.*, the detached leaf method requires complex methodology, and a short time to develop symptoms before the leaves die. For example, Afanasenko (2015) used Petri dishes with filter paper moistened with 0.004 % of benzimidazole, and sprayed the leaves with the inoculum. Leaves were scored 4 days post inoculation. Arabi and Jawhar (2007) used a method where they removed the first leaf, sterilized it, rinsed it, and dried it. Thereafter, the leaves were placed on a 1.5 % agar with 80 mg/L of benzimidazole in sterile conditions. The leaves were incubated at 20-22°C (day) and 16-18°C (night) in the Petri dishes, with a 12 h daylength for five days.

The whole plant method is simpler, plants just need to be sprayed with the fungal conidial suspension (Arabi and Jawhar, 2004; Afanasenko, 2015). This method simulates the real conditions quite closely; however, the plants need to develop the disease (infection can be quite inefficient with this method, and therefore, the

lack of disease development). The right environmental conditions for infection development (humidity and temperature) need to be maintained in order to achieve disease. Because the infection process of *C. sativus* occurs through wounds or stomata or, in their absence, by a formation of an appresorium that penetrates the cell wall of the leaf, it is quite hard to infect plants just by spraying, if there are no wounds, as it is harder for the fungus to form an appressorium. And, where susceptible plants may get infected through just one small wound, resistant plants will be hard to infect (Arabi et al., 2011).

These methodological problems are reasons why Arabi and Jawhar (2010) developed a transparent tape method that allows for the *in vivo* inoculation of individual leaves. This method was found to be highly repeatable and correlated with the results of their seedling assay. This method also allows to infect one plant with different fungal isolates *in vivo*. And, because the fungal conidia are placed only on a a small part of a leaf and covered with transparent tape, much less conidia are needed, and the site of infection is known. The leaf is not wounded, but it is subjected to a high dose of conidia on a small area of the leaf surface, and a humid environment under the tape. However, to my knowledge, this method has not yet been used in selection for spot blotch resistance in barley germplasm.

Scoring methods

Fetch and Steffenson (1999) developed a comprehensive, graphical scale to score infections. This is done according to the size of the lesions and their quantity (where they give values 1-9 to leaves with different infection responses, IRs) and shape (where they give the spots a certain qualitative score if the shape of the spots is either typical or atypical) This method is quite popular for whole plant scoring (Arabi and Jawhar, 2004; Arabi and Jawhar, 2010; Afanasenko, 2015).

The IR 1-9 scale is not linear, and, although lesions of IR 7 are bigger than IR 6 lesions, the IR value is not an absolute size value, as this encompasses a series of continuous values. For breeding purposes, Fetch and Steffenson (1999) recommend breeders to select lines with IR values 1-5, or resistant to moderately resistant (R to MR) plants. However, selection criteria depend on the level of resistance that is desired. For example, Valjavec-Gratian (1997) classified plants with IR values 1-3 as resistant, and the rest (IR>3) as susceptible.

Fetch and Steffenson (1999) also observed some other unusual reactions, called additional infection responses (AIR). Six *C. sativus* pathotype 2 isolates induced whitish gray lesions 48 h post inoculation. Mature lesions exhibited a gray center surrounded by a light brown area. This lesion type is classified as C. Some lesions lacked a chlorotic region, exhibiting a diffuse necrotic reaction (type A). Some lesions had a chlorotic halo reaction, characterized by a small necrotic spot surrounded by a larger chlorotic area (type B). The typical response is characterized by a necrotic spot surrounded by a chlorotic region, where the necrotic spot has a bigger area than the chlorotic one (type D in my classification). Some plants also exhibited no symptoms in the infection site, which could be due to resistance or to a failed infection (escape) (type E).

Objectives

The main objective of this work is to find barley genotypes that are resistant to spot blotch and are useful for breeding new barley varieties resistant to spot blotch. Identifying the most aggressive, and thus most problematic fungal isolates is also important, as this helps to develop varieties that are resistant against them.

Five fungal isolates from distinct geographical locations in Sweden (Figure 1) were used in this study, in order to have a greater chance to find isolates distinct in aggressiveness and virulence. In the first test, all five of them were used, whereas in the second one, three selected isolates were used.

The tape method is a relatively new method for inoculating barley with spot blotch published by Arabi and Jawhar in 2010, but, to my knowledge, it has not yet been used in selection for spot blotch resistance in barley germplasm. It was used to successively test three fungal isolates on the same set of plants. The viability of this method needs to be tested in my conditions.

Hypotheses

Buddy is a cultivar that could possibly be resistant to spot blotch, because it descends from a Canadian cultivar, ''Ellice'', known for its resistance to spot blotch, while Barke is a cultivar known to be susceptible to the disease. If resistance is found in Buddy, the existing doubled haploid (DH) population that comes from a cross of Buddy with another parent will contain the resistance gene. If resistance is found in the DH population, then the location of the gene could be identified later.

In order to be sure that there are one or more resistance genes in Buddy, and go further with phenotyping the DH population, a significant difference between Buddy and Barke has to be found, with Buddy being less susceptible than Barke.

The fungal isolates collected from different locations in Sweden could be genetically different, and exhibit different levels of aggressiveness. In order to study whether the fungal isolates were indeed different, a significantly different response to the fungal isolates has to be found between the barley genotypes.

The 35 genotypes selected by Nordic breeders may contain resistance genes, as some are selected for their resistance, while others are of unknown resistance, selected because they were used as parents in MAGIC (Multi-parent Advanced Generation Inter-Cross) populations. The existence of this resistance will be verified by significantly different responses of the barley genotypes to the same fungal isolate.

The tape method is a method that could work well for selection of resistant cultivars, although it has not been used for breeding purpouses before. Its validity can be tested by comparing the results obtained with this test with the results obtained by field trials or other types of greeenhouse tests.

Figure 1. The locations in Sweden where the barley leaves with the fungal isolates were collected. From north to south, Lännäs, Vreta Kloster (VK), Bjälbo, Gotland and Ystad.

Image adapted from http://www.maps-gps-info.com/mp-se.html (2016).

2. Materials and Methods

Fungus isolation and growth

The five fungal isolates used in this study were isolated in 2011 from geographically different locations (Figure 1). The leaves suspected to be infected with *C. sativus* were placed in Petri dishes with filter paper saturated with water until spores developed. Spores were then examined under the microscope, to make sure they were typical of *C. sativus*. After this, a single spore (or a few spores) from one spot in the leaf was collected using an insect pin and placed on a Petri dish with agar media. Even though in some cases more than one spore was taken, they most likely had the same genotype, because the spores were growing together, so the isolate in the plate should be genetically uniform. The fungus was then left to grow at room temperature until it developed spores, reinoculated to a new dish and stored in the freezer. Before it was used in tests, the isolate had been transferred to new plates repeatedly, but efforts were made to minimize the number of transfers.

In order to prepare the fungal agar medium, 200 g of vegetable juice (Granini) were mixed with 2 g of $CaCO_3$ (AppliChem), after which the mix was diluted to a liter with demineralized water. Two grams of plant agar (Duchefa) were added to the mixture, after which the media was autoclaved at 120 ^{O}C for 20 minutes. The hot media was then poured into Petri dishes (9 cm in diameter) in a sterile flow bench.

In order to prepare the fungal inoculum, the fungal isolate needed to be actively growing on the plate. After thawing the original fungal isolate, a subculture of it was prepared in a flow bench using an inoculation loop. This petri dish was used to make the inoculi. The fungus grew for ca. one week, in order to develop enough spores for inoculation, and the unused plates were stored at 4 ºC for posterior use.

Fungal inoculum preparation

A drop of Tween 20 Polysorbate (Merck) was added to 100 ml of deionized water, so the inoculum could stick better to the surface of the leaf. Subsequently, five ml of this solvent were added to the fungal plate with developed spores. The spores were collected in the liquid using a spreader, after which the slurry was filtered through a mesh in order to remove mycelium. The concentration of conidia was adjusted to 2 x 10^4 conidia/ml, using a hemacytometer, following the procedure by Arabi and Jawhar (2010).

Planting

For the first round of testing, barley seeds of two different barley cultivars, cv. Barke and cv. Buddy, were used.

The seeds were placed in Petri dishes on filter paper, 20 seeds per plate. The paper was then saturated with water, after which the seeds were left to stratify at 4^{O} C for 72 hours. The seeds were subsequently left to germinate 1 day, and then

planted in soil (Emmaljunga, potting soil). The germinated seeds were transferred to plastic pots (10 cm in diameter) and grown for five weeks.

For the second experiment, 35 barley genotypes were used, plus 2 control cultivars (Buddy and Barke). Some of the 35 genotypes were suspected to have resistance to spot blotch based on field trials, while others were parents of MAGIC (Multi-parent Advanced Generation Inter-Cross) populations. The seeds were allowed to germinate for 2 days after stratification, and then planted into 1.5 L plastic pots, in soil mixed with Osmocote exact fertilizer (Hi-End, 5-6 months), in a proportion of 50 L of soil per dl of fertilizer.

The plants were grown for two weeks between each inoculation, in a 22 ºC greenhouse chamber with a 16 h/ 8 h light/dark cycle. The room was aerated at a temperature higher than 24 ºC. High Pressure sodium lamps would switch off if there was enough light (light intensity was maintained > 200 w/m^2). The plants were watered from underneath at need.

Inoculation of barley leaves (tape method)

The method used in this experiment is almost the same as the tape inoculation method used by Arabi and Jawhar (2010). Contrary to the method by Arabi and Jawhar (2010), where the leaves where undamaged, the inoculation site was damaged by 8 fine insect pins attached to a holder, in order to achieve better infection.

For the inoculation, the middle part of the leaf was damaged, and 5 µl of the fungal inoculum were placed on a tape that was in turn placed on top of the damaged site on the adaxial part of the leaf (Figure 2). The infection was allowed to develop for 9 days, after which the symptoms were scored.

For the first experiment, all five fungal isolates were used simultaneously (Figure 1), in order to compare them. Eight blocks with 10 plants in each block were used (one Barke and one Buddy plant per fungal isolate in each block). The inoculation was performed on the second leaf of 2 weeks old plants.

In the second experiment, 10 seeds per genotype were used, but germination was uneven. Forty genotypes were used initially, but only 35 had 7 or more germinated plants. Seven full blocks were possible, with one plant per block, plus an incomplete eighth block. The first fungal isolate was inoculated on the second leaf of two weeks old plants (Zadoks stage 12 or higher). The second and third inoculations were performed on the upper main shoot leaf, and on the flag leaf (Zadoks stage 20 or higher and 30 or higher) (Zadoks et al., 1974).

The fungal inoculum used for the first inoculation, VK, was the most aggressive one, isolated in Vreta Kloster (Östergötland). The second inoculum was used due to its equally high aggressiveness relative to VK and its distant geographical location (isolated in Lännäs, Ångermanland). The third inoculum used was Gotland, as there was no difference between Gotland (Gotland), Bjälbo (Östergötland) and Ystad (Skåne), and Gotland was from an island, which would

make it more geographically isolated, even though Ystad was further away from the other locations (Figure 1).

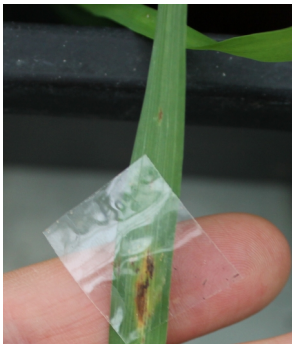

Figure 2. A leaf tested with the tape method, 9 days post inoculation.

Scoring

For the scoring of the infection response, the scale provided by Fetch and Steffenson (1999) was used, with some modifications (Figure 3). In the scale by Fetch and Steffenson, lesion size and their quantity determines the severity of the disease. But because in the tape method, only a part of a leaf is inoculated, there is a scant number of lesions, so only the size of the biggest lesion is used to determine severity of the disease.

A rate of 1-9 was assigned to an infection, depending on the infection response (IR). Additional infection responses were classified as A-E. A-C were the additional responses (AIR) specified by Fetch and Steffenson (1999). These include the diffuse necrotic reaction (A) (Figure 4), the chlorotic halo (B) and the gray center surrounded by necrosis (C) (Figure 5). The typical reaction (D) (Figure 6), or the absence of lesions (E) were also measured.

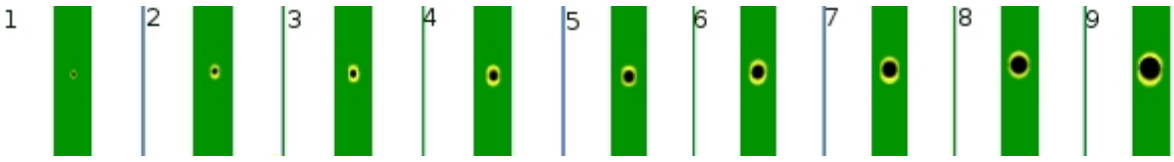

Figure 3. A slighty modified scoring scale. Whereas the scale by Fetch and Steffenson (1999) showed whole infected leaves with multiple lesions, this scale rates the degree of infection in a small section in the middle of the leaf, using the tape method. The IR score of a lesion is based on its size.

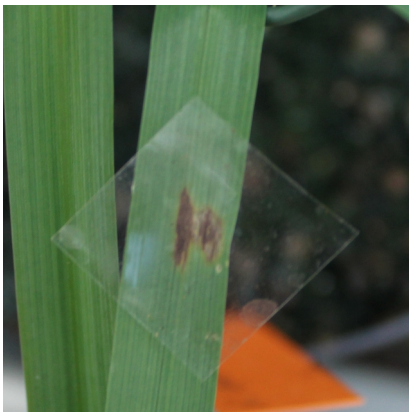

Figure 4. An example of a diffuse necrotic reaction (AIR A). The lesions are dark brown, and become greener towards the edges, with an absence of any chlorotic (yellow) halo.

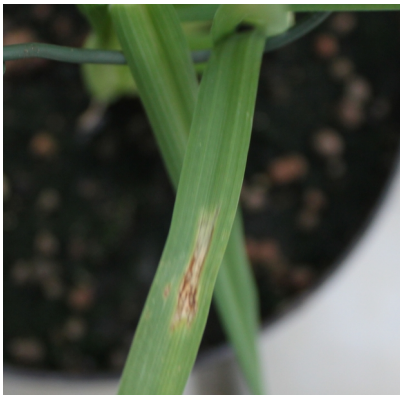

Figure 5. The whitish gray lesions that were present in some lesions, classified as AIR C. These lesions also lack chlorosis, and typically present whitish areas in the middle of a dark brown spot that becomes greener towards the edges.

Figure 6. Example of a typical lesion caused by spot blotch, characterised by a dark brown necrotic spot surrounded by a lighter area, which fades into the small chlorotic halo (yellow area), becoming green in the edges. Physiological stains look quite similar to these lesions.

Statistical analysis

There were two types of data obtained from the scoring: quantitative, parametric data (infection response, IR) and non-parametric data (additional infection response, AIR). Depending on the IR score of the lesions, the plants were classified as either resistant (R, IR score 1-3) (Figure 7), moderately resistant (MR, IR score 4-6), moderately susceptible (MS, IR score 7-8) (Figure 8) or susceptible (S, IR score 9) to a particular fungal isolate.

IR differences between cultivars, isolates and blocks were tested by ANOVA. So, after checking for normality, two two-way ANOVAs were performed with the IR as a dependent variable. In the first analysis, the factors used were the fungal isolates, the barley cultivars and their interaction, whereas in the second analysis the fungal isolates, block numbers and their interaction were used. One-way ANOVAs were also performed when appropriate. In addition to the ANOVA, a Tukey test using a p value of 0.05 was performed in order to determine which fungal isolates are the most aggressive ones and which cultivars are resistant.

In order to analyse the non-parametric data, the proportion of leaves with AIRs of a certain type was calculated for each cultivar. Then, in order to see how the AIR correlated with the degree of resistance/susceptibility, the coefficient of correlation was calculated for the proportion of AIRs in relation to the average IR using a linear model. This analysis was performed separately for each round of inoculation. All statistical analyses were performed using R 3.3.2.

Figure 7. Example of a plant that is resistant. This leaf would have an IR score 2, due to the tiny size of the lesions (less than a mm in diameter).

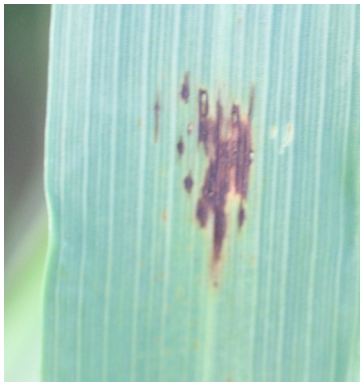

Figure 8. Example of a moderately susceptible plant. This wound would have an IR score 7, due to its size (more than 3 mm in width and around 1 cm in length).

Greenhouse and field data from Finland

The seeds I used come from a PPP in which Marja Jalli from the LUKE center in Finland is participating, and I got access to their *C. sativus* scoring data. They sprayed the plants in the greenhouse with a spore suspension that had a concentration of 40,000 conidia / ml), using a mixture of the same isolates as the ones they used in the field. The temperature there changed between the day (21 – 23 ºC) and the night (15 ºC). Humidity was kept at 100 % for 24 h after inoculation, and for 2 h daily. Scoring was performed 10 days after inoculation using the scale by Fetch and Steffenson (1999).

Field tests were also performed in Finland (Jokioinen), using a hill plot planting system, with 20 seeds of each genotype in each plot in two replicates. 100 kg N/ha were used as fertilizer. At BBCH 12-13 (two to three leaf stage) the plots were inoculated with dried infected barley leaves, at a rate of 3 g of inoculum per plot. The inoculum, prepared in the greenhouse in the winter, is a mixture of 4-6 Finnish isolates chosen based on their aggressiveness and virulence. There was no irrigation due to the rainy summer, and Ariane S herbicide was used. The scoring was performed twice, using the NIAB 0-100% scale, after flowering and at the milk ripening stage.

3. Results

Pilot experiment

Five different fungal isolates from distinct locations within Sweden (Figure 1) were tested on barley cultivars Buddy and Barke. This experiment was made to determine how to proceed further. The responses to infection were scored twice, at day 9 and day 16, to see whether the values changed significantly with time. A one-way ANOVA showed no significant change in the infection response (IR) values with time ($F = 0$, $p = 1$, $df = 1, 154$). And, as it is more convenient to do the scoring after 9 days, I decided to do that in the second experiment. There were no significant differences between the blocks either, as determined by a one way ANOVA ($F = 0.63$, $p = 0.43$, $df = 1,76$).

There were no significant differences in a two-way ANOVA in the interaction between barley genotype and fungus at day 9 ($F = 1.13$, $p = 0.347$, $df = 4, 68$), nor for the barley genotype ($F = 2.12$, $p = 0.15$, $df = 1, 68$), although the effect of the fungal isolate was significant ($F = 7.5$, $p < 0.0001$, $df = 4, 68$). Therefore, the IRs of Barke and Buddy were pooled and analysed by a Tukey test ($p = 0.05$) in order to see the effect of the fungal isolate.

There were only three pairs of fungal isolates that gave significantly different IR responses, as determined by a Tukey test: VK-Bjälbo, VK-Gotland, and VK-Ystad. VK was not significantly different from Lännäs, Lännäs was not significantly different from the other four, and Bjälbo, Gotland and Ystad were not significantly different from each other. The fungal isolate VK, from Vreta Kloster, gave the highest mean IR, while Lännäs was second, Ystad third, Gotland fourth and Bjälbo fifth (Figure 9). So, as can be seen in Figure 9, there were two groups that were distinctly different according to the Tukey test, a and b, except that isolate Lännäs occurred in both of them.

The additional infection responses (AIRs), which can be a diffuse necrotic reaction (A) (Figure 4), a chlorotic halo (B), a gray center surrounded by necrosis (C) (Figure 5), the typical reaction (D) (Figure 6), or the absence of lesions (E) were also measured. The isolate VK was special in that it was the one that had most of the lesions characterised by a diffuse necrotic reaction (A) and that it was the only one that generated a chlorotic halo (B) (Table 1).

The correlation analysis showed that there was a positive relationship between the proportion of the total number of leaves (either 7 or 8 per barley genotype) with diffuse necrotic reaction (A) in response to a certain fungal isolate and the average IR for that isolate ($r = 0.73$, $df = 3$), the chlorotic halo (B) and the IR ($r = 0.86$, $df = 3$), and a negative relationship between the proportion of leaves with lesions that have a gray center (C) and the IR ($r = -0.26$, $df = 3$), the typical lesions (D) and the IR ($r = -0.64$, $df = 3$), and the absence of lesions (E) and the IR ($r = -0.40$, $df = 3$). However, none of these relationships were statistically significant ($p > 0.05$). The results are from pooled Barke and Buddy responses, because their responses were not significantly different from each other.

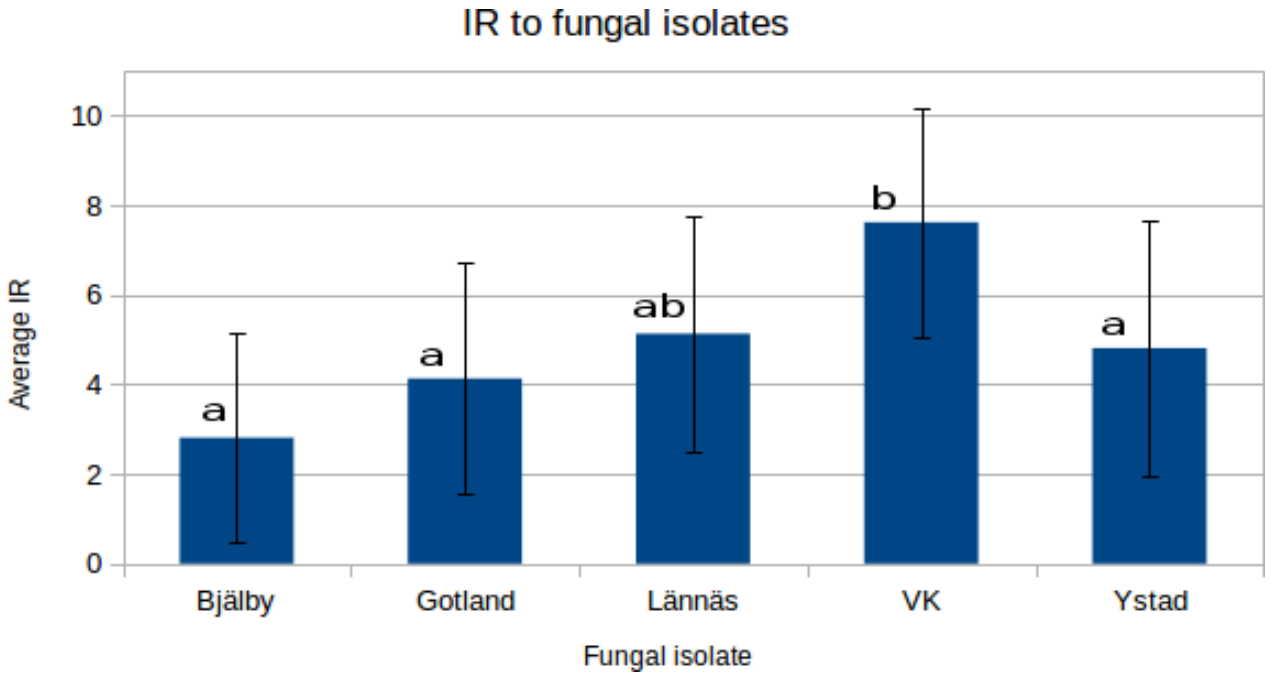

Figure 9. Average IR values from inoculations of Barke and Buddy with all five fungal isolates. Error bars indicate standard deviation.

a, b: a indicates a group of isolates, distinct from the isolates marked with b, according to a Tukey test.

Table 1. Number of leaves with specific additional infection responses (AIR) on the barley plants (the results for Barke and Buddy are pooled, as there was no significant difference) in response to each of the five different fungal isolates.

Fungal isolate	**AIR**					**Leaves counted**
	A	**B**	**C**	**D**	**E**	
Bjälbo	2	0	0	11	3	16
Gotland	0	0	2	13	0	15*
Lännäs	1	0	0	14	0	15*
VK	7	3	0	5	1	16
Ystad	0	0	1	12	2	15*

*In some cases, the total number of plants scored was lower than 16 due to problems during scoring (broken leaves, fallen tape, etc.).

Genotype selection

In the second experiment, 35 barley lines were used, plus Barke and Buddy. Many of the genotypes from the group of 35 were potentially resistant to spot blotch, while others, of unknown resistance to spot blotch, were used as parents in MAGIC populations. The plants were inoculated with the fungus from Vreta Kloster first, Lännäs in the second round, and Gotland in the third round. There was significant physiological staining due to aging on the plants in the last round of inoculation (Figure 10), so in the third round there are many data points missing, since leaf discoloration hindered scoring of symptoms.

In order to test whether there were interactions between the barley genotype and the fungal isolate, a two-way ANOVA analysis was performed, and a significant interaction was observed (Table 2).

Since there was interaction between the fungus and the barley cultivar, the resistance levels were analysed separately for each fungal isolate by a one-way ANOVA. Barley cultivars showed significantly different levels of resistance to VK ($F = 4.5$, $p < 0.001$, $df = 36, 251$), Lännäs ($F = 6.4$, $p < 0.001$, $df = 36, 250$) and Gotland ($F = 2.2$, $p < 0.001$, $df = 36, 254$). As it was determined that the differences were significant, a Tukey test (significance level $p = 0.05$) was performed to identify the pairs of genotypes that were significantly different. The result of the Tukey test showed that there were only significant differences between some of the most susceptible genotypes and some of the most resistant ones. These genotypes were grouped and marked with an a (the resistant ones) or a b (the susceptible ones) in Table 3.

The Tukey test showed that 9 cultivars were significantly more resistant to VK than the most susceptible ones, two cultivars were also significantly resistant to Lännäs and two to the Gotland isolate (Table 3). The genotypes for breeding were mostly selected among these ones, even though they may have been moderately resistant to the other isolates. However, if they were resistant to one isolate according to the Tukey test but susceptible to another one (IR score > 6), they were discarded, because consistent resistance is desirable. So the cultivars chosen for being resistant to at least one isolate and moderately resistant to the other fungal isolates are: PPP112, PPP201, PPP206, PPP207, PPP252, PPP260, PPP265, PPP269, PPP272 and PPP274 (Table 3). In addition, cultivar PPP250 was also chosen, since it was consistently resistant to all three fungal isolates (IR ≤ 4), even though its IR levels were not significantly different from the values of non-resistant barley cultivars. Again, Buddy had an IR lower than Barke, but the difference was non-significant (Table 3).

The data obtained from Finland was compared to the data obtained by the tape method in this experiment. The greenhouse method they used was quite different from ours. They sprayed whole plants, and used a spore concentration that was double the one I used; they had a cycle of day/night temperatures, whereas I maintained it constant; several isolates were mixed and humidity was kept at 100 % for 2 h per day, whereas I did not control it at all.

The field experiments in Finland are different from the experiments I performed by their very nature. In the field, light, temperature, or pathogens are much harder to control. In addition, the experiment in Finland used different fungal isolates, and a different scoring scale (a NIAB 0-100% scale); they also inoculated at a different stage and scored at different stages, compared to the tape method.

There were two scorings done in the field in Finland, and the correlation of those results with the tape method results is analysed for both scorings ($r = 0.05$, $p = 0.39$, $df = 32$ and $r = 0.02$, $p = 0.24$, $df = 22$), and shown to be not significant. The data obtained in the greenhouse in Finland and the average results of the tape method were not correlated either ($r = 0.23$, $p = 0.15$, $df = 21$). There was a significant correlation between the first field scoring and the greenhouse experiment in Finland, but not for the second field scoring ($r = 0.46$, $p = 0.014$, $df = 21$ and $r = 0.32$, $p = 0.07$, $df = 21$).

But even in the cases when the same method is used to infect the plants, there is a weak correlation or none at all between the results when different fungal isolates are used. Among the three rounds of inoculations performed using the tape method, there was only one pair that was significantly correlated (VK-Lännäs, $r = 0.28$, $p = 0.046$, $df = 35$).

Additional analyses were performed with the IR data to see whether there were environmental effects or errors (shown by the analysis of the effect of blocks) and whether the fungal isolates differed in the level of aggressiveness in this experiment too. In order to check whether there was an environmental effect, a two-way ANOVA was done with IR as dependent data, and block-fungal isolate interaction was measured. This interaction is significant ($F = 15.11$, $p < 0.0001$, $df = 2, 827$), which means that the different blocks had different effects depending on the fungal isolate, the enviromental conditions at the time of inoculation, or scoring accuracy. But this was controlled by analysing the data for each fungal isolate separately.

A Tukey test based on a one-way ANOVA for the fungal isolate (F value $= 17.3$, p value < 0.0001, $df = 2, 830$) shows that only the pairs Lännäs-Gotland and VK-Lännäs are significantly different (p value $=0.05$). VK remains the most aggressive one, while Gotland is second and Lännäs third.

A one-way ANOVA with each separate round of experiments confirms that there was a difference between the blocks in all rounds (VK, $F = 4.5$, $p < 0.0001$, $df = 7, 280$; Lännäs, $F = 6.4$, $p < 0.0001$, $df = 7, 279$; Gotland, $F = 2.2$, $p = 0.021$, $df = 7, 281$). The Tukey test ($p = 0.05$) shows the pairs of blocks that where significantly different for each experiment (for VK, blocks 1, 2 and 3 are significantly sifferent from block 8; for Lännäs, blocks 1, 2, 5, 6, 7 and 8 are significantly different from block 4, and blocks 1 and 3 are significantly different from each other; for Gotland, blocks 1 and 7 are significantly different from each other).

Most pairs in VK and Gotland that are significantly different are from blocks inoculated by different researchers. Blocks 1-4 were inoculated by one person,

while blocks 5-8 were done by another person. These differences could be due to slight differences when the inoculum was applied, to the slightly different environment in each block (water, light), or to mistakes in scoring.

These differences between the blocks will make it more difficult to find the effect of the genotypes. Another problem of the experiment is that there is a significant Genotype x Round interaction. This means that each round has to be analysed separately, as has been done in Table 3.

But IR was not the only thing measured. Additional infection responses (AIRs) were measured in a qualitative way, where lesions were classified as A (diffuse necrotic reactions) (Figure 4), B (chlorotic halos), C (a gray center surrounded by necrosis) (Figure 5), D (the typical reaction) (Figure 6) and E (absence of lesions).

There was a significant correlation between certain AIRs and the resistance/susceptibility response. There were positive correlations between VK IR and the proportion of lesions with a diffuse necrotic reaction (A) ($r = 0.75$, $p < 0.0001$, $df = 35$), Lännäs IR and lesions characterised by a gray center surrounded by necrosis (C) ($r = 0.49$, $p < 0.01$, $df = 35$), and negative correlations for VK IR and the typical response and the absence of lesions (E) ($r = -0.48$, $p < 0.01$, $df = 35$ and $r = -0.49$, $p < 0.001$, $df = 35$), Lännäs IR and the absence of lesions (E) ($r = -0.71$, $p < 0.0001$, $df = 35$), and Gotland IR and the absence of lesions (E) ($r = -0.43$, $p < 0.01$, $df = 35$).

The cultivars previously selected for their low IR show quite a high proportion of leaves without lesions (Table 4). In addition, cultivars PPP216 and PPP251 also show a high proportion of E type responses to isolate Lännäs.

Table 2. ANOVA table of the interaction in IR values between barley cv. and fungal isolate.

Factors	**df**	**SS**	**MS**	**F**	**Pr(>F)**
Fungal isolate	2	277	138.70	21.58	7.90e-10 ***
Barley cv.	36	879	24.41	3.80	2.96e-12 ***
Fungal isolate *x* Barley cv	72	1136	15.78	2.46	2.49e-09 ***
Residuals	722	4641	6.43		

***Significance level for $p < 0.0001$

Table 3. The table shows the mean infection rate of the 37 barley lines in response to each fungal isolate, as well as the total mean response. The data from Finland was obtained from Marja Jalli and is based on the first field scoring and on greenhouse testing. The last row indicates the average standard deviation for each data set.

a, b: **a** indicates that the cultivars belong to the same group and are significantly different from the cultivars marked with a **b**, as determined by a Tukey test.

Barley cv.	**VK**	**Lännäs**	**Gotland**	**Average**	**Finland Field 1**	**Finland Greenhouse**
PPP252	2.1 **a**	4.1	3.7	3.3	2.5	1.3
PPP269	2.6 **a**	2.9	3.8	3.0	3.0	1.3
PPP206	2.6 **a**	2.5	4.7	3.2	3.5	
PPP260	2.8 **a**	4.5	5.9	4.5	4.0	1.5
PPP112	2.9 **a**	3.4	5.3	3.8	3.5	
PPP265	3.0 **a**	3.5	4.3	3.6		2.3
PPP207	3.1 **a**	4.5	5.4	4.3	6.0	
PPP274	3.3 **a**	3.0	2.0 **a**	2.8	2.0	1.3
PPP272	3.5 **a**	4.4	5.6	4.4	4.0	2.0
PPP128	3.5	3.1	6.0	4.2	2.5	
PPP250	4.0	2.5	3.6	3.4	3.0	2.3
PPP256	4.1	6.5	5.6	5.4	3.0	2.3
PPP208	4.3	4.4	7.1	5.2	4.0	
Buddy	5.0	4.1	5.4	4.9		
PPP278	5.0	2.4	8.4 **b**	5.3	3.0	1.0
PPP201	5.4	1.4 **a**	2.8 **a**	3.3	4.0	
PPP257	5.5	5.5	7.0	6.0	3.0	1.8
PPP262	5.8	3.5	5.0	4.7	2.5	1.7
PPP268	5.8	2.3	2.5	3.8	2.5	2.8
PPP204	5.9	6.9 **b**	4.8	5.8	2.5	
PPP251	6.0	1.8	5.1	4.3	2.0	2.5
PPP209	6.0	4.1	6.6	5.4	4.0	
PPP253	6.1	2.9	6.7	5.2	4.0	2.8
Barke	6.3	5.0	5.0	5.3		

PPP259	6.3	4.1	4.8	5.2	3.5	2.3
PPP216	6.3	1.6 **a**	6.1	4.7	5.0	
PPP267	6.4	2.6	7.0	4.8	2.5	1.8
PPP273	6.8	6.3	4.1	5.7	2.0	1.8
PPP254	7.0	4.1	3.9	4.8	5.0	2.8
PPP205	7.0 **b**	5.1	4.8	5.6	4.0	
PPP255	7.1 **b**	2.9	2.8 **a**	4.2	6.0	3.0
PPP129	7.1 **b**	5.8	5.9	6.3	5.0	
PPP261	7.5 **b**	5.3	6.2	6.3	3.0	2.8
PPP123	7.6 **b**	4.5	4.0	5.5	4.0	
PPP276	7.8 **b**	6.9 **b**	6.7	7.1	3.0	2.5
PPP279	7.9 **b**	6.6 **b**	4.4	6.3	3.0	2.8
PPP264	8.1 **b**	3.5	5.0	5.6	3.5	2.8
Std dev.	2.8	2.9	2.8	2.9	0.7	1.3

Table 4. Proportion of leaves for each barley genotype that exhibited an absence of symptoms in response to at least one fungal isolates in 1 plant out of 8.

Barley cv.	**VK**	**Lännäs**	**Gotland**
PPP250	0.125	0.125	0.250
PPP251	0.000	0.375	0.000
PPP252	0.429	0.286	0.143
PPP260	0.125	0.125	0.000
PPP201	0.000	0.375	0.250
PPP265	0.000	0.333	0.143
PPP216	0.000	0.500	0.000
PPP269	0.286	0.250	0.000
PPP206	0.375	0.500	0.333
PPP112	0.000	0.125	0.125
PPP207	0.125	0.250	0.125
PPP274	0.000	0.000	0.167

Figure 10. Physiological staining in 2-month old barley plants that were beginning to flower. Photo taken in the greenhouse.

4. Discussion

Pilot experiment

The first experiment was used as a pilot in order to test whether it was worth pursuing tests of a set of doubled haploid (DH) lines from a cross with Buddy as a potential resistance source and to select three fungal isolates out of five, according to their level of aggressiveness, for further tests.

However, Buddy was not significantly different from Barke, a cultivar that was known to be susceptible to spot blotch in the tape assay. Although the average IR values for Buddy were lower, it is not worthwhile pursuing the testing of the existing DH lines if the differences are not significant. There was also no spatial variability (no environmental effects), as proven by the one way ANOVA for blocks. This means that if there was a significant difference between the barley genotypes, it should have been possible to detect.

For further tests, the selection of the fungal isolates was based on aggressiveness and geographical location. First, VK was chosen, as it was significantly more aggressive than the others. Then Lännäs was chosen, as it was the second most aggressive one, as well as geographically far from VK, and therefore likely to be different (Figures 1 and 9). And, because Gotland, Ystad and Bjälbo were not significantly different in aggressiveness, it was decided to select isolate Gotland for the third round of inoculation as it is from an island and therefore from a geographically isolated place, with a higher probability of having different virulence genes. It is still possible that fungal isolates spread through seeds or wind, but that is less probable because it is farther, and the use of certified seeds prevents the introduction of new pathogen strains to a field.

The reason why aggressiveness was the main factor for selecting the fungal isolates was that fungal aggressiveness is important in order to detect differences in susceptibility or resistance in the barley genotypes. The three fungal isolates were chosen for their high levels of aggressiveness and distinct geographical origin.

Genotype selection

Barley genotype-fungal isolate interactions in IR indicate that the fungal isolates have different virulence spectra. This interaction was significant for the second experiment (Table 2). This means that different isolates need to be tested on my barley genotypes in order to find lines that are resistant to a broader range of fungal isolates from the region where the newly bred cultivars will be released.

As expected when there is an interaction, there are cultivars that have different levels of resistance depending on the fungal isolate, *e.g.* PPP216 (resistant (R), to Lännäs, and moderately susceptible (MS), to both VK and Gotland), or PPP255 (R to Lännäs and Gotland and MS to VK) (Table 3). But there were also more consistently resistant genotypes.

Previous studies confirm the barley genotype-fungal isolate interactions found in this study, *e.g.*, the studies by Arabi and Jawhar (2004); Ghazvini and Tekhauz (2007); Gurung et al. (2013).

Quite a few studies have found QTLs for resistance to *C. sativus* in barley (Afanasenko et al., 2015, Bilgic et al. 2005; Bilgic et al., 2006). This would indicate that the resistance to *C. sativus* is a quantitative trait. When the spot blotch in wheat was studied, the majority of fungal isolates showed intermediate levels of aggressiveness, indicating that the resistance of wheat may be governed mainly by minor genes, or there could be different virulence factors in the fungal population (Gurung et al., 2013).

In some specific interactions, clear gene-for-gene interactions play a major role, and in some cases, a few major genes give resistance to spot blotch in some specific cultivars, either just at the seedling stage (Bilgic et al., 2005) or at both seedling and adult stages (Bilgic et al., 2006). But the general trend indicates that the different pathotypes are not different because of just one simple mechanism. The existence of complex genetic mechanisms is proven by the much more complex statistical models required to define the pathotype groups (Ghazvini and Tekhauz, 2008).

The complexity of the mechanisms that govern the resistance make it very critical to find genotypes that are resistant to more than one fungal isolate, because hopefully they will contain several different resistance genes, or they may have genes for horizontal, more general resistance. PPP112, PPP201, PPP206, PPP207, PPP250, PPP252, PPP260, PPP265, PPP269, PPP272 and PPP274 (Table 3) are the cultivars found relatively resistant to all three isolates tested in this study.

PPP265 is a well known robust and durable resistance source for *C. sativus*. Several QTLs have been localized in its genome by creating DH populations with other, susceptible barley genotypes (Bilgic et al., 2005). The other genotypes are not known for their resistance to this disease, but they are resistant to some other foliar diseases of barley. PPP201 and PPP206 are resistant to *Fusarium* head blight (McCallum et al., 2004). PPP260 is a source of resistance to powdery mildew, net blotch and scald (El Ahmed et al., 1981), and PPP207 is the spring barley cultivar that had a yield 7% higher than the highest yielding spring barley variety in the UK in recent years. This cultivar also has a good straw and is resistant to mildew, *Ramularia* and yellow rust (Shepherd, 2015). These barley genotypes are good sources for breeding, especially PPP207, which is an elite variety. This line could be crossed with PPP265, well known as a durable source of resistance.

Another objective of this study was to test how well the results of this test correlate with field tests, which are the ones that are similar to real agronomic situations. In order to test whether there was a correlation, I used data from field trials and whole plant greenhouse tests in Finland (data kindly provided by Marja Jalli in LUKE, Finland). There was no correlation between my test results and those results. There was a correlation between one of the field recordings in Finland and

the greenhouse data from Finland. The absence of correlation between my data and the data from Finland can have several explanations; it may be that the results from the method I use is different because single leaf infection is different from whole plant infection, or because wounding the leaf by punching makes it easier for the fungus to infect, or because punching the leaf and thus wounding it and giving a concentrated dose of inoculum on a single spot of the leaf activates defense responses more effectively.

It could also be because when the second and third round of inoculation were done using the tape method, it was done on plants which had leaves already infected with the fungus. This could possibly induce a response, making them more resistant or more sensitive.

The field test uses a different scale than the one I did, but that should not matter for the calculation, because if there is a correlation, it will remain there, even if the scale is another. They used plants at a different stage, and they allowed the infection to develop for a longer period. This would not matter if the infection response depended just on the virulence of the fungal isolate, a qualitative trait; however, the IR also depends on the aggressiveness of the fungus. And, as previously discussed, fungal aggressiveness can change if the plant is at a different physiological state, as well as other environmental factors (humidity, temperature...).

It could be that my test does not effectively simulate real life situations. But the problem is that I can not know which of the methods does, because the tests in Finland used a mixture of other fungal isolates, and, as we saw in the results, the type of fungal isolate used greatly influences the infection response obtained. In fact, even when the tape method is used with different isolates, most results are different. So in order to know whether the test is really similar to field results in terms of resistance, a field test with these barley genotypes should be performed using the same isolate. One problem with doing that in the field is that, although we can artificially infect the plants with a certain fungal isolate, we cannot prevent wild fungi from attacking the plants and thus changing the results. The same physiological stages should also be tested.

Environmental effects

There are significant differences in IR value between some of the blocks, even though they are composed of the same genotypes (except for block number 8, which has less genotypes). This could be due to errors or differences during inoculation, as different researchers applied the inoculum. Or it could be due to errors during scoring, where a more tired researcher makes more mistakes in the end. But the pattern of differences was not the same for the different fungal isolates, so it is probably due to small individual effects of everything previously mentioned plus slight environmental differences in the greenhouse.

Fungal isolates

The analyses of the data for the three fungal isolates show that the resistance levels differ significantly between VK and Lännäs. Besides, they have a different effect on some differential genotypes (Table 3). For example, PPP216 is resistant (R) against isolate Lännäs, and moderately susceptible (MS) to the other two fungal isolates. PPP278, for example, is also R to Lännäs, S for Gotland, MR for VK. PPP255, though, is R to Lännäs and Gotland and MS to VK.

The different barley genotype responses to these fungal isolates could mean that the isolates are different in virulence, although this should be tested on the differential lines established in other studies. The method currently used for distinguishing pathotypes of *C. sativus* uses 12 differential lines of barley (Ghazvini and Tekhauz, 2012).

If proven to be a difference in virulence, this would mean that there is a significant genetic difference between the fungal isolates, because, according to Ghazvini and Tekauz (2012), there is a close association between genetic diversity and virulence. That would in turn mean that the lines selected are resistant to a variety of *C. sativus* genotypes from the region where future offspring of the lines, if they are to be used as potential sources for breeding, will be released.

There are other barley lines in the set that are resistant to one isolate but susceptible to others. Because breeders want reliable and durable resistance sources, these lines should be discarded as sources for resistance breeding, unless they are known to carry rare resistance genes that can be pyramided with others.

AIR

The correlation between the IR and a certain type of AIR means that the AIR type is related to the plant response mechanism. Diffuse necrotic reactions and grayish areas surrounded by necrosis are associated with susceptibility, whereas the typical reaction or the absence of lesions are associated with resistance.

The association between resistance and the absence of lesions is very logical. Even though in some cases the absence of lesions may be due to an escape (a fallen tape, weakened inoculum), in most cases this will be due to resistance. Escapes were prevented to some extent by punching the leaves with the insect pins. In no case was the fungus completely avirulent on these genotypes. However, more frequent failure to infect means that the fungus would struggle to infect a field, because if the infection is completely prevented, then it cannot spread. So even if the plant is later vulnerable due to environmental effects, like climate, wet periods, etc., the absence of a compatible interaction means that the fungus cannot produce spores or spread to the field, giving field resistance.

The selected cultivars show quite a high number of incompatible responses. PPP216 and PPP251, which also show this incompatible response for a certain

isolate could be of interest for breeders in combination with other resistance sources.

I have not found explanations for the nature of the AIRs. Because they are associated with susceptibility, the diffuse necrotic reactions and gray lesions surrounded by necrosis probably indicate that the fungus is more aggressively necrotrophic. My hypothesis is that, when the lesion is a diffuse necrotic reaction, this is due to the fact that the biotrophic phase of the fungus is very short and aggressive, and the chlorosis is not visible. I think that the chlorosis is linked to the biotrophic stage of the fungus, because the chlorotic cells are still alive, although they have lost the chlorophyll.

As for the infections characterised by whitish gray centers (Figure 6), they may be due to the rapid growth of the fungus, which leads to wateriness in the tissue. The rapid growth is an indicator of aggressiveness.

Conclusions

The study of the pathogen is critical for developing plants that show durable resistance against it, because depending on from where the virulence originates (homologous recombination, mutation) the resistance may be more or less lasting.

Even though the asexual reproduction of the fungus makes it more difficult for really virulent pathotypes to predominate in the population (because mutation is rare, and sexual reproduction helps recombine the genes), modern transportation can spread the disease very quickly once it appears. If infected seeds are transported, thus crossing geographic barriers, it will not be enough to breed cutivars resistant to just local pathotypes of the disease. This is why it is important to breed for plants that are as broadly resistant as possible, and to use certified seeds, in order to prevent bringing diseased seeds to the field.

The knowledge on the mechanisms behind resistance may also help to find more different types of resistance genes. The study of the molecular mechanism behind the different types of additional infection responses could be a good way to study the pathogen and the responses it generates in the plant.

The tape method seems to be a convenient, easy way to test multiple isolates on a single plant, as long as the pathogen does not induce a systemic response. This means that this method could be used in cases when there are few seeds. But in order to do that, this method should be first verified by using the same fungal isolates in the field as in the inoculation.

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