IDENTIFICATION AND MAPPING OF QTL ASSOCIATED WITH CROWN RUST RESISTANCE IN RECOMBINANT INBRED LINES OF OAT (*Avena sativa L.)*

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Cerly Rini Yeruva

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

MASTER OF SCIENCE

SUPERVISORY COMMITTEE:

Dr. Michael McMullen
Chair

Dr Elias Elias

Dr. Upinder Gill

Approved:

April 10, 2024
Date

Dr. Richard Horsley
Department Chair

ABSTRACT

Crown rust caused by *Puccinia coronata* f.sp. *avenae* is the most destructive fungal disease threatening oat production worldwide. Therefore, a search for novel sources of genetic resistance is considered essential to combat the evolving races of pathogen. The objectives of this study were to develop two recombinant inbred line mapping populations carrying novel sources of crown rust resistance and identify the genomic regions associated with crown rust resistance. 108 and 112 $F_{5:6}$ RILs developed from each of the biparental crosses were evaluated for disease incidence in field and greenhouse under randomized complete block experimental designs with two replications. Genotyping and linkage analysis of markers associated with RILs revealed a major QTL underlying disease resistance in each population respectively. The two QTL identified in this study showed consistency across all the tested locations and the markers associated with the QTL appear to be useful for marker-assisted selection in oat breeding programs.

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INTRODUCTION

Oat (*Avena sativa* L.) is an important cereal crop grown for human consumption, forage, fodder, straw, and is highly valued for its health benefits. However, a significant decrease in oat production and quality is attributed to the leaf diseases of which, the crown rust disease caused by the pathogen *Puccinia coronata* f. sp *avenae* is of major concern. The pathogen infects the plants by forming urediniospores on surface of leaves, depriving the plant nutrients and essential elements, and thereby affecting the photosynthesis and the grain yield consequently.

The use of fungicides, as a means of disease control, is considered non-eco-friendly, and economically unjustifiable, because of the fungicide resistance that could develop in the pathogen population and the excess costs involved in the management practices. Therefore, deployment of crown rust resistant cultivars is considered as an effective alternative which is ecofriendly and economical method in practice. However, because of evolving races of pathogen, breakdown of resistance in plants is common and hence there is a need for continuous development of resistant cultivars.

More than hundred genes that show race-specific resistance to crown rust disease have been defined and some of these genes were deployed in cultivars (Admassu-Yimer *et al.,* 2022). However, the chromosomal location and the molecular markers linked to only a few of the defined genes were known. This lack of information of the crown rust resistance genes, in addition to the complexity of the oat genome, is causing difficulty in utilizing the genomic tools for crown rust resistance breeding in oats. Moreover, the effectiveness of the resistance genes is short-lived because of the continuous evolution of virulent pathotypes.

The identification of genomic regions, and the novelty of the quantitative trait loci (QTL) linked to the desired traits in oats have become possible from the year 2020, with the availability

of the first-ever sequence data of the hexaploid oat, OT3098 reference genome assembly. Consequently, the first objective of this research was to develop a biparental recombinant inbred line mapping population derived from novel sources of crown rust resistance and conducting replicated trails across different environments. Furthermore, the second objective is to identify and map the genetic regions associated with crown rust resistance in the recombinant inbred line mapping population. Findings from this research will facilitate the oat breeding programs with novel crown rust resistance genes for use in marker assisted selection as well as in pyramiding the genes for durable resistance.

LITERATURE REVIEW

Oats and its importance

Avena sativa (Common Oat) is a species of cereal grain crop cultivated worldwide, which belongs to the tribe Aveneae of the grass family Poaceae. It ranks seventh among cereals in terms of production globally. The origin of oats is Asia Minor, and they were introduced to northern part of North America by English and North Europeans in 1602 (Coffman *et al.,* 1977). Due to their greater adaptability, oats which were weeds in emmer wheat (*Triticum dicoccum*) supplanted emmer as a cultivated crop. Cultivated oats were the descendants of wild red oats (*Avena sterilis*) and common wild oats (*Avena fatua*). It is closely related to rye grass (Peng *et al.,* 2022) and was domesticated about 2000- 3000 years ago from weedy progenitor in the fields of wheat or barley (Krattinger *et al.,* 2022).

The distribution of oats is confined mainly to the temperate northern hemisphere but also include Australia and Argentina. It thrives well in cool and moist climates. The life cycle of oats consists of germination, tiller production, stem elongation, Pre-anthesis, anthesis, grain-filling and ripening termed as Zadoks Growth Stages (ZGS) (Zadoks *et al.,* 1974). These stages are followed by harvesting, cleaning, grading raw seeds, dehulling and industrial processing.

Oat kernel, termed as caryopsis or groat is a good source of protein, oil (Unsaturated fatty acids), soluble fiber (β-glucan), B complex vitamins, minerals (Butt *et al*.,2008). Continuous consumption of oats is useful for controlling diabetes and provides a healthy lipid profile. Oats are used primarily in human foods or as feed for horses or other livestock.

Globally, oats are cultivated in more than 70 countries. The rising popularity of oats as a healthy superfood, has increased global oat market size to $7.13 billion in 2023 compared to $6.67 billion in 2022 (https://www.thebusinessresearchcompany.com/report/oats-global-market-

report)**.** In the year 2022, United States ranked seventh with a production of 0.84 million metric tons (https://www.statista.com/statistics/1073550/global-leading-oats-producers/). Canada is the main exporter of oats and United States is the largest importer of oats**.**

Oat production in North Dakota

Agriculture is the leading industry sector in North Dakota and it led the nation in production of all beans, oats and wheat during 2018(https://www.dot.nd.gov/grants/2020-BUILD-improving%20resiliency/References/USDA,%20National%20Agricultural%20Statistics%20Service,%20North%20Dakota%20Field%20Office%20August%202019.pdf) In the year 2022, North Dakota ranked top as a producer of high-quality oats in United States with a production of 13.5 million bushels (https://www.cropprophet.com/us-oats-production-by-state-rankings-in-2023/#2022). Minnesota, South Dakota, Wisconsin, and Pennsylvania are the top producers of oats following the lead producer North Dakota. Of the total production in North Dakota, 66.6% is harvested for grain and the remaining is grown for hay/forage.

For milling purposes, oats with low oil content are preferred as high oil causes caking and thereby difficulty in handling. Whereas varieties with high oil content are excellent for feed purpose as they provide extra energy. Late-maturing varieties, as they produce large amounts of biomass are generally grown for forage. Hi-Fi, Beach, Souris, Rockford, Newburg, Jury, and Stark are some of the oat varieties developed by the NDSU oat breeding project(http://www.ndsuresearchfoundation.org/oats)

Early planting around May is considered ideal for effective tiller production. The ideal seeding depth is 1.5-2.5 inches. The soils of North Dakota are usually deficient in Nitrogen and Phosphorous and require careful fertility management. Care should be taken to control wild oats,

as there is not any herbicide registered that can control wild oats selectively in oat crops (Ransom *et al.,*2007). Crop rotation with noncereal crops such as dry beans, canola, potatoes can be effective to control wild oats.

Oats are a cool season annual grass, requiring temperatures between 16-23˚C and may be sensitive to hot and dry weather during heading and grain filling stages (Murphy and Hoffman 1992).

Several diseases affect the yield and damage the quality of oats. Crown rust, stem rust and barley yellow dwarf virus are the most common diseases in North Dakota. To combat the diseases, growing resistant varieties is considered effective and economical. Although the use of fungicides is not common, the best time to apply is near flowering stage. The panicles must be harvested when yellow or brown and should be dried to 14% moisture before storage.

Oat genetics

The genus *Avena* of oats has around 30 species that includes diploids, tetraploids and hexaploids (Fu, 2018). Cultivated oats is a self-pollinating allohexaploid crop with 6 sets of 7 chromosomes named 1A – 7D representing A, C, D nuclear genomes with over 80,000 genes (Kamal *et al.,* 2022). The paternal A1/As- genome diploid *A. longiglumus* on hybridization with maternal CCDD genome tetraploid *A. insularis* gave rise to origin of AACCDD genome hexaploid oats 0.5 mya (Peng *et al.,* 2022). The CCDD genome tetraploid is formed by allotetraploidization between paternal C genome diploid and maternal D genome diploid. The polyploidization events are facilitated by chromosomal doubling to stabilize the chromosomal pairing (Loskutov, 2008)

Hexaploid oats, with a genome size of ~12.5 Gb is characterized by highly (86.95%) repetitive DNA sequences, transposon rich C sub genome, high intergenomic translocations,

biased gene fractionation within three sub genomes and high homology between A & D sub genomes (Kamal *et al.,* 2022). 200 DNA motifs make up 70% of the oat genome resulting in a mosaic-like genome architecture of oats.

For introgression of alien genes from wild to cultivated ones, all the species of *Avena* are split into three gene pools (Leggett *et al.,*1995). The primary gene pool consists of hexaploids which can hybridize readily and produce fertile F_1. The tetraploids *A. maroccana* and *A. murphyii* constitute the secondary gene pool which do not hybridize readily and moreover result in self-sterile F_1. However, they are rich sources for increased protein levels in groats. All the diploids and distantly related tetraploids constitute tertiary gene pool from which production of hybrids is difficult. Embryo rescue is often required and F_1 hybrids are self-sterile.

With the intent to create genetic variability, oat improvement can be divided into prehybridization period (up to 1930) and post hybridization periods. Oat improvement mainly focuses on growing a successful crop with less use of expensive herbicides. The second target is to develop disease resistant cultivars. Increased fiber content, reduced hull percentage and uniform kernel size for better value-added processing are the tertiary targets for oat breeders (Stuthman,1995).

Crown rust disease of Oats

Oat rusts were first recorded when the crop failed throughout Iowa in North America in 1858 (Simons 1985). Crown rust, stem rust and barley yellow dwarf are the three most common diseases affecting oat yield in North Dakota. Of all the diseases, crown rust caused by *Puccinia coronata* f.sp. *avenae* (*Pca)* is a widespread devastating disease affecting grain yield, groat weight and groat percentage in oat industry (Doehlert *et al.,* 2001). In susceptible cultivars grain yields are reduced by as far as 50% (Leonard *et al.,*2005).

Finger like projections on the apex of teliospores refers to “crown” in crown rust. It is also known as leaf rust because of oval to oblong pustules formed primarily on the surface of leaves. Warm temperature(20-25˚C), high humidity favors disease development.

Life cycle and infection process

Puccinia coronata f.sp. *avenae* (*Pca*) is a basidiomycete fungus that thrives as an obligate biotroph and is characterized as a macrocyclic, heteroecious fungus. *P. coronata* has five different spore stages throughout its life cycle namely basidiospores, pycniospores, aeciospores, urediniospores and teliospores. Asexual reproduction (uredinial stage) occurs in the primary host *A. sativa*. Sexual reproduction (aecial stage) occurs in the alternate host common buckthorn, *Rhamnus cathartica* (Simons, 1985) an invasive woody shrub. After its introduction from native Europe and eastern Asia to North America, buckthorn became invasive in 1880’s.

Life cycle of *Pca* begins with teliospores in the spring. Dikaryotic thick walled teliospores germinate in spring, undergo meiosis producing haploid basidiospores which infect buckthorn (*Rhamnus* sp.) leaves. Basidiospores germinate and form appressoria and penetration peg under favorable conditions. The haploid mycelium on the upper surface of the *Rhamnus* leaf produces pycnia. Pycniospores are considered as male gametes and flexuous hyphae, the female receptive structures are formed from pycnia. Male gametes cannot mate with flexuous hyphae originating from same pycnia.

Following plasmogamy aecium develops on the abaxial surface, and these cylindrical-shaped structures release dikaryotic aeciospores, the primary inoculum which infects the *Avena* species. Appressoria formed from germinated aeciospores forms substomatal vesicles after penetration through stomata.

During summer, intercellular branching of infection hyphae gives rise to sporulating uredinia forming bright orange yellow oblong pustules forming characteristic symptom of infection (Jackson *et al.,*2008). Later in the season, black pustules containing teliospores are formed which differentiate in late summer or autumn to overwinter and germinate the next spring (Figure 1).

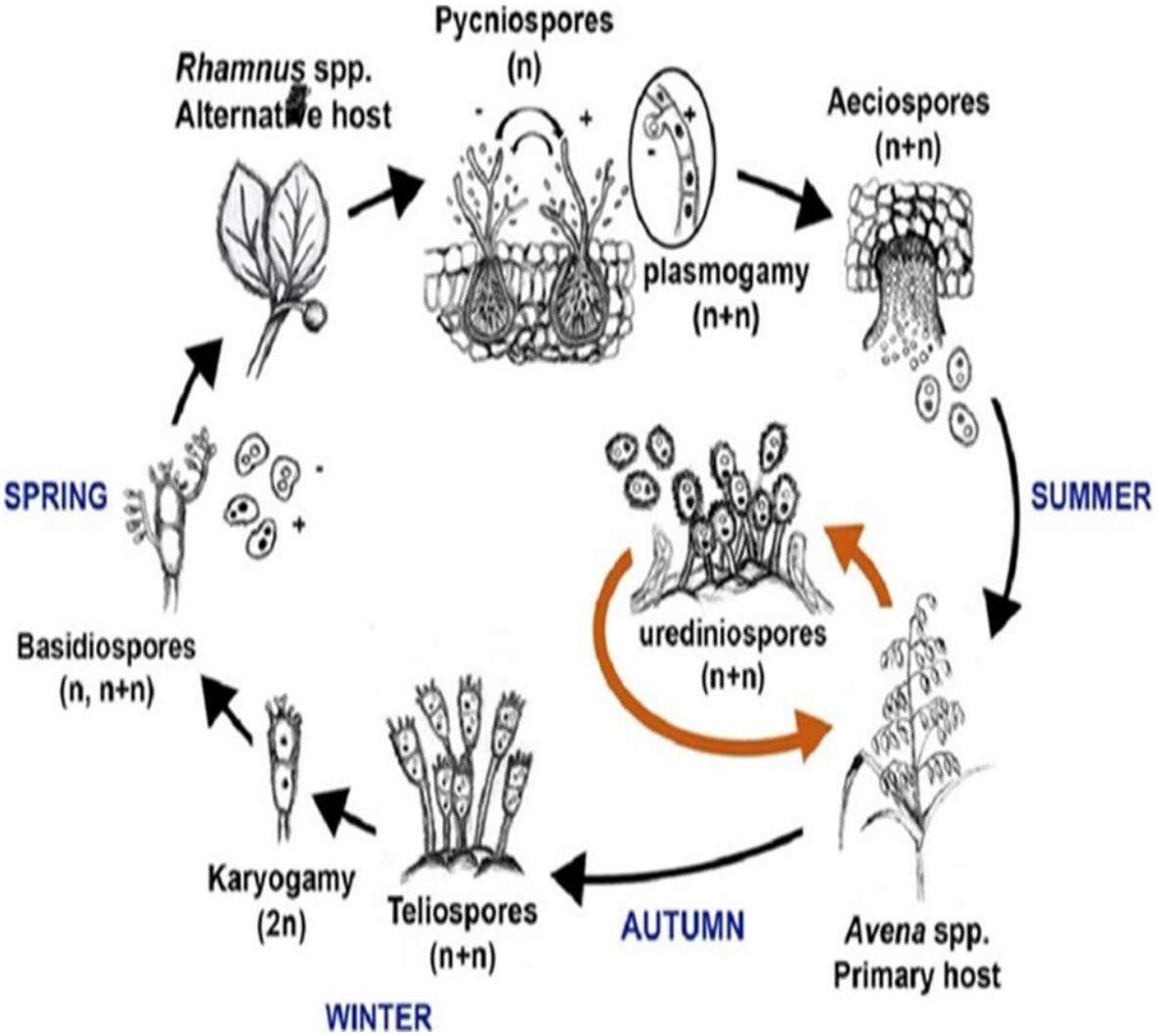


Figure 1. Life cycle of *Puccinia coronata* f.sp. *avenae,* a drawing by M. Figueroa (2017).

Disease rating

Visual rating and digital image rating are the disease rating methods used to evaluate oat crown rust among which visual assessment is most used. Digital image analysis involves

collecting and scanning infected host leaves, allowing for exact measurement of rust pustule coverage using image analysis software. Fungal DNA detection method involves amplifying FDNA from rust infected leaves using pathogen-specific primers and assessed using quantitative real-time polymerase chain reaction (q-PCR) which improves the precision of rust rating (Jackson *et al.*, 2006).

The visual disease(pustule) rating system having 0-4 scale established by Murphy (1935) where 0 [immune], ; [fleck], 1 and 2 [resistant (R)], 3 and 4 [susceptible (S)] is most often used for determining seedling resistance (Figure 2). The modified Cobb scale and infection type (IT) are commonly utilized in field evaluations (Chong *et al.,* 2011).Crown rust reaction is assessed by assigning each plot disease severity (DS) which is the % of leaf area covered by pustules and reaction class (RC); with resistant-R, moderately resistant-MR, moderately severe-MS, severe-S. Reaction class is converted to reaction score 0-1(R=0, RMR=0.1667, MR=0.3333, MRMS=0.5, MS=0.6667, MSS=0.8333, S=1).Disease severity percentage is multiplied with reaction score to obtain coefficient of infection(CI) and is regarded as superior parameter to analyze and depict actual rust damage in fields.

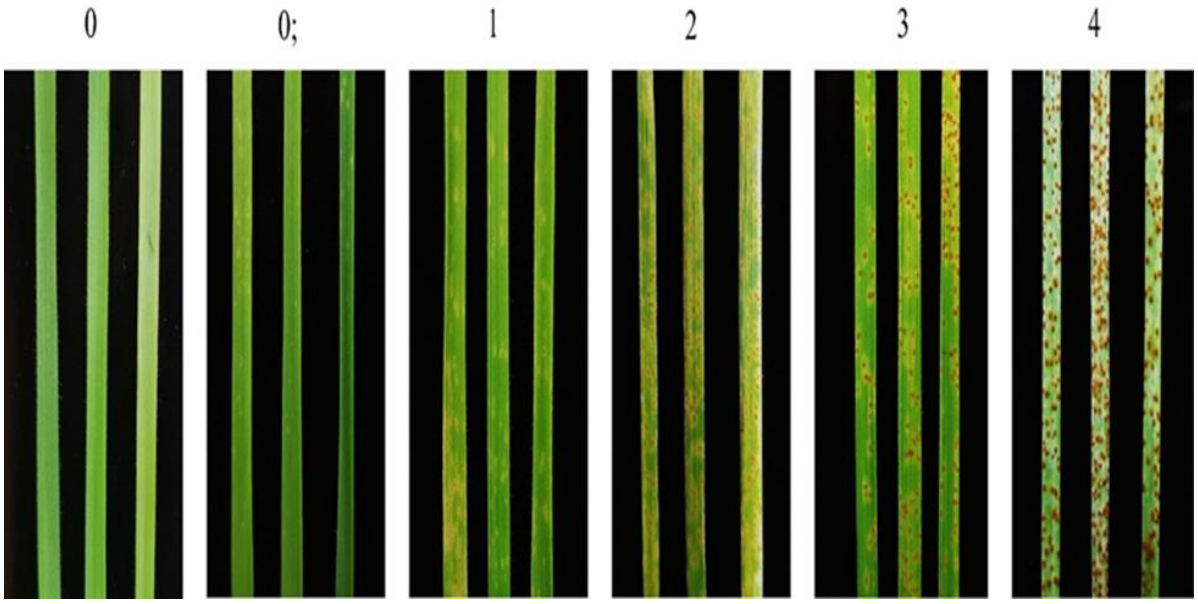


Figure 2. Murphy's disease rating scale (0-4) by Aro Lee et al., (2020)

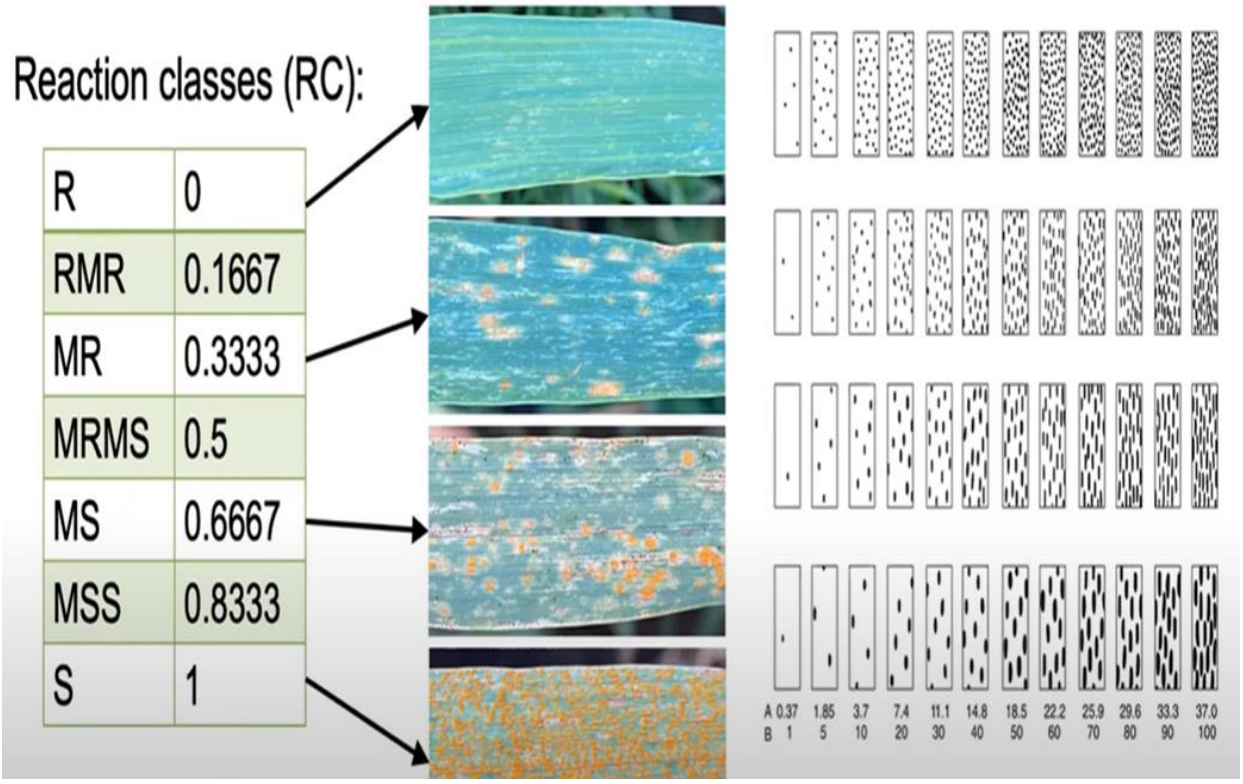


Figure 3. Modified cobb's scale for assessing crown rust in field (Lin et al.,2014)

New races of pathogen

With the rapid appearance of new virulent isolates during national and worldwide surveys Pca was considered as one of the most pathogenically diverse cereal rust fungi (Miller et al.,2020). According to Murphy (1935) evolution of new physiologically specialized forms(races) of Puccinia capable of infesting cultivars with specific resistance genes is mediated by buckthorn. Aeciospores with wide range of genotypes are produced by aecia due to following reasons: Random assortment of chromosomes and genetic recombination in teliospores results in different genotypes of basidiospores every year; several distinct genotypes of pycniospores can migrate to single aecial primordium, resulting in diverse aeciospore genotypes; Random mutation, somatic hybridization through anastomosis, pathogen cycles between wild and cultivated oats also produces new virulent Pca races (Zhao et al.,2016).

Nomenclature of pathogen phenotypes based on virulence

In North America, a new nomenclature system was adapted for *Puccinia coronata* f.sp. *avenae* from the terminology available for the wheat stem rust and leaf rust pathogens as described by Chong *et al.,* (2000). To designate the pathogen phenotype, 16 single-gene oat lines(differentials) were selected. They were chosen based on their importance in breeding in addition to stability and differentiation of Infection Types under greenhouse testing. Susceptible checks such as Starter/ Makuru/ Marvellous were used to set high Infection Type. The host differential lines were arranged in subsets of 4 that resulted in 16 unique alignments to which the consonants from B through T were assigned to specify a four-letter code for the pathogen phenotype. Each letter in the code indicates the virulence/avirulence reaction of the pathogen isolate to each of the differential line in each subset.

The nomenclature system was designed in a way that another subset of differentials can be added by placing an alphabet to the right of the existing code. Moreover, if the new subset does not have enough differential lines, the *Pc* gene to which the pathogen is virulent can be written by placing a dash following the four-letter code.

Disease management strategies

The disease caused by the crown rust pathogen can be mitigated by four ways namely: eradicating the alternate host, fluctuating planting dates, application of fungicides and by means of genetic resistance.

However, none of the disease management methods are simple. It is laborious to destroy the buckthorn around oat fields. There will be an increase in production costs by chemical methods of control. Moreover, fungicides can only reduce the disease but could not provide complete crop protection. In addition, use of chemicals will pose a potential threat to environment due to their residual effect.

As reported by McCallum *et al*., (2007), the disease escape may occur by planting early to provide required time for plant development prior to the arrival of urediniospores. To provide complete protection to the crop, Nazareno *et al.,* (2013) considered genetic resistance as a cost-effective, economical, and eco-friendly method for controlling crown rust in oats.

Defense mechanisms in plants

Failure of the plant's immune system to recognize and activate defense response against the invaded pathogens results in development of rust disease. Plants have the innate ability to defend the pathogen attack referred to as genetic resistance. The resistance that can be seen at seedling stage which continues throughout the all the growth stages was known as all-stage resistance (ASR). On the other hand, the resistance acting only during adult plant stage was

referred as adult plant resistance (APR). Genes involved in showing ASR were effective only against specific strains of pathogen and is referred as race-specific resistance. On the contrary, resistance conferred by the APR genes is effective against different pathogen strains and hence is known as race-nonspecific resistance. APR genes provide partial resistance by slowing the infection and exert low selection pressure on pathogen and were usually durable (Dinglasan *et.al.,*2022).

Furthermore, ASR is characterized by interaction of a plant dominant resistance (*R*) gene and the corresponding specific pathogen avirulence (*avr*) gene. After recognition of signal molecules(elicitors) from pathogen, a cascade of host receptor proteins encoded by the R genes will be activated to restrict the pathogen growth (Ellis *et.al.,*2014). The Avr genes can be mutated easily or eliminated, and hence the protection conferred by R genes is not long-lasting. However, the dominant R genes can be transferred into related agronomic species without much difficulty (Mysore and Ryu 2004). This type of resistance controlled by a single gene provides protection throughout plant development and has been utilized extensively to control crown rust since 1990(Ohm and Shaner 1992). Moreover, it is easy to use such plants having major gene resistance in greenhouse seedling tests and field nurseries for the breeding projects (Kebede *et al.,*2019)

Breeding for crown rust resistance

Crown rust resistance in oats is conferred by many single dominant genes following gene-for-gene characteristics (Chong *et al*.,2008). In North America, attempts to develop oat cultivars resistant to *Pca* began in late 1800s and early 1900. Genes which confer resistance to *Pca* are designated as *Pc* genes followed by a number implying a unique locus. Most of the Pc

genes used and studied in breeding programs were the results of mutation (Simons, 1970) having different specificity for each of the pathogen isolates.

'Hawkeye' is the first resistant oat cultivar developed in 1919 by crossing stem rust resistant 'Richland' variety with 'Green Russian' (Coffman *et al.,*1961). 'Victoria' (*Pc2, Pc11*) from Uruguay and 'Bond' (*Pc3, Pc4*) from Australia were used intensively in 1920s to transfer the seedling resistance to oat germplasm in United States and the varieties having the resistance derived from Victoria were released in 1940(Nazareno *et al.,*2018). However, they were devastated by Victoria blight in 1946. Similarly, the resistance derived from other varieties such as Bond, Landhafer (Pc5), Santa Fe (*Pc6, Pc7, Pc8, Pc9c, Pc21*), Ukraine (*Pc3c, Pc4c, Pc6c, Pc9*), Trispermia (*Pc6d*), Saia (*Pc15, Pc16, Pc17*) was overcome by new crown rust races by the end of 1950(Simons 1985).

With some 44 of the resistance genes catalogued from wild hexaploid oats, *Avena sterilis* has become a rich source of crown rust resistance in the beginning of 1960s (Leonard *et al.,*2004). Some of the *Pc* genes derived from *A. sterilis* include *Pc34-Pc36, Pc38-Pc43* and *Pc45*-Pc77.Cultivated oat varieties having *Pc38* and *Pc39* with resistance transferred from *Avena sterilis* released during early 1980s were defeated by *P. coronata* by the end of the decade (McCallum *et al.,*2007). Similarly, *Pc48* and *Pc68* deployed in 1990s is defeated in 2001.

Avena strigosa the diploid species of oats has become another source of crown rust resistance during the 1970s. *Pc15-Pc17, Pc19, Pc23, Pc30, Pc37, Pc81-Pc90 and Pc94* are some of the *Pc* genes identified within *A. strigosa* (Cabral *et al.,*2014). However, difficulties such as sterility of hybrids and incompatibility existed in transferring these genes from diploid to hexaploid cultivated oats. Although difficulty existed in obtaining seeds from crossing diploids ×

tetraploids and tetraploids × hexaploids; tetraploids were used as bridge to transfer the *Pc* genes from diploids to hexaploids.

As the resistance conferred by deploying single sources of resistance is not durable, there has been a reluctance in using such varieties since mid-1960's (Simons 1985).

To extend the durability of race-specific *R* genes, gene pyramiding is considered as an effective strategy. The variety 'Leggett' having two crown rust resistance genes *Pc68* and *Pc94* is released in 2004 and virulence to both genes has been reported (Chong *et al.,*2011). Virulence was detected 4 years after its release in 1995 in North America in the variety 'Ac Assiniboia' having three genes *Pc38, Pc39, Pc68*.

Only few races showing virulence to *Pc91* derived from Amagalon (*Avena magna* × *Avena longiglumis*) have been identified in United States. The "HiFi" (McMullen *et al.,*2005) having *Pc91* exhibits disease resistance and hence use of *Pc91* in combination with other *Pc* genes is of current interest in oat breeding programs (Nazareno *et al.,*2018). However, in Australia, 35% of the crown rust isolates were reported to be virulent on *Pc91*(McCartney *et al.,*2011).

Extended periods of crown rust resistance were observed by deploying Adult plant resistance genes (APR) as against seedling/race-specific resistance/all stage resistance (ASR) genes (Carson 2011). Red Rustproof, MN841801, TAM O-301 are some of the varieties exhibiting APR that remained effective and showed durable resistance for several years.

Besides use of ASR genes, APR genes, and gene pyramiding for disease control other strategies such as recurrent selection for partial resistance, cultivation of multilines, line or varietal mixtures are being practiced.

Mode of inheritance of *Pc* genes

The number of genes involved in expressing the resistance determines the inheritance of crown rust resistance. Resistance to *Pca* is conferred by the nuclear genes following meiotic inheritance (Staletic *et al.,*2009). The mode of inheritance can be dominant (*Pc68)* or incompletely dominant (*Pc56, Pc64-Pc66)* or recessive (*Pc54, Pc55*) with majority of the genes being inherited dominantly (Park *et al.,*2022). *Pc* genes could show a major effect(qualitative) or small effects(quantitative) acting additively. The segregation ratios observed in $F_1/F_2/BC_1/BC_2$ after crossing served as indicators of number of genes involved. The segregation ratios of 1:1, 3:1,1:3 is subjected to test the goodness of fit using the $\chi 2$ test. The segregation of resistant: susceptible phenotypes in the ratios of 1:1, 3:1, 1:3, 13:3, 9:7 indicates whether a single gene or two genes are acting independently or epistatically.

Genetic markers and genotyping

The DNA sequences which act as signs that symbolize the genetic variation among individuals are referred to as genetic markers. Morphological markers, biochemical markers and DNA markers are the three major genetic marker types. Morphological characters/ Phenotypes, which are easy to observe were considered as reliable indicators of genes and are used as morphological markers during the 19th century. During the later century, proteins that vary in their composition due to allelic variations in genes were utilized as biochemical markers in the era of pre-recombinant-DNA technology. However, morphological and biochemical markers were limited in number and were influenced by environmental factors and developmental stages. Therefore, DNA markers which were abundant in number were widely used in the1980s.

The variations in DNA sequences can be detected by hybridization (RFLPs) or by rapid amplification of small quantities of DNA using PCR (Microsatellites/SSRs, STSs, ESTs, RAPD,

AFLP). Some of the markers such as minisatellites, STSs and ESTs can be identified by both techniques. However, most of the markers namely RFLPs, RAPDs, SSRs can be detected through gel-based assays which was expensive and time consuming. Therefore, the current marker of choice was SNPs because of their availability throughout the genome in large numbers and detection through non-gel-based assays. SNPs represent the regions in a genome where the DNA sequence differs by a single base. It was estimated that in any genome, a single SNP can occur for every 100-300 bp (Gupta *et al.,*2001)

In the early 21st century, rapid detection of SNPs has become feasible with the introduction of NGS technologies. Identification of the genetic variation by simultaneous detection of nucleotides has become possible by a novel, rapid, low cost approach of NGS technology, referred to as genotyping-by-sequencing (GBS). As it generates many SNPs, GBS has become a unique tool for genetic analysis in genomics-assisted breeding.

The use of huge collection of SNPs generated by genotyping is based on whether the SNPs imply the genetic variations for the trait under study. Moreover, SNPs, if found in the coding sequences are very useful as they show 100% association with the trait and can be used for gene isolation. Whereas, if the SNPs are found near the coding sequence, the association of SNP with the trait will be <100%, however, they can be used for positional cloning of the gene. Rafalski., (2002) concluded that SNPs can be considered as crucial source of polymorphic markers that are inexhaustible and facilitate production of high-resolution maps for linkage and association studies.

Construction of linkage map

Sections of DNA referred to as Quantitative trait loci (QTL) are involved in controlling quantitative traits such as complex forms of plant disease resistance. As a result, it is essential to

identify the distinct sequences of DNA that act as linked markers to the QTL controlling a given trait of interest. To test the DNA markers for their likelihood of association with QTL, it is crucial to develop a linkage map. As described by Collard *et al.,* (2005) construction of linkage map involves developing a mapping population followed by identification of polymorphic markers and coding the markers to perform linkage analysis.

Different choices of mapping populations such as F_2, back cross (BC), recombinant inbred lines (RILs), doubled haploids (DHs) can be developed for phenotypic evaluation of the trait of interest. The type of mapping population determines the accessibility and efficiency of mapping information. For greater utility of the population, it is essential that the parents selected for generating the mapping population differ in one or more characters. DH and RI populations are considered as permanent populations as they produce true breeding lines that can be multiplied without any genetic changes. RI lines are constructed by inbreeding individual F_2 plants undergoing many rounds of meiosis till the homozygosity is attained. Consequently, in each line the segregation is fixed for various combinations of linked blocks derived from parental alleles. These lines not only allow replicating the experiments across different locations but also aid in examining common material if seeds are transferred to different laboratories.

The next step is to identify the DNA sequences that act as markers in distinguishing the parents. Finally, each of the identified polymorphic markers is screened across every individual in a mapping population to generate the coding data which is known as marker genotyping. The order of markers and genetic distance between them on a linkage map is influenced by the number of individuals screened in the mapping population. Linkage mapping, therefore, refers to the process of estimating genetic distance between the markers on each chromosome based on recombination values. The entire chromosome or the chromosomal segments onto which the

linked markers are grouped together are known as linkage groups. Many more genes can be accurately mapped with such molecular frameworks on each chromosome.

However, physical rearrangements, duplications, size, and plasticity of the oat genome caused difficulty in assigning the loci to chromosomes. Not enough linkage was detected between some of the genes in hexaploid oats which impeded the construction of linkage map. With the advent of molecular markers, the construction of linkage maps for oats has become possible.

The first ever linkage map in oats was constructed in the early 1990s based on F2 families of diploid *A. strigosa* group based on RFLP loci. *A. atlantica* × *A. hirtula* and *A. strigosa* × *A. wiestii* are the four diploid oat species that are used in construction of linkage map as reported by O'Donoughue et al., (1992) and Rayapati et al., (1994) respectively. Diploid species were used against hexaploid oats to generate a smaller map with a smaller number of linkage groups by avoiding homoeologous pairing thereby reducing the complexity during construction of linkage map.

Recombinant inbred lines derived from crossing the red oats *A. byzantina cv. Kanota* and white oats *A. sativa cv. Ogle* were used for constructing the first hexaploid oat linkage map (O'Donoughue et al.,1995). However, due to the small size of mapping population (71 RILs), the linkage map had shorter linkage groups with a smaller number of markers in addition to difficulty in assigning unlinked markers. As described by Rines et al., (2006), the KO map was successfully used to locate regions conferring resistance to crown rust, oat groat oil content, effects on plant height and heading date as a response to vernalization. Later, many hexaploid oat maps were generated.

The first complete, physically anchored consensus map of hexaploid oats with 985 SNPs on 21 linkage groups spanning 1838.8 cM was developed by Oliver et al., (2013) using 390 RILs derived from six biparental populations. Consensus genetic map is developed by combining and averaging the component linkage maps from various population studies.

To develop a complete genome sequence, that can be used as a framework to order and orient contigs, Chaffin et al., (2016) developed a high-density consensus linkage map of oats. It represented the most common oat varieties grown in North America, based on 12 biparental recombinant inbred line populations and is saturated with 7202 markers with a map distance of 2843 cM. Furthermore, the first haplotype map of cultivated oat lines in North America was constructed by Bekele et al., (2018) by addition of more than 70,000 GBS loci to the existing oat consensus map.

A fully annotated, chromosome scale assemblies for extant progenitors namely *A. atlantica* and *A. eriantha* that contributed As- and Cp-sub genomes was developed by Maughan et al., (2019). Nearly 50K gene models in each species that included 2965 resistance gene analogs across both the species were identified on annotation of the genome. It serves as an important genetic resource for the improvement of common oats.

The first ever genome assembly of hexaploid oat genotype OT3098 represents a breakthrough in oat genomics research. The annotated gene set, and a set of predicted transcripts and translated protein sequences mapped to OT3098 reference genome were released by PepsiCo in partnership with Grain Genes in 2021. This assembly will be spurring the genetic improvement of oats in areas such as high throughput mapping and gene cloning for traits of interest.

QTL analysis and detection of QTL

All the individuals in the mapping population are partitioned based on marker genotypes, phenotypic mean, and variances. The marker which shows a significant difference ($p<0.05$) in mean phenotype indicates that the QTL controlling the phenotype is close to the marker. Therefore, linking the phenotype and marker genotype is the principle underlying QTL analysis.

As described by Collard *et al.,* (2005), QTL can be detected using different methods based on number of markers used as a unit of analysis.

Single markers were used to locate QTL during times when linkage maps were not available. However, the position of the QTL and its effects are underestimated via single- marker analysis.

Thereafter, with the availability of linkage maps, Lander and Bolstein (1989) proposed Interval Mapping (IM) which utilizes simple linear regression to determine the maximum likelihood (LOD) of estimating a single QTL in the intervals formed by flanking markers. Conversely, effects of other QTL are ignored by IM and composite interval mapping (CIM) proposed by Zheng (1994) has become popular. It overcomes the limitation of IM by combining IM and multiple regression. In CIM, a subset of markers was fitted as cofactors to limit the residual error accounted by the effects of linked QTL. As described by Li *et al.,* (2007) an altered algorithm known as inclusive composite interval mapping (ICIM) was proposed to control the variance while choosing marker cofactors.

On the other hand, Multiple Interval Mapping (MIM) developed by Kao *et al.,* (1999) was considered accurate as it identifies multiple QTL along with their epistatic effects simultaneously by utilizing multiple marker intervals (Xu *et al.,*2017).

Interval mapping thus provides a graph of possible regions where QTL can be detected on a linkage map using LOD score. Yet, to declare a QTL as statistically significant, Churchill and Doerge (1994) proposed that permutation tests are to be carried out to ascertain the significant thresholds. Initially, to determine the false positive associations, shuffling the phenotypic values is done several times, maintaining the same values for the marker genotypes. And then, the significant threshold is determined based on level of false positive marker trait associations. If the peak of the QTL exceeds the specified LOD threshold, the QTL is then declared as real QTL with its likely position.

Nonetheless, QTL occur within the regions known as confidence intervals. As described by Hackett (2002), several methods such as bootstrapping or identifying the regions on either side of QTL peak with a decrement of LOD score value by 1 are used to calculate the confidence intervals.

Mapping of crown rust resistance QTL

Till date, 98 alleles (*Pc1-Pc85, Pc91-Pc96, Pc98*) at 92 loci that confer resistance to crown rust have been defined and accepted of which six loci confer adult plant resistance (Park *et al.,*2022). However, not many candidate avirulence (Avr) genes that correspond to race-specific *Pc* genes have been discovered (Miller et al.,2020). Moreover, many of the *Pc* genes could be the same or allelic because only a few race-specific genes namely *Pc38, Pc39, Pc45, Pc48, Pc50, Pc53, Pc54, Pc58a, Pc59, Pc68, Pc91*and *Pc98* have been genetically mapped (Nazareno *et al*.,2022).

Pc38, Pc39, Pc48 genes were mapped to the regions corresponding to KO (Kanota/Ogle) linkage groups 17, 37, 22_44+18 by Wight *et al.* (2005). The groups 17,37,22_44+18 represent chr.7D, chr.4C and chr.4A respectively according to the 2021 International Oat Nomenclature.

Kebede *et al.,* (2019) mapped *Pc45* to chr.2D and *Pc54* was mapped to chr.7D by Admassu-Yimer *et al.,* (2022). The location of *Pc58* on chr.7D and *Pc91* on chr.1D was confirmed by Klos *et al.,* (2017). Based on the consensus map, Zhao *et al.,* (2020) mapped *Pc98* to chr.4A. On the other hand, the chromosomal location of none of the APR genes namely *Pc27, Pc28*, *Pc72, Pc73, Pc74* were known (Park *et al.,*2022).

MATERIALS AND METHODS

Origin of parents

ND130020 derived from CRRSRR3/ND051306, and ND141862 developed from the cross CRRSRR1/ND080442 were the parents selected for their resistance to crown rust. Both CRRSRR1 and CRRSRR3 possess stem rust resistance and crown rust resistance and were named accordingly. CRRSRR1, CRRSRR3 were developed with the pedigree D13/Maida. Maida was derived from the cross ND873126/Assiniboia, of which Assiniboia possesses *Pc68* gene. On the other hand, D13 was developed by crossing ND873126 with Morton that has *Pc45* gene. In addition, ND873126 has genes conferring resistance to both stem rust and crown rust as it is derived from ND811386 having stem rust resistance and Valley that has *Pc38* and *Pc39* genes. Moreover, ND811386 is derived from a breeding line RL3038 received from R. McKenzie (AAFC) Winnipeg with a complex pedigree possessing *Pc38, Pc39, Pg2, Pg13*. The lineage of ND811386 is CI9221/Otee//RL3038/Dal ('Clean leaf'). ND130182 and ND141087 were the crown rust susceptible parents utilized in developing a biparental mapping populations with the crown rust resistant parents ND130020 and ND141862 respectively.

Development of biparental mapping populations

Two recombinant inbred line populations with population No. 16262 and 16205 were developed from the crosses ND141862×ND141087, ND130020×ND130182 respectively. To develop the mapping population, 150 F_2 seeds were advanced to F_5 generation using single seed descent method by planting them in cones. Later, to increase the seed for multi-location testing, F_5 seeds were sown in pots and all the seeds from each panicle were harvested and bulked as $F_{5:6}$ generation. The temperature of 21^0C was maintained all the time. High pressure sodium lamps were used to control the photoperiod maintaining 16 hours of daylight and 8 hours of night. The

$F_{5:6}$ lines were planted in a randomized complete block design (RCBD) having two replications per each line along with parents and checks. The seedling resistance was tested at NDSU Jack Dalrymple Agricultural Research Complex (Greenhouse) by planting in pots. The adult plant resistance was scored in Fargo field and Matt More Buckthorn Nursery, St. Paul Campus of University of Minnesota.

Urediniospore increase and inoculation

The mapping population was infected naturally in the field. Conversely, to test the seedling resistance in greenhouse, the crown rust spores showing virulence to the resistant cultivars namely Leggett(*Pc*94, *Pc*68), HiFi(*Pc*91), Maida(*Pc*38, *Pc*39,*Pc*68), and Morton (Pc45) were collected from field in Fargo and used as inoculum. The collected spores were placed in glass vials and mixed with soltrol-170 mineral oil and shaken thoroughly. To provide the pressure for spraying, the spray equipment was connected to nozzle with pressure pipe with a set pressure of 6psi. The nozzle top is covered with finger to release the spores while spraying on to week-old plant. Seeds of the variety 'Leggett' having *Pc*94 and *Pc*68, which is susceptible to crown rust in the field was used for increasing the spores by planting in greenhouse.

After spraying, the plants were allowed to dry for 5-10 minutes before placing them in mist chambers. To allow high humidity for spore germination, the chambers were misted continuously for 45 minutes. Later, intermittent misting of 20 seconds was provided overnight every 4 minutes for a period of 18-20 hours. Thereafter, the mist chambers are turned off and the doors are left open for an hour to allow the plants to dry. The dried plants were placed on carts and moved to greenhouse set at a temperature of 22°C.

As the flecks develop on leaves in a week or 10 days, urediniospores were collected by tilting the pots and tapping them on to aluminum foil. To remove the dirt, the spores were

cleaned thoroughly and placed in a petri dish. To allow the spores to dry, the petri dish was placed in desiccator jar having 80% Ammonium Sulfate. Subsequently, the spores were scooped using a spatula and placed in gel cap capsules. The capsules are then placed into Eppendorf tubes and were labelled with the date of collection and crown rust race. To store the spores for further use, the Eppendorf tubes were placed in an -80°C freezer. The second collection of spores on Leggett plants was done a week after the first collection.

Crown rust phenotyping

The frozen urediniospores were subjected to heat shock by placing the Eppendorf tubes in a water bath at a temperature of 42°C for 5 minutes to facilitate the germination of spores. About 5 seedlings of $F_{5:6}$ lines and parents were sown in 2 containers each for evaluating the seedling resistance in two replications. Inoculation was done on 9 days old recombinant inbred line population along with checks. A week or 10 days after inoculation, the seedlings are scored as described (Table 1).

Table 1- Scoring for seedling resistance in greenhouse.

Disease score	Symptoms on leaf
4	No necrosis, little or no chlorosis, large uredinia
3	No necrosis, Extensive chlorosis, abundant moderately large pustules
2	Necrotic or chlorotic areas, Many small to medium sized pustules
1	Necrotic or chlorotic flecks, Few small uredinia
0	Necrotic or chlorotic flecks, No uredinia

Modified cobb's scale was used for scoring the $F_{5:6}$ plants for adult plant resistance in field at Fargo and St. Paul, Minnesota sown in 2 replications. The bottom leaves of the plants were scored for reaction class along with disease severity during milk/dough stage. 60% was used as the highest disease severity score. The reaction class is converted to reaction score of 0-0.6 and is multiplied with disease severity to determine the coefficient of Infection based on the equation

$$CI = DS \times RC/100$$

where CI = coefficient of infection, DS = disease severity, RC = reaction class respectively.

Phenotypic data analysis

The experimental planting was done in a randomized complete block design with two replications each, in fields of Fargo, St. Paul Minnesota, and in the NDSU greenhouse. The disease reaction scores were multiplied with the disease severity and were divided by 100 to determine the coefficient of infection in the field. Whereas, the infection type was used for the greenhouse data. The mean values of coefficient of infection and infection type were used for performing the phenotypic analysis.

The crown rust data collected from the field was analyzed using Generalized Linear Mixed Model (GLIMMIX) procedure in SAS software version 9.4. The Laplace method of maximum likelihood technique was used for estimating the parameters of the GLIMMIX model by specifying replication and genotype as categorical variables. During the analysis, the coefficient of infection which followed a beta distribution, with values ranging from zero to one, was considered as a response variable against the genotype as the predictor variable. Additionally, replication was considered as a random effect and finally, the least square means computed from the genotypes were used for the QTL analysis.

The infection type data collected from the plants grown in greenhouse was analyzed using General Linear Model (GLM) and Linear Mixed Model (LMM) procedures in SAS software version 9.4. by including replication and genotype as categorical predictors. For the GLM model, Infection type reading was specified as dependent variable against the predictors. Sum of squares method for Type III tests was used for replication and genotype which were

considered as fixed effects and the least square mean calculated for the genotype was used for analysis. For the LMM model, replication and genotype were considered as random effects specified with unstructured covariance matrix and no fixed effects were included.

Genotyping and construction of genetic linkage map

A dime sized leaf tissue was collected from the parents and F5 plants of RIL populations at 3-4 leaf stage. The collected leaf tissues from each line were placed in a 96 well plate and stored in a freezer for a day. The plates were then sent to USDA Small Grains Genotyping Lab in Fargo, ND where the samples were run using MSC-3K chip. The Polyploid Genotyping module of the Genome Studio v2.0.5 software was used to evaluate and improve the clusters manually for SNP calling.

From a total of 2,988 SNP markers, 734 and 638 SNP markers were identified as polymorphic SNP markers distinguishing the parents in two RIL populations respectively. Of the polymorphic markers identified in both the populations, markers showing significant segregation distortion (χ^2, $p<0.01$) were eliminated and the linkage analysis was conducted using JMP Genomics v.10.2 for the final 624 and 556 SNP markers respectively. All the heterozygous calls in the RIL populations were considered as missing values during the analysis.

Initially, the markers were assigned to the linkage groups based on estimated recombination fraction cutoff at 0.4 and p=0.05, using the automated hierarchical clustering linkage grouping method. Later, linkage map orders were determined using multidimensional scaling algorithm by utilizing the kosambi mapping function. Further on, to determine the best linkage groups, the linkage maps were tested at genetic distance break values of 35cM, 30cM, 25cM, 20cM respectively in addition to 0.25 and 0.3 recombination fraction break values. Finally, the best linkage groups were merged to construct a genetic linkage map for the RIL

populations. The final genetic maps were constructed using QTL ICiMapping software version 4.2 and consisted of 35 and 42 linkage groups for the population no. 16262 and 16205 respectively.

Quantitative Trait Locus Analysis

Firstly, a genotype probability dataset was constructed in JMP Genomics software v.10.2 using the phenotypic data and the marker data. To identify and improve the precision of the QTL underlying the crown rust resistance, composite interval mapping method was deployed using the EM QTL mapping algorithm model. The CIM analysis was done by specifying 2.0cM unit of distance between two QTL testing points and maintaining the LOD threshold value 2.5. Later, to determine the flanking markers underlying the QTL, Inclusive Composite Interval Mapping with additive effects ICIM-ADD was performed by permuting 1,000 times. Finally, to determine the LOD threshold values, the linkage map files were imported to QGene v 4.4.0, and composite interval mapping (CIM) was performed by selecting the flanking markers as cofactors by iterating 1,000 times. The percentage of phenotypic variance and the additive effect of the QTL were inferred from the coefficient of determination(R^2) value and additive effect values respectively.

Prediction of advanced lines from the identified QTL markers

The SNP markers identified in the QTL analysis of both the populations were tested against the advanced lines and four randomly selected RILs from the mapping populations. The phenotypic data was aligned against the QTL marker data to test the predictive ability of the markers.

RESULTS

Reaction of parents and checks to the crown rust in field and greenhouse

The parents used in developing the recombinant inbred line populations were allowed for natural infection in the field. The resistant parents ND141862, ND130020 were scored as resistant (R) and resistant to moderately resistant (RMR) to the crown rust infection as can be observed from the reaction on the leaf (Figures 1, 2). Similarly, the susceptible parents ND141087, ND130182 showed severe infection with pustules covering the entire surface of leaves and were scored as susceptible(S) to the crown rust disease(Figures 1,2) in field. In greenhouse, chlorotic areas with many small uredinia were observed on the seedlings of the resistant parents ND141862, ND130020 and were given the infection type 2. Comparatively many uredinia were observed on the seedling of the susceptible parents ND141087, ND130182 and were scored as infection type 4. Of the checks ND200209, ND170376, ND190334 having the resistance derived from *Avena strigosa*, *Avena sterilis*, BT1020 respectively showed resistant to moderately resistant reaction in the field. The checks Legget (*Pc*94, *Pc*68), ND Heart (*Pc*91), Maida (*Pc*68, *Pc*38, *Pc*39) showed a susceptible reaction along with the susceptible check Otana. In greenhouse the checks namely, Jury (*Pc*91), Leggett (*Pc*94), HiFi (*Pc*91), Assiniboia and Maida (*Pc*38, *Pc*39, *Pc*68*)* showed a susceptible reaction for the greenhouse inoculation. However, ND131603, ND190396, ND000305 having the resistance derived from CRRSRR3, M1212 and HiFi respectively, showed resistance to crown rust inoculation in greenhouse.

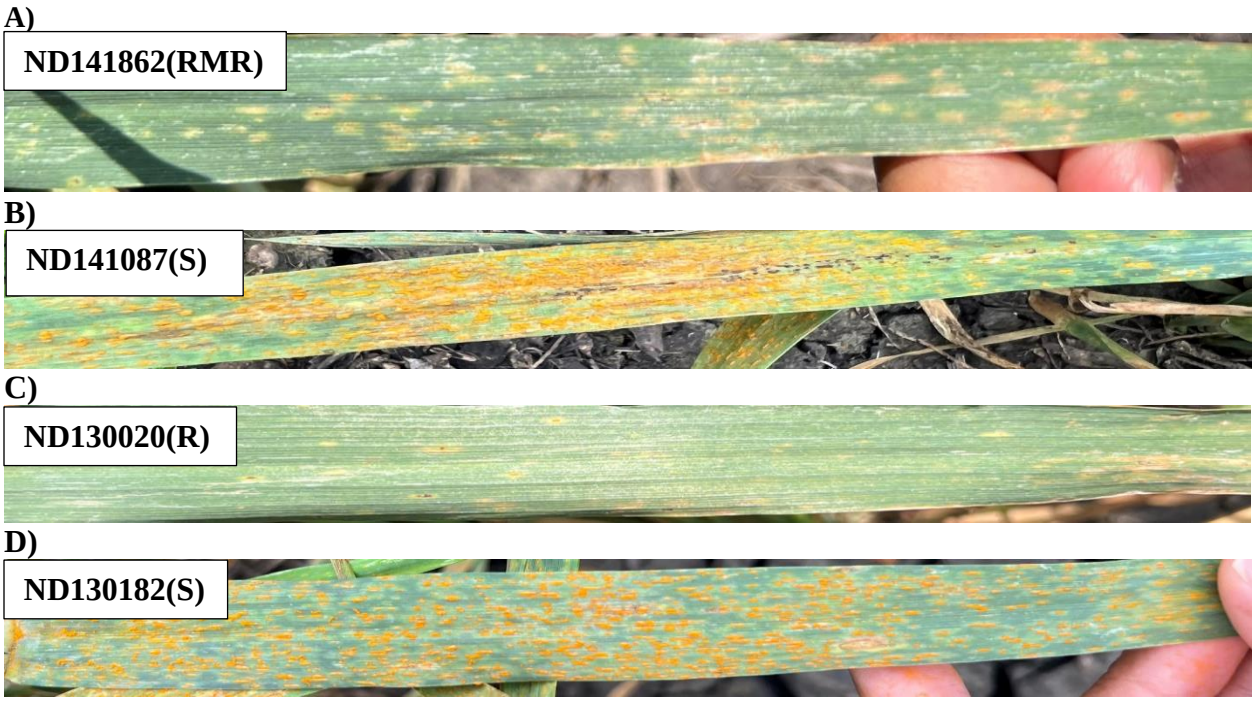


Figure 4. Reaction of the resistant parents A) ND141862, B) ND130020 and susceptible parents C) ND141087, D) ND130182 in field. RMR= Resistant to moderately resistant, R= Resistant, S= Susceptible

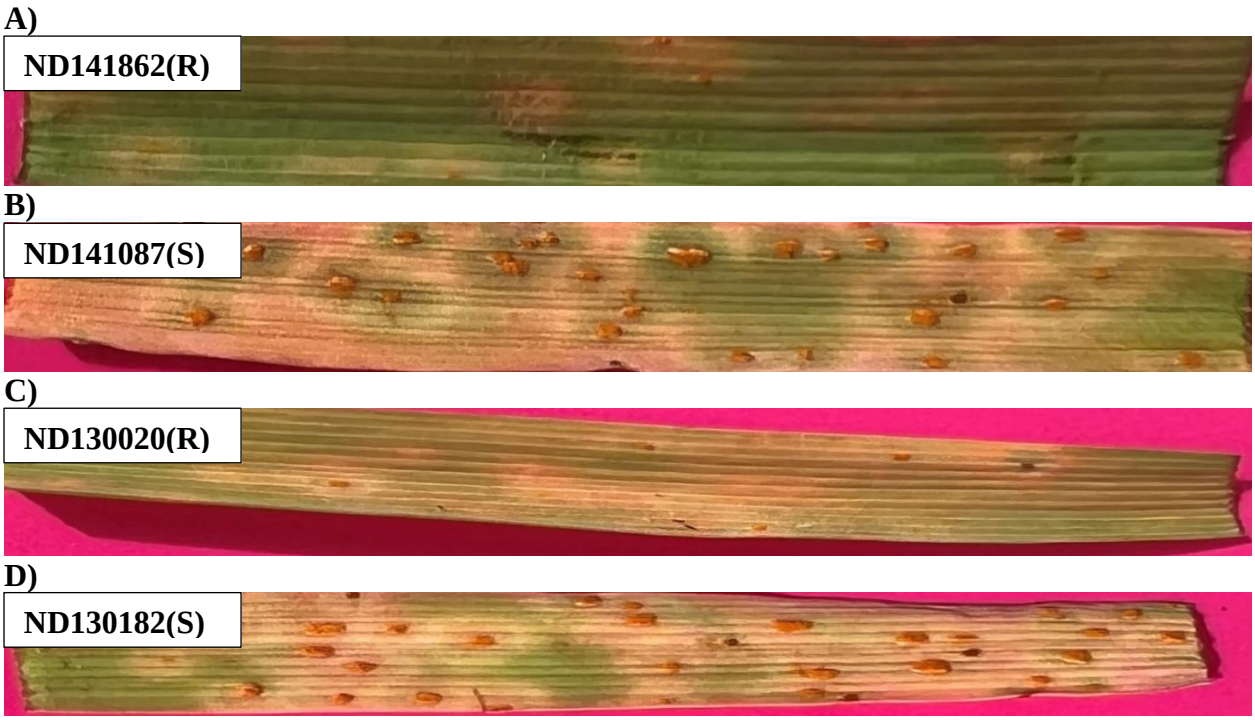


Figure 5. Reaction of the resistant parents A) ND141862, B) ND130020 and susceptible parents C) ND141087, D) ND130182 in greenhouse where R= Resistant, S= Susceptible.

Table 2. Reaction of parents and checks to crown rust disease in field.

Population	Genotype	Reaction class	CI
ID 16262	ND141862(P1)	MR	0.05
	ND141087(P2)	S	0.50
ID 16205	ND130020(P1)	R	0.00
	ND130182(P2)	S	0.50
Checks (BT)	Portage	MSS	0.34
	ND Heart	MSS	0.43
	Leggett	S	0.45
	Maida	S	0.55
	ND200209	MR	0.05
	Otana	S	0.6
Checks (FF)	Portage	S	0.50
	Deon	MS	0.27
	ND Spilde	MS	0.33
	Leggett	S	0.50
	ND170376	RMR	0.01
	ND190334	MR	0.07
	MN841801	S	0.5
	Otana	S	0.5

Note: CI= Coefficient of Infection, P1= Parent 1, P2=Parent 2, S= Susceptible, MSS= Moderately Susceptible to Susceptible, MS= Moderately Susceptible, MR= Moderately Resistant, RMR= Resistant to moderately Resistant, R= Resistant.

Table 3. Reaction of parents and checks to crown rust inoculation in greenhouse

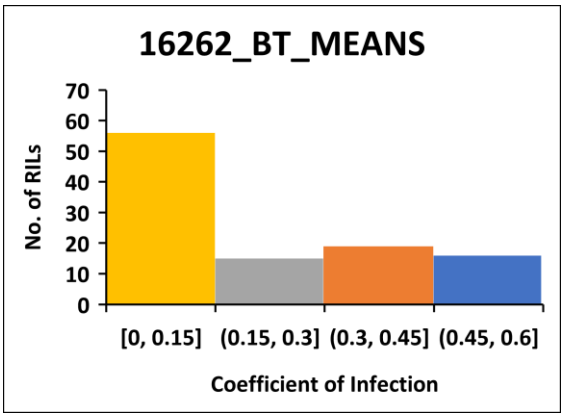
Population	Genotype	Infection type	Reaction
ID 16262	ND141862(P1)	2	R
	ND141087(P2)	4	S
ID 16205	ND130020(P1)	2	R
	ND130182(P2)	4	S
Checks	Assiniboia	3	S
	Jury	3	S
	Leggett	3	S
	HiFi	3	S
	Morton	2	R
	Maida	2	R
	ND131603	1	R
	ND190396	1	R
	ND000305	2	R
	Otana	4	S

Note: R= Resistant, S= Susceptible. Infection types of 1,2 were considered resistant and 3, 4 were considered as susceptible.

Variations in the reaction of RILs to crown rust disease

All the $F_{5:6}$ recombinant inbred lines from both the populations were evaluated for reaction to crown rust infection in Matt Moore Buckthorn Nursery at St. Paul, Minnesota and in the field and greenhouse at Fargo, North Dakota. The means of coefficient of infection (CI) from both the replications were plotted on a scale of 0 to 0.6 for the data collected from the field. The no. of recombinant inbred lines with CI value from 0-0.3 were considered as resistant and 0.3-0.6 were considered as susceptible. Similarly, the infection types 1 and 2 were considered resistant in greenhouse. In addition, the RILs that showed the infection types 3 and 4 in greenhouse were considered as susceptible. Further, nearly equal ratios (1:1) of resistant and susceptible recombinant inbred lines were identified in both populations that designated the construction of recombinant inbred line population for linkage mapping study.

A)



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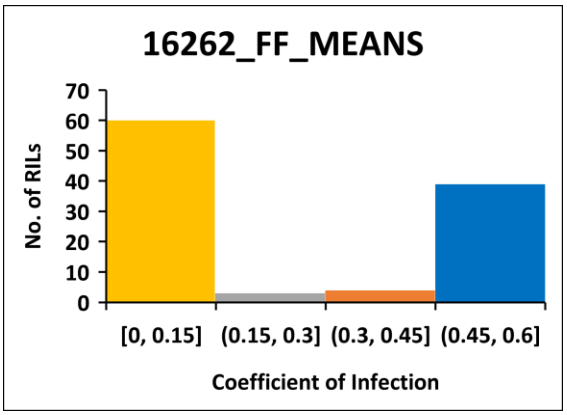

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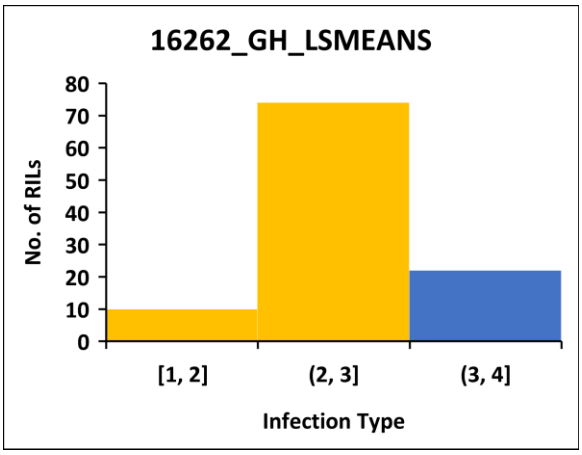
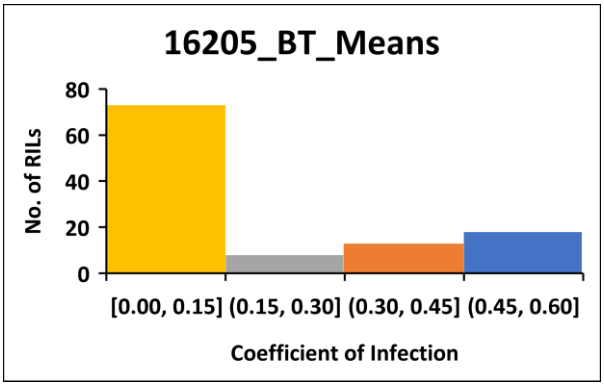
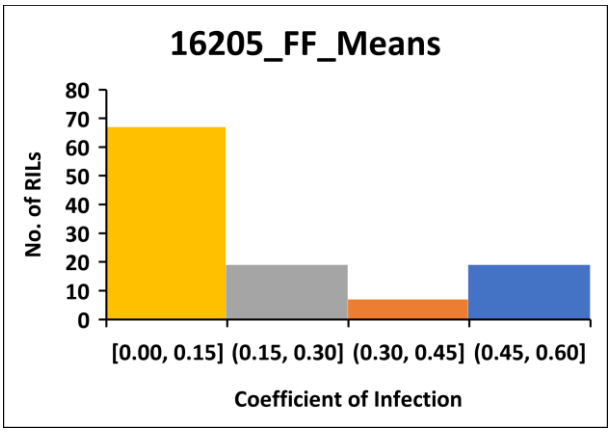


Figure 6. Frequency distribution of coefficient of Infection (CI) of recombinant inbred lines of the mapping population 16262. A) CI means of the adult plants at Buckthorn nursery (BT), St. Paul, Minnesota. B) CI means of the adult plants at field in Fargo (FF), North Dakota. C) Infection type of seedlings to crown rust inoculation in greenhouse (GH). In the histograms, gold and gray colors depict the resistant lines whereas orange and blue indicate susceptible lines.

A)



B)



C)

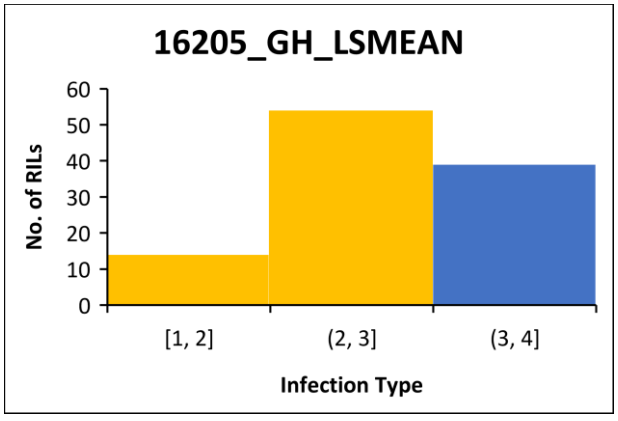


Figure 7. Frequency distribution of coefficient of Infection (CI) of recombinant inbred lines of the mapping population 16205. A) CI means of the adult plants at Buckthorn nursery (BT), St. Paul, Minnesota. B) CI means of the adult plants at field in Fargo (FF), North Dakota. C) Infection type of seedlings to crown rust inoculation in greenhouse (GH). In the histograms, gold and gray colors depict the resistant lines whereas orange and blue indicate susceptible lines.

Genetic linkage maps

A total of 2,988 SNP markers were generated for the two recombinant inbred line populations and the linkage analysis revealed 624 and 556 polymorphic SNP markers in population 16262 and population 16205 respectively. Consequently, the linkage groups with <3 markers were eliminated in the construction of the linkage map. The linkage map of population 16262 consisted of 617 markers aligned to 35 linkage groups (Figure 3A) covering a total genetic distance of 2,644.37cM representing a combination of A, C and D genomes (Table 1). A good marker density of 1.07 was observed on chromosome 3A of population 16262 consisting of 45 markers spanning 48.4cM (Table 1). The genetic linkage map of population 16205 spanned 1257.71cM consisting of 540 polymorphic SNP markers (Table 2) arranged into 42 linkage groups (Figure 3B). Highest number of markers were mapped to the D genome of the population 16205 with a good marker density on chromosome 1D having 53 markers covering 53.31cM distance.

Table 4. Summary of the genetic linkage map developed for the recombinant inbred line mapping population 16262.

Chromosome	Number of markers mapped	Genetic distance (cM)	Marker density (cM/marker)
1A	16	382.78	23.92375
2A	24	33.25	1.385416667
3A	45	48.4	1.075555556
4A	36	87.97	2.443611111
5A	6	104.06	17.34333333
6A	55	202.89	3.688909091
7A	20	77.72	3.886
1C	1	0	0
2C	42	82.42	1.962380952
3C	23	49.66	2.159130435
4C	37	99.2	2.681081081
5C	19	323.3	17.01578947
6C	48	251.11	5.231458333
7C	33	135.17	4.096060606
1D	50	138.25	2.765
2D	26	123.26	4.740769231
3D	18	47.11	2.617222222
4D	43	105.17	2.445813953
5D	33	76.38	2.314545455
6D	24	232.29	9.67875
7D	13	43.98	3.383076923
UN	5		
A genome	202	937.07	4.638960396
C genome	203	940.86	4.634778325
D genome	207	766.44	3.702608696
Total	**617**	**2644.37**	**4.285850891**

Note: cM= Centimorgan, UN = Unknown

Table 5. Summary of the genetic linkage map developed for the recombinant inbred line mapping population 16205.

Chromosome	Number of markers mapped	Genetic distance (cM)	Marker density (cM/marker)
1A	3	10.16	3.386666667
2A	9	38.72	4.302222222
3A	22	65.34	2.97
4A	52	53.31	1.025192308
5A	18	35.08	1.948888889
6A	29	58.33	2.01137931
7A	12	70.2	5.85
1C	5	11.8	2.36
2C	11	78.52	7.138181818
3C	29	81.64	2.815172414
4C	48	72.48	1.51
5C	19	92.95	4.892105263
6C	29	57.28	1.975172414
7C	38	86.37	2.272894737
1D	53	53.31	1.005849057
2D	27	64.17	2.376666667
3D	23	76.68	3.333913043
4D	40	71.97	1.79925
5D	27	97.57	3.613703704
6D	28	52.5	1.875
7D	12	29.33	2.444166667
UN	6		
A genome	145	331.14	2.283724138
C genome	179	481.04	2.687374302
D genome	210	445.53	2.121571429
Total	**540**	**1257.71**	**2.329092593**

Note: cM= Centimorgan, UN = Unknown

A) **B)**

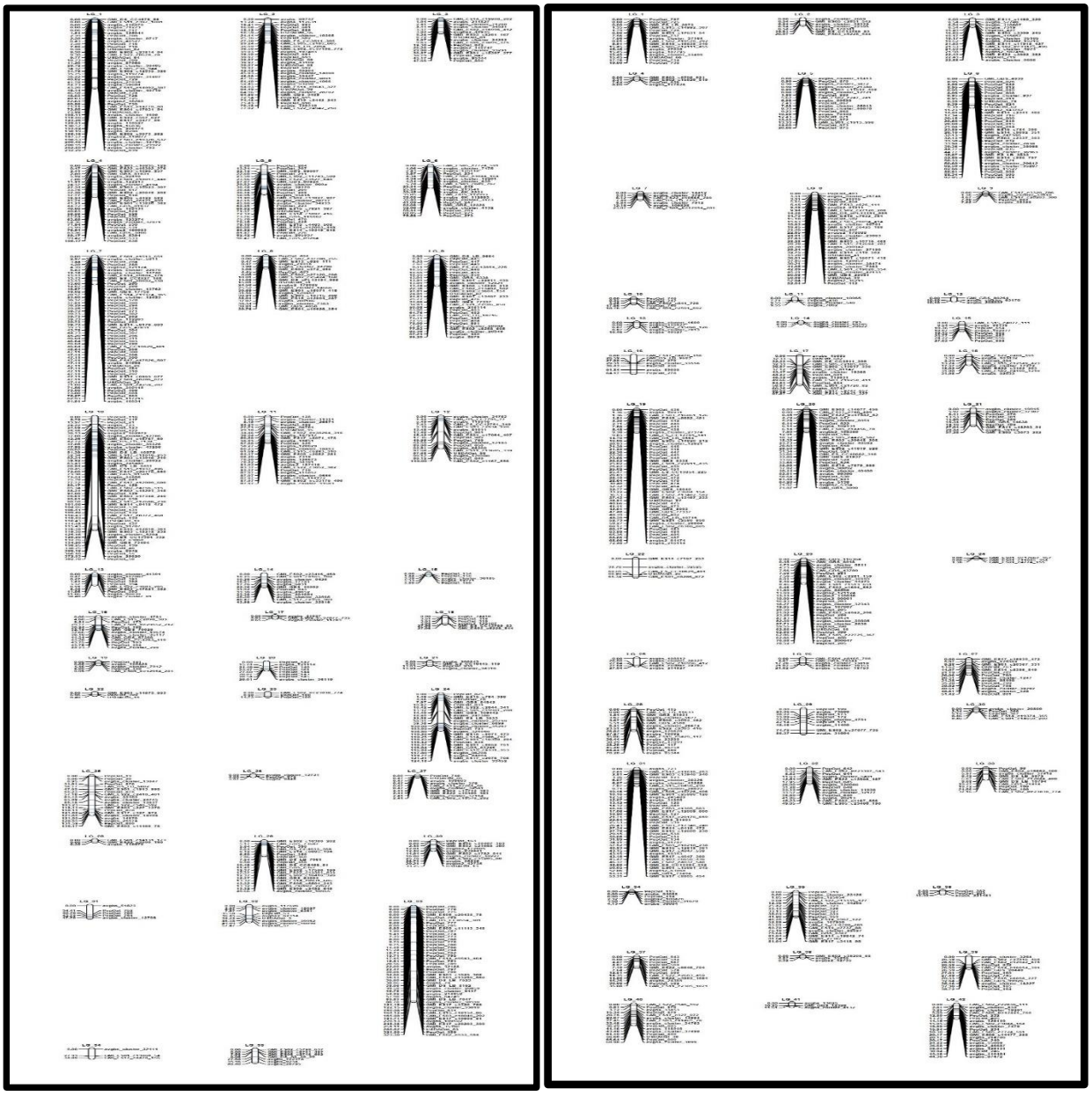


Figure 8. Linkage maps developed for the recombinant inbred line mapping populations. A)Linkage map showing 35 linkage groups of Population 16262. B)Linkage map depicting 42 linkage groups of Population 16205.

QTL analysis

A total of two QTL associated with the crown rust resistance were identified from the two mapping populations in all the three tested locations (Figure 4). One QTL was identified on chromosome 4A for the population 16262, delimited to a region of genetic distance 1.44cM and is flanked by the markers avgbs2_187416 and GMI_ES22_c3052_382(Table 3). Likewise, in population 16205, a QTL was mapped to chromosome 4D spanning a genetic distance of 2.32cM flanked by the markers GMI_ES05_c3309_245 and avgbs_115857(Table 3). The QTL identified in the mapping population 16262 explained 40-68% of phenotypic variance and reduction in the disease value ranging from 0.14-0.33 as can be inferred from the R^2 value and additive effect respectively (Table 4). Similarly, 47-77% of phenotypic variance and disease reduction range of 0.11-0.51 was interpreted from the R^2 value and the additive effect explained by the QTL identified in population 16205(Table 4). The negative values of the additive effect indicate the inheritance of resistance from the parent 1in the pedigree which in this case is the resistant parent used in the current study.

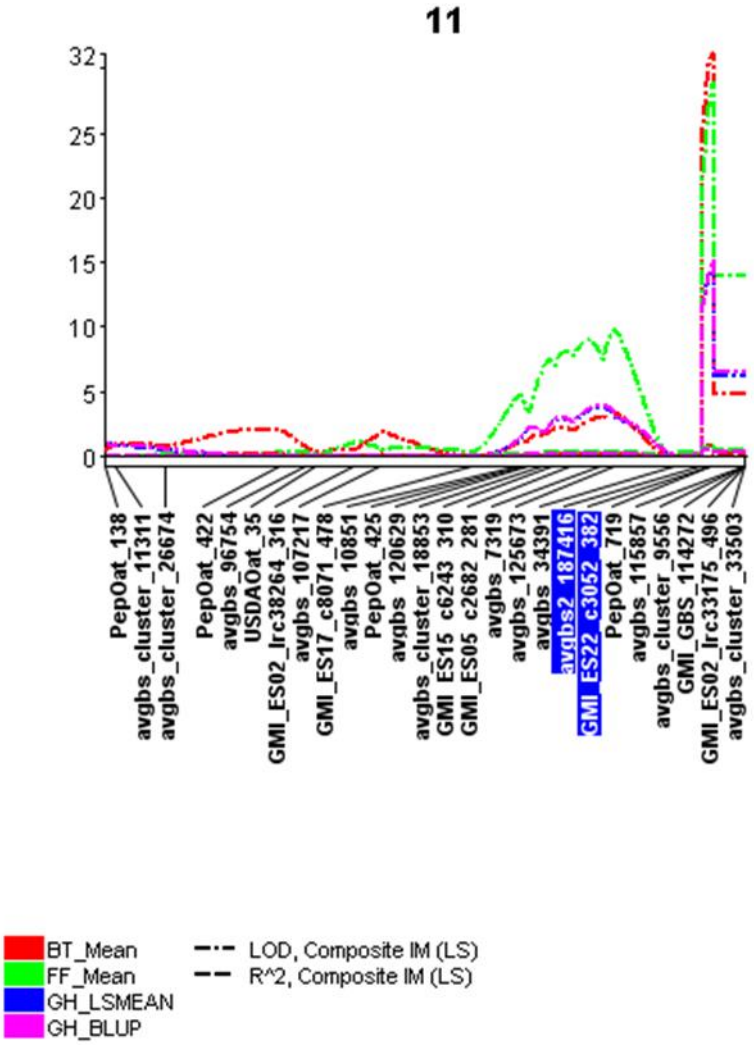


Figure 9. Composite Interval Mapping(CIM) of the QTL identified on linkage group 11 in population 16262 by implementing the least squares method. The colors indicated in the plot represent the locations mentioned in the bottom of the figure. The number on the top of the figure represents the linkage group. The markers flanking the QTL were highlighted in blue color on the linkage map on x-axis. The LOD values were represented on the y-axis. Buck thorn nursery, Fargo field and Greenhouse were abbreviated as BT, FF, and GH respectively.

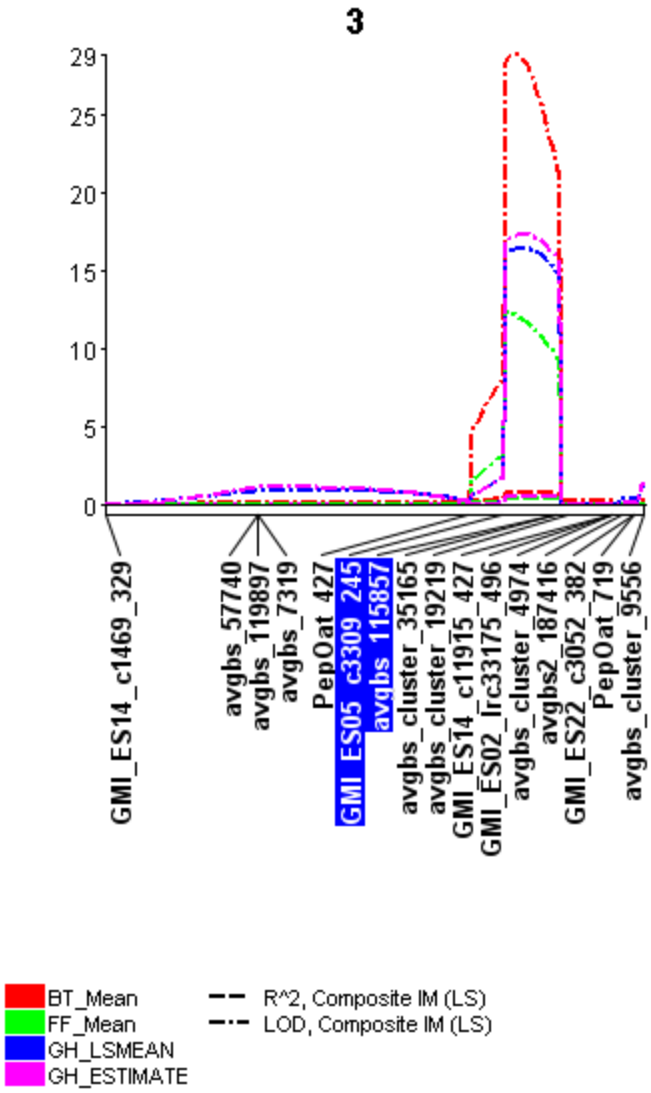


Figure 10. Composite Interval Mapping(CIM) of the QTL identified on linkage group 3 in population 16205 by implementing the least squares method. The colors indicated in the plot represent the locations mentioned in the bottom of the figure. The number on the top of the figure represents the linkage group. The markers flanking the QTL were highlighted in blue color on the linkage map on x-axis. The LOD values were represented on the y-axis. Buck thorn nursery, Fargo field and Greenhouse were abbreviated as BT, FF, and GH respectively.

Table 6. Description of QTL identified in the two mapping populations.

Parameter	Population 1	Population 2
Population ID	16262	16205
Pedigree	ND141862×ND141087	ND130020×ND130182
Resistant Parent(A)	ND141862	ND130020
Susceptible Parent(B)	ND141087	ND130182
No. of RILs	108	112
Generation of mapping population	$F_{5:6}$	$F_{5:6}$
QTL	QPc.NDSU.2023.1	QPc.NDSU.2023.2
Chromosome	4A, UN	4D
Flanking markers	avgbs2_187416 GMI_ES22_c3052_382	GMI_ES05_c3309_245 avgbs_115857
Physical position of flanking markers(bp)	445104421 463191000	405128311 450444259
Confidence Interval(cM)	81.93-83.37	16.85-19.17

Note: A= Genotype of the resistant parent, B= Genotype of the susceptible parent, RILs= Recombinant Inbred Lines, bp=Base pair, cM=Centimorgan

Table 7. LOD, R^2 and additive effects of the QTL identified in the two mapping populations.

Population	Location	LOD threshold	LOD peak	R^2	Additive effect
16262	BT	4.628	26.217	68.7	-0.148
	FF	5.103	22.255	63.0	-0.191
	GH_LSMEAN	5.046	11.926	40.4	-0.333
	GH_Estimate	5.118	12.027	40.7	-0.203
16205	BT	5.263	27.182	77.9	-0.177
	FF	5.09	11.222	41.3	-0.117
	GH_LSMEAN	4.7	15.216	48.0	-0.518
	GH_Estimate	4.46	15.8	47.8	-0.325

Note: LOD = Logarithm of Odds, R^2=Phenotypic variance, BT= Buckthorn, FF= Fargo field, GH= Greenhouse, LSMEAN= Least square mean, Estimate = Best Linear Unbiased Prediction (BLUP)

Testing the ability of the QTL markers in predicting the advanced lines

The markers identified in the QTL mapping study were tested with the disease reaction scores of the advanced lines, parents and four randomly selected RILs in each of the mapping populations. The genotypic data of the QTL markers were obviously related to the phenotypic disease reaction scores of parents, RILs and majority of the advanced lines. Of the 9 advanced lines tested in the population 16262 with the flanking markers avgbs2_187416, and GMI_ESS22_c3502_382, the advanced lines ND200655, ND200651 were predicted as susceptible lines and ND200701, ND200529, ND210626, ND210621, ND210623, ND210633 were predicted as disease resistant lines (Table 5). In the same way, the markers GMI_ES05_c3309_245 and avgbs_115857 were used to test the advanced lines of population 16205. Consequently, ND210481, ND210182, ND210356, and ND210358 were identified as the advanced lines which were resistant to the crown rust infection. However, the genotypic information of the first advanced line ND200334 of population 16205 depicted it as a susceptible line as against the highly resistant phenotypic reaction observed in different locations. However,

ND200334 was suspected to be derived from another source of resistance. Moreover, the GMI_ESS22_c3502_382 marker of population 16262 predicted the advanced line ND200703 as a resistant line on contrary to the susceptible phenotypic reaction score observed.

Table 8. Predictive ability of the QTL markers in testing advanced lines of population 16262.

Individual	BT scores	FF scores	GH scores	avgbs2_ 187416	GMI_ES22_ c3052_382
ND141862	15 MR	10 RMR	2	A	A
ND141087	50 S	50 S	4	B	B
ND200701	20 MRMS	20 MR	2	A	A
ND200529	0R	5 RMR	1	A	A
ND200703	40 MSS/S	50 S	4	B	A
ND200655	40 MSS/S	50 S	4	B	B
ND200651	40S	60 S	4	B	B
ND210626	10 MR	2 RMR	2	A	A
ND210621	0R/10 RMR	5 RMR	2	A	A
ND210623	0R/5RMR	5 RMR	1	A	A
ND210633	10 RMR/ MR	5 RMR	2	A	A
RIL Entry 15	45 S	40 S	3	B	B
RIL Entry 25	20 MRMS	_	1	A	A
RIL Entry 120	40 MSS/S	60 S	3	B	B
RIL Entry 144	5 RMR/10 MR	20 MR	2	A	A

Note: BT=Buckthorn nursery, FF=Fargo field, GH=Greenhouse, S= Susceptible, MSS= Moderately Susceptible to Susceptible, MS= Moderately Susceptible, MRMS = Moderately Resistant to Moderately Susceptible, MR= Moderately Resistant, RMR= Resistant to moderately Resistant, R= Resistant, / = segregating reaction. The genotypes of the resistant and susceptible parents are represented in green and red colored alphabets respectively. The alphabets in the blue color represent the deviation of genotype prediction from the observed phenotypic scores.

Table 9. Predictive ability of the QTL markers in testing advanced lines of population 16205.

Individual	BT scores	FF scores	GH scores	GMI_ES05_ c3309_245	avgbs_ 115857
ND130020	0 R	5 RMR	2	A	A
ND130182	50 S	30 MS	4	B	B
ND200334	0 R	0 R	2	B	B
ND210481	5 RMR	10 MR	2	A	A
ND210182	2 RMR	5 RMR	1	A	A
ND210356	-	20 MR	2	A	A
ND210358	30 MS	10 RMR	1	A	A
RIL Entry 23	10 MR	5 RMR	2	A	A
RIL Entry 48	-	2 RMR	2	A	A
RIL Entry 110	-	10 RMR	2	A	A
RIL Entry 149	40 S	60 S	4	B	B

Note: BT=Buckthorn nursery, FF=Fargo field, GH=Greenhouse, S= Susceptible, MSS= Moderately Susceptible to Susceptible, MS= Moderately Susceptible, MRMS = Moderately Resistant to Moderately Susceptible, MR= Moderately Resistant, RMR= Resistant to moderately Resistant, R= Resistant, / = segregating reaction. Hyphens (-) indicate missing data. The genotypes of the resistant and susceptible parents are represented in green and red colored alphabets respectively. The alphabets in the blue color represent the deviation of genotype prediction from the observed phenotypic scores.

DISCUSSION

To combat the evolving new races of the crown rust pathogen *Puccinia coronata* f. sp. *avenae,* breeding for crown rust resistant varieties is necessary. Therefore, to facilitate the development of durable crown rust resistant cultivars by marker assisted gene pyramiding, my research was focused on developing recombinant inbred line mapping populations carrying novel sources of crown rust resistance and identifying the markers associated with the genomic regions underlying the disease resistance.

Effective scoring of plants for a disease in addition to efficient genotyping determines the success of genetic mapping studies. The crown rust readings of the recombinant inbred lines in both the populations were collected twice in each of the two replications in three different environments. The phenotypic data collected in all the locations was highly consistent with replications suggesting the acceptability of phenotypic data. Moreover, the frequency distribution plots of coefficient of infection in both the populations showed almost an equal segregation of resistant and susceptible lines as predetermined in recombinant inbred line mapping populations.

Meanwhile, efficient genotyping and linkage analysis revealed 35 and 42 linkage groups in each population respectively, referring to relatively large genome size of oats. Yet, a greater number of markers are required to fill the intervals and precisely map the genetic regions on the linkage map. However, the linkage maps constructed for the populations in this study covered the entire oat genome with each marker lying at 2-4cM distance.

Moreover, by utilizing the composite interval mapping approach for genetic mapping, a single major QTL was precisely identified in each of the recombinant inbred line populations, derived from the pedigree of a biparental crosses involving ND141862 × ND141087 and

ND130020 × ND130182 respectively. Therefore, it can be inferred that the disease resistance is being controlled by a single or a few major genes.

Furthermore, the resistance observed in both the populations was derived from the resistant donor parents ND141862 and ND130020 respectively, as can be inferred from the negative additive effect identified in this study. Besides, the QTL observed in both the populations showed >40% of phenotypic variance, indicating it as a 'major' QTL. Also, the QTL were identified in all the three environments, referring to them as 'stable' QTL as evident from no genotype by environment (G×E) interaction. In addition, the peaks of the QTL identified in both the populations exceeded the significance threshold, declaring the QTL as 'real or statistically significant'.

In conclusion, despite both the recombinant inbred line population carried similar source of resistance, the QTL namely QPc.NDSU.2023.1 and QPc.NDSU.2023.2 were mapped to different chromosomes. QPc.NDSU.2023.1 was mapped to chromosome 4A on which the crown rust seedling resistance (ASR) genes *Pc48* and *Pc98* were previously mapped (Park *et al.*,2022). On the other hand, QTL QPc.NDSU.2023.2 of the population 16205 was mapped to chromosome 4D onto which three adult plant resistance (APR) genes were mapped by Nazareno et al., (2022). However, QPc.NDSU.2023.2 was carrying novel seedling resistance gene (ASR) on chromosome 4D. All in all, four SNP markers namely avgbs2_187416, GMI_ES22_c3052_382, GMI_ES05_c3309_245, avgbs_115857 flanking the two novel QTL associated with crown rust resistance were identified in this study. Overall, the SNP markers identified in this study could be useful for map-based cloning and after conversion to PCR based markers could be of potential use in oat breeding programs for disease resistance studies.

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