ASSOCIATION MAPPING OF RESISTANCE TO COMMON ROOT ROT AND SPOT BLOTCH IN BARLEY, AND POPULATION GENETICS OF *COCHLIOBOLUS SATIVUS*

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Title

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By

SANJAYA GYAWALI

The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

ABSTRACT

Gyawali, Sanjaya; Ph.D.; Department of Plant Pathology; College of Agriculture, Food Systems, and Natural Resources; North Dakota State University; January 2010. Association Mapping of Resistance to Common Root Rot and Spot Blotch in Barley, and Population Genetics of *Cochliobolus sativus*. Major Professors: Dr. Tika B. Adhikari and Dr. Shaobin Zhong.

Cochliobolus sativus (Ito & Kurib.) Drechsl. ex Dast. [anamorph, *Bipolaris sorokiniana* (Sacc. in Sorok.) Shoem.] is an important fungal pathogen, which causes common root rot (CRR), spot blotch (SB) and black point/kernel blight in barley in North America. Use of genetic resistance has been effective against SB presumably due to presence of durable resistance in North America. However, recently emerged virulence groups have overcome durable resistance in barley. Additionally, the genetics of resistance to CRR is poorly understood. Therefore, the objectives of current studies are multifaceted. To identify the sources of resistance to CRR, 824 contemporary barley lines from the USDA-CSREES Barley Coordinated Agricultural Project (CAP) were evaluated for resistance to CRR under natural inoculum pressure in the field during 2006 - 2008. Additionally, resistance to CRR (n = 384 lines) and SB (n = 386 lines) was also evaluated in the greenhouse. The results indicate that only 0.9% of breeding lines showed resistance to CRR in field experiments during 2006-2008. None of the genotypes showed resistance to virulent isolates in greenhouse experiments. *Hordeum jubatum* sp. *jubatum* (Accession # CGN13044) showed 12% CRR severity against highly virulent isolates. Therefore, this accession can be used as a potential source of resistance to CRR in the future. In the SB experiment, only 0.5% of the 386 genotypes showed resistant responses to isolate 4008. The barley line

NDB112 with durable resistance to SB also showed a highly susceptible reaction to this isolate. To map QTL for CRR and SB resistance, association mapping was employed using the CAP06 population (*n* = 384), CAP07 population (*n* = 384) for CRR resistance and the CAP06 population (*n* = 384) for SB resistance. In all association analyses, 3072 single nucleotide polymorphism (SNP) markers were used. The results suggest five QTL resistance to *CRR, CRR-3H-28-51, CRR-5H-180-195, CRR-6H-30-64, CRR-6H-91-97*, and *CRR-7H-50-86* were detected in chromosomes 3H, 5H, 6H, and 7H. Two QTL resistance to spot blotch, *Rcs-1H-84.6* and *Rcs-2H-106-122* were identified in chromosomes 1H and 2H, respectively. These QTL didn't coincide with any of the QTL reported earlier and confer resistance to virulence group 7.7.3.6. To investigate the virulence spectrum of *C. sativus* isolates collected from North Dakota (ND), 12 barley genotypes were inoculated with 12 virulent *C. sativus* isolates on both root and leaf. The results suggest that different virulence groups for CRR and SB diseases exist in the pathogen population. To understand the population structure of *C. sativus* populations collected from Australia and the USA (ND), 289 single-spore isolates were analyzed for amplified fragment length polymorphism (AFLP) using three AFLP primer combinations. Moderate to high gene diversity (*H* = 0.27 - 0.35) and high genotypic diversity (*GD* = 1) within *C. sativus* populations indicate occurrence of genetic recombination other than sexual in *C. sativus* populations. The moderate to high population differentiation (*Gst* = 0.196), moderate multi-locus linkage disequilibrium ($\overline{r_d}$ = 0.046 - 0.118), and low gene flow (*Nm* = 2.0) suggest the occurrence of different populations of *C. sativus* in the field.

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CHAPTER 1. GENERAL INTRODUCTION

North Dakota (ND) is the leading barley producing state in the United States, and growing over one third of the total barley production in the country (USDA, 2008). Among various biotic impediments for barley cultivation, common root rot (CRR), caused by *Cochliobolus sativus* and *Fusarium graminearum* and *Fusarium culmorum*, and spot blotch, caused by *C. sativus*, are important diseases in the Upper Midwest of the USA. *C. sativus* is an important and frequently isolated root pathogen in barley in North America (Mathre, 1982). Stack et al. (1991) estimated an annual average yield loss of 9.5% was due to CRR in barley in ND. Piening et al. (1976) reported 10% annual yield loss in barley in Canada due to CRR as early as the 1970's. Clark (1979) reported that spot blotch can cause as high as 35% of yield loss in susceptible cultivars under favorable condition. Ghazvini and Tekauz (2004) found that virulent isolates can cause as high as 30% yield reduction in barley. Spot blotch has been successfully managed by deploying durable resistance of NDB112 in the Upper Midwest of the USA and Canada for the last four decades (Steffenson, 1996). However, in recent years, virulence groups of *C. sativus* have overcome durable resistance of NDB112 in Canada (Ghazvini and Tekauz, 2008) and pose potential threat to successful barley cultivation in the region. In contrast, there is a slow progress on resistance to CRR in barley, although resistance to CRR is an effective strategy to improve barley germplasm against CRR and reduce yield losses. This can be attributed in part to lack of knowledge on the virulence spectrum of *C. sativus* as well as the effectiveness of resistance genes against the virulence spectrum of the pathogens causing CRR.

Little is known about the genetics of resistance to CRR in barley. Furthermore, breeding for resistance to CRR caused by this pathogen requires information on population structure and virulence spectrum of pathogen to develop robust screening methods for resistance to CRR and introgression of resistance gene(s) into adapted barley cultivars. *C. sativus* also causes black point (kernel discoloration leading to poor grain quality and reduced germination). Past studies showed that the host has different genes conferring resistance to CRR, spot blotch, and black point in barley and wheat (Conner, 1989; Almgren et al., 1997). However, there is limited information regarding the population structure and virulence spectrum of *C. sativus* causing these diseases. It is also unclear whether the *C. sativus* causing CRR and spot blotch have similar or different population structures. Zhong and Steffenson (2001) studied the genetic diversity of *C. sativus* based on worldwide collection of spot blotch isolates. They reported lower diversity of pathotype 2 as compared to pathotypes 0 and 1. They also suspected the low genetic diversity of pathotype 2 was due to recent origin of this pathotype from genetic exchange among other pathotypes. Most studies indicate that the resistance to CRR is attributed to be quantitative nature. Cohen et al. (1969) indicated that resistance to CRR was conditioned in a quantitative fashion. Kutcher et al. (1994) reported that resistance to both CRR and spot blotch was quantitiatively inherited in barley. They reported quantitative nature of resistance to CRR in two crosses of barley namely Fr926 x Deuce and Virden x Ellice. Bailey and Wolf (1994) also reported that the heritability of CRR resistance was medium and they estimated at least three genes were involved in resistance. In the past, bi-

parental crosses and recombinant inbred lines (RILs) had been widely used to map the resistance gene(s) and quantitative trait loci (QTL). Recent advancement in molecular markers has made it possible to use barley population for mapping resistance gene using association genetics. Association genetics is a population based survey to identify the association between phenotypic traits and genetic markers based on linkage disequilibrium (LD) (Lander and Schork, 1994) and doesn't require a population derived from bi-parental cross. The released cultivars, breeding lines, landraces and wild relatives can be used as the mapping population for association genetics. Tomassini et al. (2007) found that the resolution of map developed by association mapping was finer than a bi-parental based RIL mapping population for resistance to Stagonospora nodorum blotch in wheat. In barley, LD extends from 1 cM to beyond 10 cM based on mapping population used. In advanced breeding lines, LD extended on an average of 10 cM genome-wide in barley (Kraakman et al., 2004) however, the LD pattern has been reported variable across the barley genome. Furthermore, LD pattern was affected by the population structure. Rostoks et al. (2006) found different pattern of LD in two- and six-rowed barley. Steffenson et al. (2007) found LD decayed within 1 cM genome-wide in wild barley but was variable in different genomes. Kraakman et al. (2006) developed a high resolution map of *Rph3* (major gene) and *Rphq2* (QTL) resistant to *Puccinia hordei* in barley. Stracke et al. (2007) mapped Bymovirus resistance loci in barley using association mapping and reported that the LD extended faster than the recombination can break it. Furthermore, they suggested that the negative selection for susceptibility to Bymovirus led to the rapid decay of

LD in susceptible lines. Steffenson et al. (2007) used wild barley (*Hordeum vulgare* spp. *spontaneum*) to map stem rust resistant genes against various races using association mapping in barley. They reported novel QTL to stem rust resistance in wild germplasm. Rostoks et al. (2006) reported that with the current knowledge on LD in barley, several economically important traits can be scanned at genome level using association genetics. In the Barley CAP project, we aimed at establishing the relationship between CRR resistance gene(s) and single nucleotide polymorphism (SNP) markers using LD.

In summary, *C. sativus* is an important pathogen causing CRR and spot blotch diseases in barley in the Upper Midwest of the USA. The slow progress against this pathogen has been partly attributed to poor understanding of population structure in ND and lack of knowledge of resistance sources to *C. sativus* in barley. The emergence of new virulence groups has overcome the durable resistance in barley line, NDB112, and posed potential threat to barley cultivation in the region. Therefore, objectives of this study were to:

i. evaluate the released barley cultivars, breeding lines from four breeding programs of the Upper Midwest of the USA for resistance to CRR and spot blotch,

ii. employ association mapping to identify and map the CRR and spot blotch resistance gene(s)/QTL in barley lines, and

iii. determine the virulence pattern and population genetics of *C. sativus* causing CRR and spot blotch diseases in barley in North Dakota.

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CHAPTER 2. LITERATURE REVIEW

The Pathogen: *Cochliobolus sativus*

Taxonomy, population biology, and phylogenetic relationships of *Cochliobolus* species

Cochliobolus belongs to the phylum Ascomycota, class Dothideomycetes and family Pleosporaceae (Alcorn, 1983). Historically, *Cochliobolus* was classified under *Helminthosporium* (Alcorn, 1988) but Shoemaker reclassified its asexual stage into *Bipolaris*. *Bipolaris* and *Curvularia* are known anamorphs of *Cochliobolus* (Alcorn, 1983). The phylogenetic studies based on ribosomal DNA and other gene sequences indicated *Cochliobolus* has two distinct groups (Berbee et al., 1999). Group one composed of *Cochliobolus* species that have *Bipolaris* anamorph (relatively longer conidia) whereas group two *Pseudocochliobolus* composed of *Bipolaris* and *Curvularia* (smaller sized conidia). The closest relatives of *Cochliobolus* are *Setosphaeria* (anamorph *Exserohilum*) and *Pyrenophora* (anamorph *Drechslera*). *Cochliobolus sativus* is a heterothallic fungus (Alcorn, 1988) and has characteristic of slightly curved conidia. Although both *MAT1-1* and *MAT1-2* mating type idiomophs have been reported in *Cochliobolus*, the *MAT1* locus is responsible for the mating system in this fungus (Turgeon and Yoder, 2000). *C. sativus* is characterized by globose ascomata having a long cylindrical neck where ascospores are helically coiled filiform in asci (Alcorn, 1983). The conidia of *B. sorokiniana* (asexual stage) have typical spore germination at both

polar cells. Prehelminthosporol, Helminthosporol (host non selective) and Sorokinianin (host selective) are some of the important toxins produced by *Bipolaris sorokiniana* and cause huge losses to cereal crop production (Kumar et al., 2002). The chemical composition, information on molecular markers and/or gene(s) responsible for the toxins indicated that these genes evolved independently in *Cochliobolus*. The evolution of host non-selective toxins and some other enzymes such as Xylanase (Emami and Hack, 2002) indicated that the common ancestor of *Cochliobolus* acquired the gene before evolution of different species and these genes were conserved in many *Cochliobolus* species.

Common root rot, disease cycle, and symptoms

The fungi *C. sativus* (Ito & Kurib.) Drechsl. ex Dast., and *Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae* (Schwein.) Petch), and *F. culmorum* (W. G. Sm.) Sacc., have been reported to cause common root rot (CCR) in wheat and barley (Wiese, 1987). Among these pathogens, *C. sativus* [anamorph, *B. sorokiniana* (Sacc. in Sorok.) Shoem.], is an important and the most frequently isolated root pathogen in barley in North America (Mathre, 1982) and is widely distributed in South Asia (Kumar, 2002), Australia (Wildermuth, 1986; Whittle, 1992), and central Asia (van Leur, 1997). *C. sativus* also causes spot blotch in leaf and black point in seed (Mathre, 1992; Kumar et al., 2002). *C. sativus* has wide host range and cause diseases in wheat, barley, oat, and rye (Tinline, 1988). Therefore, this pathogen is considered a serious impediment to successful cultivation of barley.

The fungus survives in soil or crop debris (Chinn et al., 1962). The thick-walled conidia are the primary source of infection (Wiese, 1987). The disease cycle of CRR initiates with the infection on young seedling either by inoculum on seed or infection originating from conidia overwintering in soil near the seedling. Initially dark brown lesions appear on the outer layer of coleoptile and/or the first leaf sheath base near the collar region. These lesions in susceptible plants may coalesce into long areas forming necrotic brown tissues (Wiese, 1987). In severe conditions, the entire seedling may collapse. In moderately resistant cultivars, the seedlings may survive but the growth and development of the plant may reduce. *C. sativus* can penetrate the host tissue either directly through the epidermis or through the wounds or natural openings in the roots and coleoptile. The fungus forms an appresorium prior to penetration (Huang and Tinline, 1976; Clay et al., 1994). An infection peg is formed beneath the approsorium and fungus enters into the host tissue from epidermis, cortex through to endodermis resulting in disintegration of tissues in host cells. The fungus colonizes the host tissues and multiplies if the host is susceptible. The most common symptom of CRR is seen on the sub-crown internode (SCI) and roots (Sallans and Tinline, 1965). Initially, there are small oval shaped brown necrotic lesions in seminal roots, crown roots and SCIs. As the plant matures, these lesions may coalesce and the entire SCI becomes dark brown to black. In some cases, the lesion may extend to the plant crown resulting to crown root rot symptoms. In severe cases, the disease can reduce tillers resulting into plant death prior to heading. The pathogen overwinters on stubble and dead plant parts in the soil.

Spot blotch, disease cycle, and symptoms

Spot blotch, caused by *C. sativus* is an important foliar disease of small grains specially barley and wheat. It is reported everywhere barley is grown. The high humidity and 22 - 30° C temperature favor spot blotch development (Mathre, 1982). These environmental conditions are commonly found in the prairie regions of the Upper Midwest of the USA, including the red river valley, and central provinces of Canada during growing seasons. These environmental conditions predispose susceptible cultivars for disease development and cause damages on grain yield. However, spot blotch is less problematic under dry and cooler weather conditions. The inoculums of *C. sativus* may be seed- or soil-borne or may arise from the existing mycelia on crop residues. Therefore the air-borne conidia aroused from soil or crop residues or produced from wild grasses serves as source of inoculums in early spring. Foliar infections may occur in multiple cycles under favorable conditions. The disease symptoms are typically apparent in barley as oval necrotic lesions surrounded by chlorotic yellow halos (Fetch and Steffenson, 1994) on leaves. The infection on leaves results in reduced photosynthetic areas and under severe cases whole leaves are blighted which lead to premature senescence of the leaves as well as death of the plant.

Population structure, genetic diversity, and evolution

The genetic structure of fungal population describes the amount and distribution of genetic diversity within and among the populations (McDermott and McDonald, 1993). The genetic variability and evolution of plant pathogenic fungi has got more attention recently for understanding the host - parasite interaction

and disease management through host resistance. Understanding of population structure has important implication in crop improvement and disease management. Information on population structure helps design the deployment of resistance gene(s) as a disease management tactic.

Traditionally, physiological specializations of different *Cochliobolus* species to cultivated crops have been reported. Ghazvini and Tekauz (2008) used vegetative compatibility and virulence spectrum of *C. sativus* to reveal the population structure of Canadian isolates causing spot blotch. Recently, molecular markers have been used to understand the population structure of fungal pathogen. Valjavec-Gratian and Steffenson (1997), Zhong and Steffenson (2001) studied the population structure of *C. sativus* using virulence pattern of spot blotch on barley cultivars and AFLP markers. They further analyzed the genetic diversity of *Cochliobolus* species from wheat, barley, oat and corn. The population structure based on AFLP markers indicated that *C. sativus* is highly diverse. They also reported three pathotypes (0, 1, and 2) based on the differential reactions of 22 isolates on ND5883, 'Bowman' and NDB112 and pathotype 2 was dominant in North Dakota and had lower allelic diversity compared to 0 and 1.

The population structure of *C. sativus* causing spot blotch has been compared with other related species. Oliveira et al. (2002) studied the genetic diversity of *Cochliobolus* and *Bipolaris* species from rice, wheat and maize using PCR-RFLP and RAPD markers. They reported that genetic diversity was less within species compared to inter-specific diversity. Emani and Hack (2002) reported that the Xylanase genes (*XYN11A* and *XYN11B*-enzymes responsible for degrading cell

wall of plant) were conserved in *C. sativus, C. heterostrophus*, *C. spicifer* and *B. sorghicola.* They suggested that the gene was present in their common ancestors before evolution of *Cochliobolus* species. A similar pattern of Prehelminthosporol toxin was observed in *C. sativus, C. carbonum, C. victoriae,* and *C. setaria* by Apoga et al. (2002). They suggested that the gene responsible for Prehelminthosporol was acquired by common ancestor of these species before the evolution of host specific species.

The current understanding of population structure of *C. sativus* causing CRR is less compared to spot blotch in barley. It is unclear whether or not the virulence groups of *C. sativus* from leaves are similar to root. More importantly, the population structure of *C. sativus* causing CRR in North Dakota has not been examined yet.

Yield losses due to common root rot and spot blotch in barley

Both *C. sativus* and *Fusarium* spp. cause CRR disease in barley and reduce yield losses significantly. These pathogens attack other cereals such as wheat and oat. Specifically, CRR is a problem in the Upper Midwest regions of the USA and central provinces of Canada. The disease has also been reported from Australia and Asia. Piening et al. (1976) reported 10% annual yield loss in barley in Canada due to CRR. They reported as high as 26% yield loss in Saskatchewan in 1972. Stack et al. (1991) estimated 9.5% annual yield loss in North Dakota but the loss reported as high as 37% in few field plots sampled during 1980-1982. In other studies in Canada, Tinline and Ledingham (1979) found that CRR disease severity was higher in barley compared to wheat cultivars during 1973 - 1975. They

reported as high as 28% yield loss in Bonanza and Olli (barley cultivars) in Saskatoon in 1973. Furthermore, they found significant correlation between disease severity and grain yield in both wheat and barley. Smiley et al. (2005) reported 9.5% yield loss in wheat and barley in the Pacific Northwest of the USA due to crown rot caused by *Fusarium* species. They found that crown rot reduced yield as much as 35% in winter wheat in commercial fields. Clarke (1979) reported as high as 30% yield losses due to spot blotch in Canada. Ghazvini and Tekauz (2004) reported that yield losses could be as high as 30% in the susceptible barley cultivars.

The host resistance in barley

Host resistance limits pathogen growth on and/or in plants and is manifested by hypersensitive reaction (HR) (Klement, and Goodman, 1967) or programmed cell death (Dangel et al., 1996) at the site of infection. Host resistance involves series of biochemical interactions between the host and pathogen. In a review, Flor (1971) explained that pathogen avirulence (*Avr*) gene interact with resistant (*R*) gene in the host to confer resistance reaction and argued for gene-for-gene hypothesis. The host resistance is induced by the plants' ability to recognize the specific elicitors produced by the pathogen. These elicitors are produced directly or indirectly by the avirulence gene in the pathogen. In the hosts, the elicitors are recognized by the specific receptors produced by the hosts. Any failure to recognize the elicitors by the hosts lead to the susceptibility.

Johal and Briggs (1992) cloned the first plant resistance gene *Hm1* conferring resistance to race 1 isolate of *C. carbonum* in maize. The gene *Hm1* encodes for

NADPH-dependent HC toxin reductase which inactivates the HC-toxin (a cyclic tetra peptide) produced by the pathogen. Martin et al. (1993) confirmed that resistance gene *PTO* in tomato and *AvrPto* in *Pseudomonas syringae* pv. *tomato* follows classic gene-for-gene interaction. Several other *R*-genes such as *RSP2* resistance to *P. syringae* pv. *tomato* in *Arabidopsis* (Bent et al. 1994), and *N* gene resistance to TMV in tobacco (Whitham et al., 1994), *Cf-9* gene resistance to *Cladosporium fulvum,* and L^6 gene resistance to *M. lini* in Flax have been cloned.

In contrast to resistance conditioned by major genes, quantitative variation in disease resistance is controlled by many genes with small effects called quantitative trait loci (QTL). In barley, major genes such as *Rcs5*, *Rcs6* and QTL (*Rcs-qtl-7H-2-4*, *Rcs-qtl-7H-7*, *Rcs-qtl-3H-4-6*, *Rcs-qtl-3H-11-12*, *Rcs-qtl-4H-4-6*, *Rcs-qtl-1H-6-7*, *Rcs6-QTL*) have been mapped in different populations and provided resistance to spot blotch in barley (Steffenson et al., 1996, Bilgic et al., 2005, Bilgic et al., 2006, Yun et al., 2005, Yun et al., 2006). The combination of a major gene of NDB112 with QTL present in other resistant lines provided durable resistance to spot blotch in the Upper Midwest of the USA. Quantitative traits are controlled by many genes with smaller effects and they are highly likely influenced by the environmental factors. Bernardo (2008) has reviewed how the understanding, mapping and uses of QTL have been changed over last 20 years due to the advent of molecular markers. He added that in a resistance breeding program, breeder aims at selecting favorable alleles for QTL in a line and accumulating the small effects together to confer durable disease resistance.

Marker-assisted selection has enhanced the breeders' ability to select and accumulate the small favorable effects in desired breeding lines.

Common root rot resistance

Piening et al. (1976) and Stack et al. (1991) suggested that research efforts needed to focus on developing resistant cultivars to CRR in barley. The slow progress on crop improvement for resistance to CRR in barley can be attributed to following reasons. First, CRR is caused by a complex of multiple fungi with the most frequently isolated pathogens of *C. sativus* and *Fusarium sp.* from root and SCIs. Second, the earlier studies indicated that the nature of CRR resistance appeared to be quantitative in nature. Third, the population structure and virulence spectrum of *C. sativus* and *Fusarium* species, that cause CRR in barley are poorly understood.

Arabia and Jihad (2002) reported that AECS 71, 'Golf', 'Furat-2' and AECS 76 barley cultivars resistant to CRR. AECS 71, and Furat-2 were bred in Syria whereas Golf was bred in England. 'Arizona', an American cultivar, was reported to be the most susceptible cultivar. Cohen et al. (1969) suggested that CRR resistance has been attributed to many genes and quantitative inheritance. Bailey et al. (1988) found at least three genes conditioned resistance to CRR in H-105 / H-186 and H-159 / H-186 crosses in barley. Kutcher et al. (1994) and Bailey and Wolf (1994) reported that the heritability of CRR resistance was medium and conditioned by quantitative genes in barley. Bailey and Wolf (1994) studied genetics of CRR in 'Argyle' as resistant and Melvin as a tolerant cultivar in their bi-parental cross. Kutcher et al. (1994) reported that Fr926-77 and Ellice were

resistant to CRR. They suggested that the nature of resistance was quantitative and three genes could have been involved in conditioning CRR resistance in these genotypes.

Bailey et al. (1988) also found that the resistance to CRR in wheat lines (H-105 and H-159) was conditioned by three genes. Dubcek (1984) evaluated barley cultivars/lines in four locations in 1980 and 1981 in Canada. He found consistently low CRR severity in 'Bêtes', 'Fairfield' and 'Bonanza' whereas Galt was reported to be susceptible. Tinline and Ledingham (1979) reported Bonanza as resistant followed by 'Conquest' and Bêtes as moderately resistant to CRR in barley. 'Galt' was reported as the most susceptible barley cultivar. The sources of resistance to CRR in cultivated barley have been reported in few cultivars; however, this information is very scanty in wild relatives of barley.

Although the genetics of CRR resistance in wheat and its wild relatives have been explored in the past, the information on genetics of resistance to CRR in barley is still lacking. Until now, none of the gene(s)/ QTL for resistance to CRR has been mapped in barley chromosomes. Li et al. (2009) mapped crown rot (CR) resistant QTL, *Qcrs.cpi-3H* at 59.7 - 71.8 cM of chromosome 3H caused by *Fusarium* ssp. Common root rot resistance has been assessed for 14 *Triticum* species by Harding (1972). The result indicated that *T. aegilopoides* and *T. monococcum* were the most resistant followed by tetraploids. Hexaploid bread wheats were the most susceptible to *B. sorokiniana* compared to diploid wheat. In another study, the cytogenetic analysis of *C. sativus* resistance in several aneuploids of bread wheat cultivars revealed that CRR resistance is conditioned

by a gene located in 5B chromosome of 'Apex' (Larson and Atkinson, 1970). They found that the susceptibility was dominant thereby resistance was conditioned by a recessive allele in 5B chromosome.

Spot blotch resistance

Spot blotch caused by *C. sativus* is considered one of the most devastating leaf diseases of barley in the North America especially in the Upper Midwest of the USA. Spot blotch can cause as high as 35% of the yield losses on susceptible cultivars (Clark, 1979). In the Upper Midwest of the USA, the genetic resistance of NBB112 has been successfully deployed in six-rowed malting barley and spot blotch has been successfully managed for last four decades (Steffenson et al., 1996). The management of spot blotch using durable resistance of NDB112 is considered one of the best examples of disease management in cereals. The resistance of NDB112 has been effective to all pathotypes reported earlier in the region. Both major gene and QTL provided resistance to spot blotch in barley. Major genes, *Rcs5* and *Rcs6*, and other QTL such as *Rcs-qtl-7H-2-4*, *Rcs-qtl-7H-7*, *Rcs-qtl-3H-4-6*, *Rcs-qtl-3H-11-12*, *Rcs-qtl-4H-4-6*, *Rcs-qtl-1H-6-7*, *Rcs6-QTL* were mapped in different chromosomes of barley (Steffenson et al., 1996, Bilgic et al., 2006, Bilgic et al., 2005, Yun et al.. 2005, Yun et al., 2006). Furthermore, the virulence spectrums of *C. sativus* causing spot blotch has been investigated extensively in the past (Fetch and Steffenson, 1994; Valjavec-Gratian, and Steffenson, 1997; Zhong and Steffenson, 2001). However, the evolution of new virulence groups have been reported from Canada which have overcome resistance in NDB112 (Ghazvini and Tekauz, 2008). This indicates that there is a

need to monitor population structure and virulence groups of *C. sativus* on a regular basis to develop and deploy durable resistance in future.

Interrelationship of resistance to CRR, spot blotch, and black point

C. sativus can cause CRR in root, spot blotch in leaf, and black point in grain in barley and wheat. It is unclear whether or not the same pathogen can cause CRR, spot blotch and black point in barley and wheat. Mathre (1982) documented that the resistance to CRR and spot blotch were not associated. Conner (1990) did not find strong correlation between CRR, spot blotch and black point in wheat but he found a weak correlation between spot blotch and black point. A similar result was reported by Almgren et al. (1997) in barley. They found correlation neither in pathogen aggressiveness nor cultivar reactions between CRR and spot blotch in barley. However, a recent study conducted by Arabi et al. (2006) indicated that there is a strong correlation between CRR and spot blotch resistance in barley. They suggested that the resistance to CRR and spot blotch could involve similar defense mechanisms. Ghazvini and Tekauz (2007) suspected that there could be tissue specificity of CRR and aerial diseases (spot blotch and black point). This indicated that the interrelationship of CRR, spot blotch and black point in barley needs further investigation.

Mapping populations and principles of genetic mapping in self-pollinated crops

The individuals of a species furnish the mapping population in plants. These individuals are derived from crosses among the related individuals where their parents differed in traits to be studied in population. In barley, various mapping

population such as F_2 population, backcross population, recombinant inbred lines, and double haploid populations have been used to map traits of interest. These mapping populations are generated by bi-parental crosses where the parents differ in traits that are to be mapped.

F_2 population. Among various mapping populations, the F_2 population is most commonly used and the basis of this population is Mendelian genetics. To develop F_2 population, the parents with contrasting mapping traits are crossed and F_1 plants are derived. From self-pollination among F_1 plants, F_2 individuals are generated which segregate for traits that are different in parents. The degree of polymorphism in F_2 plants can be assessed for phenotypes such as disease resistance or at the genotypic level using molecular markers. Bailey et al. (1988) used a bi-parental cross to evaluate CRR resistance genes in barley. They found at least three genes conditioned resistance to CRR in H-105 / H- 186 and H-159 / H-186 crosses. The F_2 individuals are outcome of single meiosis and therefore single recombination event. One of the disadvantages of using F_2 population is that only one meiosis is available to generate map for traits of interest with low mapping resolution. The size of F_2 mapping population for gene/QTL mapping has been reported to 150-200 individuals in many studies; however, Alpert and Tanksley (1994) used more than 3400 individuals to develop high resolution map of fruit weight loci in tomato.

Backcross population. Backcross population is used to study the specific gene from one parent in the background of another parent. The population is derived by repeated crossing of F_1 hybrid to the recipient parent (also called

recurrent parent) whereas another parent becomes the donor. By repeated backcrossing, the unlinked genes of donor parent are removed from the background of recipient due to segregation whereas the linked genes are separated due to increased number of recombination. With each round of backcrossing, the genome of donor parent is decreased by 50%. Backcrossing is also used to generate near isogenic lines. Both backcross and near isogenic lines are used in mapping gene(s) with increased resolution compared to F_2 and recombinant inbred line population.

Recombinant inbred lines (RILs). Recombinant inbred lines (RILs) are developed by single seed descent method. In each generation the individuals derived from F_2 population is selfed to get the homozygous lines. In crop like barley, the self pollination within six generation can generate almost homozygous lines. The RILs is preferred over other mapping population such as F_2 for mapping genes/QTL due to the number of meiosis (recombinations) increased before the RILs reach to homozygous (Burr and Burr, 1991). Therefore, RILs population can generate higher resolution of maps than F_2 population even for tightly linked genes. Also, RILs are nearly homozygous lines and are stable. Therefore, these can be used as permanent resources for mapping genes. Due to these reasons, RILs is widely used by researchers as mapping QTL.

Double haploid. Double haploid (DH) lines are completely homozygous and thereby only one allele is available for all genes. DH are produced from haploid lines in laboratory. In barley, haploid lines either occur spontaneously or can be induced in laboratory through anther culture or by chromosome elimination using

H. bulbosum. After haploids are obtained, haploid cells can be diploidized using colchicine treatments to obtain diploid lines. The colchicine blocks the formation of spindle fibers during meiosis thereby doubling the chromosome number in the cell. The cell lines are then grown in culture medium to regenerate completely homozygous plants. DH lines are the permanent sources for mapping population and can be generated in very short period of time compared to backcross or RILs. DH lines are ideal crossing parents for generating mapping population because of complete homozygosity. The disadvantage of DH is that it can reduce the genetic variability during regeneration of materials in culture medium. In barley, selective elimination of entire chromosome set by using *H. bulbosum* (diploid wild barley) as one of the wide crosses has been demonstrated in generating successful double haploids (Kasha and Kao, 1970). During embryo development of F_1, the chromosome set of *H. bulbosum* is preferentially eliminated resulting haploid embryos of *H. vulgare* (cultivated barley). These embryos are rescued and diploidized by colchicine treatments. Anther culture is also widely used in barley for double haploid production. Heun et al. (1991) developed RFLP map of barley based on population derived from double haploid population. They developed DH population from anther culture of F_1 of cultivars 'Proctor' × 'Nudinka'. Chao et al. (1989) used DH population including other mapping population to map gene conditioning agronomic traits such as disease resistance, vernalization in wheat. Most recently, isolated microspore culture (IMC) is popular among the researchers (Davies and Morton, 1998). In IMC method, the microspores are separated from

the anther and microspores are cultured in medium. The method allows researchers to increase large number of DH compared to other methods.

Mapping population for association genetics. In contrast to bi-parental mapping, association genetics makes use of contemporary germplasm for mapping gene(s)/QTL. These germplasms could be advanced breeding lines, released cultivars, landraces, and wild relatives of cultivated crops. Kraakman et al. (2004) used spring barley to map gene(s)/QTL for yield and yield components. Rostoks et al. (2006) used released cultivars from different European barley breeding programs for association mapping and discussed the potential of association mapping at genome-wide scanning and candidate gene levels. Two- and six-rowed barley as well as spring and winter barley were used in their mapping studies. Malysheva-Otto et al. (2006) used world-wide collection of barley germplasm to understand the population structure and LD pattern in barley germplasm which included 953 barley cultivars representing Asia, Europe, North America, Middle East (fertile crescent) and Africa. Steffenson et al. (2007) used wild relatives of barley mostly *Hordeum vulgare* ssp. *spontaneum* (L.) for mapping multiple disease resistance gene(s) using association genetics. Starke et al. (2007) used 127 barley germplasm which consisted of landraces, released cultivars, and wild relatives to mapping Bymovirus resistance in barley using association mapping.

Linkage disequilibrium (LD) and association mapping (AM)

Traditionally, the gene(s) controlling a trait is mapped using bi-parental crosses. The segregating populations are developed to study the genetics of trait of interest.

There are number of limitations of bi-parental population in mapping gene(s). First, for each trait, that is to be mapped, require development of separate populations. Second, there is limitation of number of crossing over available in meiosis in bi-parental crosses. Recombinant inbred populations and isogenic lines have been used in the past to increase the number of recombinations but one has to wait several years until these populations are developed. Collins and Morton (1998) stated that AM provides a means for mapping disease resistance genes that has considerably higher resolution compared to linkage methods. AM has overcome these limitations and emerged as a powerful approach in gene(s)/QTL mining (Gupta et al. 2005; Flint-Garcia et al. 2003). AM is a population based survey to identify the phenotypic trait and marker relationship based on LD (Lander and Schork, 1994). Therefore, association genetics is based on LD which is non-random association of alleles at two marker loci or two genes/QTL or between gene/QTL and a marker locus (Gupta et al. 2005). The number of recombination that occurred during the evolutionary history of the mapping plants permits to map the gene(s) precisely and enhance the resolution of the map. LD measures the association between 2 alleles, which arises more often than can be accounted for by chance, because those alleles are physically close on a chromosome and infrequently separated from one another by recombination. The degree of LD in a population determines the resolution of the map. AM has been successfully used in mapping complex traits in humans (Lander and Schork, 1994). In recent years, it has been extended to plants and is being applied to *Arabidopsis* (Nordborg et al. 2001), maize (Yu et al. 2006; Remington et al. 2001), rice (Garris et al. 2005),

wheat (Tommasini et al. 2007), and barley (Caldwell et al. 2006; Rostock et al. 2006; Hays and Szucs, 2006). Linkage disequilibrium in wild barley, *Hordeum vulgare* ssp. *spontaneum* decays within 300 bp while it is 5-10 cM or 212 kb in elite barley (Caldwell et al. 2006; Morrell et al. 2005). Therefore, the faster the LD decay, the higher the marker density required to develop fine mapping. With the current knowledge on degree of LD decay in cultivated and wild barley, it can be used to scan the whole genome for marker trait associations (including disease resistance) but one needs to account the population structures to reduce false positive results (Steffenson et al. 2007; Kraakman et al. 2006; Rostoks et al. 2006; Garris et al. 2003; Yu et al. 2006; Lander and Schork, 1994). The false positives arise from the differences in allele frequencies in mapping populations. Garris et al. (2003) used association genetics to map bacterial leaf blight resistant gene *xa5* in rice and reported that population structure can increase the false positives of marker-trait associations. In recent years, statistical approaches such as Q- or P-matrix (structure) and K-matrix (Kinship or relatedness) have been used as covariate in the model to account and reduce the number of false positives in AM. Whole genome scanning can be performed to develop medium resolution map of candidate gene regions whereas intralocus scanning would be efficient in a population such as wild relatives of barley having higher degree of LD decay (Caldwell et al. 2006). With medium density markers, whole genome scanning would be effective to locate the candidate gene(s) whereas intralocus AM is possible with high density genetic map.

Tomassini et al. (2007) reported that 390 fold higher marker resolution can be achieved by AM of the Stagonospora nodorum blotch (SNB) resistance gene (*QSng.sfr-3BS*) in winter wheat using 44 varieties compared to QTL mapping using 240 RILs. They found LD decay in chromosome 3B was within 0.5 cM in 44 varieties compared to 30 cM in 240 RILs in a bi-parental cross. Kraakman et al. (2006) used the AM approach to confirm *Rph3* (a major gene against *Puccinia hordei*) and the QTL *Rphq2* for prolonging latency to *P. hordei*. Their finding strongly suggested the existence of a barley yellow dwarf (BYD) resistance or tolerance gene on chromosome 2, linked to SSR marker HVM054 which has previously been reported. Recently, Steffenson et al. (2007) used AM to scan various resistance sources in the whole genome of wild barley and wheat. They identified several candidate gene regions for leaf, stripe and stem rust resistance in barley and wheat.

Phenotyping of common root rot and spot blotch resistance in barley

Traditionally, barley lines have been phenotyped under natural inoculum pressure in the field for both CRR and spot blotch resistance. Tinline et al. (1975) used 1-4 scale to estimate CRR severity on sub-crown internodes (SCI) of barley. In barley, the root rot assessment is done as surrogate assessment of SCI. The disease severity on primary root and SCI is reported to be highly correlated (Wildermuth, et al., 1992). The following disease severity scale on SCI has been popularly used; 1 = 0 - 25% discoloration on SCI due to lesion, 2 = 26 - 40% discoloration on SCI; 3 = 41 - 75% discoloration on SCI due to lesion, and 4 = >75% discoloration on SCI due to lesion on SCI. van Leur (1991), and Arabi and

Jawhar (2002) modified the severity scale based on discoloration and lesion on SCI due to pathogen to adapt disease assessment under artificial inoculation in growth chamber. Disease rating is being done using 0-5 scale where 0 = immune, no lesion); 1= HR (Highly resistant), small light grown lesion covering 1-10% of the SCI; 2 = R (Resistant), light brown lesion covering 11 - 25% of SCI; 3 = MS (Moderately susceptible), light brown/black lesions covering 26 - 40% of the SCI; 4 = S (Susceptible) Black lesion covering 41 - 70% of the SCI; and 5 = HS (Highly susceptible), black lesion covering 71 - 100% of the SCI. In more precise assessment, CRR severity can be estimated as a percentage of lesions on SCI without using any scale (Horsfall and Barratt, 1945). Spot blotch severity was assessed using 0 - 9 scale of Fetch and Steffenson (1994).

Genetic markers and genotyping in plant breeding

The genetic markers are two types, morphological and molecular markers. The morphological markers has been used by plant breeders for decades as they are the phenotypic expression of gene(s) and are less reproducible in range of environments. This is because the morphological markers are influenced by environment and other genetic factors such as gene interaction and pleiotropic effects. Furthermore, it is difficult and/or time consuming to distinguish the heterozygous from homozygous using morphological markers. In contrast, molecular markers overcome these problems and offer potential use of molecular markers in plant breeding. Most molecular markers are the neutral site of DNA sequence polymorphism.

The objective of plant breeding is to improve various agronomic traits to sustain or increase crop productivity and quality. Crop improvement started with the selection of desired traits from natural populations, followed by the introgression of desired traits (genes) from one plant to another using bi-parental cross. Conventional plant breeding aimed at selecting superior recombinant among segregants which is laborious and time consuming. The recent advancement of plant breeding includes the use of molecular markers to map gene(s) of economically important traits and enhance the efficiency and/or efficacy of crop improvement techniques.

In recent years, many marker systems such as restriction fragment length polymorphism (RFLP), amplified length fragment polymorphism (AFLP), simple sequence repeats (SSR), randomly amplified polymorphic DNA (RAPD), Diversity Array Technology (DArT), and single nucleotide polymorphism (SNP) have been developed and used for mapping genes of agronomic importance in barley. RFLP was very first markers developed to map the human genome (Botsein et al., 1980) but has been extensively used to develop genetic maps in plant species later. In barley, RFLP was used for mapping genetic diversity (Saghai Maroof et al., 1994). The advantage of RFLP is that it is co-dominant marker and produces reliable and repeatable polymorphism between laboratories. However, the technique is expensive, labor intensive, time consuming and uses radio-isotopes elements. AFLP is an advancement of RFLP and is based on selective amplification of restriction fragments (Vos et al. 1995). The restriction digestion of whole genome generates large numbers of fragments of genomic DNA whereas the selective

amplification of these fragments helps detect polymorphisms. The high reproducibility, rapid generation and high frequency of detectable polymorphic bands make AFLP a suitable marker system in many organisms including plant species. A genetic map of barley has been developed using AFLP (Becker et al., 1995). In recent years, nucleotide diversity has been widely used to understand the diversity of population and how it shapes the evolution of genome in different crops. Among various markers, SNP and DArT have potential to be used in gene(s)/QTL mapping, map based cloning and association genetics (Buckler and Thornsberry, 2002). Recently, the Barley Coordinated Agriculture Project (Barley CAP) has aimed at generating 3000 SNP markers for association mapping of 40 agronomic traits in barley in USA (Hays and Szucs, 2006).

Lee and Neate (2007a and 2007c) used sequence tagged sites (STS), RAPD, and DArT markers to map three major genes *Rsp1*, *Rsp2*, and *Rsp3* conditioning resistance to *Septoria passerinii* in barley. Marker-assisted selection has become more important today because traits conditioned by QTL are more difficult to introgress into desired cultivars using conventional plant breeding techniques. Furthermore, MAS has been used as a tool to enhance the selection efficiency of plant breeding. Molecular markers linked with phenotypic markers help breeder select desired recombinants in the laboratory before they are actually evaluated in the field. For examples, de la Pena et al. (1999) mapped Fusarium head blight (FHB) resistance QTL in barley. Castro et al. (2003a and 2003b) used MAS to pyramid the stripe rust resistance QTL in barley.

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CHAPTER 3. RESISTANCE TO *COCHLIOBOLUS SATIVUS* IN NORTH AMERICAN BARLEY GERMPLASM

Abstract

Cochliobolus sativus [Anamorph: *Bipolaris sorokiniana*] causes common root rot (CRR) and spot blotch (SB) diseases in barley in North America. To search for host resistance, 1207 barley genotypes were screened for CRR resistance in the field and greenhouse conditions. Seedling resistance to SB in 384 breeding lines was also evaluated in greenhouse using *C. sativus* isolate 4008 representing virulence group 7.7.3.6. The results indicated that Canela, Eslick, Excel, FEG149-18, M123, ND25648, and Robust showed resistant reaction to CRR in the field. FEG149-18, a barley breeding line from University of Minnesota, showed less CRR severity in greenhouse experiments with artificial inoculation of *C. sativus* isolates 111, 417, 802, and 1408. Among the 384 lines screened for SB resistance, only ND23329 and ND23345 showed resistant reactions to *C. sativus* isolate 4008. This isolate overcame the spot blotch resistance found in both two- and six-rowed barley genotypes, including NDB112. The continuous distribution of disease severities of both CRR and SB suggested that resistance to these diseases could be polygenic in nature but the genetics of these resistances is unknown. The CRR and SB resistant genotypes identified in the current study will be useful in developing resistant cultivars in future.

Introduction

Cochliobolus sativus (Ito & Kurib.) Drechsl. ex Dast.[anamorph, *Bipolaris sorokiniana* (Sacc. in Sorok.) Shoem.], *Fusarium graminearum* Schwabe [teleomorph *Gibberella zeae* (Schwein.) Petch] and *Fusarium culmorum* (W. G. Sm.) Sacc., cause common root rot (CRR) in wheat and barley. *C. sativus* also causes spot blotch and black point in wheat and barley (Mathre, 1982). This fungal pathogen is frequently isolated from roots, leaves, and seeds of barley in North America (Mathre, 1982) and is widely distributed in south Asia (Kumar et al., 2002), central Asia (van Leur et al., 1997) and Australia (Wildermuth et al., 1992; Whittle, 1992), therefore influencing successful cultivation of barley worldwide.

North Dakota (ND) is the leading state for barley production in the United States, accounting for over one third of the total barley production (USDA, 2009). Stack et al. (1991) estimated that CRR resulted in an annual average yield loss of 9.5% in barley in ND. In earlier study, Piening et al. (1976) reported 10% annual yield loss in barley in Canada due to CRR. On the other hand, spot blotch can cause 10-30% yield losses in barley in North America (Ghazvini and Tekauz, 2004; Wilcoxson et al., 1990) under favorable conditions. Resistance to spot blotch has been considered an effective strategy to reduce the yield losses in North Dakota.

Traditionally, physiological specializations of different *Cochliobolus* species to cultivated crops have been reported. Recently, Gazvini and Tekauz (2008), Zhong and Steffenson (2001), and Valjavec-Gratian and Steffenson (1997) studied the population structure of *C. sativus* isolated from barley leaves using virulence pattern and AFLP markers. Valjavec-Gratian and Steffenson (1997) reported three

pathotypes (0, 1, and 2) of spot blotch associated *C. sativus* based on the differential reaction of 22 isolates on barley lines ND5883, 'Bowman', and NDB112. They reported that pathotype 1 was dominant in ND with lower allelic diversity compared to pathotypes 0 and 2. Ghazvini and Tekauz, (2008) reported eight virulence groups of *C. sativus* in Canada. Among them, virulence groups 7.7.7.5 and 7.7.5.1 were highly virulent on currently available resistance sources. Recently, the resistance to spot blotch in NDB112 has been knocked down by some highly virulent isolates from ND when tested in greenhouse. In contrast, there is a slow progress in developing CRR resistance in North America mainly due to the quantitative nature of resistance and lack of understanding of the virulence spectrum of CRR associated *C. sativus*. Arabi and Jawhar (2001) reported the virulence spectrum of CRR associated *C. sativus* from roots of barley in Syria and showed differential reactions of *C. sativus* to barley cultivars. However, the virulence spectrum of *C. sativus* isolated from roots of barley in North America has not been reported yet. The objective of this study was to investigate the resistance to CRR and spot blotch in North American malting barley germplasm.

Materials and Methods

Barley germplasm

In 2006, 56 barley genotypes, either released in USA and Canada or advanced lines from North American Barley Breeding Programs hereafter defined as Barley Coordinated Agricultural Project (CAP) core subset, were used for CRR screening in Langdon Research and Extension Center, ND (Table 3.1). University of

Minnesota (UM) six-rowed, North Dakota State University (NDSU) two-rowed (ND2), NDSU six-rowed (ND6), and Busch Agricultural Resources Inc. (BA) two- and six-rowed barley breeding programs submitted 96 lines each to Barley CAP hereafter defined as CAP06, CAP07, and CAP08. The CAP06 (*n* = 384) and CAP07 (*n* = 384) lines were screened for CRR in the same center. The CAP08 (*n* = 384) lines were screened for CRR resistance in greenhouse using *C. sativus* isolates collected from farmers field in North Dakota.

TABLE 3.1. Barley breeding lines used for screening resistance to common root rot and spot blotch during 2006-2009.

Population	North American[a]	UM[b] six-rowed	BA[b] two-rowed	BA[b] six-rowed	ND2[b] two-rowed	ND6[b] six-rowed
CAP core	55	-	-	-	-	-
CAP06	-	96	64	32	96	96
CAP07	-	96	46	50	96	96
CAP08	-	96	62	24	96	96

[a]CAP Core subset includes released cultivars in the USA and Canada as well as advanced breeding lines from barley breeding programs of the USA.
[b]Four breeding programs constitute the Upper Midwest set of Barley CAP program.

In all experiments for CRR resistance, Robust and ND20448 were common checks. Additional genotypes, 'Argyle', 'Arizona', 'Conrad', 'Eslick', and 'Golf', were included as checks in the CAP07 evaluations. In 2008, six wild relatives of barley, including *H. marinum* sp. *glucan* and *H. jubatum* sp. *jubatum*, were evaluated for CRR resistance in greenhouse. In spot blotch experiments, CAP06 (*n* = 384) lines were evaluated for seedling resistance to spot blotch caused by *C. sativus* and ND5883, Bowman, and NDB112 were used as standard checks. ND5883 is susceptible to widely distributed Pathotype 1 in ND whereas Bowman shows

differential reactions to different pathotypes. Bowman is very susceptible to Pathotype 2 isolates such as ND90Pr. NDB112 has durable resistance to *C. sativus* causing spot blotch (Wilcoxson et al., 1990) and is reported resistant to all three pathotypes reported in ND (Fetch and Fetch and Steffenson, 1994).

Inoculum preparation, inoculation, and disease severity assessment

Single spore fungal isolates were plated on potato dextrose agar (PDA) and incubated for 12 days at 22 - 24° C with an alternating cycle of 12 h light and dark. The root inoculation method developed by van Leur (1991) was used in greenhouse experiments whereas genotypes were screened under natural inoculums in the field. The conidial suspension was adjusted to 5 x 10^5 spores ml^{-1} sterile water. For root inoculation, the standardized spore suspension of *C. sativus* isolates 111, 417, 802, and 1408 were measured and mixed thoroughly. Forty-ml of spore suspension was mixed with 50 g of sterilized peat soil (Sunshine mix #1, Fiscon Horticulture Inc., Vancouver, Canada) in a beaker to prepare enough inoculum for 40 seeds. Gum Arabic 15% w/w (Sigma Aldrich Inc., USA) was mixed thoroughly with the peat conidia suspension to prepare peat-gum-conidia inoculum. Barley seeds were surface sterilized with 5% sodium hypochloride solution for 4 - 5 minutes and washed three times in sterile distilled water. Finally, 50 g of peat-gum-conidia inoculum was thoroughly mixed @ 40 seeds. Twenty to thirty seed per replicate were sown immediately into plastic containers of 7.6 cm x 21 cm (Stuewe and Sons, Inc. Corvallis, Oregon, USA) and filled with sterile Sunshine mix # 1 (Fiscon Horticulture Inc., Vancouver, Canada). Seeds were sown at 6-cm depth to promote longer sub-crown internodes (SCI). Plants were

incubated for 7 weeks in the greenhouse at 22 - 25° C and were watered as needed. Slow release fertilizer (14 - 14 - 14 N - P - K, 0.5 g $cone^{-1}$) (Scotts-Sierra Horticultural Product Company, Maryville, OH, USA) was applied once during the seven week incubation period. The SCIs were removed carefully, cleaned in running water, dried overnight, and scored for CRR severity. The CRR severity scale of van Leur (1991) was used for CRR assessment where 0% severity = immune with no lesion; highly resistant = small light brown lesions covering 1 - 10% of the SCI; resistant = light brown lesions covering 11 - 25% of the SCI; moderately resistant = light brown/black lesions covering 26 - 40% of the SCI; susceptible = black lesions covering 41 - 70% of the SCI; highly susceptible = black lesions covering 71 - 100% of the SCI. For more precise assessment of CRR severity, the percentage of lesions on SCI without using any scale was estimated according to Horsfall and Barratt (1945). In total, for CAP core subset, CAP06, and CAP07 (n = 824) lines, more than 129,000 SCIs were assessed for resistance to CRR in the field whereas for CRR experiment in greenhouse, more than 28,000 SCIs were assessed for CAP08 lines.

For spot blotch assessment, barley lines were grown in 3.8 cm x 21 cm cones (Hummert International, Earth City, MO, USA) filled with Sunshine mix #1. Three to five seedlings were grown per cone and a single cone represented a replicate. Slow release fertilizer (14 - 14 - 14 N - P - K, 0.3 g $cone^{-1}$) was applied at planting. The seedlings were grown in the green house at 22 - 25° C with a 16 h photoperiod. Leaf inoculums preparation and inoculation was done as described by Valjavec-Gratian and Steffenson (1997). At two-leaf stage (12 - 14 days),

seedlings were inoculated with a 5000 conidia ml^{-1} suspension of *C. sativus* isolate 4008. This isolates is highly virulent on ND5883, Bowman and NDB112 and has overcome all resistance sources available in current barley germplasm in North America (Unpublished data). Polyoxyethylene-20-sorbitan monolaurate (100 $\mu l\ L^{-1}$) was added to the conidial suspension to facilitate even distribution and better adsorption of conidia onto the leaf surface. The inoculum (approximately 0.15 ml $plant^{-1}$) was sprayed on the leaf surface to the point of runoff using an atomizer (DeVilbis Health Care Ind., Somerset, Pennsylvania). The inoculated plants were kept in ultrasonic misting chambers under dark for 18 h at 22° C and approximately 100% relative humidity. Plants were slowly air dried by opening the doors of ultrasonic chambers before being transferred to the greenhouse at 22 - 25° C and incubated for the next 10 days. Spot blotch severity was assessed using the 0 - 9 severity scale developed by Fetch and Steffenson (1994). Barley genotypes ND5883, Bowman, and NDB112 were repeated 16 times in each replicates as checks.

Experimental design and analysis

Both CRR and spot blotch experiments were conducted in RCBD. CRR experiments in field were conducted with 8 replicates for CAP core subset (n = 56), Barley CAP06 (n = 384), and Barley CAP07 (n = 384) whereas wild relatives of barley (n = 5) and Barley CAP 08 (n = 384) were evaluated in greenhouse in 2009 with three replicates. The percentage data of CRR were transformed using ArcSine. All statistical analysis was performed on transformed data for CRR. Barley CAP06 (n = 384) lines were screened for spot blotch resistance with two

replicates whereas standard checks (ND5883, Bowman, and NDB112) were repeated 16 times in each replicates. In total, a minimum of 96 seedlings were evaluated for spot blotch resistance in standard checks whereas 6 - 10 seedlings lines^{-1} were evaluated for spot botch resistance in CAP 06 lines. Analysis of variance (ANOVA) was conducted by means of GLM in SAS (SAS, 2002) for both CRR and spot blotch experiments. Fisher protected LSD (0.05 probability) was used to separate means of disease severity. Dunnett's test (0.05 probability level) was performed to compare the means of standard checks with CAP lines.

Results

Resistance to common root rot (CRR)

The ANOVA of CRR for the barley CAP core subset, CAP06 and CAP07 is presented in Table 3.2. The results revealed significant differences in CRR severities of barley breeding lines tested in three years. Of 824 barley genotypes evaluated in the field, only seven (0.9%) were resistant (<25% CRR severity). Majority of the genotypes (47.8%) showed moderately resistance reactions to CRR. None of the genotypes showed either immune or highly susceptible reactions in the field. 'Canela', Eslick, Excel, FEG149-18, M123, ND25648, and Robust showed resistant reactions in 2006. The frequency distribution of CRR severity of 824 genotypes showed a continuous distribution (Fig. 3.1). Argyle, a genotype bred in Canada and recorded as CRR resistant by Wolf and Bailey (1994) showed susceptible reaction in the current study. Among all checks, Argyle was the most susceptible, whereas, a two-rowed barley, Conrad, was the most resistant. The CRR assessment in greenhouse, using *C. sativus* isolates 111, 417,

802, and 1408, revealed that none of the genotypes were resistant. The resistant check ND20448 showed 55% CRR severity in this experiment, therefore, those genotypes with CRR severity less than 55% (less than the resistant check) were considered moderately resistant (Table 3.3). Using this criterion, only 12.8% of the genotypes were moderately resistant while majority of the genotypes were susceptible or highly susceptible. In this experiment, susceptible check had 78.3% CRR severity and Robust was intermediate between Arizona (highly susceptible) and ND20448 (moderately resistant).

TABLE 3.2. Analysis of variance of resistance to common root rot measured in the field experiments in Langdon Research and Extension Center, during 2006-2008.

Source of variation	df	Mean Squares	*P*-value
CAP Core subset[a]			
Replication	7	2847.6	<0.001
Breeding line	55	262.6	<0.001
Error	385	115.9	
CAP06[b]			
Replication	7	26557.3	<0.001
Breeding line	385	284.7	<0.001
Error	2695	94.3	
CAP07[b]			
Replication	7	9265	<0.001
Breeding line	389	367.1	<0.001
Error	2723	96.7	

[a]CAP Core subset includes released cultivars in the USA and Canada as well as advanced breeding lines from barley breeding programs of the USA.
[b]CAP06 and CAP07 are barley lines submitted to Barley CAP project from four breeding programs in the Upper Midwest region of the USA.

The infection responses of barley genotypes and number of resistant lines to CRR and spot blotch diseases are presented according to breeding programs

(Tables 3.3 and 3.4). The sub set of barley CAP core contains majority of the cultivars released in the USA and Canada, and also includes few advanced breeding lines. Forty seven of the 55 genotypes evaluated were moderately resistant to CRR. The majority of ND6 six-rowed, UM six-rowed, and BA six-rowed barley were moderately resistant whereas two-rowed barley from ND2 and BA breeding programs were mostly susceptible or highly susceptible to CRR. The CRR severity was higher in greenhouse experiments as compared to field experiments, therefore more genotypes showed susceptible reactions to *C. sativus* isolates. The 384 breeding lines showed a continuous distribution in CRR severity (Fig. 3.2). CRR severities of the wild relatives of barley were comparable to those of the moderately resistant lines, Eslick and ND20448, except for *H. jubatum* accession, which showed a higher level of resistance to *C. sativus* (Table 5). A very low level of disease severity was observed on primary roots, SCI, and crown regions in *H. jubatum* compared to other subspecies. The barley lines which scored CRR severity less than moderately resistant check ND20448 were considered moderately resistant.

Resistance to spot blotch caused by *C. sativus* isolate 4008

C. sativus isolate 4008 was highly virulent to majority of contemporary advanced breeding lines from the Upper Midwest of the USA. Only two lines (0.5% of the total genotypes tested) were resistant to this isolate. Twenty-five of the genotypes showed intermediate (4 - 5 severity scale) reaction and therefore were referred as moderately resistance to this isolate (Table 3.3).

TABLE 3.3. Frequency and percentage of infection response of barley genotypes to pathogens causing common root rot (CRR) and spot blotch in the field and greenhouse conditions in 2006, 2007, 2008, and 2009.

Infection response (IR)	CRR in the field [a] *n* = 824	CRR in greenhouse [b] *n* = 384	Spot blotch in greenhouse [c] *n* = 383
Immune	0	0	0
Highly resistant (HR)	0	0	0
Resistant (R)	7 (0.9) [d]	0	2 (0.5)
Moderately resistant	395 (47.9)	49 (12.8)	25 (6.5)
Susceptible (S)	422 (51.2)	233 (60.6)	241 (63.0)
Highly susceptible (HS)	0	102 (26.6)	114 (30.0)
[f]Arizona (Check)	nt [e]	78.3%	nt
Argyle (Check)	54.9%	nt	nt
Conrad (Check)	36.3%	nt	nt
Eslick (Check)	25.0%	nt	nt
Golf (Check)	40.9%	nt	nt
ND20448 (Check)	44.1%	55.0%	nt
Robust (Check)	24.0%	65.6%	nt
ND5883 (Check)	nt	nt	8.3
Bowman (Check)	nt	nt	6.4
NDB112 (Check)	nt	nt	7.8

[a]Genotypes screened for CRR resistance under natural inoculums using 0 - 100% severity scale in 2006 (*n* = 56), CAP06 screened in 2007 (*n* = 384) and CAP07 screened in 2008 (*n* = 384) in Langdon Research and Extension Center, NDSU.
[b]CAP08 genotypes screened for CRR resistance using virulent *C. sativus* isolates, 111, 417, 802, and 1457, obtained from sub-crown internodes in greenhouse in Fargo, NDSU.
[c]Seedling resistance to spot blotch screened for virulent *C. sativus* isolate 4008 using 1 - 9 severity scale of Fetch and Steffenson, (1994) in greenhouse in Fargo, NDSU.
[d]Values in the parenthesis are percentage of genotypes showing infection response to pathogen.
[e]nt = not tested.
[f]The disease severity for the checks are given in 0 - 100% scale for CRR severity and 1 - 9 scale for spot blotch.

Among the 384 lines tested, 241 (63%) had a disease score ranging from 6 to 7 whereas 114 genotypes were recorded with a score larger than 8, including the standard checks used in the experiment. NDB112, the durable resistance sources

used in the North American barley breeding programs, was highly susceptible to *C. sativus* isolate 4008.

TABLE 3.4. Number of barley genotypes/advance lines showing infection response to pathogens causing common root rot and spot blotch diseases in 2006, 2007, 2008, and 2009.

CAP Year	Breeding program	[a]Reaction to CRR (n = 1207)				[b]Reaction to SB (n = 383)			
		[c]R	MR	S	HS	R	MR	S	HS
2006	CAP Core set	2	47	7	-	nt	nt	nt	nt
CAP06	NDSU 2-rowed	-	5	83	2	-	3	72	21
	NDSU 6-rowed	-	47	49	-	2	12	63	19
	UoM 6-rowed	1	36	59	-	-	10	71	15
	Busch Ag 2-rowed	-	10	54	-	-	-	15	48
	Busch Ag 6-rowed	-	12	20	-	-	-	19	13
CAP07	NDSU 2-rowed	-	10	83	3	nt	nt	nt	nt
	NDSU 6-rowed	-	69	27	-	nt	nt	nt	nt
	UoM 6-rowed	-	61	35	-	nt	nt	nt	nt
	Busch Ag 2-rowed	-	7	39	1	nt	nt	nt	nt
	Busch Ag 6-rowed	-	25	24	-	nt	nt	nt	nt
[d]CAP08	NDSU 2-rowed	-	8	57	31	nt	nt	nt	nt
	NDSU 6-rowed	-	9	62	25	nt	nt	nt	nt
	UoM 6-rowed	-	14	60	22	nt	nt	nt	nt
	Busch Ag 2-rowed	-	16	30	16	nt	nt	nt	nt
	Busch Ag 6-rowed	-	2	24	8	nt	nt	nt	nt

[a]Number of barley genotypes/breeding lines showing infection response for CRR.
[b]Number of barley genotypes showing infection response after inoculation of *C. sativus* isolate 4008 in greenhouse. Seedling resistance to spot blotch was assessed using 1 - 9 severity scale of Fetch and Steffenson (1994).
[c]R = Resistant, MR = Moderately resistant, S = Susceptible, HS = Highly susceptible, nt = not tested, - = class not observed.
[d]Number of barley breeding lines showing infection response to the inoculation of *C. sativus* isolates 111, 417, 802, and 1408 in greenhouse.

The infection responses of lines tested for spot blotch are presented in Table 3.4 according to breeding programs. Two breeding lines, ND203329 and ND203345, from ND6 six-rowed breeding program showed resistant reactions to isolate 4008 and had a disease score less than 3. Only three genotypes from ND2

two-rowed programs were moderately resistant whereas none of the two-rowed barley lines from BA were resistant to isolate 4008. Twelve and ten genotypes from six-rowed breeding program of ND6 and UM were moderately resistant. M03 - 53 and ND23827 had a disease score of 3.1 and 3.4, respectively, indicating they also possess resistance to isolate 4008. More susceptible genotypes were recorded for two-rowed barley in both NDSU and BA breeding programs. The frequency distribution of SB severities is presented in Fig. 3.3.

TABLE 3.5. Resistance to common root rot caused by *C. sativus* isolates assessed in cultivated barley and wild relatives in greenhouse experiment in 2008.

Genotypes/species	Accessions	Disease severity (%) [b]		
		Primary roots	SCI	Crown
Arizona [a]	-	nr	84.8a	74.3a
Argyle [a]	-	nr	72.4b	53.3b
Eslick [a]	-	nr	67.0c	43.0cd
ND20448 [a]	-	nr	57.1d	38.1e
H. marinum sp. *glaucan*	CGN13067	54.7b	53.1e	42.0d
H. marinum sp. *glaucan*	CGN13077	59.0a	50.1ef	46.0c
H. marinum	CGN13088	56.8ab	48.9f	41.6de
H. marinum sp. *leporinum*	CGN13096	57.0ab	49.2f	52.2b
H. jubatum sp. *jubatum*	CGN13044	16.6c	12.2g	13.0f

[a]Genotypes are either released cultivars or advanced breeding lines of *H. vulgare*.
[b]Values fallowed by the same letter do not differ significantly at 0.05 probability level.
- = not available.
nr = not recorded.

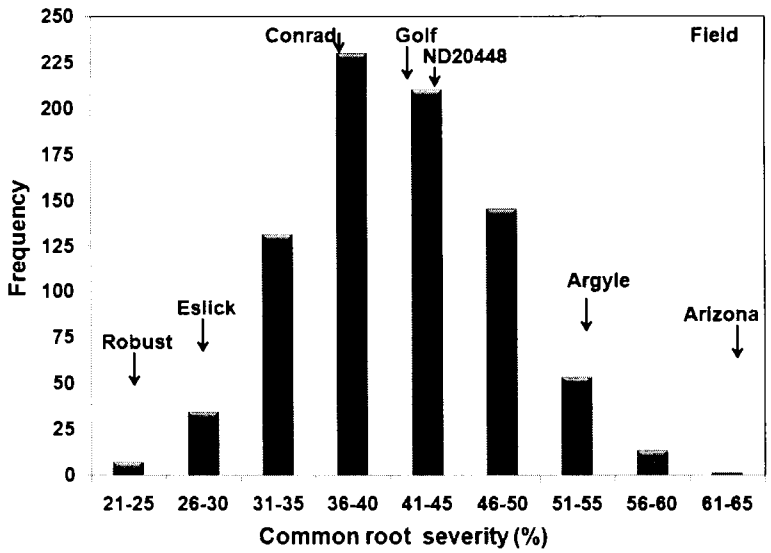

Fig. 3.1. Frequency distribution of CRR severities in 824 barley (CAP Core subset [*n* = 56], CAP2006 [*n* = 384], CAP2007 [*n* = 384]) genotypes from North America in 2006, 2007, 2008. CRR severities are the mean of eight replicates in each year. The CRR severities of standard checks, which were also evaluated in the experiments, were Arizona (78.3%), Argyle (54.9%), Conrad (36.3%), Eslick (25%), Golf (40.9%), ND20448 (44.1%) and Robust (24%).

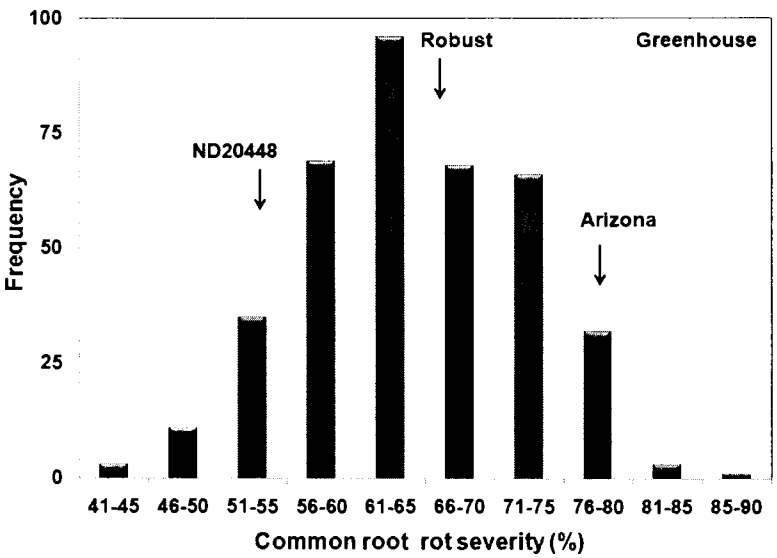

Fig. 3.2. Frequency distribution of CRR severities in CAP08 lines measured in greenhouse evaluations using *C. sativus* isolates 111, 417, 802 and 1408 causing common root rot (CRR) in 384 barley lines (CAP08) from the Upper Midwest of the USA in 2009. CRR severities are the mean of three replicates. The CRR severities of standard checks, which were also evaluated in the experiments, were Arizona (78.3%), Robust (65.5%) and ND20448 (55%).

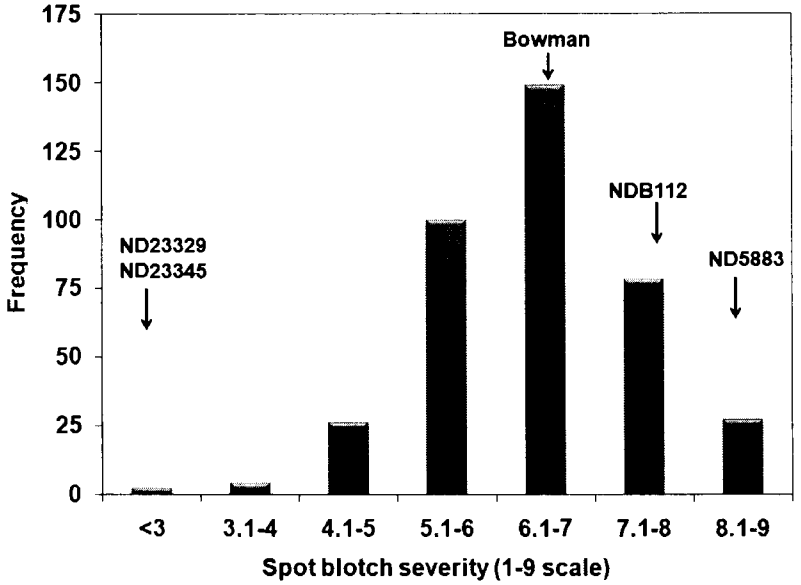

Fig. 3.3. Frequency distribution of spot blotch severities in CAP06 lines measured in greenhouse evaluations using *C. sativus* isolates 4008. Spot blotch severity are the mean of two replications where the checks, ND5883, Bowman and NDB112, were repeated 16 times in each replicates: ND5883 (8.3 severity scale), Bowman (6.4 severity scale), and NDB112 (7.8 severity scale).

Discussion

Resistance to common root rot

Use of host resistance is a viable option for management of common root rot (CRR) and spot blotch diseases in the field, however, a rigorous assessment of CRR resistance of contemporary barley germplasm of the Upper Midwest of the USA is still lacking. In this study, we evaluated the resistance to CRR in barley germplasm and advanced breeding lines from four breeding programs of the Upper Midwest region. Canela, Eslick, Excel, FEG149-18, M123, ND25648, and Robust were found to be the most resistant among 824 genotypes. Almost 48% of the genotypes were moderately resistant to CRR in the field. The continuous distribution of CRR severity of 824 genotypes indicates that the resistance to CRR

is polygenic in nature. Our findings agree with those of Bailey and Wolf (1994) and Kutcher et al. (1994) who suggested that resistance to CRR is conditioned by either oligo or polygene in barley.

Bailey and Wolf (1994) also reported that Argyle was resistant to CRR in Canada. However, our results showed that Argyle was susceptible to CRR in both field and greenhouse experiments. There could be several possible reasons for this discrepancy. First, the resistance of Argyle might have been overcome by the pathogen recently, which is most likely to occur. Second, the pathogen population of ND could be different than the Canada. Third, there could be shift in the virulence spectrum of CRR causing pathogen over time. Ghazvini and Tekauz (2008) recently reported the emergence of new virulence groups of *C. sativus* from the prairies of Canada. In ND, we have found new virulence groups which have overcome resistance to SB in contemporary germplasm of the North America. In earlier studies, only pathotypes 1, 2, and 0 were reported from ND (Fetch and Steffenson, 1994, Valjavec-Gratian and Steffenson, 1997; Zhong and Steffenson, 2001). The evidences of shift in aggressiveness of some virulence groups of *C. sativus* causing SB in North America suggest a similar shift might have overcome the resistance to CRR in Argyle. We verified the resistance of Argyle by inoculating virulent *C. sativus* isolates collected from ND and found that resistance of Argyle is overcome by isolates obtained from ND. Eslick and Conrad, the two-rowed barley cultivars released in USA, showed consistently resistant reaction in the field and greenhouse experiments. However, our results indicate that the frequencies of

two-rowed resistant genotypes were less compared to six-rowed barley in any of the breeding programs investigated in current studies (Table 3.4).

The evaluation of wild relative of barley for resistance to *C. sativus* causing CRR revealed highly resistant reaction of *H. jubatum* sp. *jubatum* whereas resistance of *H. marinum* was comparable to moderately resistant cultivars Eslick and ND20448. We speculate that smaller SCI of wild species partly contributed to higher level of resistance to CRR, however, *H. jubatum* sp. *jubatum* showed consistently lower level of disease severity in primary root, SCI, and crown region. The *C. sativus* isolate used in current studies represent highly virulent 7.7.1 among large number of isolates collected from the field (a set of seven genotypes used to define virulence groups according to Limpert and Müller, 1994). The source of CRR resistance against this virulence groups is a valuable genetic resources for future breeding efforts. Therefore, Eslick and Conrad can be used as source of partial resistance to CRR whereas *H. jubatum* sp. *jubatum* is an excellent source resistance to CRR in future.

Resistance to spot blotch

Spot blotch disease of barley has been successfully managed in North America for the last four decades by deploying durable resistance derived from NDB112 (Wilcoxson et al., 1990). NDB112 was resistant to all virulence groups found in USA and Canada until recently when Ghazvini and Tekauz (2008) reported virulence groups, 7.7.7.5, 7.7.7.1, and 7.7.5.4., that overcame resistance of NDB112 in Canada. Similarly, isolates 4008 and 1457, representing virulence group 7.7.3.6 and 7.3.3.6 from ND (similar to the virulence groups reported by

Ghazvini and Tekauz, 2008), caused severe spot blotch in NDB112 when inoculated at the seedling stage. Screening of the 384 breeding lines from the Upper Midwest of the USA, using isolate 4008, confirmed that isolate 4008 is highly virulent on most of the lines. Fortunately, two lines, ND23329 and ND23345, showed a high level of resistance to this isolate whereas the other two lines, M0-53 and ND23676, were moderately resistance with a reading of 3.4 and 3.9, respectively.

Ghazvini and Tekauz (2008) argued that a quantitative approach of data analysis is required to account the continuous nature of spot blotch severity in barley. The continuous distribution of frequency of spot blotch severities in current studies (Fig. 3.3) indicates that the resistance to this virulence group is polygenic in nature. We speculated that the resistance to *C. sativus* isolate 4008 could be conditioned by quantitative trail loci (QTL). Bilgic et al. (2005) reported three QTL in double haploid population of Diktoo/Morex cross. These QTL were mapped on chromosomes 3H and 7H. Similarly, Bilgic et al. (2006) reported more QTL for spot blotch resistance on chromosomes 2H, 3H, 4H, and 7H in barley. More recently, association mapping has been employed to map novel QTL in the USA sets as well as in a subset representing the Upper Midwest population of Barley CAP (Brian Steffenson, personal communication). To phenotype these barley lines, they used pathotypes 1 for inoculation, which is the most widely distributed pathotype in ND and routinely used for screening barley germplasms for spot blotch resistance in the region. In the current study, we used the isolate 4008 (virulence group 7.7.3.6) that has overcome both CRR and spot blotch resistance in barley

germplasm of the Upper Midwest of the USA. We have also employed the same isolate for association mapping of novel QTL against spot blotch in the same mapping population (n = 384 lines for the Upper Midwest of the USA).

Analysis of resistance lines according to breeding programs clearly indicates that the there is a lack of resistance to *C. sativus* causing spot blotch in ND2 two-rowed breeing program and both two- and six-rowed programs of BA (Table 3.4). Therefore, there is an immediate need for these breeding programs to explore resistance sources in diverse two-rowed germplasm including landraces, exotic lines, and wild relatives. Two resistant and 12 moderately resistance lines identified from the ND6 six-rowed breeding program and 10 moderately resistance lines from the UM breeding program should be potential sources of resistance to isolate 4008. To facilitate the introgression of the resistance into barley breeding programs, extensive studies on genetics of the resistance are needed.

In conclusion, barley genotypes resistant to CRR and spot blotch diseases have been identified in the Upper Midwest barley CAP germplasm. The genetics of resistance present in these lines are unknown and remains to be explored immediately for breeding resistant cultivars in future. The identification of very few lines resistant to CRR and spot blotch indicates that there is an immediate need to search for resistance to novel virulence group 7.7.3.6 in more diverse germplasm including exotic lines, landraces, and wild barley in future.

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CHAPTER 4. GENOME-WIDE ASSOCIATION MAPPING OF RESISTANCE TO COMMON ROOT ROT IN BARLEY

Abstract

Cochliobolus sativus and *Fusarium spp.* cause common root rot (CRR) in cereals and are important fungal pathogen causing CRR in small grains worldwide. Genetic resistance is a viable option for managing CRR but little information is available on mapping resistance to CRR in barley. In this study, 1152 breeding lines developed by the Barley Coordinated Agricultural Project (CAP) were phenotyped for CRR resistance in 2006, 2007, and 2008, and 3072 single nucleotide polymorphism (SNP) markers were applied to map CRR resistance. To control false positives, population structure was accounted for by using eigenvalues of principle component analysis and kinship coefficient in the mixed linear model in TASSEL 2.1. The phenotypic analysis revealed continuous distributions of CRR severity, indicating a complex polygenic nature of resistance. The pair-wise linkage disequilibrium (LD) of SNP markers varied genome-wide, with an average of 10 cM in genetic distance in 2006 and 2007. The results suggest that the quantitative trait loci (QTL), *CRR-3H-28-51, CRR-5H-180-195, CRR-6H-30-64, CRR-6H-91-97,* and *CRR-7H-50-86* were mapped in three or more mapping populations used in current study. The *q*-values (FDR) at 0.1 threshold reveal that these associations are true positives. QTL, *CRR-3H-28-51* and *CRR-7H-50-86* found associated with spot blotch resistant QTL whereas three others, *CRR-5H-180-195, CRR-6H-30-64*, and *CRR-6H-91-97,* are unique.

Common root rot may be improved by accumulating favorable alleles of these QTL in breeding lines using marker-assisted selection (MAS).

Introduction

Cochliobolus sativus (Ito & Kurib.) Drechsl. ex Dast.[anamorph, *Bipolaris sorokiniana* (Sacc. in Sorok.) Shoem.], *Fusarium graminearum* Schwabe [teleomorph *Gibberella zeae* (Schwein.) Petch] and *Fusarium culmorum* (W. G. Sm.) Sacc., can cause common root rot (CRR) in wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.). Among these pathogens, *C. sativus* is a frequently isolated root pathogen of barley in North America, and is considered a serious biotic factor limiting barley production in the region. Previous reports (Stack et al. 1991; Piening et al. 1976) indicate that CRR causes 10% annual losses to barley in North America.

Host plant resistance is one of the most effective disease management strategies for controlling many plant diseases. The bi-parental mapping population approach has been widely used to identify and map quantitative trait loci (QTL) for agronomic, disease resistance, and quality traits. The QTL with larger phenotypic effects for many traits have been successfully mapped and used for crop improvement. However, QTL that have small effects, that are conditioned by polygenes, and/or are strongly influenced by environmental conditions, have yet to be translated into crop improvement (Bernardo 2008; Xu et al. 2008). Marker assisted selection (MAS) and genome wide selection (GWS) are promising molecular approaches to translate genetic resources into crop improvement

(Heffiner et al. 2009; Xu et al. 2008). In recent years, association mapping (AM) has become an alternative approach to bi-parental mapping in order to dissect complex quantitative traits (Jannink et al. 2001). Association mapping makes use of the contemporary germplasm pool and associated data collected routinely in breeding programs, which eliminates the need to generate bi-parental mapping populations from wide crosses among parents. One advantage of using existing adapted germplasm is that the identified QTL can be assessed in a more relevant genetic background, and can be selected using MAS without major linkage drag. Other advantages of using AM include the elimination of the time needed to generate mapping populations, a larger number of traits are able to be investigated using available breeding lines, higher resolution of QTL maps, and more accurate estimation of phenotypic effects. Association mapping has been successfully used to identify candidate genes/QTL through genome wide scans (Thornsberry et al. 2001; Zhao et al. 2007b). In cereals, AM has been used in wheat (Breseghello and Sorrells 2006) and barley (Kraakman et al. 2004; Rostoks et al. 2006; Steffenson et al. 2007) to identify various complex traits.

AM is influenced by the pattern of linkage disequilibrium (LD) and population structure in the germplasm used. The level of LD is population dependent and the pattern of LD dictates the appropriateness of a candidate gene or whole genome scan approach for AM. In a study of cultivated European barley, LD was shown to extend from 1 cM to beyond 10 cM (Kraakman et al. 2004). Additionally, LD can vary between two- and six-rowed populations (Rostoks et al. 2006). In wild barley,

LD decays rapidly within 1 cM and varies across the genomes (Steffenson et al. 2007).

Population structure can affect a number of false positive marker-trait associations due to differential relatedness and allele frequencies among the subgroups (Pritchard et al. 2000). Therefore, identifying subgroups to account for population structure is very important to control the false positives in association mapping. Price et al. (2006), Patterson et al. (2006), and Zhao et al. (2007a) suggested that Principal Component Analysis (PCA) can be used to detect population structure. This information can then be used to minimize the number of false positives and maximize the power of detecting true marker-trait associations. Zhao et al. (2007a) suggested that a mixed liner model (MLM) accounts for a number of spurious marker-trait associations by the use of population structure as fixed effects and genetic relatedness (Kinship matrix) as random effects in the model (Yu et at. 2006). There has been slow progress in developing genetic resistance to CRR in barley breeding programs. This can be partly attributed to the assumed polygenic nature of CRR resistance, involvement of multiple pathogens in CRR development in the field, time consuming process of phenotyping CRR resistance in the field, and a lack of genetic information on resistance to CRR in North American barley germplasm. The main objectives of this study were to learn more about the genetics of CRR resistance by identifying QTL controlling resistance to CRR and scanning whole genome using association mapping. Materials to be used in this study are advanced breeding lines form the upper Midwest of the USA.

Materials and Methods

Plant materials

A total of 1152 advanced breeding lines from four breeding programs in the Upper Midwest of the United States were included in this study. Each breeding program, namely North Dakota State University (NDSU) six-rowed (ND6), NDSU two-rowed (ND2), University of Minnesota (UM) six-rowed, and Busch Agricultural Resources Inc. (BA) (both two- and six-rowed) submitted 96 lines in 2006, 2007, and 2008 to the Barley Coordinated Agricultural Project (Barley CAP). The BA breeding program develops both two- and six-rowed malting barley whereas the other breeding programs are specialized to either two- or six-rowed malting barley. The lines submitted were inbred lines of at least F_4 generations and selected for uniform agronomic traits. The lines were representative of other materials from each breeding program and all lines were untested previously for CRR resistance. However, all the breeding programs in the Upper Midwest have similar targets and focused on breeding malting barley. In this paper, we refer to CAP06 (n = 384), CAP07 (n = 384), and CAP08 (n = 384) as 2006, 2007, and 2008 populations, respectively.

Phenotypic evaluations

The CAP core subset, CAP06, and CAP07 populations were phenotyped for CRR resistance in field studies at the NDSU Langdon Research Extension Center (N48° 45.3', W98° 17.5'), in 2006, 2007, and 2008, respectively. The soil at this site is a Barnes / Svea complex (fine-loamy, mixed superactive Frigid, Calcic Hapludoll / mixed superactive Frigid, Pachic Hapludoll) (Soil Survey Staff, 2007).

Site preparations include fall tillage with a chisel plow and one spring tillage with a spring tooth cultivator with attached harrows. Liquid form of nitrogen (28-0-0) was applied as broadcast application before seeding to obtain an applied soil nitrate nitrogen level of 135 Kg ha^{-1} according Kandel (2007). In each year, the lines were assigned to experimental units using a randomized complete block design (RCBD) with eight replicates. In 2006, the size of an experiment unit was four rows of 1.21 m whereas in 2007 and 2008, the size of an experimental unit was 2.13 m row. In 2007, 'Robust' and ND20448 were included as checks whereas 'Argyle', Robust, 'Golf', 'Conrad', 'Eslick', and ND20448 were included as checks in 2008. The trials were conducted on field plots where the previous crop was wheat. At the early dough stage (Chan, et al. 1985) the plants were pulled from the soil and an average 20 sub-crown internode (SCI) were harvested from each experimental unit; kept in an envelope; dried over night, and stored at room temperature until they were scored for CRR severity. In 2006, approximately 9000 SCIs were evaluated for CRR resistance whereas 60,000 SCIs each collected from 3088 (CAP06 population) and 3120 (CAP07 population) experimental plots were scored for CRR severity in 2007 and 2008, respectively.

In 2009, CAP08 lines were evaluated for resistance to CRR caused by *C. sativus* in a greenhouse experiment. Virulent isolates 111, 417, 802, and 1408 were used to screen CAP08 lines for CRR resistance using the methods of van Leur (1991). Entries were assigned to experimental units using a RCBD with three replicates. *C. sativus* inoculated seeds (25 - 30 seeds) were sown into 7.6 cm x 21 cm plastic conetainers of experimental units, (Stuewe and Sons, Inc. Corvallis,

OR, USA) and filled with sterile Sunshine mix #1 (Fiscon Horticulture Inc., Vancouver, Canada). Seeds were sown at a 6-cm depth to promote longer SCI. Plants were incubated for 7 weeks in the greenhouse at 22 - 25° C and watered as needed. Slow release fertilizer (14 - 14 - 14 N - P - K, 0.5 g per conetainer) (Scotts-Sierra Horticultural Product Company, Maryville, OH, USA) was applied twice during the seven week incubation period. Disease severity was scored from 0 to 100% where 0% severity = immune with no lesion; highly resistant = small light brown lesions covering 1 - 10% of the SCI; resistant = light brown lesions covering 11 - 25% of the SCI; moderately resistant = light brown/black lesions covering 26 - 40% of the SCI; susceptible = black lesions covering 41 - 70% of the SCI; and highly susceptible = black lesions covering 71 - 100% of the SCI.

Genotypic evaluations

For molecular diversity assessment, a single plant of each line was grown in a greenhouse at the UM. The leaf tissue was lyophilized and sent to USDA-USDA laboratory of Dr. Shioman Chao in Fargo, ND for genotyping. Genomic DNA was extracted using the method of Slotta et al. (2008). The lines were genotyped using 3072 single nucleotide polymorphism (SNP) markers (Close et al. 2009). The SNP markers were designed onto Oligonucleotide Pool Assay (OPA) using Expressed Sequence Tag (EST) and sequenced amplicons at the University of California, Riverside, CA (Close et al. 2009). Based on map locations, technical performances, minor allele frequency (MAF) and biological interest of SNPs, two 1536 production OPAs named as BOPA1 and BOPA2 were produced. The Illumina Bead Station was used to generate genotypic data using Golden Gate

Assay as described by Fan et al. (2003). In total, data for 3072 SNP markers from BOPA1 and BOPA2 were generated for the CAP06, CAP07, and CAP08 populations and used in AM studies. The genotyping of all barley CAP lines (CAP06, CAP07 and CAP08) was accomplished in the USDA-Agricultural Research Service Molecular Marker Laboratory of Dr. Shiaoman Chao located in Fargo, ND.

Population structure analysis

Principal component analysis (PCA) was used to generate 'P-matrices' that was used as covariates in AM analysis. P-matrices were generated in TASSEL 2.1 The PCA was applied to genotypic data filter for minor allele frequency (MAF) of 5% to infer the genetic variations due to the differences in allele frequency in mapping populations. The PCs which explained > 50% phenotypic variation were used in AM. Therefore, the top ten to 15 principal components were used as covariates in the analysis. The PCA assignments (P-matrices) were combined with kinship coefficient matrices (K- or Kinship matrix, which is the probability that the two genes are identical by the descent) as covariates in the Mixed Linear Model (MLM) in association analysis. The kinship matrices were generated in TASSEL 2.1 using filtered SNP markers (Lynch and Ritland 1999).

Linkage disequilibrium (LD) and association mapping (AM)

Linkage disequilibrium was determined using filtered SNP markers in TASSEL 2.1 (Bradbury et al. 2007). The pair-wise r^2 was used to estimate LD between pairs of SNP markers (Pritchard and Przeworski 2001). The LD decay plots were

generated in SAS (SAS 2002) as a function of r^2 on genetic distance (cM). The average LD for each mapping population was estimated at a threshold of $r^2 = 0.2$.

To perform the AM analyses, the first three populations CAP06 (n = 384), CAP07 (n = 384), and CAP08 (n = 384) were each analyzed and they contained both two- and six-rowed barley. The CAP06, CAP07, and CAP08 populations were further subdivided into six subpopulations based on two- and six-rowed barley. These included the CAP06 six-rowed subpopulation (n = 224), the CAP06 two-rowed subpopulation (n = 160), the CAP07 six-rowed subpopulation (n = 242), the CAP07 two-rowed subpopulation (n = 142), the CAP08 six-rowed subpopulation (n = 226), the CAP08 two-rowed subpopulation (n = 158). The combined CAP06/07 six-rowed population consisted of six-rowed barley (n = 465) from the CAP06 and CAP07, the CAP06/07 two-rowed population contained two-rowed lines (n = 302) from the CAP06 and CAP07, and the combined CAP06/07 population contained all lines (n = 767) from the CAP06 and CAP07 populations.

Association analysis of each mapping population was conducted in TASSEL 2.1 (Bradbury et al. 2007). The genotypic data (filtered for 5% MAF), phenotypic data (CRR severity on SCI), P-matrices (inferred population stratification based on PCA) and K-matrices (Kinship matrices) were used to run a mixed linear model (MLM) analysis using TASSEL. The default run parameters of MLM were used, except the analysis method was set to expectation maximization (EM). Using the highly conservative p-value threshold of 0.001, markers-trait associations were declared significant. The corresponding p-values were used in further analyses such as false discovery rate (FDR) or q-value.

To contro the false positives in multiple testing, we employed a procedure that estimate the false discovery rate (FDR) explained by Benjamini and Hochberg (1995). The FDR is defined as the proportion of false positives among the significant tests. We used SAS (2002) to estimate FDR (q-value) and set $q < 0.1$ as a threshold. We analyzed genome wide AM as described previously (Kraakman et al. 2004). First we analyzed the significant marker-trait correlations. Second we examined the LD profile across genomes (chromosome wise association profiles containing –log (p) values of trait-associated markers for the CAP06 and CAP07 populations. Third, the examination of flanking markers of the trait-associated markers was done while scanning for putative QTL in the genome.

Statistical Analysis

The phenotypic data were analyzed using both descriptive (mean and standard deviation) and inferential statistics, and analysis of variance (ANOVA) in SAS (SAS Inc. 2002). The SNP markers with more than 10% missing values and less than 5% MAF were excluded from the association analysis. The filtered SNP data were created for each mapping set. Using the highly conservative p- value threshold of 0.001, markers-trait associations were considered significant.

Results

The ANOVA of the CAP06, CAP07, and CAP08 populations (two-rowed, six-rowed, and combined) revealed significant ($p < 0.001$) differences in CRR severities (Table 4.1). The average CRR severities in the CAP06, CAP07, and CAP08 populations were 43.3, 41.7, and 64.8% with standard deviation of 5.9, 6.7,

and 7.9, respectively. In the CAP06 and CAP07 populations, mean CRR severity of two-rowed barley was greater than that of six-rowed barley. The CRR severities of the CAP06 populations showed a continuous response (Fig. 4.1). A similar pattern of frequency distribution of CRR severity was observed in the CAP07 (Figure not presented).

TABLE 4.1. Common root rot (CRR) severity in barley CAP 2006, 2007, and 2008 populations evaluated in Langdon, North Dakota.

Population/subpopulation		CRR severity %		*p*-value
	n[a]	Mean	SD[b]	
CAP06	384	43.3	5.9	<0.0001
CAP06 two-rowed	160	46.5	5.6	<0.0001
CAP06 six-rowed	224	41.1	5.1	<0.0001
CAP07	384	41.7	6.7	<0.0001
CAP07 two-rowed	143	47.3	6.3	<0.0001
CAP07 six-rowed	241	38.6	4.7	<0.0001
[c]CAP08	384	64.8	7.9	<0.0001
CAP08 two-rowed	158	64.9	8.2	<0.0001
CAP08 six-rowed	226	64.7	7.7	<0.0001

[a]number of breeding lines evaluated for CRR
[b]Standard deviation
[c]CAP08 populations were screened in greenhouse using *C. sativus* isolates

Marker coverage and genome wide linkage disequilibrium (LD)

The summary of marker coverage is presented in Table 4.2. The number of SNP markers used in AM analyses ranged from 1873 to 2511 in different mapping populations after filtering for 10% missing data and MAF of 5%. In the CAP core subset, we used 2511 filtered SNPs (out of 2929 markers from Pilot OPAs 1 - 3) for association analysis. In the CAP06 population 2028 SNPs were used whereas 1923 and 1873 SNPs were used in marker-trait analysis of the two- and six-rowed subpopulations, respectively. In the combined AM analysis across row types of

2007, 1956 SNPs were used. In the analyses of two-rowed and six-rowed subsets of 2007, 1843 and 1082 SNPs were used, respectively. The CAP07 six-rowed had the lowest number of SNPs used for AM analyses among all populations evaluated in this study.

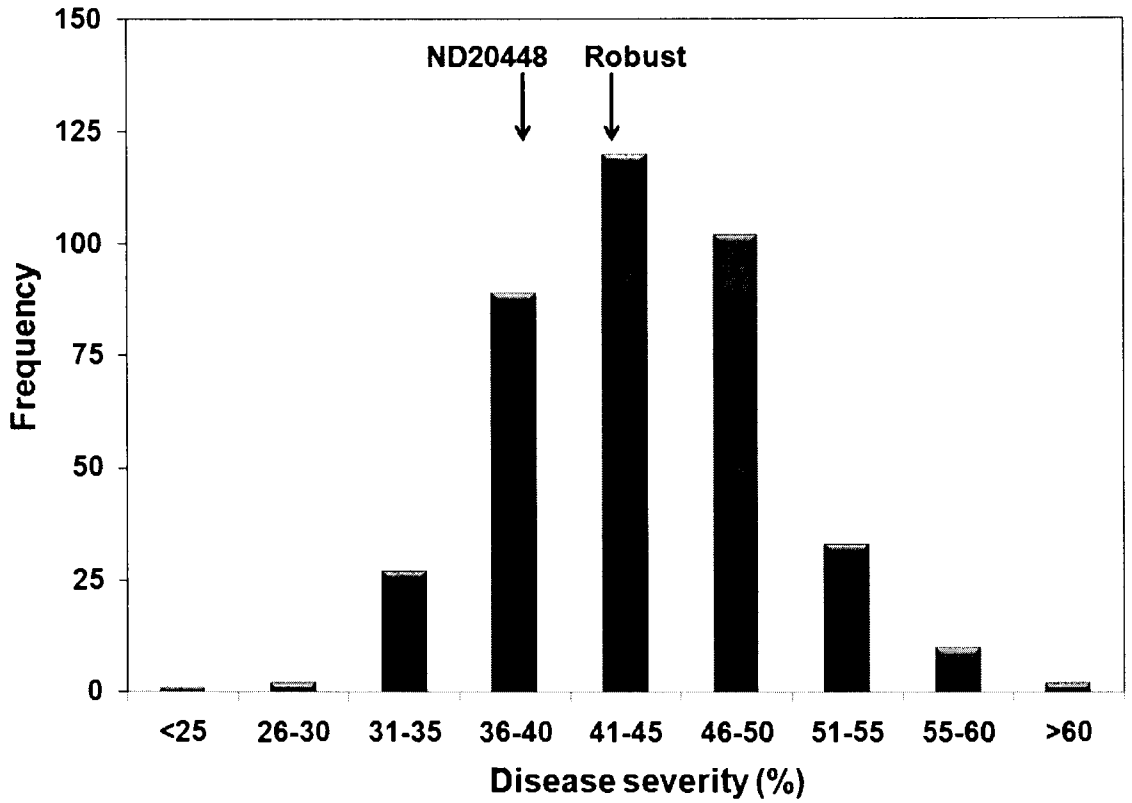

Fig. 4.1. Frequency distribution of CRR severities measured for 384 barley (CAP06) genotypes from North America. CRR severities are the mean of eight replicates and an average of 20 sub-crown internodes per entry in each replicates. The CRR severities of standard checks, which were also evaluated in the same experiment, were ND20448 (39.9%) and Robust (40.9%).

The average distances between SNP markers were 0.71 and 0.58 cM for the CAP06, and CAP07 populations, respectively. The distribution of r^2 values as a function of genetic distance in centimorgans for loci pairs are presented in Fig. 4.3. The genome wide average LD was 10 cM for CAP06 and CAP07; however, LD

differed for different chromosomes as well as subpopulations. The LD decay plot for two- and six-rowed barley for CAP06 is presented in Fig. 4.3 and a similar pattern of LD decay plots were observed for two- and six-rowed of the CAP07 (Figures not shown). In all cases, a threshold of r^2 = 0.2 was used for estimation LD genome wise as well as across chromosomes. In both the CAP06 and CAP07, the genome wide r^2 estimates across chromosomes declined rapidly to 0.2 within 10 cM of genetic distance. In the analysis of individual chromosomes for both populations, LD decayed sharply to r^2 = 0.2 within 10 cM for chromosomes 3H, 4H, and 6H chromosomes. However, in chromosomes 1H and 2H, LD extended between 10 - 15 cM in both the CAP06 and CAP07 populations. In chromosome 7H of the CAP06 population and chromosome 5H on the CAP07 population, LD extended beyond 20 cM distance (Fig. 4.3).

TABLE 4.2. Single nucleotide polymorphism (SNP) markers used in association mapping in Barley CAP lines in 2006 and 2007.

Population/ subpopulation	n[a]	No of SNP[b]	Average marker distance (cM)[c]	No of gaps > 5 cM	No of gaps > 10 cM	Average LD (cM)[d]
CAP06	384	2028	0.58	16	1	10
CAP06 two-rowed	160	1923	0.62	14	2	10
CAP06 six-rowed	224	1873	0.64	11	0	18
CAP07	384	1956	0.71	21	8	10
CAP07 two-rowed	143	1843	0.70	23	4	10
CAP07 six-rowed	241	1082	1.21	53	11	20

[a]Number of breeding lines evaluated for CRR
[b]Number of SNPs used for marker-trait association after filtering for 10% missing data and 5% minor allele frequency.
[c]Average distance estimated between all pairs of adjacent markers (cM).
[d]Average LD was calculated by making all pair-wise SNP comparisons for LD across the genome, and evaluating the moving average of r^2 values. A threshold of r^2 = 0.2 was used for LD.

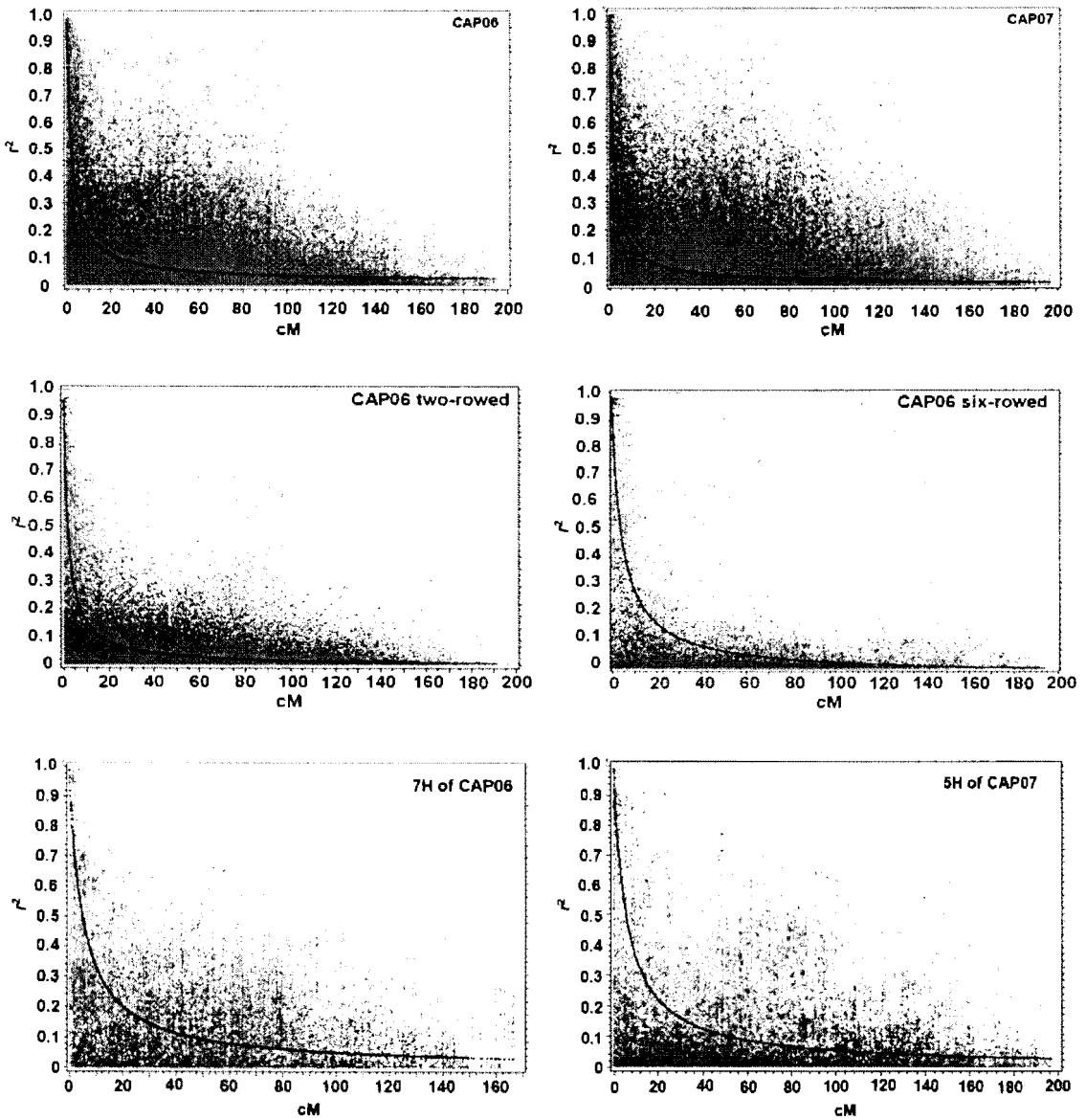

Fig. 4.2. Genome wide linkage disequilibrium among filtered SNP marker pairs as a function of genetic distance (cM) in barley in CAP06, CAP07, CAP06 two-rowed, and CAP07 six-rowed populations. The average LD across genome is presented for chromosome 7H of CAP06 and chromosome 5H of CAP07 (below). A threshold of r^2=0.2 is considered for estimating average LD.

Comparison of GLM (Q-or P-matrix) and MLM (Q- or P-matrix + K-matrix)

In genome wide association studies, population structure and allele frequency differences in mapping populations can lead to spurious associations between

markers and trait, and they needs to be accounted for before association analyses are conducted. Initially, we employed both PCA and STRUCTURE to generate P- and Q-matrices to be used as covariate in the association analyses using both GLM and MLM. Q-matrix was generated in Structure software using 70 - 80 unlinked SNP makers randomly selected at 20 cM distances from each barley chromosomes. Similarly, the number of PCs used in association analysis as covariate and their cumulative genotypic variation explained by the PCs are given in Table 4.3. The top ten PCs explained more than 50% of the genotypic variation in all mapping populations except the CAP06 two-rowed. Therefore, ten PCs were used in all MLM analyses except the CAP06 two-rowed in which 15 PCs were used in the analysis. In the CAP06/07 combined population, AM analyses were performed separately for each chromosome by using the PC estimated based on all markers due to large data set (767 breeding lines). The top ten PCs explained more than 50% genotypic variation in each chromosome in this population too, therefore, ten PCs were used in both GLM and MLM analyses. The principal component plot of the first two principal components (Fig. 4.3) shows three distinct subgroups for the CAP06 and CAP07 populations. All six-rowed lines from ND6, UM, and BA clustered together in both mapping populations. In contrast, two-rowed barley had two distinct groups, one represented by BA two-rowed breeding program and another by NDSU two-rowed (ND2) breeding program. In the current study, the options available in TASSEL such as GLM+Q, GLM+P, MLM+Q+K, and MLM+P+K were used to detect marker trait associations. The numbers of significant (p <0.001) SNP-CRR resistance associations for each option are

presented in Table 4.4. As expected, MLM had fewer significant marker-trait associations than that of GLM analyses. The results indicate that there was a general agreement in marker-trait association identified by MLM+Q+K and MLM+P+K models. However, the determination of subpopulation using Q-assignments in structure was very much subjective; therefore, only the results of MLM+P+K are discussed here. Hence, putative QTL identified in current study are solely based on results of MLM+P+K model used in TASSEL.

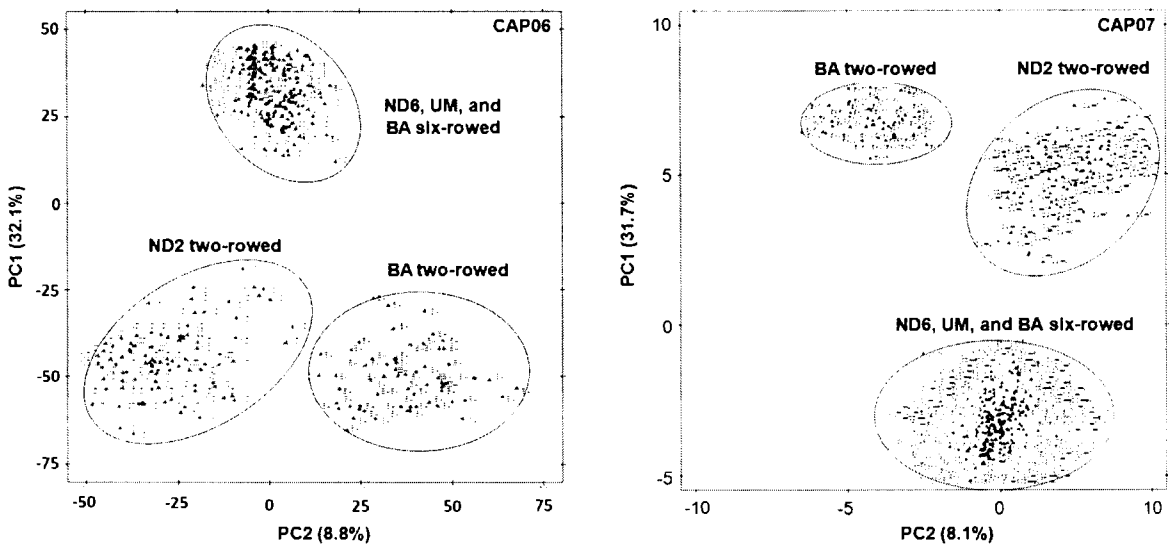

Fig. 4.3. Three subpopulations were designated as six-rowed (ND6, UM, and BA), ND2 two-rowed, and BA two-rowed barley by principal component analyses. The groups were designated by using first two principal components in the CAP06 and CAP07 mapping populations.

TABLE 4.3. The proportion of genotypic variation explained by top 15 principal components in different mapping populations used in detecting SNP-CRR resistance association analyses.

PC[a]	CAP06	CAP06 two-rowed	CAP06 six-rowed	CAP07	CAP07 two-rowed	CAP07 six-rowed	CAP08	CAP08 two-rowed	CAP08 six-rowed
					(%)[b]				
PC1	32.1	18.1	52.8	31.7	18.3	15.1	30.6	21.7	14.3
PC2	40.9	23.7	57.5	39.8	26.2	21.1	40.7	28.5	20.5
PC3	45.3	27.2	60.8	44.7	32.0	26.7	45.1	34.5	26.4
PC4	47.9	30.5	63.1	47.3	36.2	31.7	48.0	37.7	31.1
PC5	49.8	33.1	65.2	49.4	39.8	35.9	50.5	40.7	35.4
PC6	51.5	35.5	66.6	51.5	43.0	39.9	52.5	43.6	39.4
PC7	53.1	37.8	68.0	53.3	46.0	43.5	54.3	46.2	42.7
PC8	54.5	39.8	69.3	55.1	48.8	46.3	55.6	48.4	45.4
PC9	55.7	41.6	70.4	56.7	51.3	48.8	56.9	50.5	47.8
PC10	**56.8**[c]	43.2	**71.5**	**58.1**	**53.1**	**51.1**	**58.1**	**52.4**	**50.0**
PC11	57.8	44.8	72.6	59.4	54.8	53.1	59.3	54.1	52.0
PC12	58.7	46.3	73.5	60.7	56.5	55.0	60.4	55.7	53.9
PC13	59.6	47.8	74.4	61.8	58.0	56.9	61.4	57.2	55.6
PC14	60.5	49.2	75.3	62.9	59.5	58.4	62.4	58.5	57.1
PC15	61.2	**50.5**	76.1	64.0	61.0	59.9	63.3	59.8	58.6

[a]Principal components

[b]Percentage variation (Cumulative) explained by corresponding PC in mapping populations.

[c]Bold face number indicate corresponding number of PCs were used in MLM in TASSEL.

TABLE 4.4. Comparison of different methods employed in association analysis of resistance to common root rot studies in seven mapping populations in order to control false positives.

Mapping populations		Number of significant marker-trait associations			
	n^a	GLM+Q[b]	GLM+P[c]	MLM+Q+K[d]	MLM+P+K[e]
CAP06	384	30	23	6	3
CAP06 two-rowed	160	36	1	2	1
CAP06 six-rowed	224	48	26	29	18
CAP07	384	146	158	16	24
CAP07 two-rowed	142	72	19	8	10
CAP07 six-rowed	241	137	36	4	10
CAP06/07 combined	767	-[f]	172	-	43
CAP06/07 two-rowed	302	-	9	-	0
CAP06/07 six-rowed	465	-	11	-	1
CAP08	384	-	3	-	0
CAP08 two-rowed	158	-	1	-	1
CAP08 six-rowed	226	-	0	-	0

[a]*n* = number of genotypes in mapping population
[b]General linear model with Q matrices generated from Structure used as covariate
[c]General linear model with eigenvalues of PCA used as covariate
[d]Mixed linear model with Q matrices generated from Structure and Kinship matrices were used as covariate
[e]Mixed linear model with eigenvalues of PCA and Kinship matrices were used as covariate
[f]Population not tested for the AM using the given model

Genome wide scans of QTL resistance to CRR

The significant markers within 15 - 35 cM distance were considered to be in same region of a putative QTL. This criterion was based on the 10 - 25 cM distance of pair-wise LD at $r^2 = 0.2$ threshold. Significant –log (p) values as function of centimorgan distance (cM) of barley genome have been presented in Figs. 4.4, 4.5, and 4.6 for six-rowed, two-rowed and combined populations, respectively. A threshold of <0.001 [-log (p) = 3] was set for significant marker-trait associations in current study; therefore, any SNP-CRR resistance association

exceeding –log (p) = 3 was considered a putative CRR resistance QTL. The QTL that are identified in three or more mapping populations are discussed further. In six-rowed populations, the region of 28 - 51 cM on chromosome 3H, 180 - 195 cM on chromosome 5H, 30 - 64 cM and 91 - 97 cM on chromosome 6H, and 60 - 86 cM on chromosome 7H chromosome harbor significant SNP-CRR resistance associations (Fig. 4.4). Similarly, in two rowed populations, the genomic regions 28 - 51 cM on chromosome 3H and 91 - 97 cM on chromosome 6H appears important for significant association between SNP and CRR resistance (Fig. 4.5). In the combined populations (two- and six-rowed combined), the genomic regions, 28 - 51 cM on chromosome 3H, 180 - 195 cM on chromosome 5H, 30 - 64 cM on chromosome 6H, and 50 - 86 cM of chromosome 7H contains significant marker-trait associations in the CAP06, CAP07, and combined CAP06/07 populations (Fig. 4.6).

Putative QTL in CAP06 six-rowed, CAP06 two-rowed, and CAP06 populations. The significant SNP-CRR resistance associations, p-values, FDR (q-value) and R^2 (phenotypic variation explained by markers) in the CAP06 six-rowed, CAP06 two-rowed, CAP06, and CAP core populations are presented in Table 4.5. In the CAP06 six-rowed, 18 marker-trait associations were identified in 10 putative QTL regions. The fact that the FDR (q-value) of these significant associations is greater than the p-value indicates that these associations are true positives at the 0.1 threshold level. The phenotypic variations explained by SNPs varied from 3.7 - 5.4%. In the CAP06 two-rowed population, the only significant SNP-CRR resistance association was detected on chromosome 5H at 159.9 cM

distance. The FDR of this marker indicates that this association is true and marker explains 7.8% phenotypic variation. In the CAP06 combined population, three associations between SNP and CRR resistance were significant but the FDR of these associations were above 0.1 threshold level indicating false positives. In CAP core subset populations, three associations were found but analysis of FDR suggests that these associations were false positives (Table 4.5).

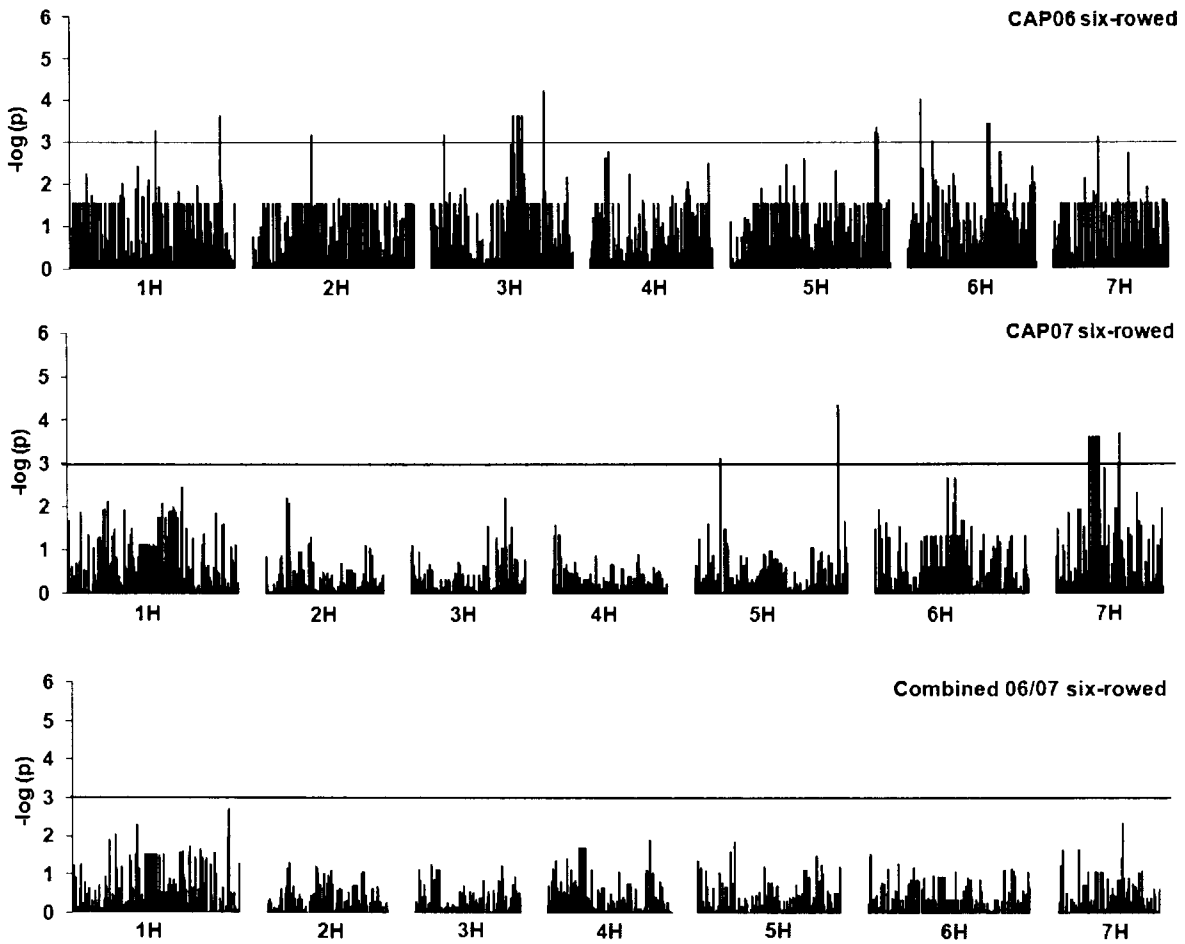

Fig. 4.4. Significant values -log (*p*) for SNP markers associate with common root rot resistance in CAP06 six-rowed (*n* = 224), CAP07 six-rowed (*n* = 242), and combined CAP06/07 six-rowed (*n* = 465) populations. A significant threshold of – log (*p*) = 3 is considered for all mapping populations. All marker trait associations are true positives.

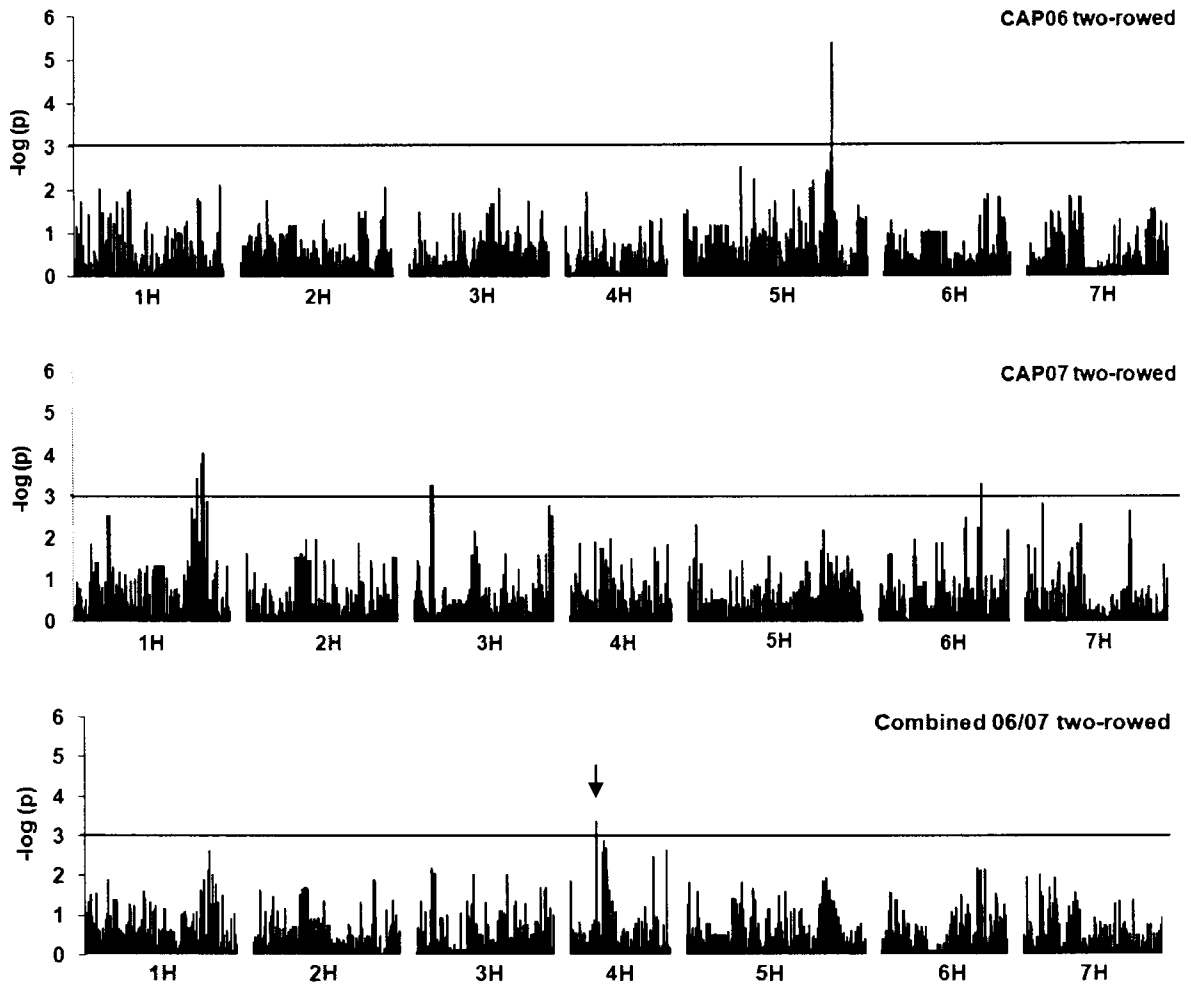

Fig. 4.5. Significant values -log (p) for SNP markers associate with common root rot resistance in CAP06 two-rowed (n = 160), CAP07 two-rowed (n = 142), and combined CAP06/07 two-rowed populations (n = 302). A significant threshold of – log (p) = 3 is considered for all mapping populations. All marker trait associations are true positives except the one which is indicated by an arrow.

Putative QTL in CAP07 six-rowed, CAP07 two-rowed, and CAP07 populations. The significant marker-trait associations, p-values, q-values, and R^2 for the CAP07 six-rowed, CAP07 two-rowed, and CAP07 are presented Table 4.6. Ten significant SNP-trait associations were detected in the CAP07 six-rowed population, two of which were mapped on chromosome 5H (37 and 180 - 195 cM regions). Other eight SNPs were mapped on chromosome 7H (78 - 86 cM region).

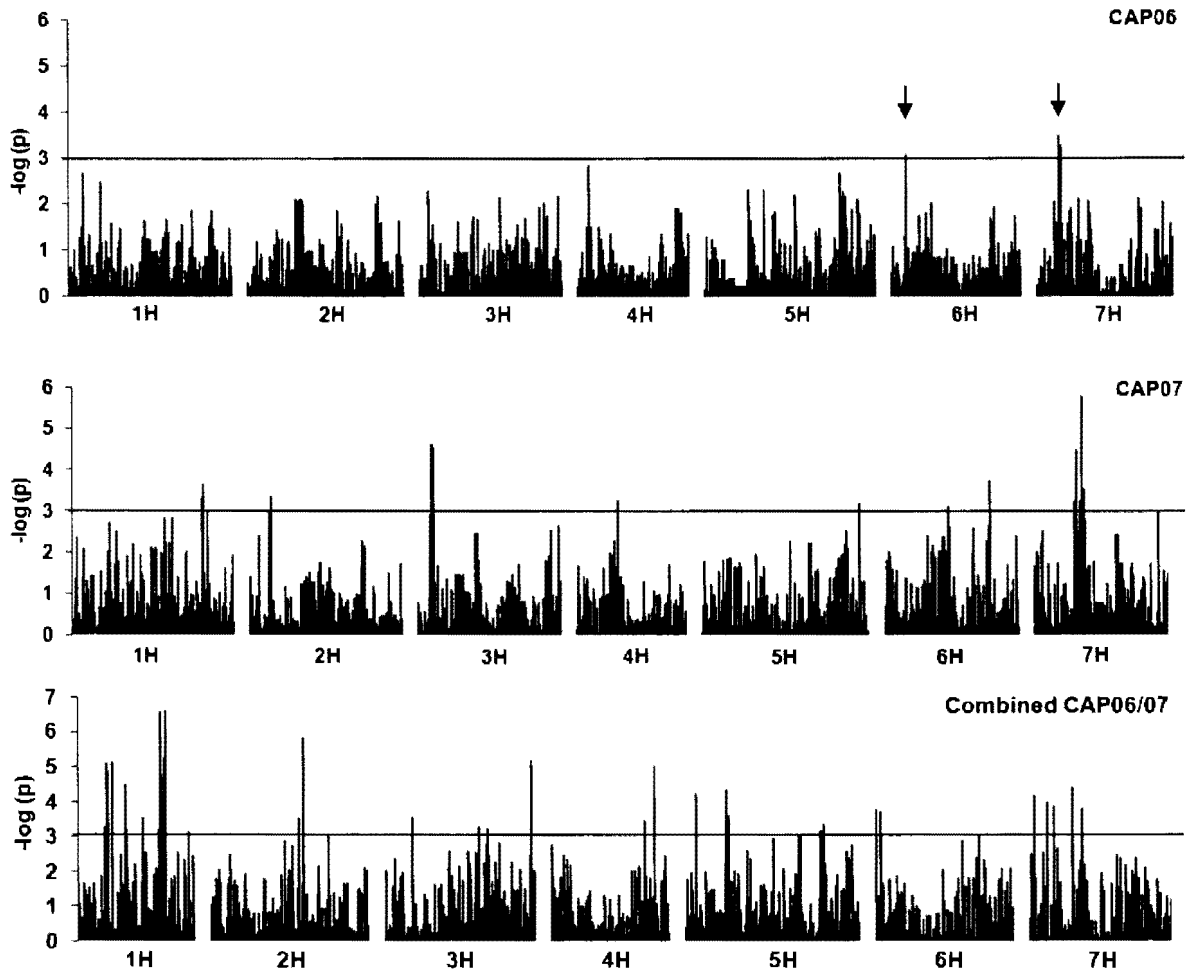

Fig. 4.6. Significant values -log (*p*) for SNP markers associate with common root rot resistance, combined for six- and two rowed barley, in CAP06 (*n* = 384), CAP07 (*n* = 384), and combined CAP06/07 (*n* = 767) populations. A significant threshold of –log (*p*) = 3 is considered for all mapping populations. All marker trait associations are true positives except the one which is indicated by an arrow.

The *q*-value (FDR) indicates that these associations are true positives (Table 4.6). Phenotypic variations explained by these markers ranged from 2.5 - 3.6%. In the CAP07 two-rowed population, ten SNPs were found to be significantly associated with CRR resistance and all associations were true positives based on the FDR (Table 4.6). These markers represent three putative QTL for CRR resistance and they mapped to chromosome 1H (86 - 94 cM region), chromosome 3H (28 - 51 cM region), and chromosome 6H (91 - 97 cM). The phenotypic

variations explained by the markers ranged from 5.05 - 6.44%. In the CAP07 combined population, 24 SNP were significantly associated with CRR resistance, of which four SNPs had unknown positions. All associations were true positives at q = 0.1 level and the phenotypic variations explained by SNPs ranged from 1.19 - 2.51%. Among 24 significant associations, three were on chromosome 1H, two on chromosome 2H, three on chromosome 3H, one in chromosome 4H, one on chromosome 5H, three on chromosome 6H, and seven on chromosome 7H, respectively.

In the combined CAP06/07 six-rowed population, no significant SNP-CRR resistance associations were detected (Table 4.7). In CAP06/07 two-rowed (302 breeding lines) population, a single significant SNP was detected on chromosome 4H. The phenotypic variation explained by this marker was 2.31%. However, FDR of this association suggests that it is a false positive (Table 4.7). In the combined CAP06/07 (767 breeding lines) population, all 43 marker trait associations were true positives at q = 0.1 level and phenotypic variations explained by them varied from 0.10-0.91%.

Putative QTL in combined CAP06/07 six-rowed, CAP06/07 two-rowed, and CAP06/07 populations. Among the putative QTL identified in current study, *CRR-3H-28-51*, *CRR-5H-180-195*, *CRR-6H-30-64, CRR-6H-91-97* and *CRR-7H-50-86* were identified in three or more mapping populations. *CRR-3H-28-51* was identified at 28 - 51 cM of chromosome 3H in the CAP06 six-rowed, CAP07 two-rowed, CAP07, and combined CAP06/07 mapping populations whereas *CRR-5H-180-195* was mapped in 180 - 195 cM of 5H in the CAP06 six-rowed, CAP07 six-

rowed and CAP07. On chromosome 6H, *CRR-6H-30-64* was mapped in CAP06 six-rowed, CAP06, and CAP07 populations. In the same chromosome, *CRR-6H-91-97* was identified on CAP07 two-rowed, CAP07, and combined CAP06/07 populations.

TABLE 4.5. Common root rot (CRR) resistant QTL, associated markers, and their phenotypic effects identified in CAP06 six-rowed, CAP06 two-rowed and CAP06 mapping populations.

Putative QTL	Marker	*p*-value	FDR (*q*-value)	R^2(%)
CAP06 six-rowed				
CRR-1H-128.1	12_10693	2.25E-04	0.041	4.59
CRR-1H-42.5	12_11266	5.30E-04	0.058	4.04
CRR-2H-40-62	11_0477	6.55E-04	0.058	3.93
CRR-3H-28-51 [a]	11_0917	6.37E-04	0.058	3.94
CRR-3H-70-104	11_1401	2.31E-04	0.041	4.54
CRR-3H-70-104	11_0762	2.31E-04	0.041	4.54
CRR-3H-70-104	12_10609	2.31E-04	0.041	4.54
CRR-3H-70-104	12_30170	2.31E-04	0.041	4.54
CRR-3H-127	11_0615	5.73E-05	0.00005	5.41
CRR-5H-180-195 [b]	12_30494	5.88E-04	0.058	3.97
CRR-5H-180-195 [b]	11_0160	4.40E-04	0.057	4.17
CRR-5H-180-195 [b]	12_30504	6.06E-04	0.058	3.98
CRR-6H-30-64 [b]	12_31485	9.29E-05	0.041	5.09
CRR-6H-30-64 [b]	11_0674	9.10E-04	0.072	3.75
CRR-6H-30-64 [b]	11_0994	3.65E-04	0.052	4.26
CRR-6H-30-64 [b]	11_0907	3.65E-04	0.052	4.26
CRR-7H-50-86 [a]	11_1213	7.24E-04	0.061	3.86
Unknown	12_10348	1.55E-04	0.041	4.78
CAP06 two-rowed				
CRR-5H-159.9	12_30759	3.44E-06	0.006	7.58
CAP06 combined				
CRR-6H-30-64 [b]	12_31485	8.32E-04	0.495 [c]	1.68
CRR-7H-12-35	11_0267	3.27E-04	0.495 [c]	1.94
CRR-7H-12-35	12_30083	5.55E-04	0.495 [c]	1.79

[a]Significant marker-trait association were identified in four out of nine mapping populations

[b]Significant marker-trait association were identified in three out of nine mapping populations

[c]False positives declared in FDR analysis

On chromosome 7H, QTL, *CRR-7H-50-86* was identified in four mapping populations. *CRR-7H-50-86* was detected in the CAP06 six-rowed, CAP07 six-rowed, CAP07, and combined CAP06/07 populations. The phenotypic variation explained by this QTL ranged from 1.30 – 3.86% in different mapping populations. The FDR at 0.1 level indicates that these associations were true positives (Tables 4.5, 4.6, and 4.7). In CAP08 populations, marker-trait associations were not detected in any population except CAP08 two-rowed barley. Marker, 11_11355, was found significantly associated with CRR resistance caused by *C. sativus* on chromosome 5H, but FDR suggested that this was a false positive. Therefore, CAP08 and sub-populations are not discussed further.

TABLE 4.6. Common root rot (CRR) resistant QTL, associated markers, and their phenotypic effects identified in CAP07 six-rowed, CAP07 two-rowed and CAP07 mapping populations.

Putative QTL	Marker	*p*-value	FDR (*q*-value)[c]	R^2(%)
CAP07 six-rowed				
CRR-5H-25-54	12_30410	7.87E-04	0.085	2.54
CRR-5H-180-195 [b]	12_30382	4.57E-05	0.030	3.65
CRR-7H-50-86 [a]	11_10073	2.52E-04	0.030	2.99
CRR-7H-50-86 [a]	11_10773	2.52E-04	0.030	2.99
CRR-7H-50-86 [a]	11_11145	2.52E-04	0.030	2.99
CRR-7H-50-86 [a]	11_11352	2.52E-04	0.030	2.99
CRR-7H-50-86 [a]	11_21335	2.52E-04	0.030	2.99
CRR-7H-50-86 [a]	12_30449	2.52E-04	0.030	2.99
CRR-7H-50-86 [a]	12_30199	2.03E-04	0.030	3.05
CRR-7H-50-86 [a]	11_20230	0.001	0.097	2.41
CAP07 two-rowed				
CRR-1H-86-99	11_10434	3.71E-04	0.075	5.41
CRR-1H-86-99	11_20792	3.41E-04	0.075	5.47
CRR-1H-86-99	12_11463	1.60E-04	0.075	5.99
CRR-1H-86-99	11_20475	9.46E-05	0.067	6.44

TABLE 4.6. (Continued)

Putative QTL	Marker	*p*-value	FDR (*q*-value)[c]	R^2(%)
CRR-1H-86-99	11_10433	9.46E-05	0.067	6.44
CRR-3H-28-51 [a]	11_10081	5.34E-04	0.075	5.09
CRR-3H-28-51 [a]	11_10710	5.34E-04	0.075	5.09
CRR-3H-28-51 [a]	11_10825	5.34E-04	0.075	5.09
CRR-3H-28-51 [a]	12_30953	5.34E-04	0.075	5.09
CRR-6H-91-97 [b]	11_20118	5.06E-04	0.075	5.21
CAP07				
CRR-1H-86-99	11_20475	4.95E-04	0.038	1.35
CRR-1H-86-99	11_10433	2.27E-04	0.028	1.51
CRR-2H-40-62	12_30420	4.33E-04	0.038	1.37
CRR-2H-40-62	11_10178	0.001	0.053	1.19
CRR-3H-28-51 [a]	11_10825	2.40E-05	0.008	1.94
CRR-3H-28-51 [a]	12_30953	2.40E-05	0.008	1.94
CRR-3H-28-51 [a]	11_20410	2.18E-04	0.028	1.51
CRR-3HS	11_21093	2.88E-05	0.008	1.92
CRR-4H-55.6	11_21481	5.70E-04	0.038	1.34
CRR-5H-180-195 [b]	11_20402	6.55E-04	0.040	1.30
CRR-6H-30-64 [b]	12_31178	7.81E-04	0.045	1.30
CRR-6H-91-97 [b]	11_20118	1.84E-04	0.028	1.54
CRR-7H-12-34	11_11348	1.67E-06	0.002	2.51
CRR-7H-50-86 [a]	11_10346	6.04E-04	0.038	1.30
CRR-7H-50-86 [a]	11_10721	5.13E-04	0.038	1.33
CRR-7H-50-86 [a]	11_10050	3.13E-05	0.008	1.91
CRR-7H-50-86 [a]	12_31441	5.72E-04	0.038	1.31
CRR-7H-50-86 [a]	11_20195	2.93E-04	0.028	1.45
CRR-7H-50-86 [a]	11_20750	5.77E-04	0.038	1.44
CRR-7H-151	12_10657	0.001	0.053	1.35
Unknown	12_31267	4.28E-05	0.009	1.84
Unknown	12_30050	1.95E-04	0.028	1.53
Unknown	12_30939	2.63E-04	0.028	1.47
Unknown	12_30908	2.74E-04	0.028	1.47

[a]Significant marker-trait association were identified in four out of nine mapping populations

[b]Significant marker-trait association were identified in three out of nine mapping populations

[c]All marker-trait association were true positives after FDR analysis

TABLE 4.7. Common root rot (CRR) resistant QTL, associated markers, and their phenotypic effects identified in two-rowed of CAP06/07 and combined population CAP06/07.

Putative QTL	Marker	*p*-value	FDR (*q*-value)[c]	R^2(%)
Combined CAP06/07 two-rowed (302 lines)				
CRR-4H-48-55	11_20853	4.22E-04	0.677	2.31
Combined CAP06/07 (767 lines)				
CRR-1H-50-65	12_30436	8.26E-06	0.002	0.69
CRR-1H-86-99	11_20475	2.67E-07	0.0002	0.91
CRR-1H-135-143	12_11496	7.22E-04	0.036	0.39
CRR-2H-81-85	11_20340	1.47E-06	0.0009	0.10
CRR-2H-116	12_30459	9.38E-04	0.043	0.49
CRR-3H-28-51 [a]	11_21197	2.76E-04	0.021	0.45
CRR-3H-70-104	11_10747	5.14E-04	0.032	0.41
CRR-3H-70-104	12_10662	6.04E-04	0.034	0.40
CRR-3H-173	11_10343	6.32E-06	0.002	0.69
CRR-4H-100-117	11_10269	9.26E-06	0.002	0.67
CRR-5H-25-54	11_10695	5.77E-05	0.007	0.73
CRR-5H-25-54	11_11128	4.34E-05	0.006	0.74
CRR-5H-142-160	11_21024	4.47E-04	0.028	0.56
CRR-6H-0-4	12_30319	1.73E-04	0.0002	0.64
CRR-6H-91-97 [b]	12_31235	9.81E-04	0.043	0.49
CRR-7H-2	11_21307	6.74E-05	0.007	0.55
CRR-7H-12-35	12_30780	1.02E-04	0.011	0.52
CRR-7H-50-86 [a]	11_10721	3.81E-05	0.005	0.58

[a]Significant marker-trait association were identified in four out of nine mapping populations

[b]Significant marker-trait association were identified in three out of nine mapping populations

[c]All marker-trait association were true positives after FDR analysis except CAP06/07 combined two-rowed population

The putative QTL, which are identified in more than three mapping populations and had lower FDR ($q < 0.1$), were only presented in Fig. 4.7. QTL, *CRR-3H-28-51*, mapped in short arm of chromosome 3H in current study and coincides with *Rcs-qtl-3H-3-4*, (QTL conferring resistance to spot botch caused by *C. sativus* in barley). *CRR-5H-180-195* was mapped on distal end of chromosome 5H. On

chromosome 6H, two QTL, *CRR-6H-30-64* (proximal to centromere) and *CRR-6H-91-97* (distal to centromere) were mapped (Fig. 4.7).

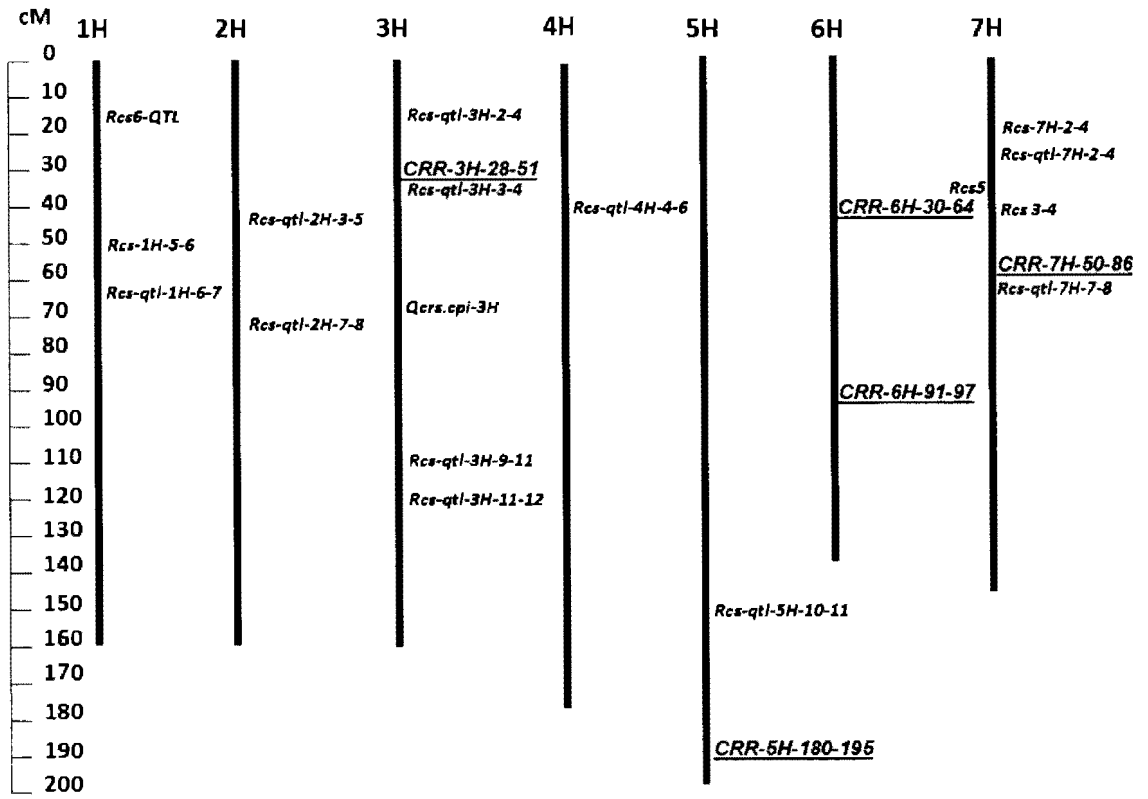

Fig. 4.7. Barley linkage map showing significant association ($p < 0.001$) of SNP markers with resistance to common root rot in different mapping populations. The marker-trait association which were identified at least two mapping populations are only presented. Each barley chromosomes are divided into approximately 10 cM genetic distance by a scale in the left. The QTL identified and mapped in current study is designated as *CRR-Genome-Genetic distance* (solid underline). The spot blotch (*Rcs*) and root rot (*Qcrs*) resistance gene(s) / QTL reported in earlier studies by Steffenson et al. (1996), Bilgic et al. (2005), Bilgic et al. (2006), Yun et al. (2005), and Yun et al. (2006) are not underlined.

QTL, *CRR-7H-50-86* was detected near centromere (50 - 86 cM region) of chromosome 7H. This QTL coincides with QTL identified for resistance to spot blotch in earlier mapping studies (Fig. 4.7). The analyses of significant –log (p)

values for different mapping populations (Figs. 4.4, 4.5, and 4.6) indicate that flaking SNP markers close to *CRR-3H-28-51*, *CRR-5H-180-195*, *CRR-6H-30-64*, *CRR-6H-91-97*, *CRR-7H-12-35* and *CRR-7H-50-86* are either significant at $p < 0.001$ or have a lower *p*-value.

Discussion

To our knowledge, this is the first report of mapping CRR resistance QTL in barley. At least five putative QTL resistance to CRR, *CRR-3H-28-51, CRR-5H-180-190, CRR-6H-30-64, CRR-6H-91-97,* and *CRR-7H-50-86* were detected in three or more mapping populations. Among these QTL, *CRR-3H-28-51*, *CRR-7H12-35*, and *CRR-7H-50-86* coincide with previous mapped spot blotch resistance QTL in barley (Fig. 4.7). We have found that the QTL are segregating in two- and six-rowed subpopulations of CAP06 and CAP07. The effects of LD pattern, population structure, marker density, and population size on marker-trait association and resolution of genome-wide scanning of CRR are discussed in current studies.

Linkage disequilibrium (LD) in CAP lines

The LD estimates are influenced by mode of reproduction of mapping populations (Nordsborg and Donnelley 1997) and markers used (Hedrick 1989). In general, the LD extends to longer genetic or physical distances in self-pollinated crops, whereas it decays rapidly in cross-pollinated crops. The estimates of LD in maize (*Zea mays* L.) ranges from 200 to 2000 bp (Tennaillon et al. 2001; Remington et al. 2001) whereas it extends to 50 kbp in the self-pollinated plants

Arabidopsis (Nordborg et al. 2002). Furthermore, the LD estimates were reported higher in advanced breeding lines compared to their wild relatives and landraces due to higher selection pressure, distinct sub-population, and specific adaptations of breeding lines (Dreisigacker et al. 2004; Melchinger et al. 1994). Steffenson et al. (2007) reported rapid LD decay within 1 cM in 318 wild barley (*Hordeum spontaneum*) accessions on all chromosomes except 4H for which LD extended beyond 40 cM. Similarly Lin et al. (2002) observed very low LD between closely linked *adh* loci on chromosome 4H in wild germplasm of barley. The LD decay in advanced breeding lines is reported variable. Malyasheva-Otto et al. (2006) estimated LD within 10 cM using SSR markers in a study that included worldwide collection of barley germplasm. Kraakman et al. (2004) reported that LD extended from 1cM to beyond 10 cM genome wide but was highly variable across the genome in barley, which is in agreement with our results. In the current studies, genome wide LD estimates were within 10 cM in both the CAP06 and CAP07 populations, but were variable between individual chromosomes. LD extended beyond 20 cM in chromosomes 7H and 5H in the CAP06 and CAP07, respectively (Fig. 4.2). Caldwell et al. (2006) suggested that selection and genome structure have effects on LD pattern. Furthermore, Stracke et al. (2007) demonstrated the effects of selection pressure on resistant lines in barley. They found that the LD extended in resistant lines further than the recombination would be able to break the linkage. In contrast, the LD in the susceptible lines decayed rapidly within 1 cM. Similarly, Rostoks et al. (2006) reported that LD was highly variable among two- and six-rowed European spring barley lines. LD extended beyond 60 cM in

spring six-rowed barley compared to 15 cM in two-rowed barley. In current study also, LD extended beyond 20 cM in six-rowed mapping populations in the CAP06 and CAP07 whereas average LD was 10 cM in two-rowed barley in both years. A variable pattern of LD estimates were reported by Somers et al. (2007) in durum (*Triticum turgidum* subsp. *durum*) and hexaploid wheat (*Triticum aestivum* L.). The genome wide LD was extended only two to three cM whereas in some genomic regions the LD extended up to 25 cM in durum and 41 cM in bread wheat.

Population structure

The principal component analysis clearly revealed three distinct groups, six-rowed barley, two-rowed barley NDSU (ND2), and two-rowed barley form BA program (Fig. 4.3). The breeding history of six-rowed barley breeding programs in the USA suggests that these breeding programs share common parents (Martin et al. 1991). Therefore, it was not unexpected that all six-rowed barley from three breeding programs (ND6, UM, and BA six-rowed) clustered together. It has been reported that the six-rowed barley of North America can be traced back to five common ancestors (Martin et al. 1991). Furthermore, Horsley et al. (1995) found that, out of seven ancestors in ND6 and six ancestors in UM breeding programs, five ancestors were common in these two breeding programs. Among the three ancestors, Mandscheuri, Lion, and Peatland shared 38 and 46% of germplasm in ND6 and UM breeding programs, respectively. Therefore, the germplasm base of North America can be considered narrow and is in agreement with current results.

The two-rowed barley of BA was separated from two-rowed breeding program of NDSU in PCA (Fig. 4.3). The breeding program of BA introduces international

germplasm into their programs; therefore, two-rowed barley from BA formed a distinct group from ND2. However, there was some overlapping between the ND2 and BA two-rowed clusters. This overlapping could be due to common parents used in both breeding programs. Price et al. (2006), Patterson et al. (2006), and Zhao et al. (2007a) suggested that PCA can be used to minimize the number of false positives and maximize the power to detect true associations. In AM of Arabidopsis, Zhao et al. (2007a) used PCs in MLM and compared the results of Q-matrices when used in MLM. They reported that the results of MLM+P+K (using P-matrices) were similar to MLM+Q+K (using Q-matrices) model which is in agreement with current results.

Putative QTL resistance to CRR

In the current study, *CRR-3H-28-51*, and *CRR-7H-50-86* were detected in four mapping populations. *CRR-5H-180-195*, *CRR-6H-30-64*, and *CRR-6H-91-97* was found in three mapping populations (Tables 4.5, 4.6, and 4.7). Furthermore, FDR of significant markers suggests that these associations were true. FDR is useful in genome wide studies in which many markers are tested for association for trait of interest (Kraakman et al. 2004; Kraakman et al. 2006). Therefore, putative QTL identified in different mapping populations and that have *q*-values less than the 0.1 threshold are only discussed further.

The QTL *CRR-3H-28-51* on chromosome 3H, was identified in the CAP06 six-rowed, CAP07 two-rowed, CAP07 combined, and combined CAP06/07 populations. In the CAP06 six-rowed population, only one marker 11_0917 was found significant (Table 4.5). The marker explained 4% of the phenotypic variation

and it had *q*-value = 0.058. In the CAP07 two-rowed population, four SNPs had significant associations with CRR resistance one of which was also found in combined CAP07 population. In the CAP07 population, three SNPs were found significantly associated with *CRR-3H-28-51.* The R^2 ranged from 1.51 - 1.92% with *q*-value varied from 0.008 - 0.028 for these associations. In the combined CAP06/07 mapping population, 11_21197 was found significantly associated with this QTL. In all associations, the FDR was below 0.1 level. The results indicate that the associations were true positive. Identifications of SNP-CRR resistance associations in 28 - 51 cM of chromosome 3H in four mapping populations was also verified by –log (*p*) analysis (Figs. 4.4, 4.5, and 4.6). In addition, this QTL is present in both two- and six-rowed germplasm investigated. These results indicate that this region likely harbors an important QTL resistance to CRR in barley germplasm from the upper Midwest of the USA. Furthermore, this QTL coincides with the QTL *Rcs-qtl-3H-2-4* and *Rcs-qtl-3H-3-4* that confer resistance to spot blotch caused by *C. sativus* in barley. Bilgic et al. (2005) mapped spot blotch resistance QTL, *Rcs-qtl-3H-3-4,* in the same region where *CRR-3H-28-51* was mapped in the current study. In another study using Calicuchima-sib/Bowman-BC population, Bilgic et al. (2006) identified the spot blotch resistance QTL, *Rcs-qtl-3H-1-2*, proximal to *CRR-3H-28-51*. Li et al. (2009) mapped the crown rot (CR) resistant QTL *Qcrs.cpi-3H* in the region 59.7 – 71.8 cM, distal to *CRR-3H-28-51*. However, another QTL detected in 70-104 cM region in chromosome 3H, *CRR-3H-70-104*, coincides with *Qcrs.cpi-3H*. However, *CRR-3H-70-104* was detected only in the CAP06 six-rowed and combined CAP06/07 mapping population only.

Therefore this QTL need to be validated further using different mapping populations.

CRR-5H-180-195 was mapped in 180 - 190 cM region of chromosome 5H in the CAP06 six-rowed, CAP07 six-rowed and CAP07 mapping populations. Three SNPs, 11_0160, 12_30170, and 12_30504 were found significantly associated with CRR resistance in CAP06 six-rowed population. The markers 12_30382 and 11_20402 were significant in CAP07 six-rowed and CAP07, respectively. These markers explained 1.30 – 4.17% phenotypic variation in different mapping populations had FDR below 0.1 level. *CRR-5H-180-195* was mapped in three independent mapping populations and SNPs associated with it were true positives. In this region, no known QTL resistant to spot blotch has been mapped. The distribution of –log (p-value) in 5H (Fig. 4.4) indicated that only one SNP, 12_30382 was highly significant (CAP07 six-rowed) while the *p*-values of nearby markers in the same bin are high; however, the FDR suggest that the associations in this regions were true positives (*q*-values = 0.04 - 0.06). Results also showed that this QTL is present predominantly in six-rowed materials studied. Bilgic et al. (2005) mapped *Rcs-qtl-5H-10-11* for spot blotch resistance in the same bin where *CRR-5H-159.9* was mapped in the current study. The QTL was identified only in the CAP06 two-rowed populations; and therefore, it needs to validate by using different mapping populations. This region also harbors other resistance sources such as stem rust resistance genes, *rpg4* and *Rpg5* (Steffenson et al., 2007).

The short arm of chromosome 7H is an important region in terms of resistance to *C. sativus* in barley because *Rcs5, Rcs-7H-2-4, Rcs-qtl-7H-2-4, Rcs-3-4,* and

Rcs-qtl-7H-7-8 were all mapped in this region. In the current study, the QTL *CRR-7H-50-86* was mapped to bins 5 - 8 region of the chromosome 7H in the CAP06 six-rowed, CAP07 six-rowed, CAP07, and combined CAP06/07 populations. The phenotypic variations explained *CRR-7H-50-86* ranged from 0.58 - 3.86%. The analyses of flanking markers of *CRR-7H-50-70* in bins 5 - 8 of chromosome 7H indicate that there are significant marker-associations in the regions (Fig. 4.6). The distribution of –log (*p*-value) in the six-rowed and combined populations (Figs. 4.5 and 4.7) also reveal that the 50-80 cM region of chromosome 7H has significant markers associated with CRR resistance. From current findings, it can be speculated that short arm of the chromosome 7H region may harbor possible multiple loci or alleles at a single locus resistance to CRR and spot blotch, and this region warrants further research. Furthermore, previous mapping studies also reveal that the region also contains the major genes *Rpg1* (*Puccinia graminis* f. sp. *tritici*), QTL loci such as *Rpt-1-2* (*Pyrenophora teres*), *Rrs-1-2* and *Rrs-2-4* (*Rhynchosporium secalis*), and *Rbg-1* (*Blumeria graminis f. sp. hordei*) (Steffenson et al. 2007, Yun et al. 2005, Yun et al. 2006). QTL, *CRR-7H-50-86* was mapped in bin 6 - 7 of 7H which was detected in the six-rowed of CAP06 and CAP07, CAP07, and combined CAP06/07 populations. Interestingly this QTL is mapped in the same region of which spot blotch resistance QTL *Rcs-qtl-7H-7-8* was mapped in Diktoo/Morex and Harrington/TR306 population conferring seedling resistance to spot blotch (Bilgic et al. 2005).

Population effects on QTL detection

Population effects on QTL detection were evident from the segregation of different QTL in two- and six-rowed barley. Previous studies indicate that two- and six-rowed barley constitute distinct populations and relatively low gene-flows found between these two gene pools (Malysheva-Otto et al. 2006). However, intercrosses have been made between two- and six-rowed barley from the Upper Midwest of the USA, especially in the NDSU two-rowed breeding program. Therefore, common QTL in these two row-types can be expected from the current study. Genetic diversity, population size, allele frequency, and LD can differ between two- and six-rowed barley, and this has implications on QTL detections in the current study. The SNP-CRR resistance associations found in the CAP07 two-rowed population were entirely different than the marker-trait associations detected in the CAP07 six-rowed population. It is possible that different QTL are segregating in the CAP06 two- and six-rowed barley germplasm used in the current study or different flanking markers may be needed for the two- and six-rowed population fi they are in the same QTL region. However, *CRR-3H-28-51* was detected in both the CAP06 six-rowed and CAP07 two-rowed populations. This may be due to the intercrosses of two- and six-rowed barley made within Midwest barley germplasm in the past. Alternatively other factors may be responsible for the fewer QTL detected in two-rowed populations including differences in phenotypic variation, allelic frequency, and LD of two mapping populations. For example, the two-rowed barley was more susceptible compared to six-rowed in both years (Table 4.1). It can be speculated that the number of QTL resistance to CRR is low in two-rowed

compared to six-rowed subpopulation. This is evidenced by the lower number of QTL detected in two-rowed compared to six-rowed in CAP06 (Table 4.5). However, the number of QTL detected was the same in two and six-rowed populations in CAP07. In contrast, a lot more marker-trait associations were found in the CAP07 that the CAP06 population. This indicated that population structure due to allele frequency differences between two-rowed and six-rowed barley may be confounding QTL detection in barley germplasm. Long and Langley (1999) argued that population size has effects on power of association to detect candidate loci of complex traits. Therefore, another possibility is that the population size of two-rowed (n = 302) was smaller compared to six-rowed (n = 465) barley populations; thereby, minimizing the power to detect marker-trait association in two-rowed germplasm.

QTL regions identified in the current study varied considerably from single marker positions to 35 cM in different mapping population and some of the markers may be in LD in this region. A number of factors such as population size, LD at QTL regions, marker density, and allele frequency might have influenced the resolution of the current association mapping. Except population size, the other factors were variable within each mapping population whereas all factors were variable among different mapping population sets. The LD pattern within and across the genome partly explains the map resolution in the current study.

The phenotypic variation of the putative QTL region explained by associated markers ranged from 0.45 – 2.45% in combined populations while it ranged from 2.30 - 7.58% and 2.50 - 5.41% in two- and six rowed subpopulations, respectively.

Many factors can be attributed to this small R^2 explained by markers. First, the resistance to CRR is a complex trait because multiple pathogens cause CRR severity in barley. Common root rot is caused by *C. sativus* and many species of *Fusarium* in the field. Previous studies have indicated that the resistance to CRR is polygenic in nature (Bailey and Wolf 1994; Kutcher et al. 1994). The continuous distributions of CRR severity in both CAP06 and CAP07 of the current study indicate that many alleles with smaller effects may be involved in resistance to CRR. Second, the mapping populations (CAP06 and CAP07) are advanced breeding lines generated from relatively narrow gene pools of the Upper Midwest. Due to strong selection for target traits in advanced breeding lines, the QTL with larger effects tends to be fixed rapidly and become difficult to detect in association studies. In contrast, bi-parental mapping populations are often generated by wide crossing to create large variations in segregating or recombinant inbred lines, which allows researchers to detect markers that predict QTL with larger genetic effects. In AM studies, the marker-trait significance may be caused by few lines segregating in the population; therefore they tend to explain smaller phenotypic effects. Third, the larger population size allows for more accurate detection of QTL effects than the smaller population size. Long and Langley (1999) estimated the effects of sample size on the power of association to detect the candidate loci to variation of complex traits. In a simulation study, they reported that with 500 individuals explaining 10% of phenotypic variation, the power of association to detect candidate loci was 0.85. To achieve the same level of power of association to detect complex genetic loci, at least 25% phenotypic variation is required for a

sample size of 100 individuals. The sampling size (n = 384) can be considered sufficient in the current study to achieve a satisfactory level of power to detect CRR resistance QTL. Furthermore, the QTL are segregating in either two- or six-rowed subpopulations in this study. Therefore, the analysis in a larger data set (combined CAP06/07) tends to detect QTL with smaller phenotypic effects with more accuracy (Table 7). This is evident from detection of QTL with small R^2 in CAP06 and CAP07, which is a combined population of both two- and six-rowed barley. In contrast, QTL with relatively larger R^2 were detected in two- and six-rowed subpopulations than the combined populations in both years. Kumar et al. (2002) reported that in the upper Midwest of the USA and prairie regions of Canada, *C. sativus* is widely distributed and is a major problem in small grain causing CRR, spot blotch and black point disease. Therefore, it can be speculated that the larger effects of CRR resistance have been fixed by intuitive selection in the breeding programs of the Upper Midwest of the USA. The more accurate assessments of QTL, though they have smaller phenotypic effects in the current study, have important implications on MAS. The QTL with larger effects can be effective in phenotypic selection whereas QTL with smaller effects are more effective through MAS only if the phenotyping for small effects can be done more accurately.

In conclusion, association mapping, using advanced breeding lines, has potential to identify novel QTL and introgress them immediately into potential adapted barley cultivars. Common root rot resistant QTL, *CRR-3H-28-51, CRR-5H-180-195, CRR-6H-30-64, CRR-6H-91-97,* and *CRR-7H-50-86* have been

mapped in the upper Midwest germplasm and are segregating in different populations. Therefore, AM can complement the bi-parental mapping approach to dissect the complex traits like CRR resistance. Although the QTL identified in this study explain small phenotypic variation, they are more accurate and can be used directly in MAS without any major linkage drag after these QTL are validated in different genetic background and environments.

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CHAPTER 5. GENOME-WIDE ASSOCIATION MAPPING OF RESISTANCE TO *COCHLIOBOLUS SATIVUS* ISOLATE 4008 CAUSING SPOT BLOTCH IN BARLEY GERMPLASM FROM THE UPPER MIDWEST OF THE USA

Abstract

Cochliobolus sativus causes spot blotch (SB) in barley (*Hordeum vulgare* L.) which is an important foliar disease of barley in North America. The disease may cause up to 35% of yield losses in susceptible cultivars. Durable resistance of NDB112 has provided protection against this disease successfully for the last four decades. Recent emergences of virulence groups, which have overcome resistance of NDB112, pose potential threat to barley cultivation in the region. Therefore, the current study was conducted to explore novel sources of resistance to virulence group 7.7.3.6 of *C. sativus*. In this study, 384 breeding lines from the Upper Midwest of the USA, were phenotyped for resistance to SB using *C. sativus* isolate 4008 in greenhouse. After filtering 3072 SNPs for 5% minor allele frequency, 1873, 1923, and 2028 markers were applied to map SB resistance in the CAP06 six-rowed, the CAP06 two-rowed, and the CAP06 populations using association genetics. To control the spurious marker-SB resistance association, P-matrices (assignments of PCA) were combined with K-matrices (Kinship) and analyzed using mixed linear model. The phenotypic analysis revealed a continuous distribution of SB severities but skewed towards susceptible lines. Only two lines from the six-rowed breeding program of NDSU showed high resistance to *C. sativus* isolate. The genome wide linkage disequilibrium (LD) was 10 cM however

the LD varied across genomes. Among five marker-trait associations detected in current study, only two associations were true positives. Among these putative QTL, *Rcs-1H-84.6* was detected in chromosome 1H whereas *Rcs-2H-106-122* was found in chromosome 2H. These QTL do not coincide with any QTL resistance to SB which were mapped in earlier studies and therefore are unique. Lack of major QTL resistance to virulence group 7.7.3.6 of *C. sativus* warrants more research on new sources of resistance to this pathogen. Although the phenotypic variations explained by the significant markers are small and QTL confers moderately resistance to SB disease, these QTL are important for providing additional protection against newly emerged virulence groups in the region. The genetics of resistance to isolate 4008 in two advanced breeding lines, ND233429 and ND23445, is unknown and warranted more research on genetics of these two lines in future.

Introduction

Cochliobolus sativus (Ito & Kurib.) Drechsl. ex Dast [anamorph, *Bipolaris sorokiniana* (Sacc. in Sorok.) Shoem.], is an important and the most frequently isolated root and foliar pathogen in barley in North America (Mathre, 1982). The pathogen is also widely distributed in small grains in other regions including South Asia (Kumar, 2002), Australia (Wildermuth, 1986; Whittle, 1992), and central Asia (van Leur, 1997). *C. sativus* causes spot blotch on leaves and black point on seeds (Mathre, 1982; Kumar et al., 2002). The infection on leaves results in reduced photosynthetic areas and under severe cases whole leave are blighted, which leads to premature senescence of the leaves as well as death of the plant.

High humidity and temperatures of 22 - 30° C favor spot blotch development (Mathre, 1982). These environmental conditions are commonly found in the prairie regions of the Upper Midwest of the USA, including the Red River Valley, and central provinces of Canada during the growing seasons. *C. sativus* has a wide host range and cause diseases in wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), oat (*Avena sativa* L.), and rye (*Secale cereale* L.) (Tinline, 1988). Spot blotch can cause as high as 30 - 36% yield losses in barley in prairie regions of North America (Clark, 1979; Wilcoxson 1990; Ghazvini and Tekauz, 2004) in susceptible cultivars. Therefore, this pathogen is considered a serious impediment to successful cultivation of barley.

Spot blotch resistance in the Midwest six-rowed barley is largely conditioned from a durable gene from the line NDB112 (Wilcoxson et al. 1990; Steffenson et al. 1996). *Rcs5*, *Rcs6*, and a number of QTL have been mapped in barley chromosomes and condition resistance to spot blotch in barley in the regions (Steffenson et al. 1996; Bilgic et al. 2006, Yun et al. 2006). The mapping of these genes and QTL were done using widely distributed pathotype 1, represented by *C. sativus* isolate ND85F. Zhong and Steffenson (2001) reported genetic and virulence diversity of *C. sativus* causing spot blotch in ND. They reported that three pathotypes of *C. sativus* in ND with pathotypes 1 widely distributed in the region. Recently, Ghazvini and Tekauz (2008) reported a new virulence pattern of *C. sativus* in Canada. They found the emergence of new virulence groups that have overcome the durable resistance of NDB112. The virulence group 7.7.3.6 isolated from barley roots in ND has overcome resistance of NDB112. Therefore, the

objectives of this study were to scan the whole barley genome for marker-SB resistance associations using advanced breeding lines from the upper Midwest of the United States and identify QTL for resistance to virulence group 7.7.3.6 of *C. sativus* specifically isolate 4008.

Materials and Methods

Barley germplasm

The barley germplasm of this study consisted of 384 advanced breeding lines developed in four breeding programs in the Upper Midwest of the USA. Each breeding programs namely, North Dakota State University (NDSU) six-rowed (ND6), NDSU two-rowed (ND2), University of Minnesota (UM) six-rowed, and Busch Agricultural Resources (BA) two- and six-rowed submitted 96 lines in 2006 to Barley Coordinated Agricultural Project (CAP). The BA breeding program develops both two- and six-rowed malting barley whereas the other breeding programs either specialized in six- or two-rowed barley cultivar development. The submitted lines were inbred at least four generation (F_4) and selected for uniform agronomic traits adapted to the Northern Great Plain. The lines were of representative to each breeding programs but these lines were not selected for resistance to newly emerged virulence group 7.7.3.6. However, all breeding programs in the Upper Midwest have similar targets and are focused to malting barley in the Northern Great Plains. In this paper, we investigated association mapping of SB resistance in three different mapping populations.

To investigate the AM for spot blotch resistance against virulence group 7.7.3.6 of *C. sativus*, the first populations, the CAP06 (n = 384), was analyzed, which

contained both two- and six-rowed barley from all four breeding programs. The CAP06 population was further subdivided into two based on two- and six-rowed barley. These included the CAP06 two-rowed (n = 160) and the CAP06 six-rowed (n = 224) subpopulations.

Phenotypic evaluations

The CAP06 lines were evaluated for SB (SB) resistance using highly the virulent *C. sativus* isolate 4008 of newly emerge virulence group 7.7.3.6. in the Department of Plant Pathology, NDSU in 2008. We used isolates 4008 because it was found highly virulent on all cultivars tested at the seedling stage including NDB112, with a reading of 7 - 9 based on the 0 - 9 scale of Fetch and Steffenson (1994). Considering the high virulence of isolate to all cultivars, the screening for SB was done in greenhouse during winter 2008 to prevent the escape of isolates during cropping season. Entries were assigned to experimental units using a randomized complete block design with two replicates of CAP06 lines. Within each replicate, the checks ND5883, Bowman, and NDB112 were repeated 16 times.

Experimental units were 21 cm x 3.8 cm containers (Hummert International, Earth City, MO, USA), filled with Sunshine mix #1. Three to five seedlings were grown per cone. Slow release fertilizer (14-14-14 N-P-K, 0.3 g per cone) was applied at seeding. The seedlings were grown in the greenhouse at 22-25°C with a 16-h photoperiod. Leaf inoculation was done as described by Valjavec-Gratian and Steffenson (1997). In brief, at the two leaf stage (12 - 14 days), seedlings were inoculated with 5000 conidia ml^{-1} suspension of *C. sativus* isolate 4008. Polyoxyethylene-20-sorbitan monolaurate (100 µl L^{-1}) was added to the conidial

suspension to facilitate even distribution and better adsorption of conidia onto the leaf surface. The inoculum (approximately 0.15 mL plant^{-1}) was sprayed on the leaf surface to the point of runoff using an atomizer (DeVilbis Health Care Ind., Somerset, Pennsylvania). The inoculated plants were kept in the dark for 18 h at 22°C and approximately 100% relative humidity using ultrasonic misting chambers. The next day, plants were air dried slowly before being transferred to the greenhouse at 22-25°C and incubated for the next 10 days before scoring SB severity. SB severity was assessed using the 0-9 severity scale of Fetch and Steffenson (1994).

Genotypic evaluations

The genotyping of all barley CAP 06 lines was accomplished in the USDA-ARS genotyping laboratory of Dr. Shioman Chao in Fargo, ND. The DNA was isolated from a single plant grown in greenhouse at the UM. The leaf tissue was freeze dried at the UM and sent to Dr. Chao in Fargo for DNA extraction using the method of Slotta et al. (2008). The lines were genotyped using 3072 single nucleotide polymorphism (SNP) markers (Close et al. 2009). The SNP markers were designed onto Oligonucleotide Pool Assay (OPA) using Expressed Sequence Tag (EST) and sequenced amplicons at the University of California, Riverside, CA (Close et al. 2009). Based on map locations, technical performances, minor allele frequency and biological interest of SNPs, two 1536 production OPAs named as named as BOPA1 and BOPA2 were produced. The Illumina Bead Station was used to generate genotypic data using Golden Gate Assay as described by Fan et

al. (2003). In total, data for 3072 SNP markers from BOPA1 and BOPA2, were generated for CAP06 and used in association mapping studies.

Population structure

To control number of false positives, principal component analysis (PCA) (P-matrices) was employed as covariates in AM analysis. P-matrices were generated in TASSEL 2.1. Therefore, PCA was applied to genotypic data (filter SNPs for 5% minor allele frequency, MAF) to infer the genetic variations due to the differences in allele frequency in mapping populations. Top ten to 15 principal components, which explained > 50% of total variations, were used as covariate in the association analyses using both GLM and MLM. The PCA assignments (P-matrices) were combined with the kinship coefficient matrices (K- or Kinship matrices), is a measure of relative kinship which is the probability that the two genes are identical by the descent) as covariate in the mixed linear model (MLM) in association analysis. The kinship matrices were generated in TASSEL 2.1 using filtered (5% MAF and 10% missing data) SNP markers (Lynch and Ritland 1999). Therefore, results based on PCA assignments in association analyses were only discussed in this study.

Linkage disequilibrium (LD) and association mapping (AM)

Linkage disequilibrium was analyzed using filtered SNP markers in TASSEL 2.1. The pair wise LD (r^2) was used to estimate LD between pairs of SNP markers (Pritchard and Przeworski 2001). The LD decay plots were generated in SAS (SAS, 2002) as function of (r^2) over genetic distance (cM). The LD pattern for SNP markers were also analyzed for each chromosome separately. Association

mapping of each mapping sets were conducted in TASSEL 2.1 (Bradbury et al. 2007). The filtered genotypic data (SNP markers), phenotypic data (SB severities), P matrices (inferred population stratification based on PCA) and K matrices (Kinship matrices) were used to run a mixed linear model (MLM) in TASSEL. The default run parameters of MLM were used except analysis method was set to expectation maximization (EM). The significant marker-trait associations were declared at $p < 0.001$. The corresponding r^2 and p-values were used in further analyses such as LD profile genome wise and false discovery rate (FDR) or q-value. To control the false positives in multiple testing, we employed a procedure that estimate the false discovery rate (FDR) explained by Benjamini and Hochberg (1995). The FDR is defined as the proportion of false positives among the significant tests. We used SAS (2002) to estimate FDR (q-value) and set $q < 0.1$ as a threshold. We analyzed genome wide AM as described previously (Kraakman et al. 2004). First we analyzed the significant marker-trait associations. Second we examined the LD profile across genomes (chromosome wise association profiles containing –log (p) values of trait-associated markers for all mapping populations. Third, the examination of flanking markers of the trait-associated markers was done while scanning QTL in the genome.

Statistical analysis

The phenotypic data were analyzed using both descriptive (mean and standard deviation) and inferential statistics analysis of variance (ANOVA) in SAS (SAS, 2002). The SNP markers with more than 10% missing values and less than 5%

minor allele frequency were excluded from the association analysis. The filtered SNP data were created for each mapping sets and used in mapping studies.

Results

Phenotypic evaluation

The average SB severity of CAP06 was 6.5 (Table 5.1). The analysis of variance suggested that there was significant difference in breeding lines for SB severity. Isolate 4008 produced severe SB severity on all three checks, ND5883, NDB112, and Bowman. The average of 32 replicates of ND5883, NDB112, and Bowman were 8.3, 7.8, and 6.4 respectively. In current study, the durable resistance of NDB112 was overcome by *C. sativus* isolate 4008. This result is consistent with other experiments conducted for SB resistance assessments where isolate 4008 was highly virulent on NDB112 (current study). Only six breeding lines had SB severity scale < 4. The SB severity of ND23329 and ND23345 were 2.6 and 2.9, respectively and were significantly lower than all three checks using a Dunnett's test ($p < 0.05$). Three other breeding lines from NDSU and one from UM had mean SB severity ranging from 3.1 - 4. Mean SB severity of six-rowed barley was lower than two-rowed barley. The frequency distribution of disease severity scale across row types is presented in Fig. 5.1. The frequency distribution revealed that disease severity had a continuous distribution.

Marker coverage, population structure, and genome wide LD

The summary of marker coverage and LD is been presented in Table 5.2. Number of SNP markers, after filtering for 10% missing and 5% minor allele frequency, ranged from 1873 to 2028 for three different mapping populations. The

average distances of SNP marker was 0.58 for CAP06 whereas it was 0.62 and 0.64 cM for two- and six-rowed barley, respectively. The number of gaps for >10 cM was less than 5 for any of the populations used for mapping. The principal component analysis revealed two distinct groups in two-rowed barley whereas all six-rowed barley from ND6, UM, and BA clustered together (Fig. 5.2). In two-rowed barley, lines from ND2 and BA barley breeding programs formed two distinct groups.

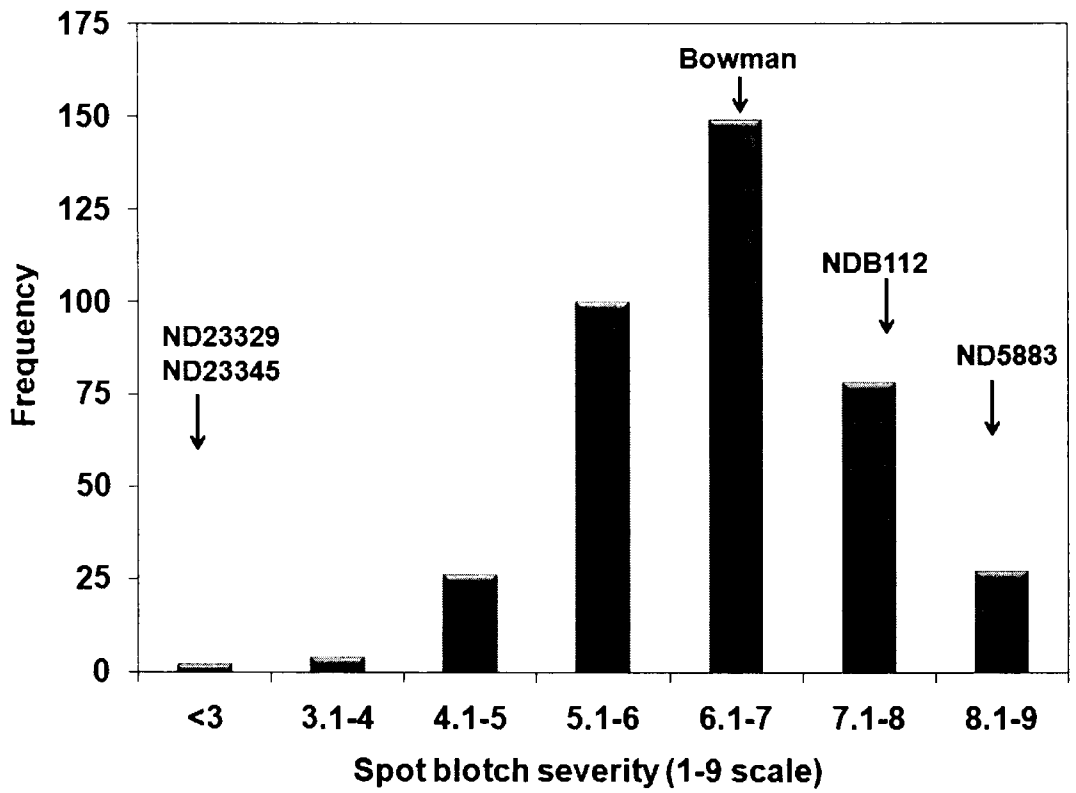

Fig. 5.1. Frequency distribution of spot blotch severities of 383 barley breeding lines from the Upper Midwest of the USA. Spot blotch severity are the mean of two replications where the checks, ND5883, Bowman and NDB112, were repeated 16 times in each replicates: ND5883 (8.3 severity scale), Bowman (6.4 severity scale), and NDB112 (7.8 severity scale).

TABLE 5.1. Phenotypic values for spot blotch severities in resistant lines and standard checks used in mapping populations of CAP06, CAP06 two-rowed, and CAP06 six-rowed.

Lines	Pedigree	SB severity	ND5883	NDB112	Bowman
ND23676	ND18546/ND20493	4	*	*	ns
ND24407	18427/3/M//16723/4/19088/5 / ND19854	3.9	*	*	ns
ND23827	ND18546/ND19655	3.4	*	*	
M03-53	M119 / M97-77	3.1	*	*	
ND23329	ND19656/ND19495	2.8	*	*	*
ND23345	ND19656/ND19495	2.6	*	*	*
ND5883	Clipper/6/Betzes//Clho 5791/2*Parkland/3/Betzes /Piroline/4/Akka/5/Centennial	†8.3			
NDB112	Kindred/CI 7117-77	†7.8			
Bowman	ND2685/ND1156//Hector	†6.4			
CAP06 combined		6.5			
CAP06 two-rowed		6.7			
CAP06 six-rowed		6.3			

* Significant at 0.05 probability level using Dunnetts' test.
ns = non significant at 0.05 probability level.
SB severity = Mean spot blotch severity of barley genotypes.
† Spot blotch severity scale of standard checks ND5883, Bowman, and NDB112. The values of checks (ND 5883, NDB112, and Bowman) were the average of 32 replicates.

The genome wide average LD was recorded 18, 10, and 10 cM at $r^2 = 0.2$ for CAP06 six rowed, CAP06 two-rowed and CAP06 populations. However LD differed for different genomes (Fig. 5.3). In CAP06, LD extended beyond 20 cM in chromosome 7H.

TABLE 5.2. Single nucleotide polymorphism (SNP) markers used in association mapping in Barley CAP 2006.

Population/ subpopulation	n^a	No of SNP[b]	Average distance (cM)[c]	No of gaps > 5 cM	No of gaps > 10 cM	Average LD[d] (cM)
CAP06 six-rowed	224	1873	0.64	11	0	18
CAP06 two-rowed	160	1923	0.62	14	2	10
CAP06	384	2028	0.58	16	1	10

[a]Number of breeding lines evaluated for spot blotch
[b]Number of SNPs used for marker-trait association after filtering for 10% missing data and 5% minor allele frequency.
[c]Average distance estimated between all pairs of adjacent SNP markers.
[d]Average LD was calculated by making all pair-wise SNP comparisons for LD across the genome, and evaluating the moving average of r^2 values. A threshold of $r^2 = 0.2$ was used for LD.

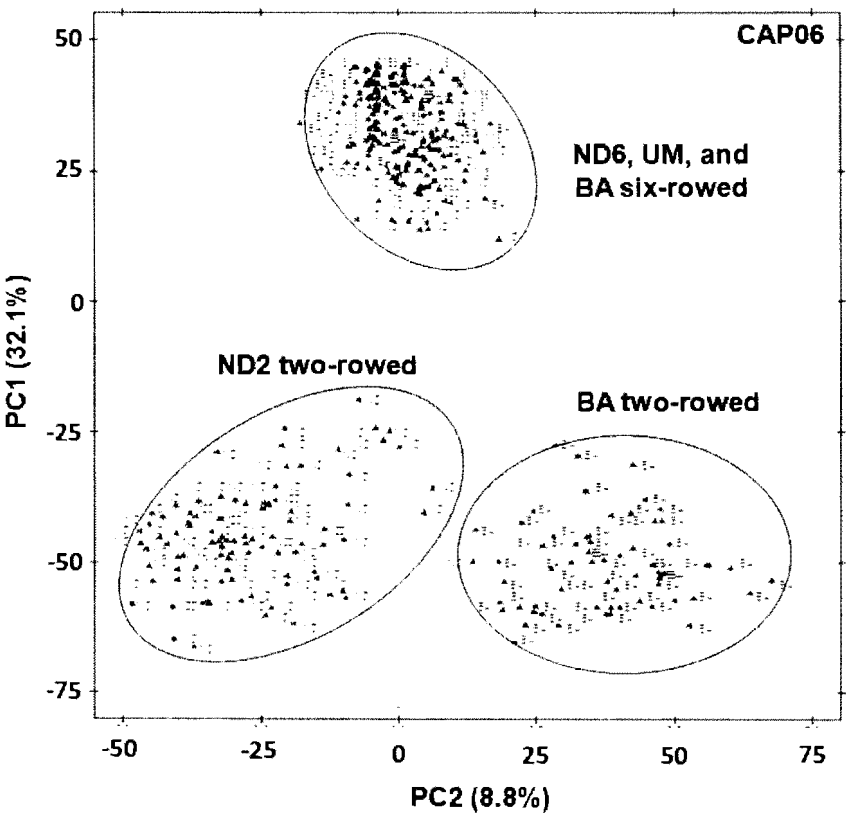

Fig. 5.2. Three subpopulations were designated as six-rowed (ND6, UM, and BA), ND2 two-rowed, and BA two-rowed barley by principal component analysis. The groups were designated by using first two principal components in CAP06.

TABLE 5.3. The proportion of genotypic variation explained by top 15 principal components in different mapping populations used in detecting SNP-CRR resistance association analyses.

PC[a]	CAP06	CAP06 two-rowed (%)[b]	CAP06 six-rowed
PC1	32.1	18.1	52.8
PC2	40.9	23.7	57.5
PC3	45.3	27.2	60.8
PC4	47.9	30.5	63.1
PC5	49.8	33.1	65.2
PC6	51.5	35.5	66.6
PC7	53.1	37.8	68.0
PC8	54.5	39.8	69.3
PC9	55.7	41.6	70.4
PC10	**56.8**[c]	43.2	**71.5**
PC11	57.8	44.8	72.6
PC12	58.7	46.3	73.5
PC13	59.6	47.8	74.4
PC14	60.5	49.2	75.3
PC15	61.2	**50.5**	76.1

[a]Principal components
[b]Percentage variation (Cumulative) explained by corresponding PC in mapping populations.
[c]Bold face number indicate corresponding number of PCs were used in Mixed Linear Model in TASSEL.

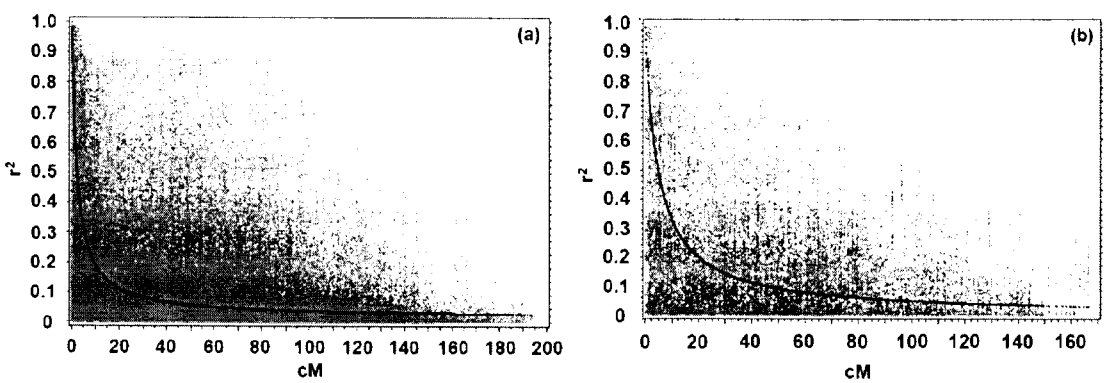

Fig. 5.3. Genome wide linkage disequilibrium among filtered SNP marker pairs as a function of genetic distance (cM) in barley CAP06. (a) LD plot for whole genome (b) LD plot for 7H chromosome of barley CAP06.

TABLE 5.4. Comparison of different methods employed in marker-spot blotch resistance association studies in three mapping populations in order to control false positives.

Mapping population	Number of significant marker-trait associations			
	GLM+Q[a]	GLM+P[b]	MLM+Q+K[c]	MLM+P+K[d]
CAP06 six-rowed	144	11	1	1
CAP06 two-rowed	4	8	2	3
CAP06	30	40	3	4

[a]GLM+Q = General linear model with four Q matrices, generated from Structure, used as covariate
[b]GLM+P = General linear model with four P matrices, eigenvalues of PCA used as covariate
[c]MLM+Q+K = Mixed linear model with four Q matrices, generated from Structure, used as covariate combined with K matrix
[d]MLM+P+K = Mixed linear model with four P matrices, eigenvalues of PCA, used as covariate combined with K matrix

TABLE 5.5. Spot blotch resistance QTL identified against *C. sativus* isolate 4008 in CAP06, CAP06 two-rowed, and CAP06 six-rowed mapping populations in barley.

Putative QTL[a]	**Marker[b]**	***p*-value**	**FDR (*q*-value)**	**R^2(%)**
CAP06 six-rowed				
Rcs-2H-106-122 [c]	11_0134	8.90E-04	0.110	3.8
CAP06 two-rowed				
Rcs-1H-84.6 [c]	11_1313	6.36E-05	0.114	4.4
Rcs-4H-96.5	12_30046	5.69E-04	0.342	3.4
Rcs-5H-102.5 [c]	11_0815	5.45E-04	0.342	3.3
CAP06 combined				
Rcs-1H-6	12_30933	7.96E-04	0.349	1.8
Rcs-1H-84.6 [c]	11_1313	6.67E-05	0.100	2.5
Rcs-2H-106-122 [c]	12_30049	1.25E-04	0.100	2.3
Rcs-5H-102.5 [c]	11_0815	3.13E-04	0.183	2.1

[a]QTL designated for resistant to *C. sativus* casing spot blotch in barley.
[b]SNP marker significantly associated with spot blotch resistance.
[c]Marker-trait association found in two mapping populations.

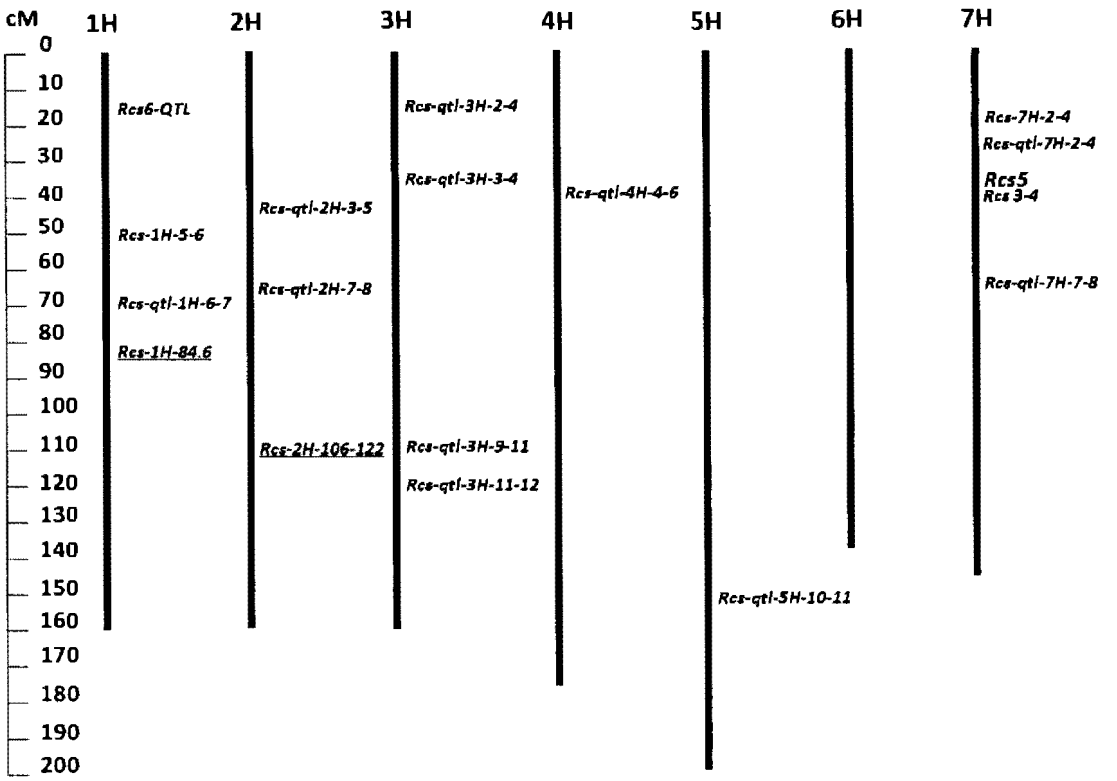

Fig. 5.4. Barley genomic map showing significant association of single nucleotide polymorphism (SNP) with resistance to *C. sativus* isolate 4008 causing spot blotch. The spot blotch resistance genes and QTL reported by Steffenson et al. (1996), Bilgic et al. (2005), Bilgic et al. (2006), Yun et al. (2005) and Yun et al. (2006) are given right side of each genome. Bin estimation for genome was used according to Kleinhofs and Garner (2001). QTL identified in current study is underlined and designated as *Rcs-chromosome-cM distance* where *Rcs* stands for resistant to *C. sativus* followed by chromosome and then by marker position in cM distance.

Marker-trait association using different models

In the current study, four different models were tested for assessing marker-trait associations. The number of significant associations between SB resistance and SNP markers using GLM and MLM are presented in Table 5.4. In GLM, 30 and 40 significant associations were found between SNP and CRR resistance

when models were GLM+Q, and GLM+P. In contrast, only three and four markers were found significant when MLM+Q+K and MLM+P+K were applied to CAP06 population. In both models, MLM+Q+K and MLM+P+K, common markers were detected which were significant associated with SB resistance.

QTL conferring resistance to *C. sativus* isolate

QTL resistance to SB, the marker position, *p*-value, *q*-value (FDR) and R^2 explained by the significant markers are presented in Table 5.5. In the CAP06 six-rowed population, the putative QTL, *Rcs-1H-84.6* and *Rcs-2H-106-122* were detected in chromosome 1H and 2H respectively. Marker associated with these QTL explained 2.38 and 2.50% phenotypic variation. The FDR analysis indicated that these associations were true positive at $q = 0.1$ level. In the CAP06 two-rowed population, three marker-trait associations were detected in chromosomes 1H, 4H, and 5H but the FDR analysis suggested that these associations were false positives.

In CAP06 population, only one SNP- SB resistance association was detected in chromosome 2H in 106-122 cM region (Table 5.5). However the FDR analysis revealed that this association was a false positive. The putative QTL identified in current study are shown relative to the SB resistant gene and/or QTL reported in earlier studies (Fig. 5.4) (Steffenson et al. 1995; Bilgic et al. 2005; Yun et al. 2005; Bilgic et al. 2006; Yun et al. 2006). The QTL identified in current study did not coincide with any QTL reported earlier. *Rcs-1H-84.6* was distal to earlier reported spot blotch resistance QTL, *Rcs-qtl-1H-6-7*.

Discussion

In association studies, population structure due to differences in allele frequency and genetic relatedness in mapping population lead to spurious association between markers and trait. Therefore, population structure needs to account for before association analysis in order to reduce the number of false positives. Price et al. (2006) and Zhao et al. (2007) suggested that principal component analysis (PCA) can be used in minimizing number of false positives and maximizing the power to detect true associations. The PCA of CAP06 population suggests three distinct subpopulations based on first two components (Fig. 5.2). All six-rowed barley from ND6, UM, and BA clustered together. The North American barley breeding programs share common ancestors and can be traced back to five to seven common lines (Martin et al. 1991; Horsley et al. 1995). Therefore, germplasm base of six rowed barley breeding in the region can be considered narrow. In contrast, ND2 and two-rowed barley from BA formed distinct groups. The BA barley breeding program introduces international barley germplasm each year and therefore was different than ND2. However, few lines from ND2 clustered into BA two-rowed barley breeding program. This may be due to sharing of common parents between these two breeding programs.

In barley, two- and six-rowed constitute distinct populations and there are not many two-rowed by six-rowed populations available to breeding programs worldwide (Malysheva-Otto et al. 2006). However, in the breeding programs of Upper Midewest of the USA, crosses have been made in two- and six-rowed

barley using East Asian Accessions while breeding for resistance to Fusarium Head Blight.

The LD of mapping population is affected by number of factors such as allele frequency, reproduction biology, genetic drift and selection pressure. Steffenson et al. (2007) reported that LD decayed within 1 cM in wild barley whereas Kraakman et al. (2004) found that genome wide LD in cultivated barley was 10 cM. Rostoks et al. (2006) found different LD pattern in European two- and six-rowed barley. They reported that LD decayed within 15 cM in two rowed barley whereas it extended upto 60 cM in six-rowed. In current study also, average LD of two-rowed barley was less compared to six-rowed barley, however, the average LD varied chromosome wise. The genome wide LD of CAP06 population was found 10 cM which is consistent with previous reports of Kraakman et al. (2004) and Rostoks et al. (2006).

The frequency distribution of SB severity scale (Fig. 5.1) indicates that the resistance to SB in current mapping populations is polygenic in nature. The frequency distribution is skewed towards susceptible infection responses due to high virulence of *C. sativus* isolate used in current study. Therefore, the SB resistance QTL detected in this study confers moderately resistance reactions. Ghazvini and Tekauz (2008) suggested that current SB severity scale is suitable for screening genetic materials if the SB resistance is conditioned by major gene. The suggested that the aggressiveness of this pathogen need be analyzed using quantitative techniques when majority of lines show infection responses between 5 - 6 (intermediate responses) severity scales. In the current study, the majority of

lines were scored between 5 - 7 severity scales. Only two advanced breeding lines, ND23329 and ND23345, from NDSU six-rowed barley breeding programs showed high level of resistance to isolate 4008 compared to two-rowed barley. The resistance present in these lines appears to be conditioned by major gene(s) based on phenotypic evaluation in 0 - 9 severity scale. Only these lines showed infection responses (< 3 disease severity scale) which is a typical hypersensitive infection response shown by the major gene in SB resistance in barley (Table 5.1). However, the resistance of ND23329 and ND23345 could not be identified in current mapping studies. This could be due to the minor allele frequency of these resistance loci present in the populations. In current studies, we discarded the markers of less than 5% minor allele frequency as one of the parameter for filtering genotypic data for association analysis. Furthermore, we observed only 6 (0.1%) breeding lines that showed some level of resistance and recorded spot blotch severity ≤ 4. The two breeding lines which showed disease severity ≤ 3 are also potential candidate of novel resistance to *C. sativus* isolates 4008. Therefore, there is an immediate need to explore the genetic basis of these breeding lines.

Five significant marker-trait associations were detected in current study, of which, only two were true positives. These two putative QTL, *Rcs-1H-84.6* and *Rcs-2H-106-122,* confer moderate resistance to SB caused by *C. sativus* isolate 4008. The frequency distribution of SB severity skewed toward moderate susceptible to susceptible infection responses (Fig. 5.1). Therefore, the resistance conditioned by these QTL is moderate. None of the QTL reported here coincided with previously mapped QTL in barley genome but *Rcs-1H-84.6* was detected

closer to *Rcs-qtl-1H-6-7* in chromosome 1H (Fig. 5.4). In our study, we employed *C. sativus* isolate 4008 to phenotype resistance to SB. In barley breeding programs in the Upper Midwest of the USA, *C. sativus* isolate ND85F (pathotype 1) is routinely used for screening SB resistance because ND85F represents the most widely distributed virulence group in the region. Bilgic et al. (2006), Yun et al. (2006), and Steffenson et al. (1996) all used isolate ND85F for mapping SB resistance in their mapping populations. The isolate used in current studies, represented the virulence group 7.7.3.6 which was similar to virulence groups 7.7.5.4, and 7.7.7.5 reported by Ghazvini and Tekauz (2008) in Canada. This virulence group is highly aggressive and has overcome the durable resistance of NDB112 (Table 5.1). The breeding line NDB112 was widely used as a source of durable SB resistance in barley breeding programs in the Upper Midwest of the USA and Canada (Wilcoxson et al. 1990; Steffenson et al. 1996). Recently, we have reported *C. sativus* isolates from ND that are highly virulent on NDB112 and other contemporary resistance sources available in barley germplasm in the regions. Therefore, moderately resistant QTL, identified in current studies, have important implication for barley improvement against spot blotch in North America.

None of the QTL reported in earlier studies were mapped in current mapping populations. This result is in agreement with the phenotypic evaluations of contemporary resistance sources available in the Upper Midwest of the USA. The infection response of NDB112, the six-rowed barley regarded as having durable resistance to SB to all virulence groups of *C. sativus*, was comparable to the highly susceptible check, ND5883. Furthermore, the infection responses of ND5883 and

NDB112 were not significantly different at $p<0.05$ (Table 5.1) indicating the resistance of NDB112 is overcome by virulence group 7.7.3.6. The short arm of chromosome 7H containing major gene *Rcs5* and QTL *Rcs-qtl-7H-2-4* appear neither in the CAP06 mapped nor the CAP06 six-rowed mapping populations. Similarly, the gene *Rcs6* and QTL present in other chromosomes were not detected in the current study. These results indicate that the virulence group 7.7.3.6 has overcome contemporary resistance sources in North American barley breeding programs. In contrast, Bowman, a two-rowed cultivar, showed less SB severity compared to NDB112 in current study. The results indicated that NDB112 provided durable resistance against the virulence groups represented by major pathotypes 1 and 2 (Steffenson et al. 1995), however, it failed to provide protection against virulence groups 7.7.3.6 recently found in ND (current study) and the virulence groups 7.7.5.4, 7.7.7.5, and 7.7.7.1 reported in Canada (Ghazvini and Tekauz, 2008). The virulence groups represented by pathotypes 1 dominated *C. sativus* isolates in the past; however, it is likely that there might be a shift in virulence of this pathogen and need to monitor in future. Therefore, the virulence groups represented by isolates 4008 is a newly emerged virulence groups and pose potential threats to barley cultivation in the regions.

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CHAPTER 6. VIRULENCE SPECTRUM OF *COCHLIOBOLUS SATIVUS* TO BARLEY CULTIVARS IN NORTH DAKOTA

Abstract

Cochliobolus sativus is an important and frequently isolated root and leaf pathogen of barley in North America. However, the virulence spectrum of *C. sativus* isolated from roots is poorly understood. Sub crown internodes (SCI) of barley were collected from farmers' fields and two North Dakota State University research stations to isolate *C. sativus* in 2006. Six to ten virulent isolates of *C. sativus* were selected to study the virulence spectrum on roots and leaves of seven to twelve barley genotypes. None of the barley genotypes showed a high level of resistance to root isolates 111, 417, 802, 1408, 1457, and 4008. The comparison of virulence groups of the same *C. sativus* isolates causing CRR and spot blotch diseases revealed that different mechanisms might operate for CRR and spot blotch resistance in barley. Isolates 4008 (Virulence group 7.7.3.6) and 1457 (Virulence group 7.3.3.6) were highly virulent on both roots and leaves of currently deployed resistant cultivars and advanced breeding lines, including NDB112 the genotype used as the source of durable resistance to spot blotch in all six- rowed breeding lines in North Dakota. Therefore, there is an immediate need to explore new and robust resistance sources to this virulence group. A careful selection of virulent isolates, representing both virulence groups causing CRR and spot blotch, is essential for screening of barley germplasm for host resistance effective in the region.

Introduction

Common root rot (CRR) is caused by *Cochliobolus sativus* (Ito & Kurib.) Drechsl. ex Dast.[anamorph, *Bipolaris sorokiniana* (Sacc. in Sorok.) Shoem.], *Fusarium graminearum* Schwabe [teleomorph *Gibberella zeae* (Schwein.) Petch] and *Fusarium culmorum* (W. G. Sm.) Sacc., in wheat and barley. Among these pathogens, *C. sativus* is an important and frequently isolated root pathogen of barley in North America (Mathre, 1982) and is widely distributed in south Asia (Kumar et al., 2002), central Asia (van Leur et al., 1997) and Australia (Wildermuth et al., 1992; Whittle et al., 1992). *C. sativus* also causes spot blotch in leaves and black point in seeds of wheat and barley (Kumar et al., 2002) and is considered a serious biotic factor influencing successful cultivation of barley.

North Dakota (ND) is the leading state for barley production in the United States accounting for over one third of the total barley production (USDA, 2009). Stack et al. (1991) estimated that CRR resulted in an annual average yield loss of 9.5% in barley in North Dakota. Piening et al. (1976), in an earlier study, reported 10% annual yield loss in barley in Canada. Under favorable conditions and on susceptible cultivars, spot blotch can cause as high as 30-35% yield losses in barley in North America (Ghazvini and Tekauz, 2004; Wilcoxson et al., 1990). Resistance to spot blotch has been considered an effective strategy to reduce yield losses in ND, but work on resistance to CRR has not been attempted mainly due to the quantitative nature of the resistance (Kutcher et al., 1994) making it more difficult to work with, and a lack of understanding of the virulence spectrum of *C. sativus* associated with CRR.

The understanding of population structure has important implications in crop improvement and disease management. Information on the population structure helps design the deployment of resistance gene(s) for disease management. Traditionally, physiological specializations of different *Cochliobolus* species to cultivated crops have been reported. Recently, Valjavec-Gratian and Steffenson (1997) and Zhong and Steffenson (2001) studied the population structure of spot blotch associated *C. sativus* isolated from barley leaves using virulence pattern and AFLP markers. Valjavec-Gratian and Steffenson (1997) reported three pathotypes (0, 1 and 2) of spot blotch associated *C. sativus* from leaves based on the differential reaction of 22 isolates on barley lines ND5883, Bowman, and NDB112. They reported that pathotype 2 was dominant in North Dakota with lower allelic diversity compared to pathotypes 0 and 1. More recently, Ghazvini and Tekauz (2008) reported that eight virulence groups of spot blotch associated *C. sativus* in Canada and that some groups were highly virulent on contemporary resistance sources. In CRR, Arabi and Jawhar (2002) reported the virulence spectrum of *C. sativus* from roots of barley in Syria, showing differential reactions of *C. sativus* to barley cultivars. However, the virulence spectrum of *C. sativus* isolated from roots from the Northern Great Plains of the USA has not been reported. The objective of this study was to investigate the virulence spectrum of CRR associated *C. sativus* isolated from SCIs of barley collected from different locations in North Dakota and to compare it with the virulence spectrum of similarly collected *C. sativus* causing spot blotch on leaves of barley.

Materials and Methods

Fungal isolates and barley genotypes

C. sativus was isolated from the SCIs of diseased barley samples that were randomly collected from experimental plots in Langdon in 2004 and 2005 around plant maturity. In 2006, more than 400 isolates of *C. sativus* were isolated from SCIs of barley, wheat, and oat using systematic sampling scheme at Feekes growth stage 11.1-11.3 (Large, 1954) from farmers' fields and from Langdon and Minot Research Extension Centers in North Dakota (Fig. 6.1). At each of the 17 sites sampled in 2006, 20-25 plants were uprooted randomly and SCIs were collected. The SCIs were surface sterilized in 5% sodium hypochlorite solution for 4 minutes, washed three times for 3 minutes in sterile distilled water and dried between sterile whatman filter paper. The SCIs were cut into pieces of 1.5 mm and incubated for 72 h in a growth chamber at 22±1° C in the dark on PDA (Difco, Detroit, MI, USA) amended with Kanamycin sulphate (13 mg L^{-1}). *C. sativus* was identified based on colony morphology and spore type. Single-spores of *C. sativus* were isolated and grown in PDA plates. The single spore isolates were labeled, cut into small blocks, air dried under a laminar flow hood for three days and stored at -30° C for later use.

In a preliminary CRR greenhouse screening in 2006, 35 randomly selected *C. sativus* isolates from North Dakota and five isolates from Australia were evaluated for CRR virulence on Robust and ND20448 (Table 6.1), the two barley genotypes found to be moderately resistant to CRR in the field in 2006 at Langdon Research Extension Center, Langdon North Dakota (data not shown). From the 35 isolates,

111, 417, 802, 1408, 1457, and 4008 were selected for the CRR and SB experiments due to their higher CRR virulence on Robust and/or ND20448. The barley lines used to study the virulence spectrum of *C. sativus* in North Dakota are listed in Table 6.2. The lines comprised of both two-rowed and six-rowed cultivars commonly grown in the Upper Midwest of the USA, cultivars with putative resistance based on previous research and advanced breeders lines being considered for cultivar release.

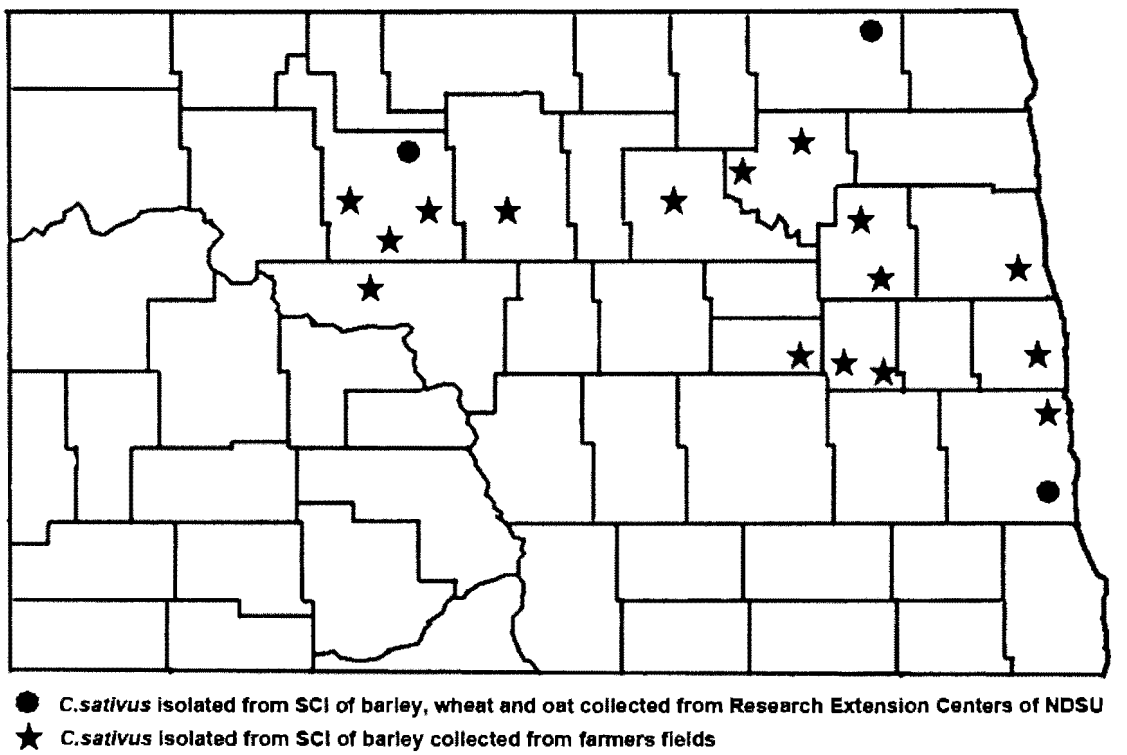

Fig. 6.1. Distribution of *Cochliobolus sativus* isolates collected in counties of North Dakota in 2006.

TABLE 6.1. Isolates of *C. sativus*, source of origin and responses to two barley genotypes measured to determine the virulence of *C. sativus* isolates in 2006.

Isolate	Crop	Location, year, state, country	Disease severity %[c]	
			Robust[d]	ND20448[d]
111[a]	Barley/root	Ramsey 2006, ND, USA	85	16
209	Barley/root	Grand Forks 2006, ND, USA	39	3
302	Barley/root	Nelson 2006, ND, USA	74	5
408	Barley/root	Nelson 2006, ND, USA	63	1
417[a]	Barley/root	Nelson 2006, ND, USA	82	2
617	Barley/root	Traill 2006, ND, USA	66	24
628	Barley/root	Traill 2006, ND, USA	74	2
646	Barley/root	Traill 2006, ND, USA	69	8
657	Barley/root	Traill 2006, ND, USA	34	2
702	Barley/root	Ramsey 2006, ND, USA	52	2
722	Barley/root	Ramsey 2006, ND, USA	70	14
802[a]	Barley/root	Ward 2006, ND, USA	85	12
811	Barley/root	Ward 2006, ND, USA	47	8
1313	Barley/root	Griggs 2006, ND, USA	42	6
1408[a]	Barley/root	Griggs 2006, ND, USA	67	26
1411	Barley/root	Griggs 2006, ND, USA	46	1
1432	Barley/root	Griggs 2006, ND, USA	68	9
1447	Barley/root	Griggs 2006, ND, USA	53	7
1451	Barley/root	Griggs 2006, ND, USA	50	11
1457[a]	Barley/root	Griggs 2006, ND, USA	83	12
1502	Barley/root	Ward 2006, ND, USA	60	5
1516	Barley/root	Ward 2006, ND, USA	20	5
4008[a]	Barley/root	Cavalier, 2004, ND, USA	84	31
4023	Barley/root	Cavalier, 2004, ND, USA	71	4
4028	Barley/root	Cavalier, 2004, ND, USA	36	2
4032	Barley/root	Cavalier, 2004, ND, USA	9	2
5001	Barley/root	Cavalier, 2005, ND, USA	51	12
5003	Barley/root	Cavalier, 2005, ND, USA	70	12
5007	Barley/root	Cavalier, 2005, ND, USA	58	20
7003	Barley/root	Cavalier, 2006, ND, USA	24	1
7011	Wheat/root	Cavalier, 2006, ND, USA	8	2
7026	Wheat/root	Cavalier, 2006, ND, USA	8	2
8013	Oat/root	Cass, 2006, ND, USA	23	2
8014	Oat/root	Cass, 2006, ND, USA	11	1
8022	Oat/root	Cass, 2006, ND, USA	7	8
9006	Barley/root	Moonta, 2006, SA, Australia	44	2
9027	Barley/root	Port Vincent, 2006, SA, Australia	52	7
9030	Barley/root	Nyngan, 2006, NSW, Australia	22	3
WAC8151	Barley/Leaf	-, 1985, WA, Australia	35	2
WAC8152	Barley/Leaf	-, 1985, WA, Australia,	60	2
Control[b]	-	-	0	0

Table 6.1 (Continued)

[a]Isolates selected on the basis of higher disease severity on both Robust and Eslick.
[b]Control is mock inoculation with sterile water
[c]Disease severity assessed for 36 SCIs for each cultivar and isolates.
[d]Robust and ND20448 were selected for preliminary screen based on the results of field experiment conducted in 2006.
- = not available

TABLE 6.2. Barley genotypes used in a study of virulence spectrum of *C. sativus* isolated from 19 fields in North Dakota.

Genotypes	PI Number	Row type	Origin	Reaction to Spot Blotch[a]	Reaction to Common root rot[b]
Argyle	PI496255	six-rowed	Canada	--	R
Arizona	PI583667	six-rowed	USA	--	HS
Bowman	PI 483237	two-rowed	USA	MR-S	--
Conrad	--	two-rowed	USA	--	--
Eslick	--	two-rowed	USA	--	--
Golf	PI485529	two-rowed	England	--	R
ND20448	--	six-rowed	USA	--	--
ND23329	--	six-rowed	USA	--	--
ND23345	--	six-rowed	USA	--	--
ND5883	PI 349366	two-rowed	USA	HS	--
NDB112	--	six-rowed	USA	R	--
Robust	PI 476976	six-rowed	USA	--	--

R = Resistant, MR = Moderately resistant, MS = Moderately susceptible, S = Susceptible, HS = Highly susceptible, -- = Not available.
[a]Fetch and Steffenson (1994); Valjavec-Gratian and Steffenson (1997),
[b](Arabi and Jawhar (2002); Bailey and Wolf (1994)

To study the virulence of *C. sativus* causing CRR, seven barley genotypes, Argyle, Arizona, Conrad, Eslick, Golf, ND20448, and Robust were selected to determine their differential reactions to *C. sativus* based on SCIs. The six isolates used are listed above (Table 6.2). The experiment was done in 2007 and repeated in 2008. To study the virulence of *C. sativus* causing SB in 2007, the seven barley genotypes listed above were used as well as the checks ND5883, Bowman and NDB112. The experiment was repeated in 2008 using the 2007 barley genotype

set with the inclusion of ND23329 and ND23345 (Table 6.3). In 2007 the isolates used were the 2007 CRR set with the inclusion of the leaf pathotypes ND93-1 (pathotype 0), ND85F (pathotype 1) and ND90Pr (pathotype 2). In 2008, the 2007 SB isolate set was used and a root isolate, 4032, was also included.

Inoculum preparation, inoculation, and disease severity assessment

Single-spore-fungal isolates were grown on PDA and incubated for 12 days at 22 - 24° C with an alternating cycle of 12 h light and dark. For CRR, the root inoculation method developed by van Leur (1991) as modified by Arabi and Jawhar (2002) was used. The conidial suspension was adjusted to 5 x 10^5 spores ml-1. Forty-ml of spore suspension was mixed with 50 g of sterilized peat soil (Sunshine mix #1, Fiscon Horticulture Inc., Vancouver, Canada) in a beaker to prepare enough inoculums for 40 seeds. Gum Arabic 15% w/w (Sigma-Aldrich Inc., St Louis, MO, USA) was mixed thoroughly with the peat conidia suspension to prepare peat-gum-conidia inoculum. Barley seeds were surface sterilized with 5% sodium hypochlorite solution for 4 - 5 minutes and soaked three times in sterile distilled water. Peat-gum-conidia inoculum was thoroughly mixed with 25 - 36 seeds for each cultivar which when sown was considered an experimental unit. Experimental units were replicated 8 times in both the 2007 experiment and the 2008 experiment. Seeds were sown immediately into plastic conetainers of 7.6 cm x 21 cm (Stuewe and Sons, Inc. Corvallis, OR, USA) and filled with sterile Sunshine mix #1 (Fiscon Horticulture Inc., Vancouver, Canada). Seeds were sown at 6 cm depth to promote longer SCI. Plants were incubated for 7 weeks in the greenhouse at 22-25° C and watered as needed. Slow release fertilizer (14 - 14 -

14 N - P - K, 0.5 g per conetainer) (Scotts-Sierra Horticultural Product Company, Maryville, OH, USA) was applied twice during the seven week incubation period. The SCIs were removed carefully, cleaned in running water, dried overnight, and scored for disease severity. The dark brown-to black lesions on SCIs were scored visually on a 0 - 100% severity scale which represented the percentage of SCI discoloration. Isolates causing average SCI severity 20 - 40% moderately virulent, 41 - 70% virulent and >70% highly virulent.

For SB assessments, barley lines were grown in conetainers of 3.8 cm x 21 cm size (Hummert International, Earth City, MO, USA) filled with Sunshine mix #1. Three to five seedlings were grown per cone and a single cone represented an experimental unit. Each experimental unit was replicated three times in the 2007 experiment and four times in the 2008 experiment. Slow release fertilizer (14 - 14 - 14 N - P - K, 0.3 g $cone^{-1}$) was applied at planting. The seedlings were grown in the greenhouse at 22 - 25° C with a 16 h photoperiod. Leaf inoculation was done as described by Valjavec-Gratian and Steffenson (1997). In brief, at two-leaf stage (12 days), seedlings were inoculated with a 5000 conidia ml^{-1} suspension of an individual isolate of *C. sativus*. Polyoxyethylene-20-sorbitan monolaurate (100 µl L^{-1}) was added to the conidial suspension to facilitate even distribution and better adsorption of conidia onto the leaf surface. The inoculum (approximately 0.15 ml $plant^{-1}$) was sprayed on the leaf surface to the point of runoff using an atomizer (DeVilbis Health Care Ind., Somerset, Pennsylvania). The inoculated plants were kept in the dark for 18 h at 22° C and approximately 100% relative humidity using ultrasonic misting chambers. The next day, plants were air dried slowly before

being transferred to the greenhouse at 22 - 25° C and incubated for the next ten days before scoring disease severity. SB severity was assessed using the 1 - 9 severity scale of Fetch and Steffenson (1994). Average infection responses of < 4.5 were considered to be resistant and ≥ 4.5 were considered susceptible.

Virulence group designation

To describe the virulence groups of *C. sativus* causing CRR and SB diseases, we used the triplet coded nomenclature system of Limpert and Müller (1994). According to this system, the infection response of a differential genotype to each isolate is designated by binary values (1 for resistant and 0 for susceptible reactions). The resultant binary values were then converted to decennary values by conversion factors 2^0 (equal to 1), 2^1 (equal to 2), and 2^2 (equal to 4) for each set of three differential hosts in a subset. The sum of all decennary values in each subset is calculated and separated by dots. A group of isolates with similar three (in CRR studies) or four digit codes (in SB study) were considered a virulence group. The differential lines were ordered alphabetically and then divided into subsets of three. For CRR where there were seven differentials, the third triplet code was the value for only one barley genotype.

Results

Common root rot virulence

All isolates tested for CRR severity produced light brown to dark black lesions on SCI's. The symptoms varied from small to large necrotic lesions representing differential severity on SCI's. CRR severity was isolate and cultivar specific. In

susceptible cultivars Arizona and Argyle, lesions appeared in the collar region as early as three weeks after inoculation, but seven weeks of incubation was optimum to differentiate the resistant and susceptible reactions on SCIs of the barley genotypes used in this study.

Isolates 111, 417, 617, 802, 1408, 1457, and 4008, all collected from barley roots were the most virulent from a screening of 40 isolates in 2006, where they produced higher disease severity on Robust, ND20448 or both (Table 6.1). In isolates from North Dakota, CRR severity ranged from 7 - 85% in Robust and 1 - 31% in ND20448. *C. sativus* isolated from roots of wheat and oat were less virulent to barley SCI. The disease severity percentage induced by isolates obtained from wheat and oat ranged from 7 - 24% on Robust and 1 - 8% on ND20448. One isolate, 4032, from barley roots showed low virulence on both Robust and ND20448 and was comparable to wheat and oat isolates. Two Australian leaf isolates WAC 8151 and WAC 8152 were virulent on SCI of the susceptible cultivar, Robust, but less virulent on the more resistant ND20448. The virulent isolates were evenly distributed over the different collection sites. The average disease severity across all isolates was 50% on Robust compared to 7% in ND20448.

In the 2007 and 2008 CRR study, cultivars Arizona and Argyle showed higher CRR severity across all isolates compared to other genotypes (Table 6.3). ND20448, Eslick, and Conrad produced moderate CRR severity when challenged with virulent isolates, but disease severity was less on these cultivars when challenged with less virulent isolates. In both years, 4008 was the most virulent isolate causing 57 - 95% CRR severity in different barley genotypes. Isolates 111

and 417 were considered moderately virulent because they produced higher CRR severity on susceptible to moderately susceptible cultivars such as Arizona, Argyle, Robust, and Golf than on the more resistant ND20448, Eslick, and Conrad. Isolates 802, 1408, and 1457 were less virulent on SCI of ND20448, Eslick, and Conrad. Barley genotypes ND20448, Eslick, and Conrad experienced less severe CRR when inoculated with virulent isolates 111, 417, and 4008 whereas Arizona, Argyle and Robust were highly susceptible to the majority of isolates. Virulent isolates induced moderate to high CRR severity on Golf, but when challenged by less virulent isolates, CRR severity was less. All isolates used in the CRR study belonged to a single virulence group (7.7.1) although isolate 1457 was marginally less virulent on ND20448.

Spot blotch virulence

In the 2007 and 2008 SB study, the ten isolates tested on the twelve barley genotypes varied lesion types. The symptoms differed from minute necrotic lesions with no, or only slight chlorotic halos, to large necrotic lesions surrounded by distinct yellow halos (a typical SB symptom). Isolate 1457 induced infection responses (IRs) of four and five more often than other isolates. In some instances, the necrotic lesion surrounded by chlorotic halo was not as typical as described by Fetch and Steffenson (1994). The IRs of ND5883, Bowman, and NDB112 genotypes to isolates ND85F, ND90Pr, and ND93-1 were in agreement with earlier reports by Fetch and Steffenson (1994), and Zhong and Steffenson (2001). Isolates 111, 417, 802, and 1408, which were virulent on SCIs, were less virulent on leaves with the majority of genotypes showing resistant IRs (Tables 6.4).

However, isolates 4008 and 1457 were highly virulent on the leaves causing severe SB to all barley genotypes except ND23329 and ND23345 (six-rowed advanced breeding lines from NDSU). Argyle, which showed higher CRR severity to most isolates tested in the CRR study, exhibited resistant IRs to all isolates except 4008 and 1457 in the spot botch study. There were four distinct virulence groups of *C. sativus* isolates. Group 0.0.0.0 was composed of isolates 111, 417, 802, 1408, 4032, and ND93-1. Isolates ND90Pr fall under group 5.0.0.0 which is the pathotype 2 of Valjavec-Gratian and Steffenson (1997). Group 1.3.3.0 comprised of only one isolate, ND85F, which is the pathotype 1 described previously in North Dakota (Valjavec-Gratian and Steffenson, 1997). The most virulent isolates, 1457 (7.3.3.6) and 4008 (7.7.3.6) overcame the resistance of NDB112 the resistant check.

Discussion

The virulence spectrum of *C. sativus* causing SB on leaves has been reported in North Dakota and Canada, but there is a lack of information on virulence of *C. sativus* causing CRR. Valjavec-Gratian and Steffenson, (1997) studied the virulence of *C. sativus* from SB of barley from North Dakota and reported pathotypes 0, 1 and 2. Zhong and Steffenson (2001) studied the virulence spectrum as well as genetic diversity of *C. sativus* and other *Cochliobolus* species using AFLP markers. This is the first comprehensive report in the USA of the virulence spectrum of *C. sativus* isolated from roots of barley and compared with isolates from leaves.

TABLE 4.3. The proportion of genotypic variation explained by top 15 principal components in different mapping populations used in detecting SNP-CRR resistance association analyses.

PC[a]	CAP06	CAP06 two-rowed	CAP06 six-rowed	CAP07	CAP07 two-rowed	CAP07 six-rowed	CAP08	CAP08 two-rowed	CAP08 six-rowed
					(%)[b]				
PC1	32.1	18.1	52.8	31.7	18.3	15.1	30.6	21.7	14.3
PC2	40.9	23.7	57.5	39.8	26.2	21.1	40.7	28.5	20.5
PC3	45.3	27.2	60.8	44.7	32.0	26.7	45.1	34.5	26.4
PC4	47.9	30.5	63.1	47.3	36.2	31.7	48.0	37.7	31.1
PC5	49.8	33.1	65.2	49.4	39.8	35.9	50.5	40.7	35.4
PC6	51.5	35.5	66.6	51.5	43.0	39.9	52.5	43.6	39.4
PC7	53.1	37.8	68.0	53.3	46.0	43.5	54.3	46.2	42.7
PC8	54.5	39.8	69.3	55.1	48.8	46.3	55.6	48.4	45.4
PC9	55.7	41.6	70.4	56.7	51.3	48.8	56.9	50.5	47.8
PC10	**56.8**[c]	43.2	**71.5**	**58.1**	**53.1**	**51.1**	**58.1**	**52.4**	**50.0**
PC11	57.8	44.8	72.6	59.4	54.8	53.1	59.3	54.1	52.0
PC12	58.7	46.3	73.5	60.7	56.5	55.0	60.4	55.7	53.9
PC13	59.6	47.8	74.4	61.8	58.0	56.9	61.4	57.2	55.6
PC14	60.5	49.2	75.3	62.9	59.5	58.4	62.4	58.5	57.1
PC15	61.2	**50.5**	76.1	64.0	61.0	59.9	63.3	59.8	58.6

[a]Principal components

[b]Percentage variation (Cumulative) explained by corresponding PC in mapping populations.

[c]Bold face number indicate corresponding number of PCs were used in MLM in TASSEL.

TABLE 6.4. Mean infection response and standard deviations for 12 barley genotypes inoculated with ten *C. sativus* isolates causing spot blotch in 2007 and 2008.

Cultivars		Isolates									
	Rep[a]	111	417	802	1408	1457	4008	4032[b]	ND85F[c]	ND90Pr[c]	ND93-1[c]
ND5883	7	3.2±0.5	3.1±0.8	3.0±0.8	3.9±2.0	6.2±1.5	6.7±1.0	3.4±0.4	7.3±1.3	2.1±1.0	2.7±0.5
Bowman	7	3.2±1.2	2.4±0.9	2.6±1.6	2.8±0.6	5.0±1.1	6.0±0.2	2.4±0.7	2.3±0.7	8.8±0.1	1.6±0.6
NDB112	7	2.7±0.9	2.2±0.8	2.4±0.8	3.3±1.5	5.6±2.2	6.1±1.2	2.3±0.7	1.7±0.5	1.9±0.7	1.7±0.8
Argyle	7	2.6±0.8	2.3±0.5	2.3±0.6	2.3±0.8	5.1±0.7	6.3±0.6	1.9±0.6	2.2±0.5	2.1±0.8	1.3±0.4
Arizona	7	3.9±0.7	2.9±0.9	2.7±0.6	3.3±1.0	5.6±1.9	7.0±1.1	2.2±0.6	5.1±1.6	6.7±2.3	3.0±0.5
Robust	7	2.9±0.7	2.5±0.4	2.5±0.9	2.8±1.0	5.7±1.9	7.1±0.6	2.4±0.8	2.6±1.2	1.9±0.8	1.9±0.8
Conrad	7	2.4±1.2	2.1±0.8	2.3±0.5	2.5±0.9	5.3±1.3	6.5±1.1	1.7±0.2	7.0±2.0	2.4±1.4	2.1±0.8
Eslick	7	2.1±0.9	1.9±0.5	2.0±0.5	2.6±1.0	4.9±1.1	5.3±1.7	1.1±0.8	6.0±1.7	1.8±0.9	1.9±0.8
ND20448	7	2.6±0.9	2.1±0.7	2.7±0.7	2.6±1.0	5.4±1.5	6.2±1.3	1.7±1.1	5.3±1.7	2.2±0.9	1.7±0.6
Golf	7	2.5±1.0	2.4±0.2	2.6±0.9	2.9±0.5	4.0±1.0	6.1±0.5	1.5±0.6	3.6±0.6	1.4±0.4	1.7±0.5
ND23329[b]	4	3.4±1.5	2.9±1.6	2.0±0.8	2.7±0.9	3.2±0.6	2.7±0.7	1.8±0.7	2.0±0.9	1.9±0.8	2.1±1.0
ND23345[b]	4	2.5±1.0	2.7±0.6	2.4±0.4	2.9±0.7	3.7±0.8	3.8±1.0	1.9±0.4	1.9±0.3	2.2±0.5	2.0±0.7

[a]Rep = replication. Mean infection responses were calculated by averaging infection responses of the corresponding replications (3 and 4 replications in 2007 and 2008) using 1-9 rating scale of Fetch and Steffenson (1994).
[b]Isolate 4032 was included in 2008 only, therefore all genotypes were evaluated in four replicates to this isolates. Genotypes ND23329 and ND23345 were included in 2008 only.
[c]ND85F, ND90Pr, and ND93-1 were previously designated as pathotypes 1, 2, and 0 respectively.

TABLE 6.5. Virulence group of *C. sativus* causing common root rot and spot blotch in barley.

Isolates	Virulence group	
	Common Root Rot[a]	Spot blotch[b]
111	7.7.1	0.0.0.0
417	7.7.1	0.0.0.0
802	7.7.1	0.0.0.0
1408	7.7.1	0.0.0.0
1457	7.7.1	7.3.3.6
4008	7.7.1	7.7.3.6
4032	nt[c]	0.0.0.0
ND85F[d]	nt	1.3.3.0
ND90Pr[d]	nt	5.0.0.0
ND93-1[d]	nt	0.0.0.0

[a]Virulence group of isolates causing CRR was defined by infection response of *C. sativus* on seven barley genotypes, the third triplet therefore contain only one genotype
[b]Virulence group of isolates causing spot blotch was defined by infection response of *C. sativus* on 12 barley genotypes,
[c]nt = not tested.
[d]ND85F, ND90Pr, and ND93-1 were previously designated as pathotypes 1, 2, and 0 respectively.

Arabi and Jawhar (2001) reported the virulence of *C. sativus* on roots of barley from Syria. They found that virulence of *C. sativus* isolates were highly variable on barley cultivars possessing different levels of resistance. Our study demonstrated that *C. sativus* isolates from barley SCIs in North Dakota were virulent on moderately resistant to susceptible cultivars (Table 6.3).

A single virulence group of *C. sativus* (7.7.1) was identified from the infection response of isolates on seven barley cultivars on SCIs. This group was highly virulent on SCIs as severe symptoms were recorded on many of the seven barley cultivars tested, two of which were previously identified as resistant (Arabi and Jawahar, 2002). The isolates used in this study were selected for higher virulence

to SCIs in order to compare the ability to cause CRR with the ability to cause SB on leaves in barley. In CRR study, we did not find any cultivars highly resistant or immune to the *C. sativus* isolates used. One isolate, 4008, was highly virulent to all genotypes tested. Arabi and Jawhar (2002) also reported an isolate 'CRR16' highly virulent to all cultivars tested in Syria and while it is unknown if there is any genetic relationship between these isolates it is of concern that there is such high virulence in the *C. sativus* CRR population. The infection responses of Golf and Arizona are different from those recorded by Arabi and Jawhar (2002) who found Golf resistant and Arizona highly susceptible to Syrian isolates. In our experiments, Golf and Arizona showed similar susceptible reactions. In bi-parental genetic studies in Canada, Bailey and Wolf (1994) used Argyle as their CRR resistant parent. Based on this information, we initially included Argyle as a resistant genotype in current CRR studies. However, we observed that Argyle consistently showed a susceptible reaction to all isolates evaluated. It may be that the isolates used in the current studies were either more virulent than the Canadian isolates used by Bailey and Wolf (1994) and/or that a different population exists in Saskatchewan compared to North Dakota, or that a shift in the virulence spectrum of *C. sativus* has taken place since their study. The isolates identified in this study are useful in screening CRR resistance in barley germplasm in North America. Recently, we successfully used four of these virulent isolates 111, 417, 802, and 1408 to screen for CRR resistance in 768 barley breeding lines (Data not presented here).

Recently, Ghazvini and Tekauz (2008) identified eight virulence groups of *C. sativus* causing SB in twelve barley cultivars from Canada. In the current study, we

identified four virulence groups of *C. sativus* causing SB using twelve genotypes (Table 6.4). Of twelve genotypes, four (Bowman, NDB112, ND5883 and Robust) were common to the differential set used by Ghazvini and Tekauz (2008). Based on the low virulence pattern, isolates 111, 417, 802, 1408, 4032 and ND93-1 have been grouped into low virulent types (0.0.0.0). Isolates 4008 and 1457 showed high virulence on the majority of the cultivars evaluated. Therefore, these two isolates were grouped as 7.3.3.6 and 7.7.3.6 and can be considered highly virulent groups. Both 7.3.3.6 and 7.7.3.6 have overcome the resistance of NDB112, which had previously been considered to have durable resistance to SB in North America (Wilcoxson et al., 1990). The existence of highly virulent isolates that are able to overcome the resistance in NDB112 is consistent with the results of Ghazvini and Tekauz (2008) who have also recently reported highly virulent groups in the prairie regions of central Canada. The barley breeding programs in the Upper Midwest of the USA had been using NDB112 as the resistance source to SB for many years. Our findings suggest that there is an immediate need to explore new sources of resistance for SB caused by these virulence groups.

The virulence groups of *C. sativus* were strikingly different in their ability to cause CRR and SB severity in barley (Table 6.5). Isolates 111, 417, 802, and 1408 were virulent on SCIs but were weakly virulent on leaves (virulence group 0.0.0.0). The results are consistent with the hypothesis that the genetic basis for virulence in *C. sativus* isolates causing CRR and SB to corresponding IRs in barley genotypes, are different. Almgren et al. (1999) in Sweden reported no correlation in aggressiveness between isolates and leaves, seeds, and roots in barley.

Kutcher et al. (1994) in Canada did not find relationships between CRR and adult plant resistance to SB while studying the genetics of resistance to these diseases. Similarly, Conner (1990) also did not find strong correlations between CRR and SB or black point in wheat but he found a weak correlation between SB and black point. In contrast, more recently Arabi et al., (2006) found correlation between CRR and SB resistance among Syrian isolates. Our findings supports the hypothesis that *C. sativus* possesses different virulence factors for causing CRR and SB severity and barley genotypes possess different mechanisms that condition resistance to CRR and SB in barley. The majority of isolates obtained from SCIs were in virulence group 0.0.0.0 and isolate ND93-1 (pathotypes 0) used routinely in SB studies in North America also fell into this group.

Interestingly, isolate ND93-1 originated from barley kernels whereas other isolates, ND85F (pathotype 1) and ND90Pr (pathotype 2), were obtained from barley leaves (2001). Veljavec-Gratian and Steffenson (1997) indicated that *C. sativus* isolates originating from aerial parts are unlikely to develop tissue specificity in barley. More recently, Ghazvini and Tekauz (2008) also argued that tissue specificity is unlikely to occur in *C. sativus* isolates originating aerially in barley. However, they suspected that tissue specificity may occur in isolates originating below ground (roots) and aerial (leaf and kernel) in barley. In the current study, we examined the specificity based on IRs of root and leaf isolates on barley genotypes. Six out of eight isolates originating from SCIs showed tissue specificity to leaves (Table 6.4). However, two isolates, 1457 and 4008, acquired a broader adaptation to both roots and leaves casing severe CRR and SB in all

genotypes used in the study. These findings indicate that *C. sativus* the virulence group 7.7.1 might have different adaptations to root and leaf, and futther investigation is needed with more root and leaf isolates.

The IRs induced by *C. sativus* isolates on SCIs, causing CRR on barley genotypes, appeared to be continuously distributed (Table 6.3). The continuous responses of genotypes to virulence of *C. sativus* suggest that the resistance to CRR could be of polygenic nature. Kutcher et al. (1994) and Bailey et al. (1988) reported that CRR resistance in barley was quantitative. Arabi and Jawhar (2002) also speculated that the resistance to CRR could be polygenic. The polygenic nature of disease resistance will make it difficult to select for resistance to CRR in barley breeding programs. Therefore, the slow progress in achieving resistance to CRR may be attributed to complex pathosystem of *C. sativus*. The use of populations derived from bi-parental crosses has been less attractive for mapping QTL conferring resistance in complex pathosystems but the approach has been useful in mapping resistance loci of *C. sativus* causing SB due to its less complex nature. Valjavec-Gratian and Steffenson (1997) found a major gene in ND5883 against *C. sativus* isolate ND90Pr inoculated onto leaves. They reported a single virulence gene in isolate ND90Pr associated with high virulence on cultivar Bowman. A major single gene *Rcs5* and other QTL on 7H chromosome have been mapped by Bilgic et al. (2005). Methods for mapping QTL associated with resistance to CRR are now required.

In conclusion, our results demonstrate that a complex host-pathogen interaction exists between *C. sativus* isolates and barley genotypes in CRR and

SB diseases. The different IRs of the same *C. sativus* isolate on SCIs and leaves indicate that different resistance mechanisms are involved for CRR and SB in barley. The occurrence of both tissue specific and non specific isolates of *C. sativus* in root and leaf indicate that further research is warranted on tissue specificity between isolates originating from root and aerial parts. None of the barley genotypes had high or complete resistance to CRR caused by *C. sativus*. The continuous range of CRR severity suggested that resistance to CRR is polygenic in nature and more complex than resistance to SB in barley. ND20448 and Eslick showed lower CRR severity across the majority of virulent isolates; therefore, they should be investigated for use as resistance sources in Upper Midwest barley germplasm as they are already in elite germplasm adapted to the region. However screening of additional germplasms needs to evaluate to find genetic material with higher sources of resistancemin future. Isolates 4008, 111, and 417 were highly virulent on roots and should be used for screening barley germplasm for resistance to CRR. A careful selection of virulent isolates, representing both virulence groups causing CRR and spot blotch, is essential for screening of barley germplasm for host resistance effective in the region.

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CHAPTER 7. GENETIC STRUCTURE OF *COCHLIOBOLUS SATIVUS* POPULATIONS SAMPLED FROM ROOTS AND LEAVES OF BARLEY AND WHEAT IN NORTH DAKOTA AND AUSTRALIA

Abstract

Common root rot (CRR) and spot blotch, caused by *Cochliobolus sativus* (Ito & Kurib.) Drechsl. ex Dast., are important diseases of barley (*Hordeum vulgare* L.) and wheat (*Triticum aestivum* L.). However, the population biology of *C. sativus* is poorly understood. In this study, the genetic structure of four *C. sativus* populations (NDBleaf, NDBroot, NDWroot, and AusBroot) isolated from roots and leaves of barley or wheat in Australia and North Dakota were analyzed with amplified fragment length polymorphism (AFLP) markers. A total of 127 AFLP loci were generated among 289 isolates of *C. sativus* analyzed with three primer combinations. Gene diversity (H = 0.27 - 0.35) and genotypic diversity (GD = 1) were high in all four populations. Genetic variation among *C. sativus* individuals within population accounts for 78% whereas 22% of the genetic variation was explained among populations. The high population differentiation, (Gst) = 0.196, was observed across all populations, suggesting low gene flow, (Nm) = 2.0, occurred among populations. The multilocus linkage disequilibrium (LD) ($\overline{r_d}$ = 0.046 - 0.118) was moderate in *C. sativus* populations. These results support the hypothesis that *C. sativus* reproduce asexually in the field populations. Mechanisms other than sexual recombination such as transposon mediated

genetic rearrangement and parasexual recombination may play an important role in maintaining high genetic diversity in *C. sativus*.

Introduction

Cochliobolus sativus (Ito & Kurib.) Drechsl. ex Dast. [anamorph, *Bipolaris sorokiniana* (Sacc. in Sorok.) Shoem.], *Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae* (Schwein.) Petch), and *Fusarium culmorum* (W. G. Sm.) Sacc., are the major causal agents of common root rot (CCR) in barley and wheat (Mathre, 1982). Among these, *C. sativus* is the most frequently isolated root pathogen of barley (*Hordeum vulgare* L.) in Australia (Wildermuth, 1986; Whittle, 1992), North America (Mathre, 1982), and South Asia (Kumar, 2002). The fungus also causes spot blotch and black point in small grains (Mathre, 1982; Kumar et al., 2002). The diseases caused by *C. sativus* are most common in weather conditions with high humidity and temperature (22 - 30° C) (Mathre, 1982), which occur in the prairie regions of the Upper Midwest of the USA, including North Dakota (ND). The spot blotch disease results in reduced photosynthetic areas and leads to premature senescence of the infected leaves as well as death of the plant under severe cases. It can cause 30 - 36% yield losses in susceptible barley cultivars in the prairie regions of North America (Clarke, 1979; Wilcoxson 1990; Ghazvini and Tekauz, 2004). Annual yield loss of barley due to CRR was 9.5% in North Dakota, but it could be as high as 37% in few field plots sampled during 1980 - 1982 (Stack et al. 1991).

Despite the importance of the diseases caused by *C. sativus*, information on population biology of the fungus is very limited. The genetic structure of fungal population describes the amount and distribution of genetic variation present within and among the fungal populations (McDermott and McDonald, 1993). The understanding of population structure and virulence pattern of *C. sativus* has implication in devising robust breeding program for disease resistance and deployment of resistance gene. Zhong and Steffenson (2001) studied the genetic and virulence diversity of *C. sativus* causing spot blotch in ND using AFLP markers and infection responses on barley genotypes. Recently, Ghazvini and Tekauz (2008) reported virulence pattern of *C. sativus* from Canada. However, population structure of *C. sativus* originating from roots and causing CRR is poorly understood. Especially, there is limited information about genetic structure of *C. sativus* from barley. Also, it is not known whether the population structure of *C. sativus* isolates originating from root and aerial parts of barley is similar or different. The objectives of this study were to investigate the genetic structure of *C. sativus* populations collected from different tissues (root and leaf) of the hosts (barley and wheat) sampled from different geographic regions (Australia and ND, USA) and to determine relationships between the genetic structure and the origins of the pathogen.

Materials and Methods

Fungal isolates and populations

Cochliobolus sativus isolates used in the study were from infected root or leaf samples of barley and wheat randomly collected from North Dakota (ND) and

Australia. Four fungal populations (NDBleaf, NDBroot, NDWroot, and AusBroot) were defined based on geographic locations (ND or Australia), host (wheat or barley) and tissue (leaf or root) origins. Population NDBleaf was from the barley leaf samples collected from different locations of ND in 2002, 2003, and 2005. Population NDBroot originated from barley roots sampled from 18 locations during 2004 - 2006 in ND. Population NDWroot was from wheat root samples collected from Langdon, ND, in 2006. Population AusBroot was from barley root samples collected from five locations in Australia (Table 7.1).

For fungal isolation, symptomatic subcrown internodes (SCIs) of barley or wheat root samples were surface sterilized in 5% sodium hypochlorite solution for four min, washed three times for three min in sterile distilled water and dried between sterile Whatman filter paper (Whatman Inc. Piscataway, NJ, USA). The SCIs were cut into pieces of 1.5 mm and incubated for 72 h in a growth chamber at 22 ± 1° C in the dark on PDA (Difco, Detroit, MI, USA) amended with kanamycin sulphate (13 mg L^{-1}). Identification of *C. sativus* isolates was based on colony morphology and spore type. All isolates were derived from single spores of the fungus and grown on PDA plates. For long-term storage, the fungal isolates were grown on PDA, which was cut into small blocks, air dried under a laminar flow hood for three days and stored in a freezer at -30° C.

Genomic DNA

Individual *C. sativus* isolates were inoculated into potato broth medium (HIMEDIA Lab. Mumbai, India) and grown for 2 - 3 days by continuous shaking (150 rpm) in a shaker. The mycelia were harvested by filtration through 4 layers of

TABLE 7.1. *Cochliobolus sativus* isolates used in the population genetics study.

Population	Tissue/Host	Year	Location	Source	No. of isolates
NDBroot1 [a]	Root/Barley	2006	Ramsey/ND/USA	S. Gyawali	15
NDBroot2	Root/Barley	2006	Grand Forks/ND/USA	S. Gyawali	10
NDBroot3	Root/Barley	2006	Nelson, ND, USA	S. Gyawali	8
NDBroot4	Root/Barley	2006	Nelson, ND, USA	S. Gyawali	8
NDBroot5	Root/Barley	2006	Benson, ND, USA	S. Gyawali	7
NDBroot6	Root/Barley	2006	Traill, ND, USA	S. Gyawali	3
NDBroot7	Root/Barley	2006	Ramsey, ND, USA	S. Gyawali	2
NDBroot8	Root/Barley	2006	Ward, ND, USA	S. Gyawali	7
NDBroot9	Root/Barley	2006	Ward, ND, USA	S. Gyawali	6
NDBroot10	Root/Barley	2006	McHenry, ND, USA	S. Gyawali	1
NDBroot11	Root/Barley	2006	Foster, ND, USA	S. Gyawali	4
NDBroot12	Root/Barley	2006	Foster, ND, USA	S. Gyawali	12
NDBroot13	Root/Barley	2006	Griggs, ND, USA	S. Gyawali	6
NDBroot14	Root/Barley	2006	Griggs, ND, USA	S. Gyawali	6
NDBroot15	Root/Barley	2006	Ward, ND, USA	S. Gyawali	3
NDBroot16	Root/Barley	2006	Ward, ND, USA	S. Gyawali	4
NDBroot17	Root/Barley	2004	Cavalier, ND, USA	S. Gyawali	6
NDBroot18	Root/Barley	2005	Cavalier, ND, USA	S. Gyawali	4
NDBleaf1 [b]	Leaf/Barley	2002	--, ND, USA	Y. Sun	50
NDBleaf2	Leaf/Barley	2003	--, ND, USA	Y. Sun	8
NDBleaf3	Leaf/Barley	2005	--, ND, USA	Y. Sun	28
NDWroot [c]	Root/Wheat	2006	Cavalier, ND, USA	S. Gyawali	37
AusBroot1[d]	Root/Barley	2007	Moonta, SA, Australia	S.M. Neate	10
AusBroot2	Root/Barley	2007	Port Vincent, SA, Australia	S.M. Neate	1
AusBroot3	Root/Barley	2007	Munno Para, SA, Australia	S.M. Neate	5
AusBroot4	Root/Barley	2007	Hermitage, QLD, Australia	S.M. Neate	5
AusBroot5	Root/Barley	2007	Nyngan, NSW,Australia	S.M. Neate	33

[a]NDBroot 1 - 18 contain *C. sativus* isolates isolated from sub-crown internodes (SCIs) of barley root samples collected at different locations of ND.
[b]NDBleaf 1 - 3 include *C. sativus* isolates isolated from leaf samples collected in ND during 2002 - 2005.
[c]NDWroot was from SCIs of wheat root samples collected from two adjacent farmers' fields in ND.
[d]AusBroot 1 - 5 were *C. sativus* isolates from SCIs of barley root samples collected from Australia.

cheese cloth, frozen at -30° C, lyophilized, and ground to a powder. Genomic DNA was isolated from the ground mycelia either by a procedure described by Yoder (1988) and modified by Zhong and Steffenson (2001) or using FastDNA kit

(QBiogene) according to the manufacturer's protocol. Genomic DNA samples were quantified using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA), diluted to 50 ng μl^{-1} in nanopure water and stored at -20° C for further use.

AFLP analysis

AFLP analysis was performed by a modification of the method of Vos et al. (1995). The AFLP Core Reagent Kit (Invitrogen Life Technologies, CA) was used to prepare DNA templates for pre-selective amplification according to the manufacturer's protocol. Briefly, genomic DNA (200 to 500 ng) of *C. sativus* was digested with *EcoR*I and *Mse*I at 37° C for 3 hrs. The 16 bp adaptors supplied in the kit were used to ligate to the restricted DNA fragments at 20° C for 3 hrs. The ligation products were diluted 1: 2 in nanopure water. Primers complimentary to the adapter sequences plus one selective base at 3' end were used for pre-amplifications. The pre-amplification was performed in a reaction of 20 µl containing 1: 2 diluted DNA template, 36 ng of each *EcoR* I primer (E-A) and *Mse* I primer (M-C), 0.2 mM dNTPs, 1× buffer, 1.5mM $MgCl_2$, and 1 unit of Taq DNA polymerase at 94° C for 30 s, 56° C for 60 s, and 72° C for 60 s for 20 cycles. For selective amplification, primers combinations with two selective bases (E-AN and M-CN) were used with the E-primers labeled by IRD700 or IRD800 (Li-COR, Lincoln, NE). The PCR conditions for selective amplifications were the same as described by Vos et al. (1995). After selective amplifications, the reactions were mixed with 5 - 10 µl of formamide loading dye, denatured at 95° C for 4 min, chilled immediately on ice, and then loaded (0.7 µl each) in an polyacrylamide gel in the

Li-COR system (Li-COR, Lincoln, NE). The polyacrylamide gel was prepared by mixing 20 ml of RapidGel-XL 6% liquid acrylamide (USB Corporation, Cleveland, OH) with 150 µl of 10% fresh ammonium persulfate and 15 µl of tetramethylethylenediamine (TEMED). The amplified fragments with a size ranging from 145 to 700 bp were scored manually scored with reference to a 50 bp ladder (Li-COR Biosciences, Lincoln, Nebraska). The DNA fragments were scored in a binary system where presence of a band was coded as 1 and absence as 0. A band absent in at least one isolate but present in all other isolates was considered polymorphic.

Population genetic analyses

Data generated from the AFLP analysis were used to analyze the genetic diversity and genetic structure of the *C. sativus* populations. Each unique multilocus haplotype was considered as a genotype or an individual and the number of distinct genotypes was calculated for each population. The Nei's genetic diversity (*H*) (Nei, 1973; Nei, 1978) and Shannon's Information Index (*I*) were calculated using POPGENE 1.3.2 (Yeh et al. 1997). The analysis of molecular variance (AMOVA), population genetic differentiation (ΦPT), Nei unbiased genetic distance (*D*), Nei unbiased genetic identity (I_d), and gene flow (*Nm*) were estimated using GenAlex 6.3 (Peakall and Smouse, 2006). The AMOVA (Exocoffier et al. 1992) was conducted for hierarchical partitioning of genetic variation among population and individuals within populations of *C. sativus* using Φ statistics. The Φ fixation indices and their level of significance ($P < 0.001$) were determined by 1,000 permutations. Variance was partitioned between populations (AusBroot,

NDBleaf, NDBroot, and NDWroot) and individual isolates within populations. Multilocus linkage disequilibrium (LD) was estimated using Multilocus 1.3 (Agapow and Burt, 2001). The linkage disequilibrium (LD) is non-random association between two marker loci and was calculated using $\overline{r_d}$. The test of significance of all analyses was performed at 1,000 randomizations for all populations.

The population structure of *C. sativus* was analyzed using the STRUCTURE 2.3.2 software and individual isolates were assigned to genetic clusters based on inferred genetic ancestry (Pritchard et al. 2000). Structure is a Bayesian model based on clustering method, which assigns the multilocus genotypes to user defined clusters (K) or sub-populations. Furthermore, structure is based on maximization of linkage equilibrium (LE) within population but minimization of LE between populations. The parameters of structure analysis were set to admixture model, number of sub-populations (K) = 1 - 10 with five iterations, burn in period of 10,000 and run length 100,000. The sub-populations number (K) was determined by considering various factors as suggested by Camus-Kulandaivelu et al. (2007). First, the ln (P) curve was generated in excel using the data of structure showing the probability of each subpopulation number. Second, the assessments of inferred ancestry of structure were judged based on prior knowledge of origin of *C. sativus* isolates (geographic regions, crops, and host origin). Finally, a neighbor joined tree was generated in TASSEL 2.1 (Baradbury et al., 2007) and used to decide number of K sub-populations.

Results

AFLP analysis

Initially, 16 primer combinations were screened for polymorphism between two *C. sativus* isolates (111 and 4008), which were selected on the basis of their different virulence patterns on barley genotypes. Isolate 111 collected from Ramsey county of ND in 2006 (Table 7.1) is highly virulent in causing CRR but less virulent in causing spot blotch whereas isolate 4008 collected from Cavalier county of ND in 2004 is highly virulent in causing both CRR and spot blotch. Each of the AFLP primer combinations produced 50 - 56 bands ranging from 145 to 700 bp in size. Three primers combinations (*Eco*RI-AA / *Mse*I-CA, *Eco*RI-AA / *Mse*I-CC, and *Eco*RI-AC / *Mse*I-CC) were selected based on their high polymorphism demonstrated in this study and in a previous report (Zhong and Steffenson 2001) and subsequently used to analyze the 289 isolates of *C. sativus* (Figs. 7.1 and 7.2). A total of 127 AFLP markers were generated across four populations of *C. sativus* using the three primer combinations.

Genetic diversity, population differentiation, and linkage disequilibrium

No clones (with the same haplotype) were identified in any of the populations. The AMOVA revealed that genetic variation among populations (AusBroot, NDBleaf, NDBroot, and NDWroot) accounted for 22% at a probability level of 0.001 (Table 7.2). However, most of the genetic variation (78%) was from individual isolates within populations.

The average gene diversity (Nei's gene diversity) with the three primer pairs and across populations was 0.307 (H = 0.271 - 0.350) (Table 7.3). NDBroot

showed the highest gene diversity (H = 0.350) whereas the lowest gene diversity was found in NDWroot (H = 0.271). Gene diversities were 0.317 and 0.292 for AusBroot and NDBleaf, respectively. The Shannon's information index also revealed a similar pattern of gene diversity in the four populations analyzed (Table 7.3).

Pair wise comparisons of Nei's unbiased genetic distance (D) and genetic identify (I_d) are presented in Table 7.4. The genetic distances ranged from 0.104 (between AusBroot and NDBleaf) to 0.187 (between AusBroot and NDWroot). Similar results were found based on the Nei's genetic identity between pairs of populations. The pair wise comparisons of population genetic differentiations (ΦPT) and gene flow (Nm) among the four populations are presented in Table 7.5. The overall gene flow (Nm) across four populations was 2.0 (Table 7.5). Significant departures from gametic phase equilibrium were observed in all populations at $p <$ 0.001 based on multillocus LD. The LD measured by $\overline{r_d}$ ranged from 0.046 to 0.118. AusBroot had the lowest LD and NDWroot had the highest LD. (Table 7.5). These results are in agreement with the higher gene flow between AusBroot and NDBleaf, and AusBroot and NDBroot populations than that observed between AusBroot and NDWroot. The population genetic differentiation between the AusBroot isolates and the ND isolates (Barley leaf and root isolates) were lower than those found among NDBroot, NDBleaf, and NDWroot populations within ND. The genetic differentiation and gene flow averaged across all populations were 0.196 and 2.0, respectively.

Population analysis using STRUCTURE 2.3.2 and neighbor joined tree generated in TASSEL 2.1 also indicate that genetic distances between AusBroot and NDBroot, and AusBroot and NDBleaf populations were low (Figs. 7.3 and 7.4). The structure analysis revealed that NDBroot population had three distinct subgroups, one closer to NDWroot population whereas other two groups were distinct (Fig. 7.3). The AusBroot population had the highest admixture compared to all other populations in the current study. The neighbor joined tree also revealed a similar grouping of *C. sativus* populations into five sub-populations (Fig. 7.4).

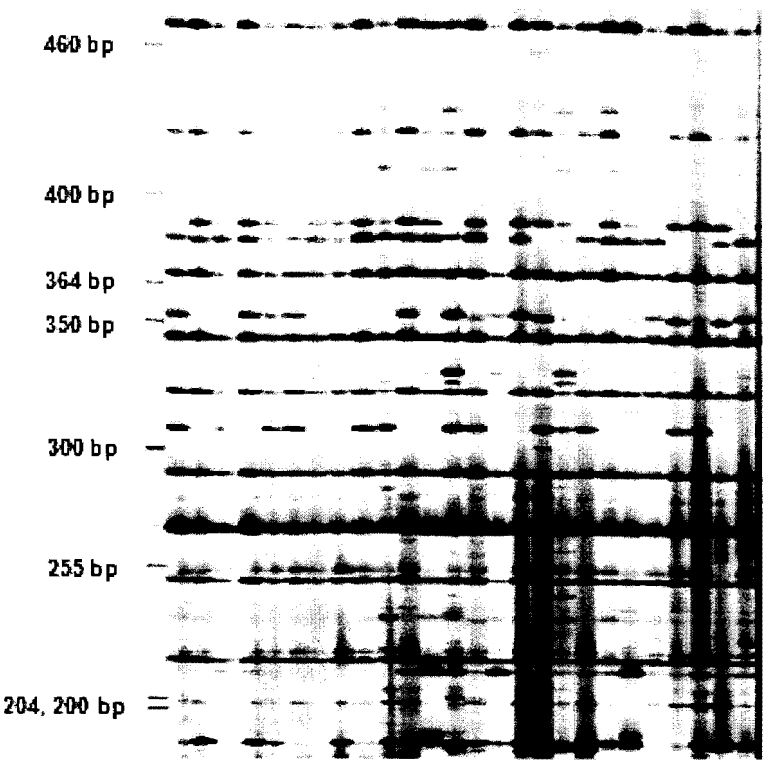

Fig. 7.1. AFLP analysis of *Cochliobolus sativus* isolates recovered from sub-crown internodes of barley in North Dakota, USA, using primer pair *Eco*RI-AA/*Mse*I-CC. Lane 1 is the 50 - 700 bp DNA sizing standard (Li-COR Biosciences, Lincoln, Nebraska). *C. sativus* isolates representing NDBroot population from ND are in lane 2 to 25.

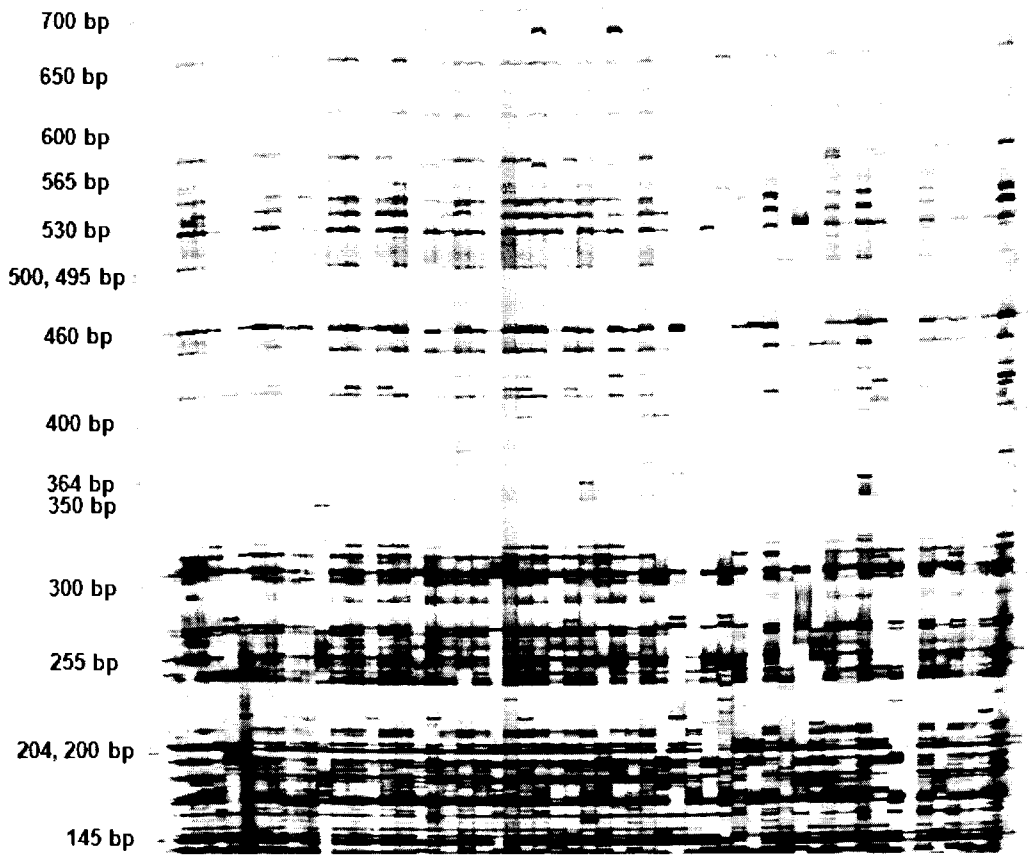

Fig. 7.2. AFLP analysis of *C. sativus* isolates recovered from sub-crown internodes of barley in Australia using primer pair *Eco*RI-AA/*Mse*I-CC. Lanes 2 to 44 are the *Cochliobolus sativus* isolates representing AusBroot population. Lane 1 is the 50 - 700 bp DNA sizing standard (Li-COR Biosciences, Lincoln, Nebraska).

TABLE 7.2. Analysis of molecular variance (AMOVA) for *Cochliobolus sativus* populations collected from Australia and North Dakota (barley and wheat) during 2002 - 2007.

Source of variance[a]	df	Estimated variance	Variation (%)	Φ^b	*p*-value[c]
Among population (ΦPT)	3	5.714	22	0.219	0.001
Individuals within populations	287	20.397	78		

[a]Variance was partitioned into two groups as among population (AusBroot, NDBroot, NDBleaf, and NDWroot); individuals within each population sampled from four populations (AusBroot, NDBroot, NDBleaf, and NDWroot).
[b]ΦPT was calculated as the proportion of the variance among the populations, relative to the total variance.
[c]Probability of obtaining equal or lower Φ values was determined by 1,000 random permutations.

TABLE 7.3. Population diversity of all *Cochliobolus sativus* isolates sampled from Australia and North Dakota in barley and wheat, calculated using amplified fragment length polymorphism (AFLP) markers generated by three primer pairs.

Population	n^a	H^b	I^c	LD^d
AusBroot	56	0.317	0.482	0.046***[e]
NDBleaf	86	0.292	0.447	0.111***
NDBroot	112	0.350	0.522	0.075***
NDWroot	37	0.271	0.429	0.116***

[a]Sample size for each population
[b]Nei gene diversity (1973) within population
[c]Shannon's information index
[d]The multilocus linkage disequilibrium (LD) was measured by $\overline{r_d}$ (Agapow and Burt, 2001)
[e]*** significant at 0.001 probability level.

TABLE 7.4. Pairwise comparisons of Nei unbiased genetic distance[a] (*D*) (above diagonal) and genetic identity (I_d) (below diagonal) among the four *C. sativus* populations.

Population	AusBroot	NDBleaf	NDBroot	NDWroot
AusBroot	-	0.104	0.134	0.187
NDBleaf	0.901	-	0.134	0.153
NDBroot	0.874	0.875	-	0.181
NDWroot	0.829	0.858	0.835	-

[a]Nei's unbiased genetic distance (*D*) and genetic identity (I_d) (Nei, 1978) was calculated in GENALEX 6.

TABLE 7.5. Pairwise comparisons of population genetic differentiation[a] (ΦPT) (above diagonal) and gene flow[a] (*Nm*) (below diagonal) among the four *C. sativus* populations.

Population	AusBroot	NDBleaf	NDBroot	NDWroot
AusBroot	-	0.183	0.195	0.281
NDBleaf	2.23	-	0.206	0.258
NDBroot	2.07	1.93	-	0.253
NDWroot	1.28	1.44	1.48	-

[a]Gene flow was calculated as $Nm = [(1 / \Phi PT) - 1] / 2$ using AFLP data in GENALEX 6.2 (Peakall and Smouse, 2006), where ΦPT is population genetic differentiation (an analogous of *F*st) was calculated as the proportion of variance among populations relative to the total variance. The probability of obtaining equal or lower ΦPT value was determined by 1,000 randomizations by permuting individuals within populations.

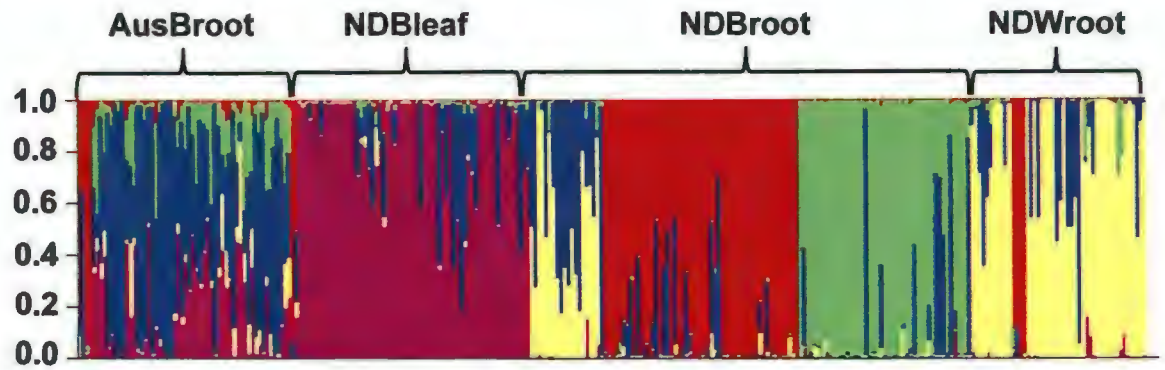

Fig. 7.3. Population structure of *C. sativus* collected from different geographic regions and crops. Inferred ancestry of each *C. sativus* isolates was considered with K = 5 subpopulations. Each sub-population is represented by a different color. AusBroot (isolates from barley root from Australia); NDBleaf (isolates of barley leaf from ND collected during 2002 - 2005), NDBroot (isolates of barley root from ND collected during 2004 - 2006; this subpopulation is represented by three distinct subgroups), and NDWroot (isolates of wheat root from ND).

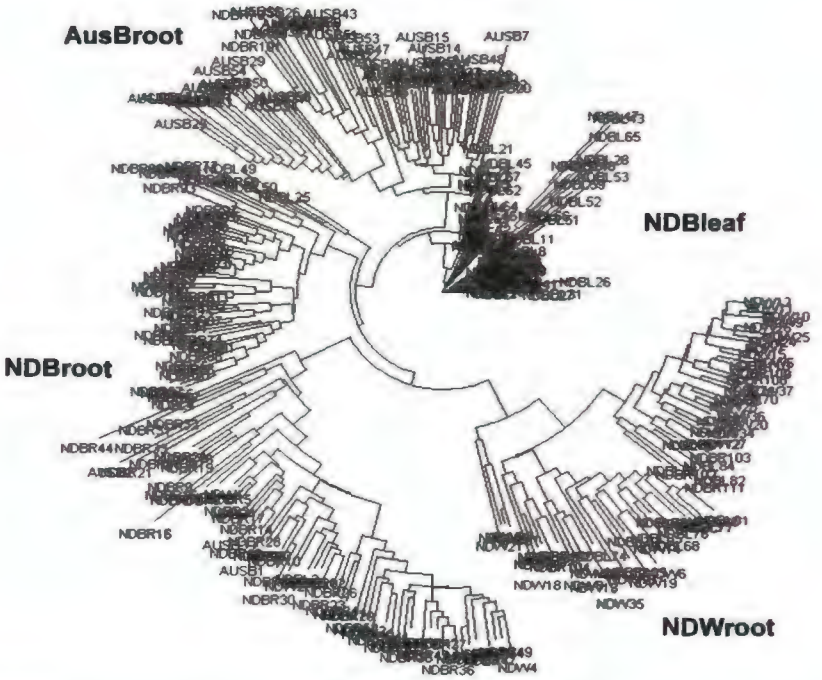

Fig. 7.4. Neighbor joined tree of 289 *C. sativus* isolates using AFLP markers generated by the three AFLP primer combinations (*Eco*RI-AA/*Mse*I-CC, *Eco*RI-AA/*Mse*I-CA, and *Eco*RI-AC/*Mse*I-CC). AusBroot (isolates from barley root from Australia); NDBleaf (isolates of barley leaf from ND collected during 2002 - 2005), NDBroot (isolates of barley root from ND collected during 2004 - 2006; this subpopulation is represented by three distinct subgroups), and NDWroot (isolates of wheat root from ND).

Discussion

The emergence of highly virulent *C. sativus* groups in the North America, including the Upper Midwest of the USA and prairie regions of Canada, has raised questions regarding the effectiveness of durable resistance to SB in the regions. The highly virulence groups 7.7.3.6 found in ND (Gyawali et al. 2010) and 7.7.7.5 and 7.7.5.1 found in Canada (Ghazvini and Tekauz, 2008) pose a potential threat to barley cultivation in the region. The emergence of highly virulent groups from the North America also suggest that *C. sativus* has a higher potential to shift the aggressiveness than previously thought. In this study, the genetic diversity of *C. sativus* populations from different geographic regions, hosts, and plant tissues was analyzed using 127 AFLP loci generated by three AFLP primer pair combinations. Majority of the genetic variation (78%) was accounted for by individual isolates within populations. The genetic variation among populations accounts for only 22% and was significant at 0.001 probability level. The results suggest that *C. sativus* differentiated into populations based on geographic regions, crops and tissue specificity.

Analyses of pair wise genetic distances (*D*) and genetic identify (I_d) among geographically separated and host specific isolates of *C. sativus* suggest that these populations are different. It appears that the AusBroot population is different from those collected from barley and wheat in ND. The *C. sativus* isolates collected from root and leaf also grouped into different populations. Moderate genetic differentiation was identified between *C. sativus* populations originated from the two hosts (barley and wheat). The genetic differentiation estimated for *C. sativus* across all populations was 0.196. This value can be considered moderate

for *C. sativus* compared to those commonly found in other ascomycete fungi in the region. Lee and Neate (2007) reported overall *G*st = 0.238 in *Septoria passerinii* in the upper Midwest of the USA. Adhikari et al. (2008) found mean *F*st = 0.288 across all pairwise comparisons of *Phaeosphaeria nodorum* from the upper Midwest of the USA. Milgroom et al. (1995) reported a similar level of population differentiation (*G*st = 0.20 to 0.31) in chestnut blight caused by *Cryphonectria parasitica* in the eastern North America. The population genetic differentiation observed in *C. sativus* could be due to two reasons. First, it is possible that *C. sativus* is adapted to different environments represented by Australia and ND. Barley and wheat are grown under hot and dry environments in Australia whereas in ND, these crops are cultivated in warm and humid environments. Second, the crop and tissue specificity of *C. sativus* partly explain the population differentiation. *C. sativus* is adapted to many small grains, including barley and wheat, grown around the world. In ND, *C. sativus* is more problematic to barley causing severe spot blotch compared to wheat (Dr. S. Ali, personal communication). In contrast, *C. sativus* is reported most frequently from SCI causing CRR from in wheat and barley in North America (Tinline et al., 1994). Spot blotch is most frequently reported in wheat in warm and humid regions around the world, in the area where traditionally wheat is not grown, (Saari, 1985). In another study, we demonstrated some level of tissue specificity in *C. sativus* collected from barley root and leaves (Gyawali et al., 2010). However, the crop specificity of *C. sativus* in wheat and barley has not been explored rigorously yet. The population genetic differentiations across regions (Australia and ND, USA), crop (barley and wheat) and tissue

specific isolates (root and leaf) were supported by pair-wise comparisons of gene flow between these populations. The lowest gene flow between AusBroot and NDWroot population was due to a combination of both crop specificity and geographic separation. In contrast, barley and wheat is often rotated in the same fields within a few years of interval in ND. The lower gene flow between NDWroot and NDBroot, and NDWroot and NDBleaf partly explains the crop specificity of *C. sativus* populations. It was interesting to note that the gene flow between root and leaf isolates was found lower compared to gene flow between AusBroot with NDBroot and NDBleaf.

The LD can provide important information on biology of fungal population such as sexual reproduction and random mating populations. In current study we found low to moderate multilocus LD in *C. sativus* populations and were significantly different than 0 at a probability level of 0.001. The NDWroot population had a higher $\overline{r_d}$ compared to other three populations. This is probably due to the small sample size and locations. The wheat samples were collected from two farmers' field in Langdon, ND and only 37 isolates were analyzed in NDWroot. The AusBroot population was also small but the isolates were collected from diverse farmers' field representing seven locations in four states (South Australia, Queensland, New South Wales, and West Australia) (Table 7.1). Brown (1975) reported that the sample size should be >100 for more accurate estimation of multilocus LD. Population NDBleaf had a higher LD compared to other two populations (NDBroot and AusBroot). Zhong and Steffenson (2001) found that some isolates from different regions of the world clustered into the same AFLP

group. They suggested that the migration of fungal pathogens in these regions might have occurred with the infected seed because *C. sativus* is a seed-borne pathogen (Mathre, 1982). Therefore, population admixture and small sample size might have been responsible for higher LD in NDWroot and NDBleaf.

The sexual stage of *C. sativus* has not been reported in nature, although it can be induced in culture. Therefore, asexual reproduction is considered as the major mode for the life cycle of the fungus. However, a higher genetic diversity was found in field population of *C. sativus* (Zhong and Steffenson, 2001). Our results also showed high gene and genotypic diversity (Tables 7.3) in *C. sativus* populations. The high genetic diversity found in *C. sativus* could be due to several reasons. First, the transposon-mediated rearrangement of genetic materials could potentially induce genetic variation in ascomycete fungi reproducing asexually in the field (Kistler and Miao, 1992; Fierro et al., 1999; Daboussi and Capy, 2003). The higher karyotypic variation and/or chromosomal length polymorphism is correlated with lack of sexual stage in ascomycetes fungi. Zhong and Steffenson (2007) reported highly polymorphic chromosomes among *C. sativus* isolates using contour-clamp homogenous electric field (CHEF) electrophoresis. They reported chromosomal aberrations such as translocation, deletion, and duplication in *C. sativus* isolates (Zhong et al. 2002). High level of karyotypic variation has been reported in model fungi such as *Fusarium oxysporum* and *Magnaporthe grisea* (Boehm et al., 1994; Alves-Santos 1999; Nitta et al., 1997). Transposon mediated variation has been reported in *Tox1* locus of *Cochliobolus heterostrophus* (Kodama et al., 1992) and *TOX2* locus of *Cochliobolus carbonum* (Ahn et al.

1996). Kistler and Miao (1992) suggested that a higher level of karyotypic variation would be observed in fungi reproducing asexually whereas sexually reproducing fungi would show lower level of karyotypic variation. In several asexual *Fusarium* species (Boehm et al., 1994, Alves-Santos et al., 1999), higher levels of karyotypic variation have been reported whereas karyotypic variation was lower in *Aspergellus nidulans* (Geiser et al., 1995) and *Gibberella fujikuroi* (Xu et al., 1995), which reproduce sexually. The evidence of higher karyotypic variation in *C. sativus* (Zhong and Steffenson, 2007) and the lack of sexual stage in the field populations support the hypothesis that transposon-mediated genetic variation might be one of the factors generating the high genetic diversity in *C. sativus*.

Second, high genetic variation might also be due to parasexual recombination among individuals within populations in *C. sativus*. This is an agreement with Zhong and Steffenson (2001) who also speculated that there could be genetic exchange between *C. sativus* population through parasexual recombination. Parasexual recombination is initiated by the fusion of the two hyphae belonging to the same vegetative compatibility groups followed by formation of successful heterokaryon (Pontecorve, 1956). During parasexual recombination, the two haploid nuclei fuse to form a diploid nucleus where the homologous chromosomes in the diploid nucleus synapse and undergo genetic recombination. Eventually, the diploid nucleus begins to lose the chromosome through the mitotic division, forms intermediate stage of partial diploids in some individuals before returning to haploid state. Parasexual recombination has been demonstrated in *C. sativus* in laboratory (Tinline, 1988), although it is unknown if it occurs in nature. If parasexual cycle has

important role in higher genetic diversity within *C. sativus* populations, then further study is warranted to demonstrate that individuals with partial diploids occurs in nature by using co-dominant markers such as RFLP, SSR or SNP. Salamati et al. (2000) reported partial diploids in some isolates collected from field in *R. secalis* using RFLP markers in Australia. They demonstrated that parasexual cycle occurred in five isolates and found intermediate diploids. Our population genetic analyses of *C. sativus* reveal strong evidence for mechanisms other than sexual recombination. The moderate population genetic differentiation (*Gst* = 0.196), moderate *LD* ($\overline{r_d}$ = 0.046 - 0.118) among populations and lower gene flow (*Nm* < 2.5) found in the *C. sativus* populations support the hypothesis that transposon-mediated genetic rearrangement and/or parasexual genetic recombination occur during the asexual reproduction of the fungus.

In summary, *C. sativus* is composed of diverse genotypes in field populations. The Nei's genetic diversity and Shannon's information index (Table 7.3) of *C. sativus* populations observed in this study offers the pathogen a greater evolutionary potential compared to clonal genetic structure of other fungal populations. There is evidence that new virulence groups, 7.7.3.6 from ND, and 7.7.7.5 and 7.7.5.1 from prairie regions Canada (Ghazvini and Tekauz (2008) have emerged which have overcome the durable resistance in barley line, NDB112. Earlier, Fetch and Steffenson (1994) detected only three pathotypes in ND using three differential barley lines. The genetic plasticity of *C. sativus* can be attributed to higher genetic diversity and can result in the emergence of new virulence

groups quickly. This poses a potential threat to barley production in the Upper Midwest of United States.

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