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Exploiting the Genetic Diversity of Wild Ancestors and Relatives of Wheat for its Improvement

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EXPLOITING THE GENETIC DIVERSITY OF WILD ANCESTORS AND RELATIVES OF WHEAT FOR ITS IMPROVEMENT

BY

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This thesis is approved as a creditable and independent investigation by a candidate for the Master of Science in Plant Science degree and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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This thesis is dedicated to my respected father Mr. Amrik Singh Sidhu, mother Mrs. Harjit Kaur, my dear sister Sukhdeep Kaur and cute niece Samreet.

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ABSTRACT

EXPLOITING THE GENETIC DIVERSITY OF WILD ANCESTORS AND RELATIVES OF WHEAT FOR ITS IMPROVEMENT

JAGDEEP SINGH SIDHU

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Wheat is the third most staple food worldwide but current 1% annual improvement in the wheat production is insufficient to meet the growing demands in future. The narrow genetic base of wheat limits continuous improvement in wheat productivity and tolerance to biotic and abiotic stresses under changing climate. Wild ancestors and relatives of wheat hold a potential in widening the genetic pool of wheat and enhance its resilience to biotic and abiotic stresses. This study was focused towards characterizing the genetic diversity in wild relatives of wheat for disease resistance and efficient association with diazotrophs. In the first study, we evaluated a mini core set of *Triticum turgidum* subsp. (tetraploid wheat, AABB) for resistance to *Fusarium* head blight (FHB), leaf rust and tan spot. Three, six, and nine accessions showed resistance response to *Fusarium* head blight (FHB), leaf rust and tan spot respectively. These germplasm resources could be further exploited in wheat breeding. In the second study, in addition to tetraploid wheat, diploid and hexaploid germplasm of both wild and adapted species were evaluated for efficient association with diazotrophic bacteria by analyzing the N content. We observed significant differences for ^{15}N content among different species, represented as average $\sigma^{15}N$. Lower $\sigma^{15}N$ indicates a higher possibility of biologically fixed nitrogen (BNF). Wild accessions both in diploid (*T. boeticum,* A^mA^m, $\sigma^{15}N = 20.85$) and tetraploid species

(*T. turgidum* subsp. *dicoccoides*, AABB, $\sigma^{15}N$ = 16.44) showed significantly better associations with diazotrophs as compared to domesticated species (*T. monococcum*, A^mA^m, $\sigma^{15}N$ = 26.67) and modern hexaploid varieties (*T. aestivum*, AABBDD, $\sigma^{15}N$ =31.74). Our study shows that the wild species hold a promise in identification and characterization of efficient association with diazotrophic bacteria and this interaction can be recovered in modern cultivars of wheat to enhance the performance of wheat in marginal soils. In the final study, we analyzed the genetic diversity in the global collection (178 accessions) of rye using 4,037 high-quality SNPs and developed of a mini core set of 32 accessions of rye that represents more than 95 % of the allelic diversity (PIC = 0.25) of our collection (PIC = 0.26). Genome-wide association study (GWAS) was performed on 160 accessions (*Secale cereale* subsp. *cereale*) with 4,037 high-quality SNPs to identify genomic regions conferring tan spot resistance. Nearly 32%, 27%, 24%, and 17% accessions showed resistant, moderately resistant, moderately susceptible and susceptible reaction to *Pyrenophora tritici-repentis* race 5 (*PTR* race 5) respectively. Two QTLs conferring resistance to tan spot (*PTR* race 5) were identified ($p = <0.001$) using mixed linear model (GAPIT) on chromosomes 5R and 2R. The QTLs *QTs-sdsu-5R* and *QTs-sdsu-2R* explained 13.11% and 11.62 % of the variation. In conclusion, wild relatives and ancestors of wheat hold a potential for wheat improvement especially for tolerance to abiotic and biotic factors.

INTRODUCTION

Wheat (*Triticum aestivum* L.), the third most staple food worldwide; provides one-fifth of the calories and 20% of the protein to more than 4.5 billion people [1]. But annual wheat yield improvement of an average 1% will be insufficient to feed the rising population [2]. Climate change, soil degradation, loss of arable land, unavailability of irrigation waters, increasing fertilizer costs and above all, evolving pathogens further aggravate this scenario [3]. A steady increase in productivity of at least 2% per year is required to meet the growing wheat demands [4]. Furthermore, wheat improvement must be resource efficient and sustainable.

Part of the reason for stagnant wheat yield is the lack of genetic diversity in the gene pool of wheat germplasm used worldwide. This is partially because of how wheat originated; wheat evolved from a miraculous and spontaneous cross between cultivated emmer (*Triticum dicoccon* - AABB) and goatgrass (*Aegilops tauschii* - DD) approx. 8000 years ago [5,6]. This event happened only once or twice and the reproductive isolation of wheat from its wild parents lead to a narrow genetic base of wheat [5]. In order to identify novel high yielding varieties with improved abiotic and biotic stress tolerance we have to broaden the genetic base of wheat.

One of the feasible and best approach is to incorporate genetic diversity from wild ancestors and other relatives of wheat such as tetraploid wheat (*T. turgidum* subsp.) and rye (*Secale cereale L.*). As opposed to domesticated varieties – being pampered by breeders and farmers – wild species are challenged by a spectra of abiotic and biotic stresses, leading to the survival of the fittest with the best tolerance ability [7]. Proving their potential, wild relatives of wheat has contributed so many important genes into

wheat germplasm such as drought tolerance [8], salt tolerance [9], O_3 tolerance [10], powdery mildew resistance, stem rust resistance, leaf rust resistance, yield and grain quality traits [7]. These wild species can also be a great source for novel traits such as better interaction with beneficial microbes e.g. diazotrophs which can relieve our dependency from fertilizers and lead to a better environment friendly ecosystem.
To exploit this useful genetic diversity of wild relatives we have to identify core sets covering vast genetic and geographic diversity of corresponding species; and then characterize those sets for different useful traits, and finally mobilizing the useful genes through wide hybridization or alien gene introgression into adapted cultivars.

This study was focused on characterization of diverse mini-core set of tetraploid wheat lines (includes wild and cultivated) for disease resistance; characterization of diverse germplasm of wheat (diploid, tetraploid and hexaploid) for an efficient interaction with diazotrophic bacteria; and assessment of genetic and geographic diversity in a global set of rye (includes cultivated and wild), and mapping of novel resistance loci conferring resistance to tan spot.

OBJECTIVES:

1. Characterization of wild tetraploid wheat mini-core set for resistance to *Fusarium* head blight (FHB), leaf rust and tan spot.
2. Evaluation and identification of ancestors and wild relatives of wheat for their interaction with diazotrophs.
3. Assessment of genetic and geographic diversity in a global set of rye and characterization of genomic regions conferring resistance to tan spot.

Chapter 1

Literature review

1.1 Wheat – a general introduction

1.1.1 Importance of wheat

Wheat has been cultivated for 8000 years and from then to now it has been a staple food for so many great civilizations of Europe, West Asia and North America [11]. It provides $1/5^{th}$ of the calories and 20% of the protein to more than half of the world [1]. Wheat is grown on more than approx. 220 million ha, spanning on more agricultural land than any other crop. Signifying its importance, wheat's world trade is larger than all other crops combined together. Wheat-based foods are rich in carbohydrates, vitamins, and minerals; added with meat or legumes it makes good satisfying and balanced diet. Wheat is also favored by farmers because of easy agronomic practices, storage of grains and grinding for flour making [11].

1.1.2 Rising Wheat demands vs stagnant yields

World population is rising day by day and according to current pace, there will be 9 billion people by 2050 [12]. There are already 1 billion hungry people in the current world who don't get proper nutrition and by 2050 this number is predicted to rise [13]. Even to maintain present food demands, we have to increase food production at least by 70%, with a special focus on increasing yield of staple crops such as wheat, rice and maize [12]. In specific, wheat has to be produced 20 times more by 2050 as compared to current yearly average production of 735 million metric tons [14]. This scenario of wheat production demand is further aggravated by loss of arable land, soil degradation, loss of irrigation water, stagnate yields, and an expected 20 to 30% loss in wheat production due

to increasing temperatures [3]. In nutshell, for food security, total wheat production over the next 50 years needs to exceed the total production in the last 10,000 years since agriculture began.

1.1.3 Lack of genetic diversity in wheat germplasm

For genetic improvement in any crop, genetic diversity is the key [7]. Genetic diversity is the base on which breeders can make selections for the superior traits of interest. Genetic diversity is progressively lost during the crop domestication and further in the breeding programs which are focused towards few traits, hindering long-term crop improvement [7]. For wheat, along with domestication, the way it originated can also be blamed for its narrow genetic base. As wheat evolved from a miraculous and spontaneous cross between cultivated emmer (*Triticum dicoccon*, AABB) and goatgrass (*Aegilops tauschii*, DD) making wheat a hexaploid with a genome composition of AABBDD [5]. This event happened only a few times in history, coupled with reproductive isolation it led to the narrow genetic base of wheat. It is apparent that in order to meet global food security, we have to develop high yielding cultivars with better stress tolerant capacity. Do that, we have to increase the aura of genetic diversity in wheat germplasm.

1.1.4 Origin of wheat

Modern day wheat is a hexaploid (AABBDD) with three sets of homeologous chromosomes designated A, B and D. Its origin is unique, as it involved a whole-genome hybridization of the ancestral species. Approx. 500,000 years ago a spontaneous cross between *T. urartu* (2n=2x=14, A^uA^u) and an unknown species carrying B genome (2n=2x=14, BB) gave birth to wild emmer, *T. turgidum* subsp. *dicoccoides* (2n=4x=28,

AABB) [15]. Wild emmer was further domesticated into *T. turgidum* subsp. *dicoccon* (2n=4x=28, AABB) [16,17] and is aptly called as cultivated emmer.

During the time period of cultivation of tetraploid species of wheat such as cultivated emmer, crops were grown in close proximity to wild relatives. Less than 8,000 years ago, tetraploid (*T. turgidum* subsp. *dicoccon*) wheat spontaneously got hybridized with D genome species – *Aegilops tauschii* (2n=2x=14, DD) and gave rise to the modern bread wheat, *T. aestivum* (2n=6x=42, AABBDD) [5,6]. The addition of DD genome provided wheat with an enhanced geographic and environmental adaptability; enhanced yield and quality; made wheat the one of the most staple food.

1.2 Wild relatives of wheat

1.2.1 Gene pools of wheat

Gene pool (GP) word come from a Russian word *genofond* (given by Aleksandr Sergeevich Serebrovskii) which refers to complete set of genes or genetic information found in a population [18]. Gene pool of a particular species also includes its wild relatives as genetic information can be shared between them, though these events may be rare due to reproductive isolations. In 1971 Jack Harlan and Jan de Wet divided the gene pool into sub-gene pools based on crop wild relative's relatedness with cultivated species and easiness of sharing genetic information with cultivated species [19]. Primary gene pool (GP1 or 1°) includes most genetically close relatives (readily crossed), secondary gene pool (GP2 or 2°) includes less close relatives (difficult to interbreed) and tertiary gene pool (GP3 or 3°) includes distant relatives (natural crossing not possible). In case of wheat, Jian *et al.* adapted the gene pool concept of Harlan and de Wet but they coupled that concept with chromosome homology [20]. Though the base is easiness for

hybridization but to a larger extent, wheat gene pools are based on similarity of the chromosome sets [21].

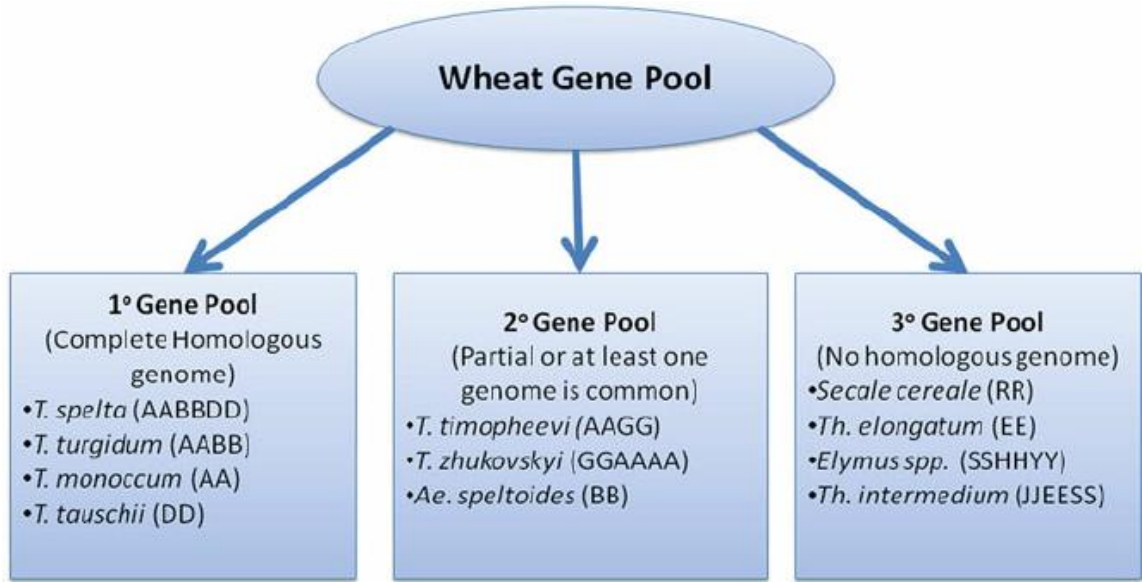

Figure 1-1: Gene pools of wheat [22]. The gene pool concept of wheat is based on homology between chromosome sets.

1.2.1.1 Primary gene pool of wheat

The primary gene pool of wheat includes wild species which share a complete homologous genome with wheat. It includes *Triticum spelta*, *Triticum monococcum* subsp., *Triticum turgidum* subsp., and *Aegilops tauschii*. Though the hybridization is not that easy due to ploidy discrepancies still genes can be transferred from these wild relatives via direct cross with wheat (amphidiploid) or through bridging species such as the development of synthetic hexaploid wheat by crossing *Triticum turgidum* subsp., and *Aegilops* tauschii.

1.2.1.2 Secondary gene pool of wheat

The secondary gene pool of wheat includes species which have at least one genome in common. It includes *T. timopheevi* (AAGG), *T. zhukovsyi* (GGAAAA), and *Aegilops speltoides* (BB). Genes can only be transferred through biotechnological approaches and to a lesser extent through direct hybridization with wheat.

1.2.1.3 Tertiary gene pool

It includes species of wheat relatives which don't have any homologous genome common with wheat. It includes *Secale cereale* (RR), *Thinopyrum elongatum* (EE), *Elymus* subsp. (SSHHYY), and *Thynopyrum intermediatum* (JJEESS). Gene transfer is possible only through biotechnological approaches or through bridging species such as X *Triticosecale* (AABBRR) in case of rye.

1.2.2 *Triticum turgidum* subsp.

To broaden the genetic base of wheat, tapping the diversity of its wild relatives seems feasible and best approach [21]. Though every species in wheat gene pool has its own importance, in this study, we focused towards most closely related species, *Triticum turgidum* subsp. (AABB) – from the primary gene pool and other towards distant relative *Secale cereale* (RR) – from the tertiary gene pool.

1.2.2.1 Origin of tetraploid species

1.2.2.1.1 Wild emmer:

All diploid species of wheat with A, B, D and S can be traced back to a common ancestor from which they originated 2.5 to 6 million years ago. Among these species, *Triticum urartu* (AA) and other unknown species of sitopsis section hold their important place as their hybridization around 0.36 to 0.5 million years ago gave birth to tetraploid species of

wheat, *T. turgidum* subsp. *dicoccoides* (2n=4x=28, AABB) – wild emmer [15,23]. Wild emmer lines are hulled (hard glume) and they shatter freely. Natural stands of wild emmer are still found in the Fertile Crescent region [24].

1.2.2.1.2 Cultivated emmer

Cultivated emmer evolved from wild emmer and was domesticated in the Fertile Crescent around 9,500 to 9,000 years back [16,17]. Two populations of wild emmer are found in the Fertile Crescent, northern and southern. Based on genetic analysis (Özkan et al. 2002; Wunder 2005), chloroplast DNA microsatellite variations [28], and RFLPs [29], it is suspected that northern population of wild emmer (South turkey, Iran and Iraq part of the Fertile Crescent) is real progenitor of cultivated emmer and location of this domestication correspond to the focal point where agriculture started, Karacadag region in Southeast Turkey [24]. Brittle rachis was the main trait that was altered through domestication, thus cultivated emmer has non-brittle rachis that helps to keep spikelet's intact on spike until manually harvested [24,30]. Based on my experience with tetraploids species of wheat, cultivated emmer is easy to thresh as compared to wild emmer but still cannot be freely threshed, as it is hulled too. Soon after domestication, cultivated emmer wheat expanded towards east through Mesopotamian plain to India, towards west through Anatolia to Europe [24]. For almost 6,000 years it stayed as one of the most important grain crops in these regions [17,31].

1.2.2.1.3 Diversification of free-threshing tetraploids:

Origin of free-threshing tetraploid (AABB) species is still a matter of debate, whether they originated from wild emmer or they originated from cultivated emmer. In most of the studies, it is shown that these free-threshing tetraploids evolved from the natural

stands of cultivated emmer [32,33]. Their origin was a result of post-domestication diversification [24]. This diversification happened either due to the pressure of local agro-ecological conditions or driven by natural hybridization. *Triticum turgidum* subsp. *durum* is suspected to have evolved from domesticated emmer in the eastern Mediterranean region due to the adaptation to the local ecological conditions (Dvorak 2007; Feldman 2007). A similar theory is applicable to the other free-threshing tetraploid species namely, Rivet wheat (*T. turgidum L*. subsp. *turgidum*), Polish wheat [*T. turgidum* L. subsp. *polonicum* (L.) Thell.], and Khorasan wheat [*T. turgidum* L. subsp. *turanicum* (Jakubz). These species might have also emerged due to agroecological pressures too. Another possibility of species diversification is interploidy introgression. During early periods of agriculture, crops were grown in close proximity to their wild relatives, therefore, there was always a chance of cross-pollination even among different polidy levels [37,38]. Two subspecies of *T. turgidum* are suspected to emerge likewise, Georgian wheat [*T. turgidum* subsp. *paleocolchicum* (Menabde)] emerged from a cross between wild emmer and *T. aestivum* [39] and Persian wheat [*T. turgidum* subsp. *carthlicum* (Nevski)] is believed to be a segregant from a cross between domesticated emmer and *T. aestivum* [40]. Morphologically, Persian wheat is really similar to *T. aestivum*. These introgressions from hexaploid wheat point out that there has been a role of *T. aestivum* in the diversification of tetraploid species [24].

1.2.3 Importance of different *Triticum turgidum* subsp. in wheat improvement

1.2.3.1 Wild emmer (*T. turgidum* subsp. *dicoccoides*)

Many important genes especially related to stress tolerance has been transferred from wild emmer to wheat. To name few, Leaf rust resistance QTL [41], stripe resistance genes [42–44], septoria leaf blotch resistance [45], and *fusarium* head blight [46].

1.2.3.2 Cultivated emmer (*Triticum turgidum* subsp. *dicoccon*)

Similar to wild emmer, cultivated emmer has also contributed a number of important genes into the wheat gene pool. To name few resistance to *Septroria nodorum* leaf blotch [47], [48], resistance to Russian wheat aphid [49] and resistance to Greenbug [50].

1.2.3.3 Durum wheat (*T. turgidum* subsp. *durum*)

Durum wheat has freely threshable heads and non-brittle rachis. It is today's most cultivated tetraploid species of wheat with total 17 million ha of cultivation. Durum is mainly produced in European Union countries, Canada, Syria, USA, Algeria, and Morocco; and to a smaller extent in the Russia, Turkey, Tunisia, Mexico, and India [51,52]. It is mainly used for pasta, macroni and semolina etc. Regarding the potential of durum wheat as a relative to wheat, mostly it is used as a bridge to transfer genes from other diploid species such as *A. tauchii* (DD). Thousands of durum based synthetic hexaploid wheat lines have been developed [53,54]. Several important genes have been incorporated into wheat from durum wheat e.g. Hessian fly resistance genes were transferred from durum line PI134942 [55], stem rust resistance [56], and *Fusarium* head blight resistance [57].

1.2.3.4 Rivet wheat (*T. turgidum* subsp. *turgidum*)

Rivet was once cultivated in Mediterranean region but slowly it disappeared as a crop [58]. To my best knowledge, no report of introgression from rivet to wheat has been reported.

1.2.3.5 Khorasan wheat (*T. turgidum* subsp. *turanicum*)

Kohrasan wheat was first described as *T. orientale* [59] and then treated as a variety of durum wheat [60]. Eventually, it was given its current name *T. turgidum* subsp. *turanicum*. Common name Khorasan was given based on its natural diversity in the Persian province of Khorasan [61]. It is also interesting that Khorasan wheat has not been cultivated beyond the limits of Near and central Asia (Turkey, Mesopotamia, Iran, Kazakhstan), and northern Africa) [61]. Due to its nutritional qualities Khorasan wheat was adapted in organic farming and mainly grown in upper mid-west areas of North America (borders of Montana, North Dakota, Alberta, and Saskatchewan) [61]. Kamut® is the most popular variety of Khorasan wheat and it is believed to be a segregant from a cross between *T. turgidum* subsp. *polonicum* and *T. turgidum* subsp. *durum* which occurred spontaneously in the Fertile Crescent region. [62]

1.2.3.6 Polish wheat (*Triticum turgidum* subsp. *polonicum*)

Polish is not that popular as a crop though it is grown sporadically in warm climates of southern Spain. Italy, Ukraine and warmer parts of Asia, Algeria, and Ethiopia [63]. It is characterized by large glume size up to 4.5 cm, long seeds and thousand kernel weight may reach upto 80 gm. Hybrids developed by crossing polish wheat with *Aegilops* species record yielded 80 tonnes/hac but had high fertilizer needs. Based on similar

discoveries it can be said that polish wheat is a great source for high yielding wheat varieties characterized by plump grain [64].

1.2.3.7 Persian wheat (*T. turgidum* subsp. *carthilicum*)

Persian wheat has not been exploited that much for wheat improvement. There are only a few reports for novel disease sources, such as *fusarium* head blight resistance sources [65]. Species like *T. turgidum* subsp. *carthilicum* are being underexplored and studied, a better evaluation of their germplasm may provide us with important sources for abiotic and biotic stress tolerance.

1.2.3.8 Georgian wheat (*T. turgidum* subsp. *paleocolchicum*)

This tetraploid species is endemic to Georgia and is locally known as colchic emmer [66]. Taxonomy wise, it was first classified as a subsp. *T. dicoccum*, then V. Menabde considered it as *T. paleocolchicum* (Menabde) [66]. In this study, Van Slageren's classification was considered in which he described georgian wheat as *T. turgidum* subsp. *paleocolchicum* [67]. *T. turgidum* subsp. *paleocolchicum* is of great interest from the evolution point of view as it combines the free-threshing traits with other wild traits of wild emmer and is considered as a bridge between wild and emmer wheat [66].

1.2.4 Mini core collections

Most of the plant genetic resources are preserved as accessions in the gene banks [68]. A number of accessions for particular species may go up to thousands. Owing to the large number of accessions, management in gene banks and utilization by breeders has always been a challenge [68]. One of the strategies to handle such large number of accessions is Core Collections (CC) and minicore collection (MC). First proposed by Frankel and Brown the concept of core collections implies to keep only a few (10 percent of full

collection) diverse lines from the full collection which can represent the genetic diversity of full set to the best [69,70]. Based on that concept there are core collections for a number of crops including wheat [71] [72], rice [73], maize [74] and soybean [68]. In case a full set is too large then core collection will still be large to be handled efficiently by breeders or in gene banks. In that case, mini core collections are the answer, mini core is only 10 percent of core collections which means the only a percent of the full set [75]. Mini core is much easier and efficient to handle as compared to full set as well as core set.

1.2.5 *Triticum turgidum* subsp. mini core or core collections

For *Triticum turgidum* subsp. of wheat, only few core collections have been developed that too for elite varieties of durum [76,77]. Others core collections which include few wild species of *T. turgidum* subsp., are based on one or few target traits and not with a intent to cover the genetic diversity of these species, such as Santra *et al*. focused to cover locations with least chronic diseases [78], Sanguineti *et al*. selected lines for better root architecture [79]. Therefore, there is need to develop core sets and mini core sets for other species of wheat which can be better exploited for wheat improvement.

1.3 Rye (*Secale cereale L.*)

1.3.1 Importance of Rye as a crop

Rye (*Secale cereale* L.) belongs to the Triticeae tribe in the family Poaceae [80] and is believed to share a common ancestor with wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) [81]. Germany, Poland, Russian federation, Belarus, and Ukraine are the major producers of rye and it is also produced to some extent in China, Canada, Turkey and USA [82]. Around the globe, rye is cultivated mainly for/as food, feed,

pasture, as a cover crop or a green manure crop [83]. It is one of the main sources of carbohydrates for northern and eastern Europe [81], [84]. Several types of rye-based bread are eaten around the world and mainly in Europe e.g. German bread are made up of 70% wheat flour and 30% rye flour [85]. Rye based products are a rich source of nutritionally important compounds like minerals (Zn, Fe, and P), beta-glucans, resistant starch and dietary fiber [86]. In North America, rye is preferably grown as a cover crop or as pasture, and its grains are used in livestock feed and in alcohol distilling. In drylands of southern Australia, it is grounded to prevent wind erosion. Furthermore, due to its sturdiest, it is also considered as a good pioneer crop to restore the fertility of waste lands [83].

1.3.2 Origin and dissemination of rye

Most of the *Secale* sp. originated in the Middle East, eastern Turkey in specific. Wild species *Secale strictum* Presl. (Syn. *Secale montanum Guss*. *emend*. Sencer) is believed to be the ancestor of rye [87–90]. *Secale strictum* along with other wild species first invaded wheat and barley fields as a weed and from these weedy species of rye, farmers consciously or unconsciously selected a variant with a non-brittle rachis and larger seeds, now classified as *Secale cereale*, only cultivated species of rye [88]. Exact geographic origin of cultivated rye is still a matter of debate but mostly proposed to be around Mt. Ararat and Lake Van area in eastern Turkey [88,91]. Thereafter, along with the dissemination of wheat and barley to Europe and Western Mediterranean, rye first came as a weed to these places [88], [91]. Due to its resiliency, it then adapted as a secondary crop in the areas with the harsh environment (cold and heat stress), where other staple crops like wheat were not able to survive. Eventually, seeing its versatility, people started

cultivating rye in Canada and northern parts of the US. Species are also found in Russia, Japan, Australia and an isolated population can be found in South Africa as well.

1.3.3 Taxonomy of rye

The taxonomic classification of genus *Secale* has been a matter of debate. Reviewed by Sencer and Hawkes [88], Roshevitz [92], and several other studies, different systems have been proposed to classify species of genus *Secale*. Lately, Frederiksen and petersen [93] concluded only three *Secale* subsp. *i.e. S. sylvestre, S. strictum* (including *subsp. strictum* and subsp. *africanum*; and *var. strictum*, *a*nd *var. ciliatoglume)* and *S. cereale* (which encompasses subsp. *cereale* and subsp. *ancestrale*). This classification is in accordance with the classification of Sencer and Hawks [88]. Based on reproductive mode, growth habit and wild/domesticated behavior the taxonomic system of American Germplasm Resources Information Network (GRIN) classifies the genus *Secale* into four species. *S cereale* annual allogamous species, *S. sylvestre* and *S. vavilovii* annual autogamous species and last is perennial wild-type allogamous *S. strictum* [94]. Among all *Secale* sp., *S. cereale* is the only cultivated species.

1.3.4 Stress tolerance in rye

Rye (*Secale cereale L.*) is known for its stress tolerance and hardiness. In adverse environments such as marshy lands [83], cold [95–97], drought [98], salt stress [99,100] and aluminum stress [101–103] rye is reported to perform much better than other cereals. Rye is also a well-documented source of tolerance/resistance to many pathogens as well. Crespo-Herrera *et al.* reviewed the important pathogen resistance genes transferred to wheat from rye [104]. One of the important examples signifying the pest resistance of Rye is 1BL.1RS translocation in wheat. Petkus rye chromosome arm 1RS carries savior

genes conferring resistance against stem rust (*Sr31*), leaf rust (*Lr26*), powdery mildew (*Pm8*) and yellow rust (*Yr9*) [105–107]. Another important translocation is 1AL.1RS from Insave rye transferred into wheat variety Amigo which carries stem rust resistance gene $Sr1RS^{Amigo}$ and powdery mildew resistance gene *Pm17,* allelic to *Pm8* [106].

Along with abiotic and biotic stress tolerance/resistance, rye chromatin in wheat also contributes to an increase in grain yield and adaptation potential. [108–110]. During 1990's, 60 percent of the wheat varieties at International Maize and Wheat Improvement Center (CIMMYT) carried 1BL.1RS translocations [111] and 40 percent such varieties were also developed in China [35]. Though there are some cases where rye chromatin is reported to negatively impact agronomic traits in wheat e.g. 1RS translocation have negative impacts on yield [109], quality [112] in few wheat backgrounds. These negative effects may be due to suppressors in certain wheat backgrounds or due to linkage drag from rye [104]. Associated negative impacts can be mitigated by switching different wheat backgrounds or by targeting fine translocations from rye or by changing the target wheat chromosome to be translocated as in some cases rye chromosome arm 1RS performs differently depending on which wheat chromosome arm it is replaced e.g. 1AS or 1BS or 1DS [108,113].

Triticale (*X. Triticosecale*), a cross between durum wheat (AABB) and rye (RR) further signifies stress tolerance potential of rye by producing relatively higher biomass and grain yield over other cereals in dry and cold environments [114]. Via triticale or chromosome substitutions/translocations important genes (as above discussed) from rye has been exploited for the improvement of other cereals like wheat. Still, there may be many more important genes in rye that can be explored for wheat improvement [115]. To

better access them, genetic diversity analysis and characterization of those genes is a crucial step.

1.3.5 Genetic diversity analysis in Rye

Among the diploid species of Poaceae family, rye has the largest genome (~7.9 Gbps) [116] and about 90% of the genome is occupied by repetitive sequences [117]. Due to the complex genome, coupled with the regional cultivation of rye, its genome has not been extensively studied, unlike other related grasses. Still, many important genetic diversity studies analyzing the relationship between/within *Secale* sp. have been conducted. Due to technological limitations, these studies were based on small number of molecular markers, covering a small portion of the genome, e.g. 11 PCR-RFLPs [118]; 14 allozyme, 3 SSR [119]; 15 SSR [120], 24 SSR [94]; 20 isozyme loci, 14 ISSR, 38 SSR [121]; 242 ISSRs and 169 RAPDs [122]; 779 AFLP [123]. Above that most of the markers used in these studies were not mapped to their corresponding chromosome locations [124]. To address this issue of anonymous and less number of markers, so far only single study has been conducted by Targonska et al [124]. They used 1054 DArT markers approx. equally distribution on all 7 chromosomes of rye and concluded that these SNPs provides a better picture of genetic diversity in rye gene pool. This achievement can be attributed to comparatively high number of markers used in this study and well distribution of markers of all chromosomes.

1.3.6 Association mapping for tan spot resistance

Along with genetic diversity analysis, characterization or mapping of genes for important traits is also critical. Finding the underlying gene(s) for a phenotype helps in its manipulation and efficient transfer from one plant or species to other. Genetic mapping

can be done using either bi-parental mapping or association mapping. Bi-parental mapping involves the use of recent recombination frequency among markers and trait in any segregating generation in order to find markers linked with a trait. Though bi-parental mapping is a robust technique, it is laborious and time-consuming as in order to study linkage disequilibrium between marker and traits, one has to develop a segregating population that may take at least 2 years. More importantly, there are only few recombination events during the development of a mapping population [125]. An alternative approach is Genome wide association studies (GWAS) which take the advantage of historical recombination. A diverse germplasm is collected (GWAS panel) in order to cover a maximum diversity of a species, it is genotyped to get genome-wide distributed markers, any association between the phenotype of interest and genotype is detected using regression-based models. One of the challenges for GWAS is underlying population structure and/or kinship among genotypes which can lead to false positives [126]. To tackle that, many statistical approaches have been developed which incorporate the knowledge of population structure and kinship as covariates into the model [95], linear mixed models (lmm) are one of the good choices among other statistical models. Lmms are known to take care of population structure as well as Kinship [127,128]. With the advancement in next-generation sequencing techniques and phenotyping platforms underlying genes of many traits have been mapped using genome wide association studies [129–132]. But in rye, so far only one association study has been done that too is candidate gene-based association mapping [95].

1.4 Wheat diseases

Since 1990s cultivated area under cereal crops like wheat and barley is decreasing in the US. Farmers planted 29 million ha in 1992 as compared to 20 million ha in 2016/17 and this trend is going down as the estimation of area under wheat for 2017/18 is 18 million ha [133]. This is partly because of the change in agricultural support policies that give farmers more planting flexibility, because of competition in international agriculture markets, introduction of genetically modified soybean and corn – which made cultivation of these crops easier, increasing demand for ethanol, shifting diet choices towards low carbohydrate foods and partly because of emerging diseases like Fusarium head blight FHB, leaf rust and emerging diseases like tan spot [134].

1.4.1 Leaf rust

1.4.1.1 Importance

Leaf rust is the foliar rust disease of wheat and it leads to significant yield losses worldwide [135]. During 2007, leaf rust caused 13.9% of the winter wheat yield loss in Kansas (Kansas Department of Agriculture), the chief wheat-producing state in the US. Yield losses are attributed to less number of kernels and are aggregated by lower kernel weight [135].

1.4.1.2 Causal agent

Leaf rust is caused by a fungus named *Puccinia triticina* Eriks, belongs to order Uredinales in the family Basidiomycetes [135]. It was first assigned to *P. recondita* species complex [136] then seeing it's sexual incompatibility with fungi of this group it was classified as *Puccinia triticina* Eriks.

1.4.1.3 Host Range

Wheat is the primary host of *Puccinia triticina*. It can also infect tetraploid species of wheat namely durum wheat (*Triticum turgidum* subsp. *durum*), wild emmer wheat (*Triticum turgidum* subsp. *dicoccoides*), cultivated emmer wheat (*Triticum turgidum* subsp. *dicoccon*) and triticale (X *Triticosecale*) [135]. *Thalictrum speciosissimum* hosts sexual spore stages of *P. triticina*. Natural stands in North America are resistant to *P. triticina* that's why fungal infections in North America are the dependent on the asexual spores only [137]**.**

1.4.1.4 Life cycle

Puccinia triticina is a macrocyclic (5 spore stages) and heteroecious (two different hosts) rust fungi. Wheat is the primary host, urediniospores, teliospores, and basidiospores are produced on it and *Thalictrum speciosissimum* is the alternate host which harbors pycniospores and aceiospores. Primary infection on wheat is caused by aeciospores (from alternate host) or urediniospores (from volunteer grasses). Post-infection, urediniospores are developed which act as a source of secondary inoculum provided favorable conditions prevail (10-25ºC, free water on leaf surface). During unfavorable conditions, fungus produces teliospores, which act as dormant spores for overwintering. Via meiosis teliospores gives rise to basidiospores. Basidiospores are the final spores to be produced on primary host and are carried by wind to the nearby alternate host (*Thalictrum speciosissimum*). Infection leads to sexual spores - pycniospores (male spores) and receptive hyphae (female spores), followed by fertilization and development of diakaryotic hyphae. This leads to aeciospores, which are wind born and once they infect the primary host (wheat) again, the life cycle of leaf rust is complete [135].

1.4.1.5 Resistance types

The genetic resistance characterization can be based on seedling stage or Adult Plant called Adult Plant Resistance (APR). Seedling resistance is race specific, vertically controlled by a single major gene and hypersensitive in reaction. On the other side, APR is polygenic, race non-specific, horizontally controlled by many genes, partial and durable in nature [138]. Major resistance genes follow gene for gene hypothesis and for the protection of plants they lead to hypersensitive response or programmed cell death in tissue surrounding the site of infection.

1.4.1.6 Resistance sources/genes

By now, about 80 major resistance genes have been identified [139]. They are designated *Lr1* to *Lr 78*, *Lrac 104* and *Lrac 124*. Out of the total, 24 genes confer APR and rest are expressed at seedling stage. Most of the genes have been mapped and have been located on 20 of 21 chromosomes of wheat with an exception of 3A [138].

In 1926 [140] identified wheat cultivars Malakof and Webster resistant to leaf rust. Upon genetic analysis, their corresponding genes were designated *Lr1* and *Lr2* respectively (Ausemus et al from [135]). From then till today approx. 34 resistance genes are identified from hexaploid wheat cultivars [138].

Approx. 56 genes have been identified and characterized in wild species of wheat. *Aegilops* subsp. have contributed approx. 18 genes. Namely, *Lr9* from *A. umbellulata*; *Lr19, Lr24,* and *Lr29* from *A. elongatum*; *Lr28, Lr35*, *Lr36*, *Lr47*, and *Lr51* from *A. speltoides*; *Lr 37* from *A. ventricosa*. *Aegilopes tauschii* donated seven genes - *Lr21, Lr22a*, *Lr32*, *Lr39*, *Lr41*, *Lr42,* and *Lr43*. *Lr44* comes from *Triticum spelta*. *Triticum monococum* gives *Lr 50*. 6 genes have been identified in tetraploid species of wheat.

Lr14a, Lr53, and Lr64 come from *Triticum dicoccoides*. Lr72, Lr61, and Lr23 were found in *Triticum turgidum* subsp. *durum*. *Lr25*, *Lr26*, and *Lr45* come from *Secale cereale*.

1.4.2 *Fusarium* Head blight

1.4.2.1 Importance

Fusarium head blight is caused by *Fusarium graminearum* which infects the heads of wheat and leads to distorted (lower test weight) and degraded (mycotoxin contaminated) seeds or in severe cases, no seed set at all [134]. Infection is aggravated by prolonged humid and wet conditions. Due to lower test weight, yield losses can toll up to 80% [141]. FHB is a worldwide problem and in the US alone total economic losses due to FHB from 1993 to 2001 were estimated at $7.67 billion [142]. Seeds contaminated with myctoxins like Dieoxynivalenol (DON) and nivalenol (NIV) are harmful to human and animal health.

1.4.2.2 Causal organism

Fusarium head blight is predominantly caused by *Fusarium graminearum* (teleomorph: *Gibberella zeae*), especially in North America (page 1715, in [134]). Based on the sexual stage *Gibberella zeae* (Schwein.) Petch, it belongs to order Hypocreales in family Nectriaceae [143].

1.4.2.3 Host range

The pathogen is mainly reported to cause head blight in wheat and barley but it is capable to infect rice and oats as well [143]. It is also one of the main pathogens causing ear, stalk, and root rot of maize [134]. Wheat and corn both being the host of *Fusarium graminearum* makes it a bigger concern in corn-wheat-soybean cropping rotations [144].

1.4.2.4 Life cycle

Fusarium graminearum overwinters as saprophytic mycelia on crop debris and in case of corn-wheat-soybean cropping rotation, it mainly overwinters of corn residue [143]. In spring, sensing favorable weather conditions, generally, fungi develops perithecia which wear sexual spores known as ascospores. Ascospores are forcibly discharged from the perithecia [145] and with the aid of air currents it infects the wheat heads which are at anthesis stage [146]. Secondary infection from plant to plant is the result of conidia (produced on sporodochium), which can be windborne or spread by the rain splashes [146–148].

1.4.2.5 Resistant types

Host resistant to Fhb is the best sustainable and environment-friendly tool – as for any other pathogen [149–151]. In case of wheat – Fhb relationship, host resistance is complex and host can have resistance to one or another step in *Fusarium* infection process. Therefore it is divided into four main types: Type I – resistance to initial infection, Type II – resistance to fungal spread from the initial site of infection, Type III – Resistance to DON production and Type IV – Resistance to seed colonization. Among these, Type I and II are more extensively studied, mostly because these resist the fungus at an early stage of infection. Type III and IV have not been investigated deeply [152–154].

1.4.2.6 Resistance sources/genes

Many strains or races of *Fusarium graminearum* have been reported but no specific host – strain specific system has been recognized, in other words, virulence in *Fusarium graminearum* is not host-specific and resistance in cultivars is not strain specific thus it is of horizontal, quantitative and non-specific in nature [155]. So far 52 Fhb QTL mapping

studies have been done, out of total 52, 42 are performed using hexaploid wheat and rest on relative species. So far more than 400 QTLs have been identified on all chromosomes of wheat except 7D [156]. Loci *Fhb1* (Qfhs.ndsu-3BS) is the major Type II resistance source used worldwide. It hails from highly resistant Chinese cultivar named Sumai-3 [157–159]. Based on line Wangshuibai lin *et al.* 2006 identified QTL on 2D, 4B and 5A [160] and Jia *et al.* identified QTL for Type I resistance on chromosome 2B, 3BS, 4B, 5B and 7A [161]. Brazilian cultivar Forntana possess Type I resistance [153] and is found carry resistant QTL on 3A, 5A, 2B, 6B and 7A [162]. Forntana's resistance may be due to hard glumes and narrow flower opening [155]. Swiss winter wheat cultivar Arina is also reported to carry major resistance QTLs on chromosome 4AL, 6DL, 1BL and 6BS, 4DS [163–165].

As compared to hexaploid wheat, there has not been much success in identifying resistance among durum or tetraploid wheat species. There are only few resistance QTLs identified only from wild species. *T. turgidum* subsp. *dicoccoides* accession FA-15-3 (syn. Israel A) was found be resistance [166] and based on single chromosome recombinant population, QTL on 3AS was located [167]. *T. turgidum* subsp. *dicoccoides* accession PI478742 is found to carry resistant QTL on 7AL [168]. *T. turgidum* subsp. *durum* cultivar Strongfield is reported to carry resistance QTL on 2BS and *T. turgidum* subsp. *carthilicum* cultivar Blackbird have resistance QTL on 6BS [169].

1.4.3 Tan spot

1.4.3.1 Importance

Tan spot is an important disease of wheat caused by necrotrophic fungus *Pyrenophora tritici-repentis*. Tan spot is reported nearly everywhere where wheat is cultivated [170].

Disease symptoms on susceptible host appear as tan colored oval shaped necrotic and/or chlorotic spots with a black pinhead spot in the center. In highly susceptible genotypes these lesions may coalesce and cover the larger/whole leaf surface area [170,171]. Up to 49% yield loss has been attributed to tan spot during favorable disease conditions [172]. Losses due to tans pot are attributed to low thousand kernel weight, less number of kernels per head, if the infection is early then less number of tillers, low biomass, and low leaf area index [173]. Due to its overwintering habit on crop residues or stubles tan spot is of major concern in sustainable agricultural systems which are based on no-tillage as the inoculum of primary infection is always there in the field [170].

1.4.3.2 Causal organism

Pyrenophora tritici-repentis is the main causal organism of tan spot. *P. tritici-repentis* was first named as *Pleospora trichostroma* [174] and from that time its nomenclature has changed many times. Presently, its sexual stage is called as *Pyrenophora tritici-repentis* (Died.) Drechs and the asexual stage is called as *Drechslera tritici-repentis* (Died.) Drechs [175].

1.4.3.3 Host range

The main host for *P. tritici-repentis* is wheat and it can infect all species of wheat including tetraploid and hexaploid species [170]. Along with wheat, it can infect many other grass species like barley (*Hordeum vulgare*), oats (*Avena sativa*), wild oats (*Avena fatua*), rye (Secale cereale) and many other [176,177]. These alternative hosts though help in spread and survival of inoculum but they are also a great source of novel resistance genes.

1.4.3.4 Life cycle

P. tritici-repentis overwinters on crop residue by forming sexual fruiting bodies called pseudothecia. In spring (favorable weather) it produces ascospores bearing asci. Asci contains ascospores and are the primary source of inoculum. Another primary source of inoculum can be seed born conidia, conidia from alternative hosts etc. After initial infection conidia are produced in large numbers and these are blown to nearby plants by wind or rain causing secondary infection and the cycle continues till favorable conditions (high humidity, temp above 15ºC) sustain [178,179].

1.4.3.5 Host selective toxins (HSTs) and Race classification

Different isolates (races) of *P. tritici-repentis* have been reported to produce one or more of the three host selective toxins (HSTs), which are designated as Ptr ToxA, Ptr ToxB, and Ptr ToxC. (Ciuffetti et al. 1998). These Isolates are classified into different races based on their reaction on the differential checks which is determined by their corresponding HST. There are four hexaploid wheat differential checks namely salamouni (universal resistant check), glenlea (Necrotic reaction to Ptr ToxA), 6B635 (Cholortic reaction to Ptr ToxC) and 6B622 (Cholortic reaction to Ptr ToxB). Till date 8 races have been classified based on which host selective toxin they produce, Race 1 (Ptr ToxA and Ptr ToxC), Race 2 (Ptr ToxA), Race 3 (Ptr ToxC), Race 4 (none), Race 5 (Ptr ToxB), Race 6 (Ptr ToxB and Ptr ToxC), Race 7 (Ptr ToxA and Ptr ToxB), Race 8 (Ptr ToxA, Ptr ToxB, and Ptr ToxC).

Ptr ToxA and Ptr ToxB are known proteins and are both known to activate host defense mechanism that is employed to defend against biotrophic pathogens. On the other side, Ptr ToxC is not a protein rather it is a non-ionic molecule.

1.4.3.6 Resistance sources/genes

Though fungus can be controlled using cultural and chemicals controls, host resistant against tan spot is most cost-effective and environment-friendly way to limit yield losses[180]. As described above, fungus produces at least 3 host-selective toxins (HSTs): Ptr ToxA, Ptr ToxB, and Ptr ToxC, that interact directly or indirectly with dominant host genes *Tsn1* [170,181], *Tsc2* [182] and *Tsc1* [183,184] respectively. Recessive counterparts of these genes are characterized as insensitive genes to the corresponding toxin. Along with these insensitive genes, few resistance genes (*tsr2*, *tsr3*, *tsr4*, *tsr5*), and QTLs associated with tan spot resistance have also been discovered. *Tsr2* hails from *T. turgidum* subsp. *turgidum* accession (PI 352519), mapped on chromosome 3BL and confers resistance to race 3 isolates causing necrosis in tetraploid wheat [185]. *Tsr3* resists isolates of race 1. It is mapped on chromosome 3D and it was reported in synthetic hexaploid wheat lines [186]. *Tsr4* confers resistance to another isolate of race 1 and mapped on chromosome 3A. *Tsr4* comes from resistant cultivar salamouni [187]. Tsr5 is reported to resist race 5 isolates causing necrosis on tetraploid wheat lines, mapped on 3B [188]. Though some major tan spot resistance genes have been reported but tan spot resistance is majorly considered as quantitative as its reaction varies with genetic background, environment, and experimental error. Based on the quantitative behavior of tan spot resistance, many QTLs associated with tan spot resistance [170] has been reported by many independent studies such as [26,189–193].

If we look at sources of resistance they are mostly from tetraploid (*T. turgidum* subsp.) [180] and hexaploid (*T. aestivum*) [194,195] wheat, with few from D genome donor

species *A. tauchii* [196,197]. Still, there is a lot of scope in the identification of tan spot resistance sources from relatives of wheat [170].

1.5 Exploiting wheat-diazotrophic interactions

1.5.1 Impact of Nitrogen fertilizer uses

Nitrogen is an essential element in plant growth, representing 2% of the total plant dry matter in the food chain [198]. Though N makes about 80% of the atmosphere, the inability of the plants to fix free nitrogen makes them dependent on synthetic fertilizers [199]. More than 60% of the synthetic fertilizers produced worldwide are consumed in cereal production only and the yield increments due to these fertilizers come with considerable environmental impacts [200]. Nitrogen is applied in the plant available form - NO_3 or NH_4. On an average only 30 – 50% of the applied nitrogen is observed by the plants; rest leads to water resources contamination through leaching of nitrates and surface runoff; global warming and ozone layer depletion through ammonia volatilization [201]. Additionally, increase in the production costs of the inorganic fertilizers makes them unaffordable for many farmers. Therefore, we must remove our dependency on synthetic fertilizers to have sustainable agriculture. One of the alternatives is to exploit the association of cereals with nitrogen-fixing bacteria.

1.5.2 Biological nitrogen fixation

Diazotrophs are bacteria or archaea which can fix atmospheric nitrogen via a process known as biological nitrogen fixation. They can enzymatically (nitrogenase) reduce atmospheric N_2 to ammonia, making it accessible to the plants [202]. This process is unique to bacteria and archaea [203]. Plants benefit from this process by developing endosymbiotic, associative or entophytic relations with bacteria.

1.5.3 Endosymbiotic associations

Endosymbiotic associations between legumes and nitrogen-fixing bacteria (*Rhizobium*) are the most efficient associations. Bacteria reside inside the root nodules, which provide oxygen-free conditions for nitrogen fixation and in exchange bacteria provides fixed nitrogen in the form of ammonia. Up to 70% of the nitrogen needs for associated plants are fulfilled by these bacteria, making them independent of synthetic nitrogen fertilizers [204].

1.5.4 Endophytic and associative associations

Highly efficient endosymbiotic relationships have not been reported in the gramineae family. However, numerous studies have reported endophytic or associative associations of plant growth promoting bacteria's with grass family. Lines or varieties of sugarcane cultivated under low nitrogen conditions can obtain a substantial percentage of their nitrogen from associations with endophytic and associative diazotrophic bacteria.

1.5.5 Wheat diazotrophic studies

A few wheat-bacterial association studies have been conducted and showed a promise for biological nitrogen fixation in wheat. Webster *et al.* reported that bacteria *Azorhizobium caulinodans*, which forms nodules on the stems and roots of the tropical legume *Sesbania rostrate*, can colonize the wheat roots internally [205]. Lethbridge *et al.* showed BNF nitrogen acquisition in two spring wheat cultivars through bacteria mixture including *Azotobacter* and *Azospirillum* [206]. Schloter *et al.* presented different patterns of wheat root colonization by *Azospirillum barsilense* [207]. Best example proving the potential of BNF in wheat is a study done by Iniguez *et al.* [208]. Using nitrogen-fixing *bacteria Klebsiella pneumoniae* 342 (Kp342) authors showed a significant gain in wheat nitrogen

plants from biologically fixed nitrogen. Kp342 relieved nitrogen (N) deficiency symptoms and increased total N concentration in the plant. Similar to these there are many studies which have shown the potential of nitrogen fixation in wheat – diazotroph systems.

1.5.6 The Potential in wild relatives-diazotroph interactions

Problem with already done wheat-diazotrophs studies is the amount of fixed nitrogen is not enough that can suffice the N needs of modern varieties. Therefore a better system needs to be discovered. One of the approaches would be to test the wild relatives of wheat. No study has been conducted to see diazotroph interaction with wild relatives of wheat. We hypothesize that as these wild relatives are present in wild and their nitrogen requirements to some extent must be meant by diazotrophic bacteria. Therefore we hypothesize that wild relatives of wheat provide us with a hope to explore wheat – diazotrophic associations. These systems may not be applied directly to modern agriculture but these can help to unravel the genetic basis of the efficient associations with diazotrophs which can then be exploited for restoring this ability in the modern cultivars.

Chapter 2

Characterizing wild and domesticated tetraploid wheat species (*Triticum turgidum* subsp.) for resistance to *Fusarium* head blight, leaf rust, and tan spot.

2.1 Abstract

The narrow genetic base of wheat germplasm limits a continuous improvement in the wheat productivity and limits its ability to perform under stressed environments. Wild ancestors of wheat harbor under-exploited genetic diversity which can be used for wheat improvement. In this study, we evaluated a mini core set (95 accessions) of *T. turgidum* subsp. for resistance to *Fusarium* head blight (FHB), leaf rust and tan spot. The mini core represents a genetic and geographic diversity of 1,890 accessions of *T. turgidum* subsp. from NBRP Kyoto gene bank. Upon screening for Type II resistance against FHB using single spikelet inoculations in a greenhouse, we identified three resistant accessions of *T. turgidum* subsp. *dicoccon* viz. MG 5293-1, KU 1058, and MG 5416-1 with disease severity of less than 15 percent. All three accessions were collected from different geographic backgrounds suggesting the possibility of diverse sources of resistance. Upon comparison among subspecies, higher disease severity was observed on *T. turgidum* subsp. *dicoccoides* as compared to *T. turgidum* subsp. *dicoccon*. The accessions were also evaluated for their response to leaf rust both at seedling stage in the greenhouse and at adult stage in the field. We identified three accessions of *T. turgidum* subsp. *durum* viz. Cltr 4071, PI 244061, and PI 185233 that were resistant at seedling (HR - R) as well as at adult stage (5R – 10R). In addition, a few accessions were susceptible at seedling stage but demonstrated resistant reaction in the field, could possess adult plant resistance genes against leaf rust. Screening against tan spot (*PTR* race 5) also yielded interesting results:

of the 84 accessions screened nine accessions were found to be resistant and all are *T. turgidum* subsp. *dicoccoides*. Among susceptible accessions *T. turgidum* subsp. *dicoccoides* showed chlorotic reaction but *T. turgidum* subsp. *dicoccon* responded with necrotic reaction. As *T. turgidum* subsp. *dicoccoides* is the wild ancestor of *T. turgidum* subsp. *dicoccon*, this divergence in response to tan spot could yield a good system to study the plant-fungi co-evolution. All resistance sources identified in our study could be exploited for wheat improvement via synthetic hexaploid wheat or direct cross with modern wheat cultivars.

2.2 Introduction

It is essential to increase the wheat production in order to meet the ever-growing food-feed needs of the growing population [2]. Simultaneously, changing climate leading to recurrent biotic (evolving pathogens) and abiotic stresses (fluctuating weather, increased greenhouse emissions) is challenging the current wheat production [3]. Recent origin of polyploid wheat (bread wheat AABBDD) evolved from a low frequency of historic hybridization events between *T. turgidum* subsp. *dicoccon* (AABB) and *Aegilops tauschii* (DD) [15,23]. This low frequency resulted in a narrow genetic base of wheat germplasm and as a hurdle for continuous increase in wheat productivity [209]. Wild ancestors of wheat are a great trove of genetic diversity that can be exploited for broadening the genetic base of wheat (Cox 1998, Sehgal 2005, Sehgal et al 2011). Wild relatives are still found in the natural habitats e.g. Fertile Crescent and they are also conserved in the seed banks as germplasm collections. Though a series of efforts have been made to utilize the genetic diversity in *Ae. tauschii* (DD) through the development of synthetic hexaploid wheat [46,210], but tetraploid wheat (*T. turgidum* subsp.) has been relatively less

exploited for bread wheat improvement. In order to effectively access the diversity from the wild ancestors of wheat, we need to identify mini core sets with a smaller number of lines which can represent the genetic diversity of their corresponding species to the best. Equally important is the characterization of these mini core sets in order to identify lines with valuable traits which can then be exploited for wheat improvement.

Fungal head and leaf diseases cause a significant loss in yield as well as grain quality of wheat [211]. FHB is caused by *Fusarium graminearum* which infects the spikes (heads) of wheat and leads to distorted (lower test weight) and degraded (mycotoxin contaminated) seeds or in severe cases, no seed set at all [134]. Host resistance against FHB is complex therefore divided according to at which stage of *Fusarium* infection process plant defense is active. Two main types are Type I – resistance to initial infection and Type II – resistance to fungal spread from the initial site of infection [152,153]. Several sources of resistance carrying major resistance genes originated from China like hexaploid line Sumai 3 (*Fhb1*) [157–159] and from alien species like *Leymus racemosus* (*Fhb3*) [212], *Elymus tsukushiensis* (*Fhb6*) [213], and *Thinopyrum ponticum* (*Fhb7*) [214] however, very few reports of resistance in tetraploid wheat have been reported [166,168,169].

Leaf rust, another important fungal disease, is caused by *Puccinia triticina* Eriks, which mainly infects leaves and interferes with the photosynthetic efficiency of diseased plants [135]. Resistance against leaf rust can be qualitative, race specific which is called seedling resistance or/and it can be adult plant resistance which is polygenic and race nonspecific [138]. More than 80 leaf rust resistance genes have been identified, located on 20 of 21 chromosomes of wheat except for 3A [138]. Most of the genes are effective

at seedling stage or are race specific and only a hand full have shown adult plant resistance (e.g. *Lr34*, *Lr46*, and *Lr67*) [215,216]. Species-wise, approx. 34 resistance genes have been identified from the hexaploid wheat background, 18 from *Aegilops* subsp. and only six from *Triticum turgidum* subsp. [138,217,218].

Tan spot is also an important foliar disease of wheat caused by necrotrophic fungus *Pyrenophora tritici-repentis*. Tan spot is reported nearly everywhere where wheat is cultivated [170]. Fungus produces three host-selective toxins (HST) Ptr ToxA, Ptr ToxB, and Ptr ToxC, that interact directly or indirectly with dominant host genes Tsn1 (Stock et al. 1996; Faris et al. 2013), Tsc2 (Effertz et al. 2001) and Tsc1 (Orolaza et al. 1995; Friesen and Faris 2004) respectively. Based on HST, so far eight races of *Pyrenophora tritici-repentis* have been reported. In this study, we primarily investigated resistance against race 5 which produces HST Ptr ToxB. Along with major insensitivity gene *tsc2*, resistance genes like *tsr5* and few other QTLs have been reported to resist *PTR* race 5 [182,188].

For all three diseases, *Triticum turgidum* subsp. gene pool has not be exploited to its potential. Therefore the overall objective of our study was to characterize a diverse mini core set of tetraploid wheat for their reaction to important fungal diseases of wheat namely *Fusarium* head blight (FHB), leaf rust and tan spot and identify sources of resistance that can be exploited for wheat improvement.

2.3 Material and methods

2.3.1 Mini core collection

Mini core set used in this study consists of 95 accessions of *T. turgidum* subsp. (Table 2-1, Appendix Table 3). Five accessions – with higher protein content – come from CNR

Institute of Plants Genetics (Bari, Italy). 90 accessions come from gene bank collection of NBRP Kyoto, Japan representing the genetic and geographic diversity of 1890 accessions. Briefly, 1890 accessions of *T. turgidum* subsp. were genotyped using 275 DArT (Diversity arrays technology) markers and a core set of 380 accessions was extracted. This core set was further genotyped using genotype by sequencing (GBS) based 6,000 SNP and a mini core set of 90 accessions best representing the geography and genetic diversity was extracted. Detailed information about all accessions is provided in Appendix Table 3.

2.3.2 *Fusarium* head blight screening

2.3.2.1 Plant material

From the mini core set, 39 accessions in total were screened, 28 of *T. turgidum* subsp. *dicoccoides* and 11 of *T. turgidum* subsp. *dicoccon* (Table 2-1). Detailed information for each accession is in Appendix Table 3. Flourish and AC Emerson were the susceptible and resistant checks respectively. For each accession approx. six vernalized seedlings were transplanted with two seedlings per pot containing soil mix - Sunshine 365 (Sun Gro Horticulture, Agawam, MA). Plants were grown in a greenhouse with 14 hours of the day at a temperature of 21-25ºC and 10 hours night at a temperature of 18-20ºC till they were inoculated. For FHB data analysis each head/spike was considered as one rep. Mostly 10 heads were scored for each genotype. Consensus score of disease rating is based on the average of all reps.

Table 2-1: *Triticum turgidum* subsp. accessions in mini core collection and number of accessions screened for each disease.

Sr.no.	SubSpecies	Minicore	FHB	Leaf rust	Tan spot
1	*dicoccoides*	33	28	28	28
2	*durum*	28	-	26	25
3	*dicoccon*	14	11	11	12
4	*turgidum*	7	-	7	7
5	*turanicum*	4	-	4	4
6	*polonicum*	4	-	4	4
7	*carthilicum*	2	-	1	2
8	*paleocolchicum*	1	-	1	1
9	*pyramidale*	1	-	1	1
10	*abyssinicum*	1	-	1	-
TOTAL		95	39	84	84

2.3.2.2 Inoculations

Fungal cultures were grown on PDA (Potato dextrose agar) media by placing single fungus plug on each media plate under sterile conditions. After culturing, plates were placed in controlled conditions, temp 18-20ºC with 12 hours of light and 12 hours dark. Seven days later, plates were washed with sterile water to make a conidial solution (Figure 2-1A) and concentration was adjusted to 50,000 macro-conidia per ml. For every inoculation, fresh inoculum was prepared the same day. At anthesis, 10ul of macro-conidia inoculum was injected (Figure 2-1B) into two florets of middle spikelet using a

pipette following the protocol of Stack *et al* [219]. Following a gentle spray inoculated head was covered with a transparent zip-lock bag (Figure 2-1C). For rest of the growth period plants were kept in a greenhouse at 22-25ºC day (14 hours) temperature and 18-22ºC night (10 hours) temperature. Ziplock bags provided humid conditions for initial infection of fungus and were removed 3 days after inoculation.

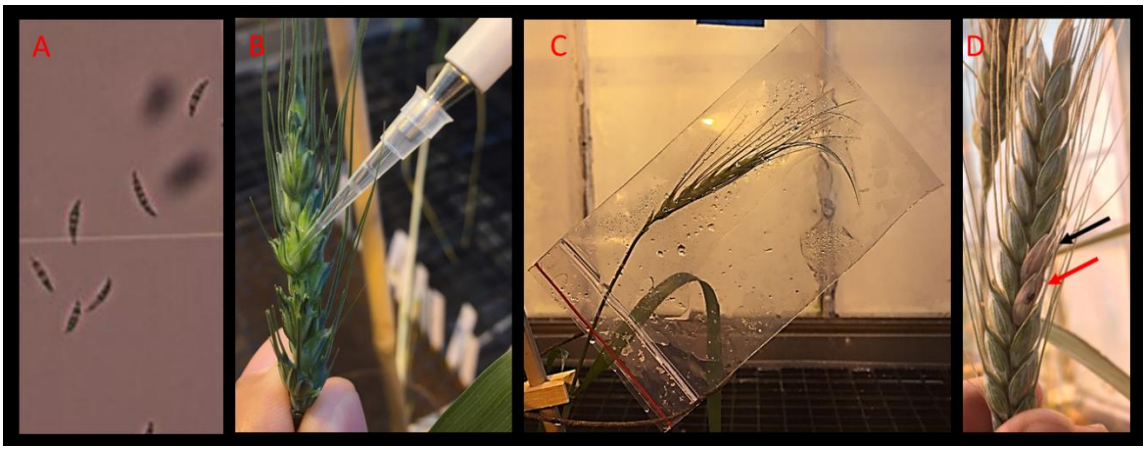

Figure 2-1: Single spikelet inoculations for FHB. A) Sickle-shaped macro conidia of *Fusarium graminearum*. B) Single spikelet inoculations with 10ul of macro-conidia solution (50,000 spores/ml) of *Fusarium graminearum*. C) Inoculated spike covered with ziplock to maintain humidity. D) The response of a resistant line (KU1058) after Single spikelet inoculation (red arrow). The disease was able to spread only to one spikelet (black arrow) from the point of inoculation and rest of the spike is healthy and green.

2.3.2.3 Disease Scoring

Three weeks after inoculation, inoculated spikes were scored for disease severity (Figure 2-1D) on a 0-100% scale [220,221]. Total spikelets on a spike and diseased – water soaked, bleached or red-brown discoloration – spikelets were counted and the ratio of

diseased/total spikelets was calculated. Response categories were divided based on percentage severity, R: 0-15%, MR: 15-30%, MS: 30-50% and S: >50%.

2.3.3 Leaf rust screening

2.3.3.1 Plant material

Leaf rust screening was done both at seedling stage in greenhouse and adult stage in the field. 84 accessions from mini core were scored for seedling response and due to germination issues in the field, only 74 accessions were scored in field conditions (Table 2-1). Detailed information for each accession is presented in Appendix Table 3. For seedling screening, each genotype was planted in three cones, three seeds in each cone (two inches in diameter and six inches height) and each cone was considered as one replication. SY Wolf was used as a resistant check, McNair and Morocco were used as susceptible checks. Plants were grown in a greenhouse with 14 hours a day at a temperature of 21-25ºC and 10 hours night at a temperature of 18-20ºC till they were inoculated.

For field screening, winter type seedlings were transplanted after vernalization and spring type accessions were planted directly in the field in the spring of 2017. Each accession was planted in two reps (4 feet rows) with approx. 5-10 seeds per rep at Brookings, SD.

2.3.3.2 Inoculations

For seedling screening, *Puccinia triticina* urediniospores were collected in glycine capsules from the naturally infected winter wheat breeding trials in Brookings, SD. The collected spores were dehydrated in a desiccator for about 24 hours and were stored at 80ºC for later use. On the day of inoculations, spores were heat shocked in a water bath at 42ºC for 10 min, while they were still in a glycine bag. Spore concentration was

normalized to 2-3 mg spores per ml of soltrol [222,223]. At 2-3 leaf stage (10-15 days old), plants were inoculated using atomizer with a pressure of five psi (Figure 2-2B). Spore germination was tested on water agar (Figure 2-2C). Soltrol was allowed to evaporate for 20-30 min and inoculated seedlings were placed for 24 hours in a humidity chamber. For further development of the disease, plants were moved into a growth chamber with 14 hours light at a temperature of 24 ºC and 10 hours dark at 18ºC.

No artificial inoculations were done in the field, all infections were due to natural disease occurrence because of spreader rows planted in observation nursery.

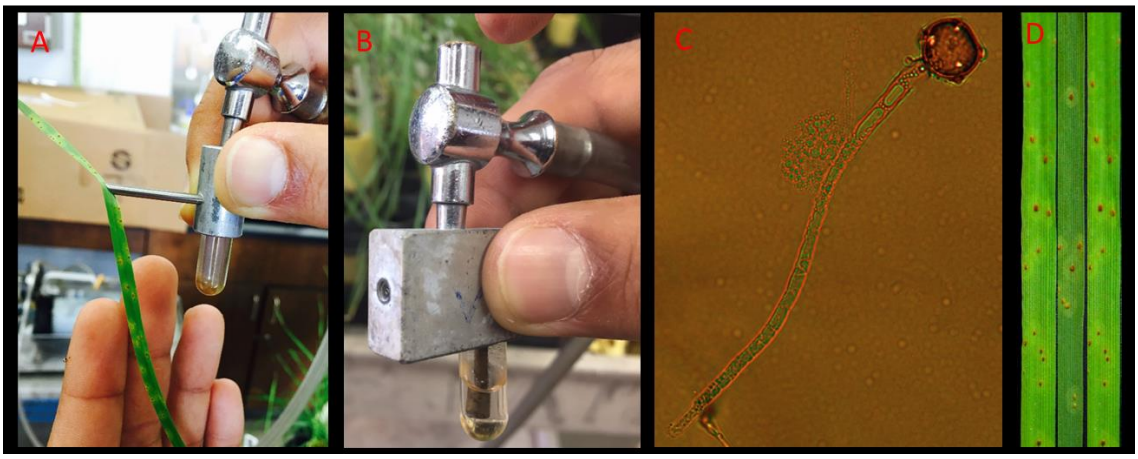

Figure 2-2: Inoculations for leaf rust at seedling stage. A) Collection of urediniospores from the infected wheat leaves. B) The front end of atomizer used for spraying inoculum. C) Germinating urediniospores. D) Susceptible reaction and developing urediniospores in pustules.

2.3.3.3 Scoring

For seedling screening, 10 days post inoculations, plants were rated for infection type (IT) on a 0 – 4 stakman scale [224] Stakman and Levine 1944). On this scale, 0: nearly

immune; 1: very resistant; 2: moderately resistant; 3: moderately resistant to moderately susceptible; and 4: very susceptible.

For field screening, scoring was done on flag leaf stage, mostly after heading based on infection type and percentage severity scale [224]. Severity was rated on a percentage scale of 5, 10, 20, 40, 60, 80, and 100. Infection types were O - immune response, R - resistant, MR - moderately resistant, MS - moderately susceptible and S - susceptible. The final score was the combination of severity and infection type.

2.3.4 Tan spot screening

2.3.4.1 Plant material

84 mini core accessions were screened for resistance against *Pyrenophora tritici-repentis* race 5. Seeds of each genotype were planted in three cones, three seeds in each cone (two inches in diameter and six inches height). Each cone was considered as one replication. Salamouni was used as a resistant check and 6B662 was used as a susceptible check. Plants were grown in a greenhouse with 14 hours of day at a temperature of 21-25ºC and 10 hours of night at a temperature of 18-20ºC till they were inoculated.

2.3.4.2 Inoculations

Inoculum preparation and inoculations were performed according to Ali and Francl, 2001 [225]. *Pyrenophora tritici-repentis* race 5 frozen plugs were plated on fresh V8-PDA media plates. For mycelium growth (Figure 2-3A), plates were kept in dark for five days (generally colonies reach 1 to 1.5-inch diameter). After that plates were flooded with sterile water, fungal mycelium was knocked down using sterile test tubes (Figure 2-3B). For conidial production (Figure 2-3C), plates were kept under light at room temperature for 24 hours and then incubated at 16ºC under dark for 24 hours. Plates were flooded

with sterile water and spores were collected using sterile loop wire (Figure 2-3D). Spore concentration (Figure 2-3E) was adjusted to 3000 spores/ml as described by Jordahl et al. 1992 [226]. At 2nd leaf stage, plants were inoculated with *Pyrenophora tritici-repentis* race 5 by using spore suspension of 3000 spores/ml (Figure 2-3F). Inoculated plants were moved to mist chamber (18°C) for 24 hours and grown for five days in a greenhouse at 21-25° C and 14 hours photoperiod.

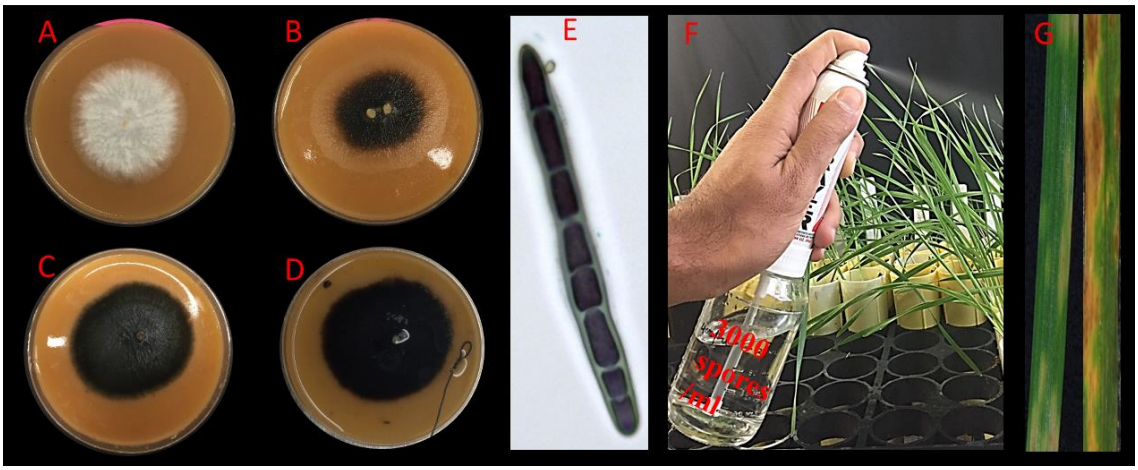

Figure 2-3: Inoculations with *P. tritici-repentis* race 5. A) Mycelium growth after 5 days of plug plating on V8 PDA. B) After mycelium was knocked down. C) Conidial production post light/dark cycle for 24 hours. D) Preparation of conidial solution using a sterile loop wire. E) Conidia stained with trypan blue – for visibility – otherwise conidia are colorless. F) Inoculum spray using 3000 spores/ml G) Chlorotic and necrotic susceptible responses.

2.3.4.3 Scoring:

Five days post inoculation, disease lesions were rated on a qualitative scale of 1 to 5 [177]. 1: resistance response, 2: moderately resistance, 3: moderately susceptible

response, 4: susceptible (some lesions coalescent) and 5: highly susceptible (all lesions generally coalescent).

2.4 Results

2.4.1 *Fusarium* head blight (FHB) screening

Average disease severity among the screened mini core accessions ranged from 14.7 to 100%. Susceptible check Flourish showed average disease severity 70% (range 60 to 80%), as expected. Average disease severity on the resistant check (AC Emerson, Cantera seeds) was 11%, ranging from 6 to 23%. Table 2-2 summarizes the distribution of different accessions among different response category. Of 39 accessions from the corset screened, three accessions showed a resistance response (Table 2-3). Interestingly these three accessions belong to *T. turgidum* subsp. *dicoccon*. Average disease severity on MG5293-1, KU1058, and MG5416-1 was 14.7, 14.2 and 15% respectively. In addition another three accessions from *T. turgidum* subsp. *dicoccon* and one accession from *T. turgidum* subsp. *dicoccoides* showed moderately resistant (MR) response (Table 2-3). Eight accessions fall into moderately susceptible (MS) category, including five *T. turgidum* subsp. *dicoccoides* accessions and three *T. turgidum* subsp. *dicoccon* accessions. The other 24 accessions were susceptible (S), the majority (22) of the accession were from *T. turgidum* subsp. *dicoccoides* and two accessions were from *T. turgidum* subsp. *dicoccon*.

Species-wise comparison of disease severity suggested that *T. turgidum subsp. dicoccoides* showed statistically higher disease severity (average 79.9% infected spikelets per spike) as compared to *T. turgidum* subsp. *dicoccon* (average 36.1 % infected spikelets per spike) (Table 2-2, Figure 2-4).

Table 2-2: Distribution of *Triticum turgidum* subsp. *dicoccoides* and *Triticum turgidum* subsp. *dicoccon* accessions among different response categories against FHB.

Category (Severity)*	***dicoccoides* no. (%)**	***dicoccon* no. (%)**	**Total (%)**
R (0-15%)	0 (0%)	3 (27%)	3 (7.5%)
MR (15-30%)	1 (3.5%)	3 (27%)	4 (10.2%)
MS (30-50%)	5 (17%)	3 (27%)	8 (20.5%)
S (>50%)	22 (78%)	2 (18%)	24 (61.5%)
Total	28	11	39

***Categories: R: 0-15%, MR: 15-30%, MS: 30-50% and S: >50%.

Table 2-3: Resistant and moderately resistant accessions to FHB identified from the mini core set of *T. turgidum* subsp.

Accession	*T. turgidum* subsp.	Origin	Disease category†	Severity*
KU1058	*dicoccon*	Spain	R	14.7
MG5293-1	*dicoccon*	Italy	R	14.8
MG5416-1	*dicoccon*	-	R	15.0
KU124	*dicoccon*	-	MR	20.5
PI355497	*dicoccon*	Soviet Union	MR	26.0
PI428105	*dicoccoides*	Israel	MR	29.1
Cltr4013	*dicoccon*	India	MR	30.0

*Average disease severity. †Categories: R: 0-15%, MR: 15-30%, MS: 30-50% and S: >50%.

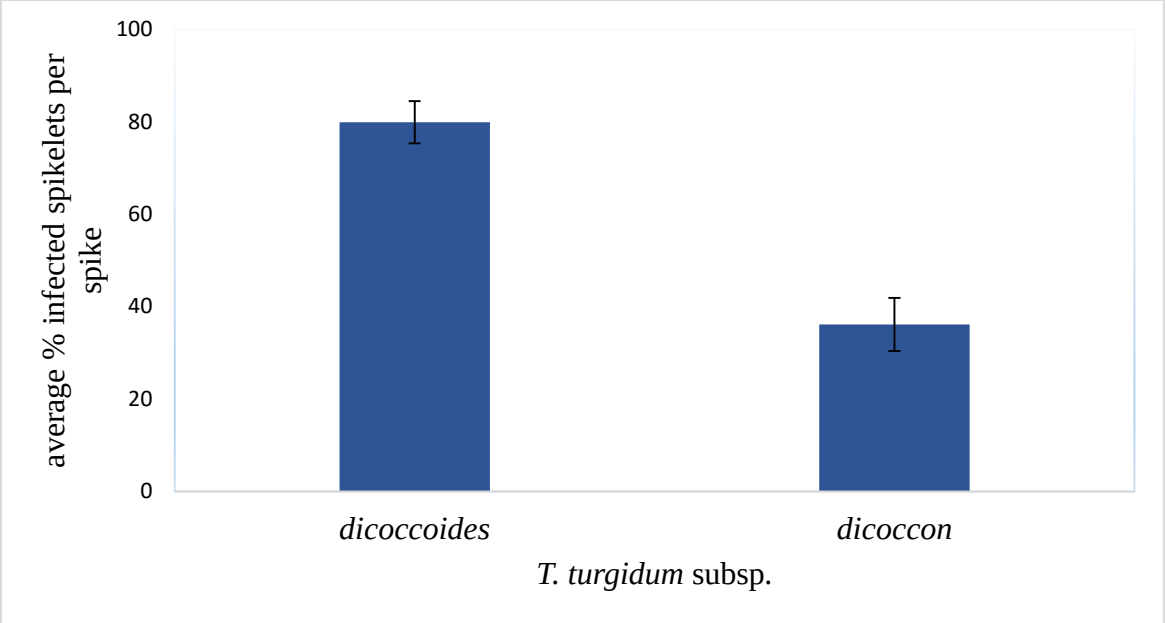

Figure 2-4: Comparison of response to FHB inoculation among *T. turgidum* subsp. *dicoccon* and *T. turgidum* subsp. *dicoccoides.* X-axis: percentage infected spikelets, Y-axis: *T. turgidum* subsp. Bars represent standard error.

2.4.2 Leaf rust screening

At seedling stage, genotypes varied in response to *P. triticina*. The resistant check SY Wolf, and susceptible checks McNair and Morocco gave highly resistance and susceptible reaction respectively as expected (Figure 2-5). Out of 84 mini core accessions screened, only two accessions (2.6%) viz. KU11830 and PI244061 were found to be highly resistant. Another six accessions (7.9%) demonstrated resistant reaction. All resistant accessions belong to subspecies *durum*. In addition, nine accessions (11.8%) were moderately resistance, 18 accessions (23.7%) were the moderately susceptible type and majority 49 (64.5%) of the accessions were highly susceptible to leaf rust (Figure 2-6). Only a few *T. turgidum* subsp. *durum* accessions were resistant whereas the majority of the *T. turgidum* subsp. *dicoccoides*, *T. turgidum* subsp. *dicoccon* and other accessions

which include (*T. turgidum* subsp. *polonicum*, *carthilicum*, *turanicum*, *paleocochicum*, *abyssinicum* and *pyradmidale*) were moderate to highly susceptible (Figure 2-6).

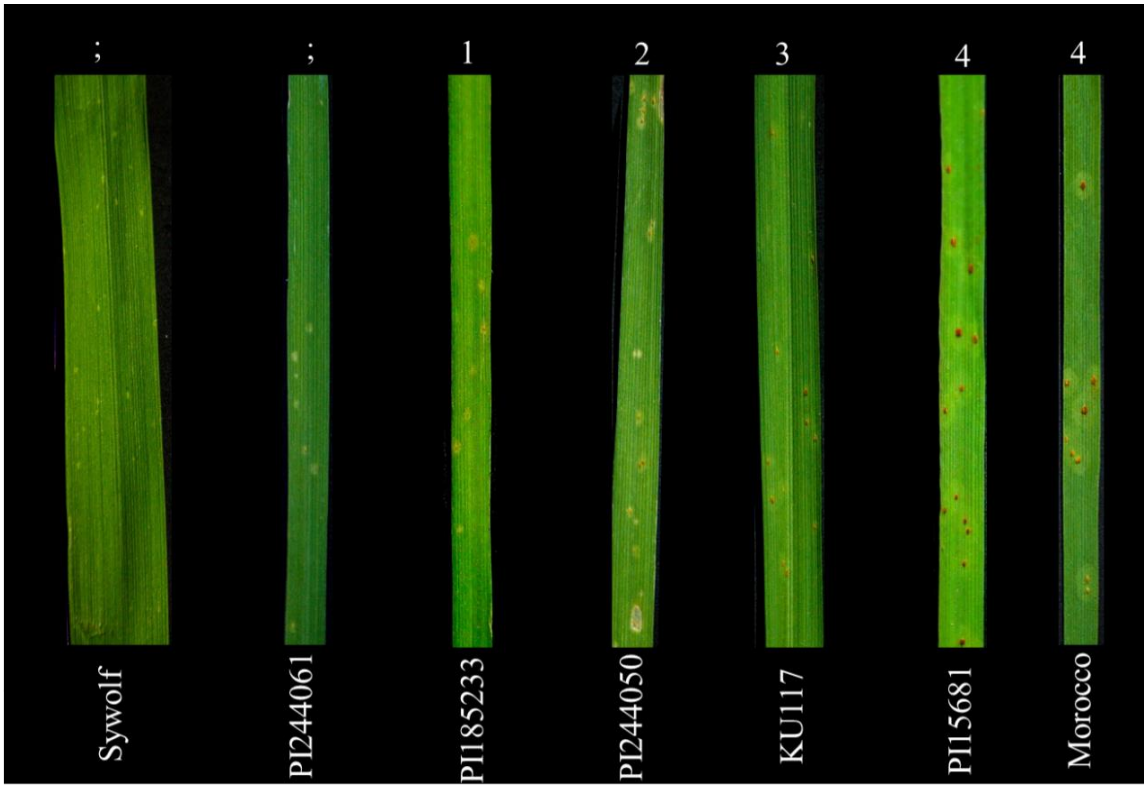

Figure 2-5: Response to leaf rust screening at seedling stage. Scoring scale is based on [224]. SY Wolf is the resistant check and Morocco is the susceptible check.

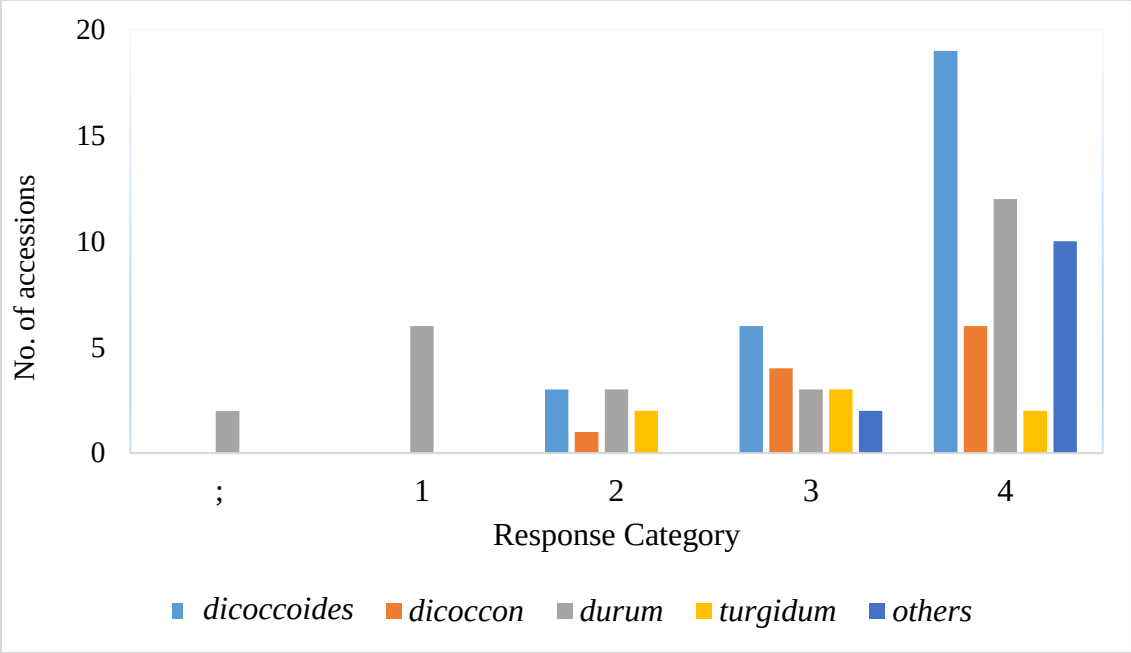

Figure 2-6: Distribution of accessions of different *T. turgidum* subsp. among different response categories of seedling leaf rust. Others include *T. turgidum* subsp. *polonicum*, *carthilicum*, *turanicum*, *paleocochicum*, *abyssinicum* and *pyradmidale*.

In field screening, a wide range in disease severity ranging from 5 to 100% was observed among 74 genotypes evaluated. Six accessions (8.2% of total) were found to be resistant including one - *T. turgidum* subsp. *abyssinicum* (KU7348), four - *T. turgidum* subsp. *durum* (Cltr1471, PI244061, PI185233, Cltr6870), and one - *T. turgidum* subsp. *turgidum* (PI134951). Severity in these accessions ranged from 5 to 10%. Another 14 accessions (19.1% of total) were found to be moderately resistant, seven (9.5% of total) moderately susceptible and majority 36 accessions (49% of total) were susceptible. Species-wise distribution among different response categories is presented in Figure 2-7. Similar to seedling screening, majority of the resistant accessions were from *T. turgidum* subsp. *durum* and most of the *T. turgidum* subsp. *dicoccoides* accessions were susceptible.

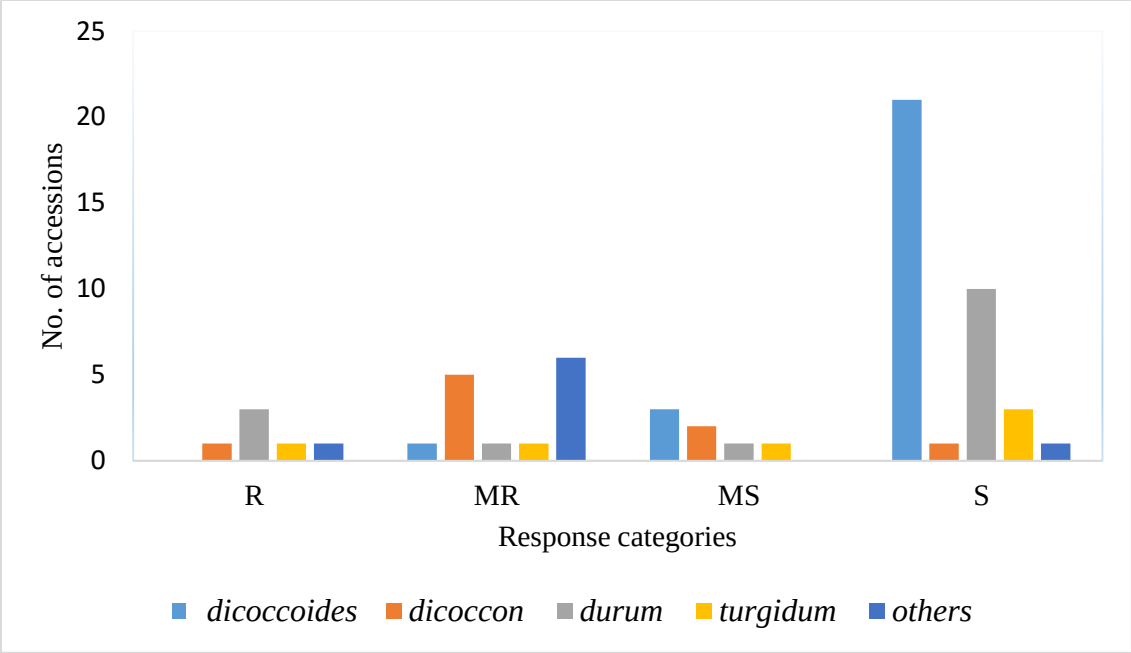

Figure 2-7: Distribution of accessions of different *T. turgidum* subsp. among different response categories of leaf rust resistance in the field. Others species includes *T. turgidum* subsp. *polonicum*, *carthilicum*, *turanicum*, *paleocochicum*, *abyssinicum* and *pyradmidale*.

Three T. *turgidum* subsp. *durum* accessions viz. Cltr4071, PI244061, and PI185233 showed consistent resistant reaction at seedling (greenhouse) and at adult plant stage (field). In addition, another 3 accessions (Cltr6870, KU7348, and PI134951) showed susceptible reaction at seedling stage but showed a resistant response at adult plant stage, this could be further investigated for adult plant resistance genes (Table 2-4).

Table 2-4: Resistant and moderately resistant accessions to leaf rust at seedling stage and at adult plant stage identified from the mini core set of *T. turgidum* subsp.

Accession	*T. turgidum* subsp.	Origin	Seedling response	Adult stage response
Cltr1471*	*durum*	Algeria	1 - R	5R
PI244061*****	*durum*	Yemen	; - HR	5R
PI185233*****	*durum*	UK	1 - R	10R
KU15591	*durum*	Egypt	1 -R	20S
PI60741	*durum*	Egypt	1 - R	20S
PI8898	*durum*	India	1 - R	10S
KU3701	*durum*	Turkey	1 - R	40MS
KU11830	*durum*	Greece	; - HR	-
Cltr6870†	*durum*	Tunisia	2.1 - MS	5R
KU7348†	*abyssinicum*	Ethiopia	3 - MS	5R
PI134951†	*turgidum*	Portugal	2.2 -MS	10R

* Resistant at both seedling stage and adult stage, † Resistant at adult stage but susceptible at seedling stage

2.4.3 Tan spot screening

Among the 84 accessions screened from the mini core, a varied response from susceptible to resistance was observed against *Pyrenophora tritici-repentis* race 5 (*PTR* race 5). Nearly 15% (13 accessions) of the accessions were resistant with the majority (nine) of the accessions of *T. turgidum* subsp. *dicoccoides* and two each of *T. turgidum* subsp.

dicoccon and *T. turgidum* subsp. *turgidum*. Another 18% (15 accessions) showed moderately resistance response whereas 29 accessions (34%) and 27 accessions (32%) showed moderately susceptible and susceptible response respectively. Among subspecies majority of the resistant or moderately resistant accessions belonged to *T. turgidum* subsp. *dicoccoides* and majority of the susceptible or moderately susceptible accessions belonged to *T. turgidum* subsp. *durum* (Figure 2-9).

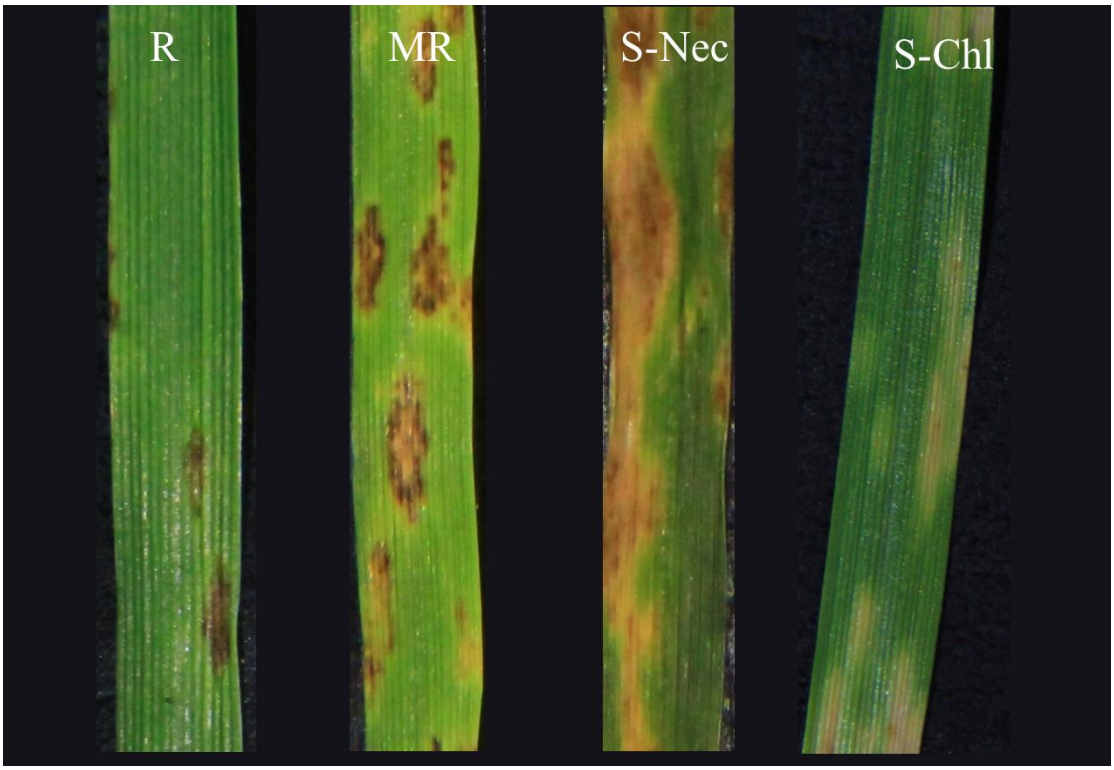

Figure 2-8: Response to *Pyrenophora tritici-repentis* race 5 (*PTR* race 5). Left to right: R is the resistant reaction on KU1974 (*T. turgidum* subsp. *dicoccoides*), MR reaction on MG43330-66 (*T. turgidum* subsp. *dicoccoides*), S-Nec is a necrotic susceptible reaction on MG5293-1 (*T. turgidum* subsp. *dicoccon*) and S-Chl is a chlorotic reaction on KU15493 (*T. turgidum* subsp. *dicoccoides*)

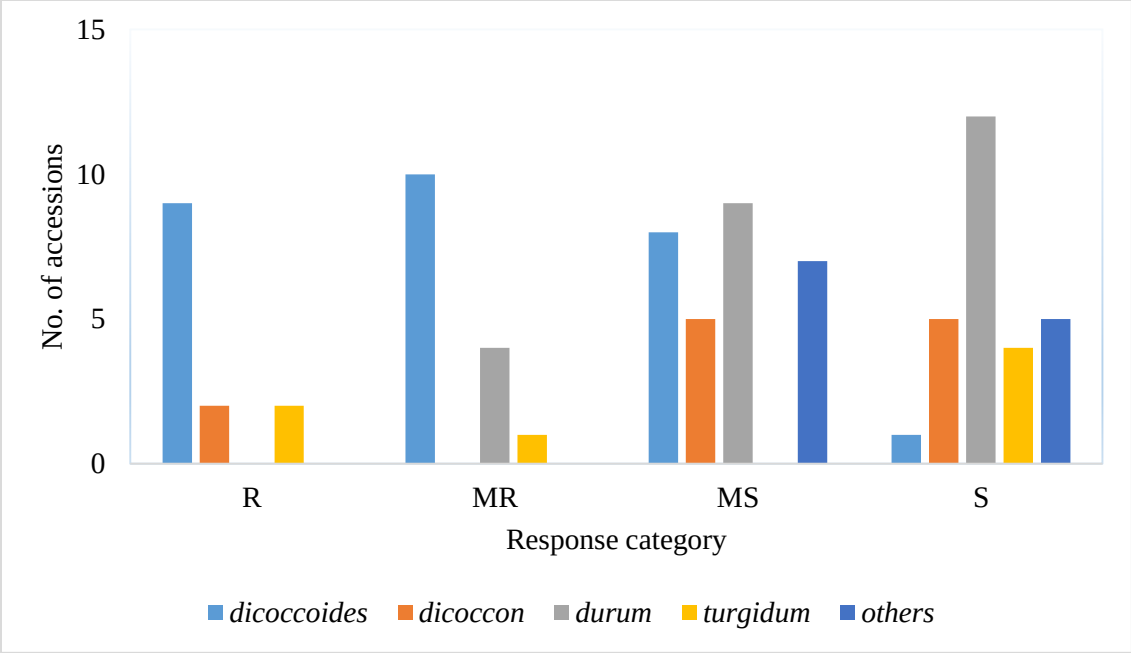

Figure 2-9: Distribution of accessions of different *T. turgidum* subsp. among different response categories of *Pyrenophora tritici-repentis* race 5 (*PTR* race 5) resistance. Others species includes *T. turgidum* subsp. *polonicum, carthilicum, turanicum, paleocochicum, abyssinicum* and *pyradmidale*.

Two types of susceptible reactions were reported against *PTR* race 5, chlorotic and necrotic (Figure 2-8). Among 84 accessions evaluated, 37 accessions showed a chlorotic reaction and 38 showed a necrotic reaction. Interestingly, all the *T. turgidum* subsp. *dicoccoides* susceptible accessions showed chlorotic reaction as opposed to *T. turgidum* subsp. *dicoccon* accessions which showed a necrotic reaction (Figure 2-10). For rest of the subspecies, no conclusive result was obtained either because number accessions were low or accessions were equally distributed in both necrotic and chlorotic categories.

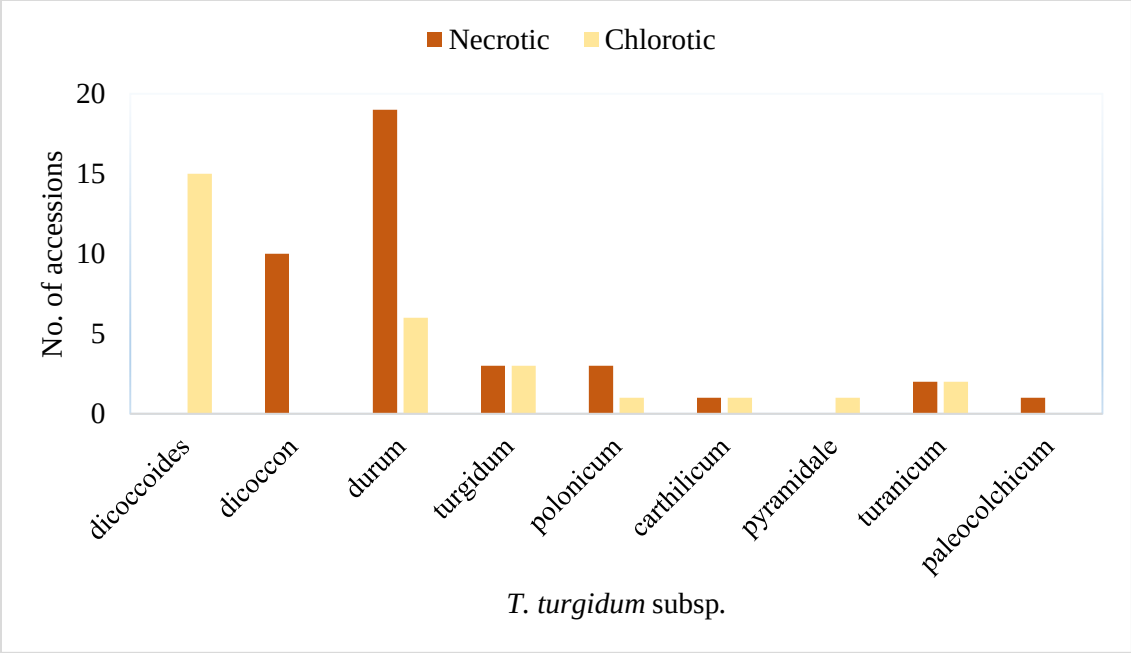

Figure 2-10: Distribution of susceptible accessions among necrotic and chlorotic response category towards *Pyrenophora tritici-repentis* race 5 (*PTR* race 5).

2.5 Discussion

Fusarium head blight, leaf rust, and tan spot are among the major devastating diseases of wheat leading to significant yield losses in the USA as well as worldwide [134,135,170]. Host resistance is one of the best approaches to combat these ever-evolving fungal diseases. Integrating QTLs/genes from diverse backgrounds increases the durability of resistance. Identification of novel sources of resistance against all three diseases from tetraploid ancestral species (*T. turgidum* subsp.) can help in enhancing the level of resistance in durum and bread wheat. We evaluated the mini core set of 95 accessions representing the genetic and geographic diversity of 1,890 accessions, which likely had a good chance of identification of resistance sources while reducing the workload [75].

Currently, for FHB most of the resistance comes from the hexaploid background [156] with only a few examples in tetraploid species [227]. Identification of resistant *T.*

turgidum subsp. sources is not only important for durum wheat breeding but these sources will also increase aura of resistance diversity in hexaploid wheat germplasm. After screening mini core set accessions for Type II resistance against FHB we identified three resistant accessions namely MG5293-1, KU1058 and MG5416-1 with disease severity less than 15%. Interestingly, all three accessions belong to *T. turgidum* subsp. *dicoccon.* So far only two sources of resistance have been identified in *T. turgidum* subsp. *dicoccon* background [228,229], thus our identified accessions will add to that scarce list. MG5293-1 is an accession from Italy, KU1058 is from Spain and MG5416-1 is of unknown origin. As most of the FHB resistance sources are of Chinese origin, diverse origin of our identified FHB resistant accessions suggests these accessions may carry novel resistance QTL/genes. Also, MG5293-1 and MG5416-1 were reported to be high in protein [230], therefore the transfer of resistance genes/QTLs into adapted germplasm of wheat may lead to increased protein content too, provided genes underlying these two traits are linked.

Identification of novel resistance sources against leaf rust is also very crucial, as *P. triticina* is known for high levels of virulence and wide adaptation in different climatic conditions [231]. In present study resistance against leaf rust was investigated both at seedling stage as well as an adult stage in the field conditions. We identified six accessions demonstrating resistance response at adult stage and eight at seedling stage. Among these, three accessions viz. Cltr4071, PI244061, and PI185233 were resistant at both seedling and adult stage. All three accessions are of *T. turgidum* subsp. *durum* type thus can be directly incorporated in durum wheat breeding. Other accessions which were

susceptible at seedling stage but resistant at adult stage may carry resistance genes/QTLs for adult plant resistance, however, this may need further investigation.

Resistance against tan spot is also important especially in the light of fact that it is caused by necrotrophic fungus *Pyrenophora tritici-repentis* (*PTR*) which can survive on residues as saprophytes thus can be a devastating disease in conservative agriculture systems [170]. In our study, we evaluated 84 accessions of a mini core set against *PTR* race 5 and identified 13 resistant accessions. The majority are from the *T. turgidum* subsp. *dicoccoides* suggesting resistance is much more prevalent in *T. turgidum* subsp. *dicoccoides* as compared to other subspecies. Another interesting finding in this study was the necrotic or chlorotic response of different species. It has been reported in a number of studies that *PTR* race 5 can cause necrotic response in the tetraploid wheat background and chlorotic in the hexaploid wheat background [188]. In our study, all the *T. turgidum* subsp. *dicoccoides* demonstrated a chlorotic response but *T. turgidum* subsp. *dicoccon*, which is cultivated form of *T. turgidum* subsp. *dicoccoides* were found be necrotic. It has been confirmed that fungus carries two virulence genes, one causing necrosis and other causing chlorosis and susceptibles genotypes carry corresponding susceptible genes [188]. From our results, it seems plausible that domestication and further evolution played a role in this host-fungus interaction leading to contrasting response in *T. turgidum* subsp. *dicoccon* as compared to *T. turgidum* subsp. *dicoccoides*.

2.6 Conclusions

In this study, a mini core collection of *T. turgidum* subsp. was characterized for devastating diseases of wheat viz. *Fusarium* head blight, leaf rust, and tan spot (race 5). Resistant sources against each disease were identified; three accessions against FHB,

three accessions against leaf rust and nine accessions against the tan spot. It was discovered that resistance against FHB is more prevalent in *T. turgidum* subsp. *dicoccon* as compared to *T. turgidum* subsp. *dicoccoides.* Resistance against leaf rust was majorly found in *T. turgidum* subsp. *durum* and resistance against tan spot (*PTR* race 5) is most prevalent in *T. turgidum* subsp. *dicoccoides*. We also propose that different response of *T. turgidum* subsp. *dicoccoides* (chlorotic) and *T. turgidum* subsp. *dicoccon* (necrotic) susceptible accessions against *PTR* race 5 can be used as a model to study the plant-fungi coevolution.

Chapter 3

Evaluation and identification of ancestors and wild relatives of wheat for their interaction with diazotrophs

3.1 Abstract

Nitrogen is an essential element in plant development and a limiting factor in plant growth. The inability of modern wheat cultivars to interact with nitrogen-fixing bacteria (diazotrophs) makes them dependent on synthetic fertilizers. Excessive fertilizer use impacts our environment in every possible way. Exploiting natural diversity of wild relatives of wheat is one the feasible approach to identify genotypes with efficient association with diazotrophs. In this study, we investigated modern cultivars and wild/domesticated relatives of wheat for their interaction with diazotrophs using ^{15}N dilution technique. Soil mixture (soil + growing mix) was used to mimic natural soil conditions with the addition of *Azosprillium sp.* We observed significant differences for $\sigma^{15}N$ (^{15}N content) among different species in 30-days old seedlings. Lower $\sigma^{15}N$ indicates a higher possibility of biologically fixed nitrogen (BNF). All wild species, diploid or tetraploid, had a significantly low concentration of ^{15}N as compared to modern cultivars and their corresponding domesticated species, indicating that wild species have gained a portion of N requirement as BNF. *Triticum boeticum* (A^mA^m, $\sigma^{15}N = 20.85$) accessions gained a higher proportion of N as BNF as compared to domesticated form *T. monococcum* (A^mA^m, $\sigma^{15}N = 26.67$). Similarly, *T. turgidum* subsp. *dicoccoides* (AABB, $\sigma^{15}N = 16.44$) gained larger proportion of N from BNF as compared to domesticated *T. turgidum* subsp. *dicoccon* (AABB, $\sigma^{15}N = 26.32$). Modern cultivars (*T. aestivum,* AABBDD, $\sigma^{15}N = 31.74$) and landraces ($\sigma^{15}N = 30.81$) were unable/less efficient to

interact with diazotrophs. We also identified two accessions of *T. turgidum* subsp. *dicoccoides* which gained much higher proportion of N ($\sigma^{15}N$ = 9.35 and 10.03) from BNF then all other accessions. These efficient accessions can be further investigated to identify underlying genes, which can be exploited for the improvement of modern cultivars. We also propose that identification of novel soil diazotrophs from the niche of these wild relatives also holds a potential.

3.2 Introduction

Wheat (*Triticum aestivum* L.), the third most staple food worldwide; provides one-fifth of the calories and 20% of the protein for more than 4.5 billion people [1]. Annual wheat yield improvement of an average 1% will be insufficient to feed the rising population [2]. Climate change, soil degradation, loss of arable land, unavailability of irrigation waters, evolving pathogens and increasing fertilizer costs further aggravate this scenario of the demand-production gap [3]. A steady increase in wheat productivity of at least 2% per year is required to meet the growing wheat demands [4]. Furthermore, wheat improvement must be resource efficient and sustainable.

Production of wheat and other cereals is highly dependent on synthetic nitrogen fertilizers, and the yield increments due to these fertilizers come with considerable environmental impacts [200]. Nitrogen is applied in the plant available form -NO_3 or NH_4. On an average only 30-50% of the applied nitrogen is absorbed by the plants, rest leads to water contamination through leaching of nitrates and surface runoff; and global warming and ozone layer depletion through ammonia volatilization [201]. Freshwater contamination with nitrogen leads to algal blooms, which results in loss of marine life and if this contaminated water is ingested by infants it may lead to lethal diseases such as

blue baby syndrome. Furthermore, just in U.S. corn production alone, synthetic fertilizer (Urea) production consumes more than 30% of the non-renewable energy and leads to 70% of the greenhouse gas emissions [232]. Additionally, increasing production cost of the inorganic fertilizers makes them unaffordable for many farmers. Therefore, we must remove our dependency on synthetic fertilizers to have sustainable agriculture.

Exploiting wheat-diazotrophic associations can be one of the sustainable approaches. Diazotrophs are bacteria or archaea which can fix atmospheric nitrogen [202]. They can enzymatically reduce atmospheric N_2 to ammonia, making it accessible to the plants, this process is unique to bacteria and archaea [203]. Plants benefit from this process by developing endosymbiotic, associative or endophytic relations with bacteria. Endosymbiotic associations between legumes and nitrogen-fixing bacteria (*Rhizobium*) are the most efficient associations. Bacteria reside inside the plant root nodules, which provide oxygen-free conditions for nitrogen fixation and in exchange bacteria provide plants with fixed nitrogen in the form of ammonia. Up to 70% of the nitrogen needs for symbiotic plants are fulfilled by these bacteria, making them independent of synthetic nitrogen fertilizers [204].

But these highly efficient endosymbiotic relationships have not been reported in the family Gramineae-grass family. However, numerous studies have reported endophytic or associative associations of plant growth promoting bacteria's with grass family. Among them, a few wheat-bacterial associations also have been reported such as *Azorhizobium caulinodans* colonization [205] and others. These studies demonstrate the potential of diazotrophs interactions in wheat. All these studies were based on modern wheat cultivars and no study has been done to see variation in wild relatives of wheat. We hypothesize

that more efficient diazotrophic associations in wild relatives are more likely to occur as compared to modern cultivars as they grow in natural ecosystems independent of synthetic fertilizers. In this study, an attempt was made to see diazotrophic interaction diversity among different wild species of wheat in contrast to modern and cultivated species. By studying these variations we can identify underlying genes and transfer those genes to high yielding cultivars.

In order to quantify biologically fixed nitrogen and discriminate it from added soil nitrogen or synthetic fertilizer, ^{15}N dilution technique [233] can be used. There are two stable isotopes of nitrogen: ^{14}N and ^{15}N. In the atmosphere, the heavy isotope, ^{15}N, occurs at a constant abundance of 0.3663 atoms%. If the ^{15}N abundance in plant-available soil or growing media is higher than 0.3663 atoms%, then we can estimate the plant N derived from each source (atmosphere and soil). Plants with lower ^{15}N atom% as compared to soil are likely to have obtained fixed N from associated bacteria (Figure 3-1). There is the only small difference between the natural abundance of ^{15}N between soil N and atmospheric N_2. For more precise and accurate quantification of biologically fixed N soil is enriched with labeled ^{15}N fertilizer.

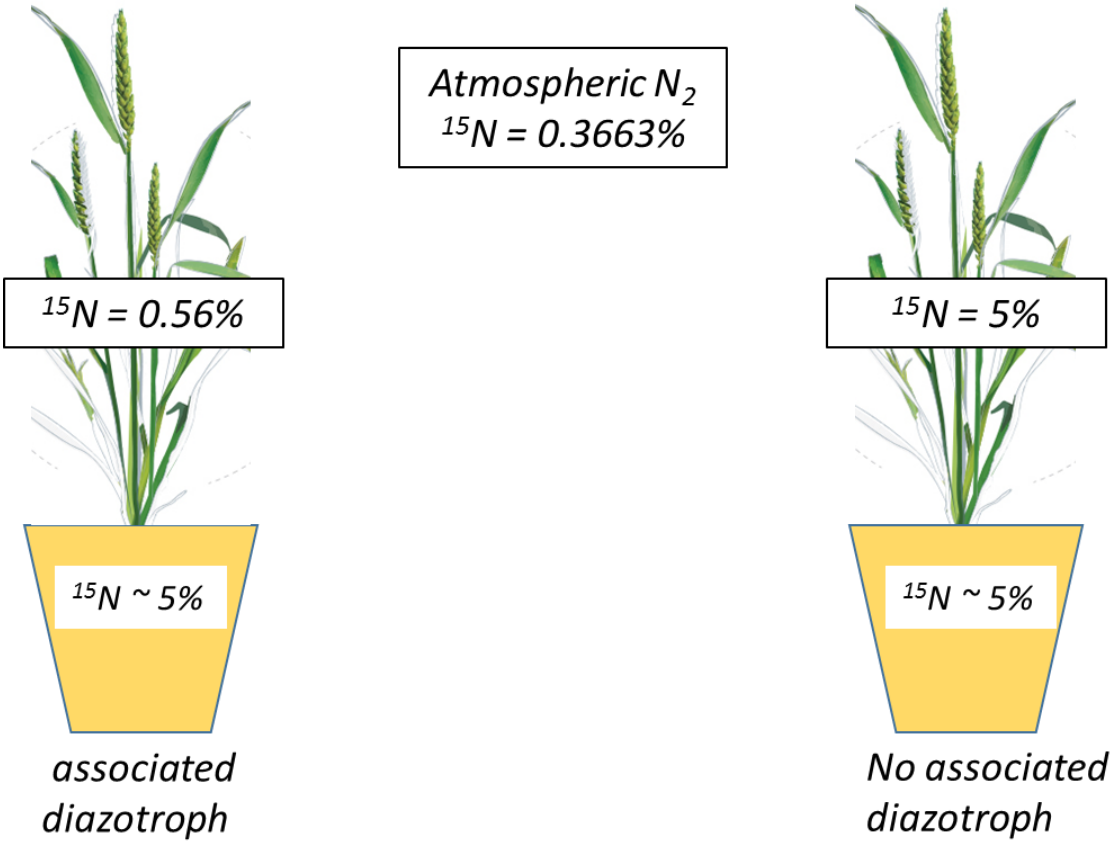

Figure 3-1: Diagrammatic representation of principle behind the ^{15}N dilution technique. Soil (in pots) is enriched with 5% ^{15}N labeled fertilizer. (A) Plant inoculated with diazotrophs have lower ^{15}N content (0.56%) indicating N derivation from the atmosphere. (B) The uninoculated plant has higher ^{15}N content (5%), which indicates that no nitrogen fixation in this system.

3.3 Material and methods

3.3.1 Plant material

A diverse germplasm set of wheat and its relatives was collected to have a good coverage of the genetic diversity of the wheat gene pool (Table 3-1). It includes pre-domesticated "A" genome diploid species [*Triticum urartu* (A^uA^u) and *Triticum boeticum* (A^mA^m)],

post domesticated "A" genome diploid species [*Triticum monococcum* (A^mA^m)], pre-domesticated tetraploid species (*Triticum turgidum* subsp. *dicoccoides* (AABB)], post domesticated tetraploid species [*Triticum turgidum* subsp. *dicoccon* (AABB)]. To have a comparison with wild relatives, pre-green revolution (Watkin collection) and modern-day hexaploid wheat varieties (AABBDD) were also introduced in the set. The only man-made cereal triticale [X *Triticosecale* (AABBRR)] was also included in the set for its stress tolerance abilities.

Table 3-1: A diverse set of *Triticum* species evaluated for association with diazotrophs. This includes pre domesticated diploid (A^uA^u and A^mA^m) and post domesticated diploid (A^mA^m) wild relatives of wheat; pre and post domesticated tetraploid wild relatives (AABB), and hexaploid wheat landraces (AABBDD) and cultivars (AABBDD). We also screened triticale (X *Triticosecale*) due to its stress tolerant abilities.

Genus	***Species***	***Genome***	***Type***	***No. of accessions***
Triticum	*monococcum*	A^mA^m	Domesticated	4
Triticum	*boeticum*	A^mA^m	Wild	4
Triticum	*urartu*	A^uA^u	Wild	4
Triticum	*aestivum*	AABBDD	Landraces	4
Triticum	*aestivum*	AABBDD	modern wheat	4
Triticum	*t.* subsp. *dicoccon*	AABB	Domesticated	4
Triticum	*t.* subsp. *dicoccoides*	AABB	Wild	4
X Triticosecale	-	AABBRR	Domesticated	4
Total				32

3.3.2 Growth medium

In order to mimic natural soil conditions, a mixture of garden soil and sungro 360 growing mixture was used as a growth medium for plants. Garden soil:sungro 360 were mixed in 1:1 ratio by volume and then continuously mixed for 3-4 times. In garden soil, there is 0.09% N, 0.05% P_2O_5 and 0.07% of K_2O. 360 growing mixture contains 35 - 45% sphagnum peat moss, composted bark, and vermiculite. Soil mixture was then filled into small cones (2 inches in diameter and 8 inches in height) which were used for planting.

3.3.3 Plant growth conditions

Each accession was repeated twice, each replication consist of three plants in a single cone. After planting, plants were watered with distilled water and grown in a greenhouse at 22 - 25 ºC day (14 hours) temperature and 18 - 22 ºC night (10 hours) temperature. After plants finished their seed reserves for nutrients and have a well-developed root system, approx. 10 days after planting, root zones were spiked with 1ml of labeled ammonium nitrate (1% ^{15}N). Following the spiking, 1ml inoculation containing *Azosprillium* subsp. was poured into each pot.

3.3.4 Tissue collection and ^{15}N analysis

Approx. four weeks after planting, young and healthy leaf tissue from each cone was collected in glass vials and dried at 65ºC for 48h. Dried leaf tissues were ground using a tissue lyser and 10mg of tissue powder for each rep was assayed for ^{15}N content by using Isotope Ratio Mass Spectrometry (IRMS) at SDSU.

3.3.5 Statistical analysis

$\sigma^{15}N$ value for each replication of each accession was calculated using following equation [234] :

$$\delta 15N\ (‰) = [(\text{sample atom\%15N} - 0.3663)/0.3663] \times 1000$$

Data was analyzed in R for differences among species and among accessions by performing Analysis of variance (ANOVA) based on linear mixed model 1. Accessions were considered to be nested under species. Species effect was treated as fixed effect and accessions effect was treated as a random effect. Pairwise comparison among species and among accessions was performed using Fisher's least significant differences (LSD) test.

$$Y_{ij} = \mu + S_i + L_{j(i)} + e_{ij} \qquad \textit{Model 1}$$

Y_{ij} : ^{15}N value for i^{th} species, j^{th} accession.

μ : Population mean or grand mean.

S_i : i^{th} species effect.

$L_{J(i)}$: J^{th} accession effect nested under i^{th} species.

e_{ij} : random error.

3.4 Results

A large variation for $\sigma^{15}N$ measurements was found between different species as well as within species. Total nitrogen uptake did not vary significantly between or within species. Approx. 61% of the total variation for $\sigma^{15}N$ values was explained by the species and approx. 29% of the variation was explained by the accessions (Table 3-2). Lower the $\sigma^{15}N$ value, larger is the likelihood that plant is getting a share of N from biologically fixed nitrogen. Among all species, the average ^{15}N concentration was found to be the

lowest in *T. turgidum* subsp. *dicoccoides* and highest in modern cultivars of winter wheat (Figure 3-2).

Table 3-2: ANOVA table describing variance explained by the species and the accessions for ^{15}N values. Each accession was replicated twice. The analysis is based on nested CR design, accessions being nested under species.

Source	Df	TSS	MSS	F-value	P-value
Species	7	1643	234.71	4.60	$6.17e^{-5}$ *
Accessions	25	771.9	30.88	3.97	$1.83e^{-5}$*
Residuals	31	240.9s	7.77		

*Significant at α-level of 0.05.

Diploid wild species, *T. boeticum* and *T. urartu* had significantly low σ ^{15}N concentration than domesticated diploid species (*T. monococcum*). Similarly, σ ^{15}N concentration in wild tetraploid (*T. turgidum* subsp. *dicoccoides*) was significantly lower than domesticated tetraploid (*T. turgidum* subsp. *dicoccon*). ^{15}N values in modern winter wheat cultivars, landraces (Watkin collection), accessions of triticale, *T. monococcum*, and *T. turgidum* subsp. *dicoccon* were significantly higher than other wild species except for *T. turgidum* subsp. *dicoccon*'s overlap with *T. turgidum* subsp. *boeticum* (Figure 3-2).

Based on pairwise comparison among all accessions, two accessions of *T. turgidum* subsp. *dicoccoides* (PI538719 and PI428057) had significantly less accumulation of ^{15}N than rest of the tested accessions. Watkin collection accession - 1190004, Triticale accession - PI547164 and winter wheat variety - Alliance had significantly high ^{15}N values than rest of the group (Figure 3-3).

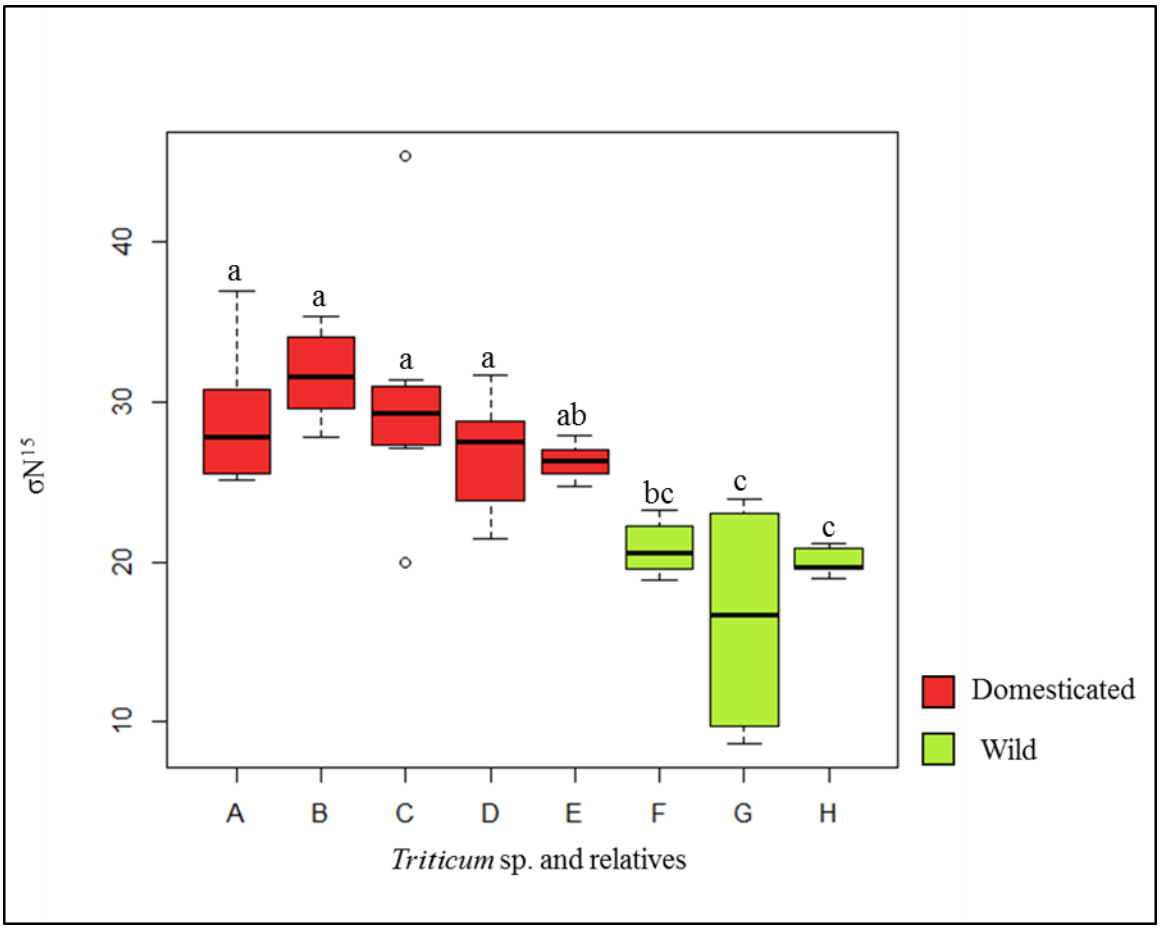

Figure 3-2: Boxplot representing species average for $\sigma^{15}N$ values. A) X *Triticosecale*, B) modern winter wheat cultivars, C) landraces from Watkin collection, D) *T. turgidum* subsp. *dicoccon*, E) *T. monococcum*, F) *T. boeticum*, G) *T. turgidum* subsp. *dicoccoides*, and H) *T. urartu*. Color code – green: wild species and red: domesticated species. Associated small letters with boxes denote different groups based on LSD values, different letter groups are significantly different.

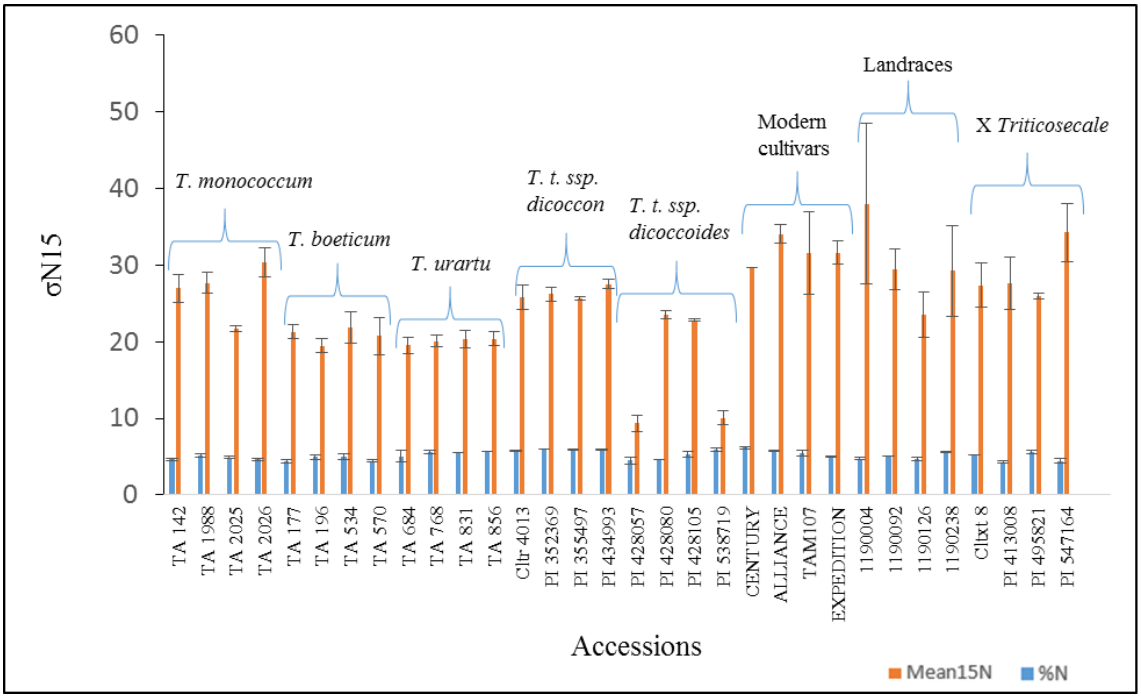

Figure 3-3: Variation for $\sigma^{15}N$ and total %N values among accessions of different species tested in this study. Note: %N is approx. same in all accessions but the large variation for $\sigma^{15}N$ can be seen.

3.5 Discussion

Nitrogen is an essential element in plant growth, representing 2% of the total plant dry matter in the food chain [198]. Though N makes about 80% of the atmosphere, the inability of the plants to fix free nitrogen makes them dependent on synthetic fertilizers [199]. Due to adverse effects of nitrogen fertilizers, we have to cut down the use of synthetic fertilizers [200]. Exploiting natural ability of wild relatives to better access available soil nitrogen and/or to interact with diazotrophs can be one of the sustainable ways. In the current study, we assessed the variation for diazotrophic interaction among different wild relatives of wheat as well as in modern wheat varieties using ^{15}N dilution technique. Among the analyzed species we observed not much variation for total nitrogen

content suggesting no species were better or worse at up taking and utilizing nitrogen. However, there was a large variation in ^{15}N content among different species.

It is interesting to note that all the wild species gained much nitrogen from low ^{15}N source as compared to modern or domesticated species (Figure 3-2). *T. boeticum* is wild form of *T. monococum* and *T. dicoccoides* is wild form of *T. dicoccon,* both of the wild species have gained more nitrogen from low ^{15}N nitrogen sources as compared to their corresponding domesticated parents. It is possible that wild relatives were able to better interact with diazotrophs which in our case were *Azosprillium* sp. or other soil-borne bacterial species. This points out that cultivation or domestication might have broken the beneficial plant-diazotrophic bond or with the application of synthetic nitrogen fertilizers, we have been unknowingly selecting against such associations. As resource allocation due to domestication changes, therefore, it is possible that nutrient supply to root associating bacteria might be cut down by the plants and that portion was transported to seed reserves.

If we look at the modern wheat varieties, landraces and triticale, similar trends are observed as with domesticated species. These accessions are so dependent on synthetic fertilizers and their ^{15}N values are much higher than wild species. It is clear as these accessions are bred to be fertilizer responsive and they are found to behave as fertilizer dependent in this experiment.

A better interaction among the wild relatives of wheat and diazotrophs can be a great source of developing synthetic nitrogen independent (or at least less dependent) wheat cultivars. As fertilizers costs are going high and for exploiting the marginal lands we need wheat cultivars that can better interact with the diazotrophs. Our study suggests that

we should conduct broad analysis of wild wheat species to identify better genotypes that could help in understanding the mechanism of wheat diazotrophs interaction.

At last, we also like to point out the limitations of ^{15}N dilution technique as a way to quantify biologically fixed nitrogen. Though this method can estimate the BNF nitrogen, it is a costly ($15/sample) and needs a lot of precise addition of all other nutrients along with nitrogen. This technique works perfectly for legumes or in case symbiotic associations but for associative systems where BNF is fixed in traces, this method should be chosen carefully. Also, it may be better to use gene expression analysis in the rhizosphere for associative interactions.

3.6 Conclusions

In this study interaction between diazotrophs and wild/domesticated relatives of wheat was assessed. Interestingly, we found that wild relatives of wheat can interact better with diazotrophs as compared to domesticated or cultivated species. This suggests that domestication might have impacted the wheat-diazotrophic interactions in a negative way. We also identified two accessions of *T. turgidum* subsp. *dicoccoides* (PI 428057 and PI 538719) which gained much higher BNF fixed nitrogen than any other accession tested. These accessions may be a great source for efficient diazotrophic associations.

In order to restore this great association ability in the modern wheat cultivars, wild relatives such as *T. dicoccoides, T. urartu,* and *T. boeticum* seems a promising source. Novel species of diazotrophs can be discovered from natural soil conditions and tested with specific wild species and eventually, underlying genes of association with diazotrophs can be transferred to modern cultivars.

Chapter 4

Assessing genetic diversity in rye and characterizing genomic regions conferring resistance to tan spot

4.1 Abstract

Rye (*Secale cereale* L.) is known for its wide adaptation due to its ability to tolerate harsh winters and grow in semiarid areas. To better assess the diversity in rye and to utilize it for wheat improvement we genotyped by sequencing (GBS) 178 geographically diverse accessions of *Secale* sp. from U.S. National Small Grains Collection. We analyzed the genetic diversity in the set using 4,037 high-quality SNPs (single nucleotide polymorphisms) and developed a mini core set of 32 accessions of rye that represents more than 95% of the allelic diversity (PIC = 0.25) of *Secale cereale* subsp. *cereale*'s global collection (PIC = 0.26). Three major clusters separating *S. cereale L.* from *S. strictum and S. sylvestre* were observed by PCA and STRUCTURE analysis, however, no correlation of genetic clustering with geographic origins and growth habit (spring/winter) was observed. The collection was evaluated for response to *Pyrenophora tritici-repentis* race 5 (*PTR* race 5) and nearly 32% and 27% accessions were resistant and moderately resistant respectively, whereas 24% and 14% accessions were moderately susceptible and susceptible respectively. Genome-wide association study (GWAS) was performed on *S. cereale* subsp. *cereale* using 4,037 high-quality SNPs. Two QTLs conferring resistance to *PTR* race 5 were identified (p= <0.001) using mixed linear model (GAPIT) on chromosomes 5R and 2R. The QTLs *QTs.sdsu-5R* and *QTs.sdsu-2R* explained 13.11% and 11.62% of the variation respectively. Comparative rye-wheat syntenic analysis showed a high correspondence between rye-wheat with known rearrangements as

expected. *QTs.sdsu-2R* is mapped in the syntenic region corresponding to the chromosome group 2 of wheat which harbors tan spot (*PTR* race 5) insensitivity gene (*tsc2*) and several other tan spot resistance genes/QTLs. The rye association set and the mini core set identified in our study could be utilized for genetic characterization of useful traits and genetic improvement of rye, triticale, and wheat.

4.2 Introduction

Rye (*Secale cereale* L.) belongs to the Triticeae tribe of the family Poaceae [80] and is believed to share a common ancestor with wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) [81]. Most of the species of Genus *Secale* originated in the Middle East, modern-day Turkey [88]. Later along with the dissemination of wheat and barley to Europe and the Western Mediterranean region, rye first came as a weed to these places. From the weedy species of rye, farmers consciously or unconsciously selected a variant with a non-brittle rachis and larger seeds. This selected variant is now classified as *Secale cereale*, the only cultivated species of rye. Due to its resilience, rye first adapted as a secondary crop in the areas with the harsh environment (cold and heat stress), where other staple crops like wheat and barley were not able to survive [88]. Eventually, seeing its versatility, people started cultivating rye in Canada and northern parts of the United States of America. Species of rye are also found in Russia, Japan, Australia and an isolated population is also present in South Africa [83].

In general, the genus *Secale* is classified into four species (GRIN, http://www.ars-grin.gov): *S. cereale* - annual allogamous species, *S. sylvestre and vavilovii* - annual autogamous species and last is perennial wild-type allogamous *S. strictum* [94]. Around the globe, rye is cultivated mainly for food, feed, and pasture; as a cover crop or green

manure crop. Rye based products are a rich source of nutritionally essential compounds like minerals (Zn, Fe, and P), β-glucan (1.3-2.7%), resistant starch and dietary fibers [86,235]. In Europe, rye grain forms a substantial portion of the human (as bread) and animal diet. In North America, rye is preferably grown as a cover crop or as pasture, and its grains are used in livestock feed and alcohol distillation. In drylands of southern Australia, it is grounded to prevent wind erosion. Furthermore, due to its sturdiness, it is also considered as a good pioneer crop to restore the fertility of waste lands [83]. Triticale (X *Triticosecale* Wittmack), a cross between durum wheat (AABB) and rye (RR) further signifies the stress tolerating ability of rye by producing relatively higher biomass and grain yield over the other cereals in dry and cold environments [114]. Through chromosome substitutions or translocations, important genes from rye have been exploited for the improvement of other cereals especially wheat. Crespo-Herrera *et al.* overviewed the rye's importance as a source of biotic stress tolerance [104]. One of the important examples signifying the pest resistance of rye is 1BL.1RS translocation in wheat. Rye chromosome arm 1RS carries savior genes conferring resistance to stem rust (*Sr31*), leaf rust (*Lr26*), powdery mildew (*Pm8*) and yellow rust (*Yr9*) [105–107]. Likewise, there are many other wheat-rye translocations harboring stress-resistance genes that aided in increasing the grain yield and the adaptation potential of bread wheat [109,236–238].

Rye offers a great potential for wheat improvement and should be further explored [115]. Assessing the genetic diversity in rye can aid in broadening the genetic base of rye, better accessing the important genes and easy gene bank management [239]. Genetic diversity analysis involves the comparison of accessions for their similarities and dissimilarities at

the molecular level, to determine the degree of diversity present in the set of accessions. Mining a large collection of accessions could be costly and laborious. Therefore extracting a core set which represents a majority of the entire set's genetic diversity can be a promising methodology [75,124,240]. As, core set or mini core set eliminates redundancy, simplify the exploration of important genes and systematic utilization of germplasm in breeding programs [75].

Among the diploid species of Poaceae family, rye has the largest genome (~7.9 Gbps) [116] and about 90% of the genome is occupied by repetitive sequences [117]. Due to the genome complexity and coupled with regional cultivation, the rye genome has not been extensively studied, unlike other related cereals. Nonetheless, many important genetic diversity studies in rye have been conducted using different marker systems like SSR [94,118–122], AFLP [123], DArT [124,241] and recently SNPs [242]. Majority of these studies either used a limited number of markers covering a small portion of the genome or may have ascertainment bias. GBS (genotyping by sequencing) provides an opportunity for simultaneous SNP discovery across the genome and enables analysis of the genetic diversity, population structure and evolution processes in the crop species. Identifying gene(s) and linked molecular markers to important phenotypic traits could help in crop improvement through marker-assisted tracking of important traits in breeding and wide hybridization. Gene identification also helps in the understanding the molecular mechanism of gene action. Several genetic linkage maps have been developed in rye [243–246] and recently a draft sequence of the rye genome has been produced to facilitate the molecular characterization of economically important traits. Several genes/QTLs have been mapped in rye like plant height [247,248], length of spikes [248]

and the number of spikelets per spike [248], benzoxazinoid content, rust resistance, α-amylase activity, and preharvest sprouting [249]. Further, the availability of large-scale SNP data will enable the characterization and mapping of the genes for important traits using genome wide associate studies (GWAS).

GWAS is based on a simple principle of linkage disequilibrium, tightly linked genes show low linkage disequilibrium (LD), and it is maintained over generations. On the other side, loosely linked loci, present distantly from each other are in linkage equilibrium [250]. GWAS has been used to characterize several economically important traits like yield, disease, pest resistance, and abiotic stress tolerance in many crop species such as rice [251–255], maize [256–262], barley [263–269], wheat [270,271,280,281,272–279]. However, the ability to identify genes/QTLs and linked markers using GWAS has not been exploited in rye. In this study, an attempt was made to map genes/QTLs responsible for tan spot resistance using GWAS methodology. Tan spot is an important disease of wheat caused by a necrotrophic fungus *Pyrenophora tritici-repentis* (*PTR*) causing up to 49% yield loss during favorable conditions [172]. Previously we have reported that though rye can be infected with tan spot, there is a good degree of resistance *to PTR* race 1 and *PTR* race 5 in rye [282]. Identification of genes/QTLs for tan spot resistance in rye could facilitate the development of tan spot resistant wheat, rye and triticale varieties. In this study, we characterized the genetic diversity in the geographically diverse set of rye accessions to develop a mini core set for genetic improvement of rye and wheat. Further, we evaluated the potential of GWAS in identifying genes/QTL conferring resistance to *PTR* race 5 in rye.

4.3 Material and methods

4.3.1 Plant materials

We selected a set of 178 geographically diverse (70 countries) accessions of *Secale* sp. from the USDA National Small Grains Collection (NSGC). A majority of the accessions are from the Middle East (primary center of origin) and Europe (secondary center of origin) (Figure 4-1). Species-wise, 160 accessions are of cultivated rye (*Secale cereale* subsp. *cereale*), nine of wild *S. cereale* subsp., five of *S. strictum*, and two each of *S. sylvestre and S. vavilovi* (Appendix Table 1 and Appendix Table 2). Only *Secale cereale* subsp. *cereale* accessions were employed in developing a rye genome wide association mapping panel and extracting a mini core set.

Figure 4-1: Geographic diversity covered by the selected accessions of the global set as well as accessions of the mini core set. Color code: Red, blue, green, yellow map pin and overlaid yellow triangle correspond to *Secale cereale* subsp., *Secale strictum* subsp., *Secale vavilovii*, *Secale sylvestre* and accessions in mini core respectively. Note: the mini

core set was selected based on hierarchical clustering of 160 *Secale cereale* subsp. *cereale* accessions.

4.3.2 Genotyping and SNP discovery

For DNA isolation we collected young leaf tissues from three-week-old plants of each accession. After isolation of DNA by CTAB method [283], the DNA of each genotype was quantified and normalized to 20ng/ul. GBS was performed by following the double-digestion enzyme protocol on an Ion Proton system for next-generation sequencing [284] at USDA Central Small Grain Genotyping Lab, Manhattan KS. Briefly, the 20ul of thr normalized DNA from each accession was double-digested with restriction enzymes, *PstI* and *MspI* and labeled with two adapters [284,285]. Once the adapters were ligated, the samples were pooled together for PCR amplification and sequencing was done on two flow cells of an Ion Proton Next Generation Sequencer. Non-reference SNP calling was performed using TASSEL 3.0 Universal Network Enabled Analysis Kit (UNEAK) pipeline. Reference-based SNP calling was done with TASSEL 5; as a reference genome, a custom reference genome was constructed from rye genome assembly of 454 sequences available at Plant Genome and Systems Biology (PGSB) website (http://pgsb.helmholtz-muenchen.de/plant/rye/gz/download/) [286].

4.3.3 Population structure and genetic diversity

Basic genetic diversity indices: polymorphic information content (PIC) and Shanon's diversity index (I-index) were calculated. For each SNP, PIC value was calculated using the formulae:

$$PIC = 1 - (p^2 + q^2)$$

Where p and q correspond to the major and minor allele frequency [287]. I-Index for each marker was calculated as follow:

$$I = -\Sigma p_i log_2 p_i$$

Where p_i is the allele frequency of the i^{th} allele at a particular locus [288]. Percentage dissimilarity based principal coordinate analysis (PCA) among and between the species was performed using R-package prcomp [289]. For comparison among accessions, a pairwise genetic dissimilarity (GD) matrix was computed using R-package ape [290]. GD was employed for hierarchical clustering and a neighbor-joining (NJ) tree was constructed using R-package fastcluster [291]. Finally, the tree was pictographically developed using an online tool, Tree of life (iTOL) [292].

Population structure among all *Secale* sp. accessions was analyzed using STRUCTURE software [293]. To decide an optimum number of clusters, we employed DeltaK method described by Evano *et al.* [294]. This method is based on a change in the log probability of the data in question, moving from successive K values. Cluster (K) with the highest value of DeltaK – the estimated likelihood [LnP (D)] – was preferred.

4.3.4 Mini core set of rye

A mini core set was extracted to represent the diversity of 160 accessions of *S cereale* subsp. *cereale*. The accessions were classified into distance based clusters. Accessions within a cluster are more similar to each other as compared to accessions in different clusters. From clusters containing less than 10 accessions, a single accession (best representing the corresponding cluster) was picked. Clusters with larger than 10 accessions were further sub-clustered such that each sub-cluster has less than 10

accessions. Then, the best accession among the sub-clusters of each cluster was selected based on PIC value of resulting mini core set after adding the tested accession.

4.3.5 Inoculations and evaluation of reaction to *Pyrenophora tritici repentis* (*PTR*) race 5

Seeds of each genotype were planted in three cones, three seeds in each cone (3.8 cm in diameter and 20 cm in length) and each cone considered as one replication. 6B662 and Salamouni were the respective susceptible and resistant checks. Till inoculation, plants were grown in a greenhouse at an average temperature of 21 °C and 16-hour photoperiod. At the second leaf stage, plants were inoculated with *PTR* race 5 by using spore suspension of 2500 spores/ml. Inoculated plants were moved to mist chamber (18 °C) for 24 hours and later grown for seven days in a greenhouse at 21 °C and 16 hours photoperiod. Seven days post-inoculation, disease lesions were rated on a qualitative scale of 1 to 5 [177]. On this scale, 1 is considered as resistant, 2 as moderately resistant, 3 moderately susceptible, and 4 and 5 as susceptible (Figure 4-7). The experiment was repeated twice while maintaining same growing conditions to ascertain consensus response to *PTR* race 5. For GWAS analysis the average of both experiments was used (Appendix Table S2).

4.3.6 GWAS analysis

Genome-wide association mapping for *PTR* race 5 resistance was primarily conducted using R package GAPIT (Genome Association and Prediction Integrated Tool) [295]. Three linear models were tested namely, GLM (Generalized Linear Model), MLM (Mixed Linear Model), and CMLM (compressed mixed linear model). GLM is based on the least square fixed effects; therefore we cannot use the information on the random

effects [295]. MLM includes both fixed and random effects. Fixed effects in our case were the SNP marker effect and population structure, and the random effect is relatedness of the individuals (kinship). MLM model is mathematically denoted as:

$$y = X\beta + Zu + e$$

where y is the vector of phenotypic values (categorical values in our case), "β" is the vector containing fixed effects namely SNP effects and population structure (Q), "u" is the random effects vector, which in our case is random genetic effects from multiple background QTL not controlled by markers (kinship). "X" and "Z" are known incidence matrixes for corresponding vectors. Kinship matrix was calculated using GAPIT's kinship algorithm which is based on VanRaden method [296] and Q matrix was obtained using principal component analysis [297]. CMLM is just an extension of MLM, which clusters the individuals into groups and uses the group based kinship matrix rather than individual based [298]. We primarily focused on MLM. Markers with p-value < 1.0×10^{-3} or log (p-value) > 3 were considered to be significant. For confirmation of the significant markers, 5-fold jackknife method was employed [299]. Briefly, the entire set of 160 accessions was divided into five sub-groups and four groups were used for association analysis, each time leaving one random group out. Results were also compared with the results from TASSEL 5.0 [300].

4.3.7 Comparative analysis of rye and wheat

To study the synteny among wheat and rye chromosomes, specifically for genomic regions conferring resistance against *PTR* race 5 in rye, comparative analysis between the wheat genome and rye genome was conducted. Flanking sequence (150 base pair) of each 4,037 SNP including the candidate SNPs identified in marker-trait analysis (MTA) were

retrieved from the rye reference genome. The 300bp long sequence for each SNP was compared with IWGSC wheat genome assembly TGACv1 [301] (http://plants.ensembl.org/Triticum_aestivum/Info/Index), using BLASTn [302]. Finally, results were visualized using a Perl based software Circos [303].

4.4 Results

4.4.1 Genotype by sequencing-based genome-wide SNPs

We obtained a total of 178,598,329 reads from two GBS libraries prepared from 178 rye accessions. Using UNEAK pipeline in TASSEL we identified 20,928 SNPs with 80% or less missing genotypes, whereas, with the reference-based pipeline, 27,882 SNPs with 80% or less missing genotypes were identified. For further analysis, the reference based SNPs were used. On average each chromosome has 4,000 SNPs (Table 4-1), with maximum (5,505) on chromosome 5R and minimum (2,536) on the chromosome 6R. To keep only the most informative SNPs, we removed 7,113 markers with indel as one allele. The high-quality SNPs (4,037) with less than 20% missing genotypes, heterozygotes less than 40% and MAF (minimum allele frequency) above 5% were retained for further analysis. Interestingly, like the total identified SNPs, the filtered set of 4,037 SNPs were also distributed similarly on all of the 7 chromosomes, with an average per chromosome of 577 and maximum (734) on chromosome 5R and minimum (358) on chromosome 6R (Table 4-1).

Table 4-1: SNPs discovered by genotyping-by-sequencing of 178 rye accessions along with their corresponding chromosome.

Chromosome	Total SNPs	Filtered SNPs*
1R	3,468	504
2R	3,914	600
3R	3,916	605
4R	5,505	685
5R	4,774	734
6R	2,536	358
7R	3,892	551
Total	**28,005**	**4,037**

***** SNPs with 20% or less missing genotypes, heterozygotes less than 40% and MAF >5%

4.4.2 Genetic variability in rye germplasm

The average PIC value for the 4,037 SNPs present in 160 *S. cereale* subsp. *cereale* accessions was 0.26 with a range from 0.09 to 0.5. A higher proportion of SNPs (38%) had PIC value ranging from 0.1 to 0.2, 26% had 0.2 to 0.3, 19% had 0.3 to 0.4, 14% had 0.4 to 0.5 and minimum, only 1% of the SNPs had PIC value of less than 0.1. PIC values for SNPs for each chromosome followed the similar pattern of distribution as genome-wide SNPs. Average PIC value for 1R, 3R and 5R was 0.27; 0.25 for 6R, 7R and 4R; and 0.26 for 2R (Fig 4-2). The Average I-index for 4,037 SNPs in 160 *S. cereale* subsp. *cereale* accessions was 0.48. Among wild species (18 accessions), average PIC value and I-index were 0.25 and 0.57 respectively.

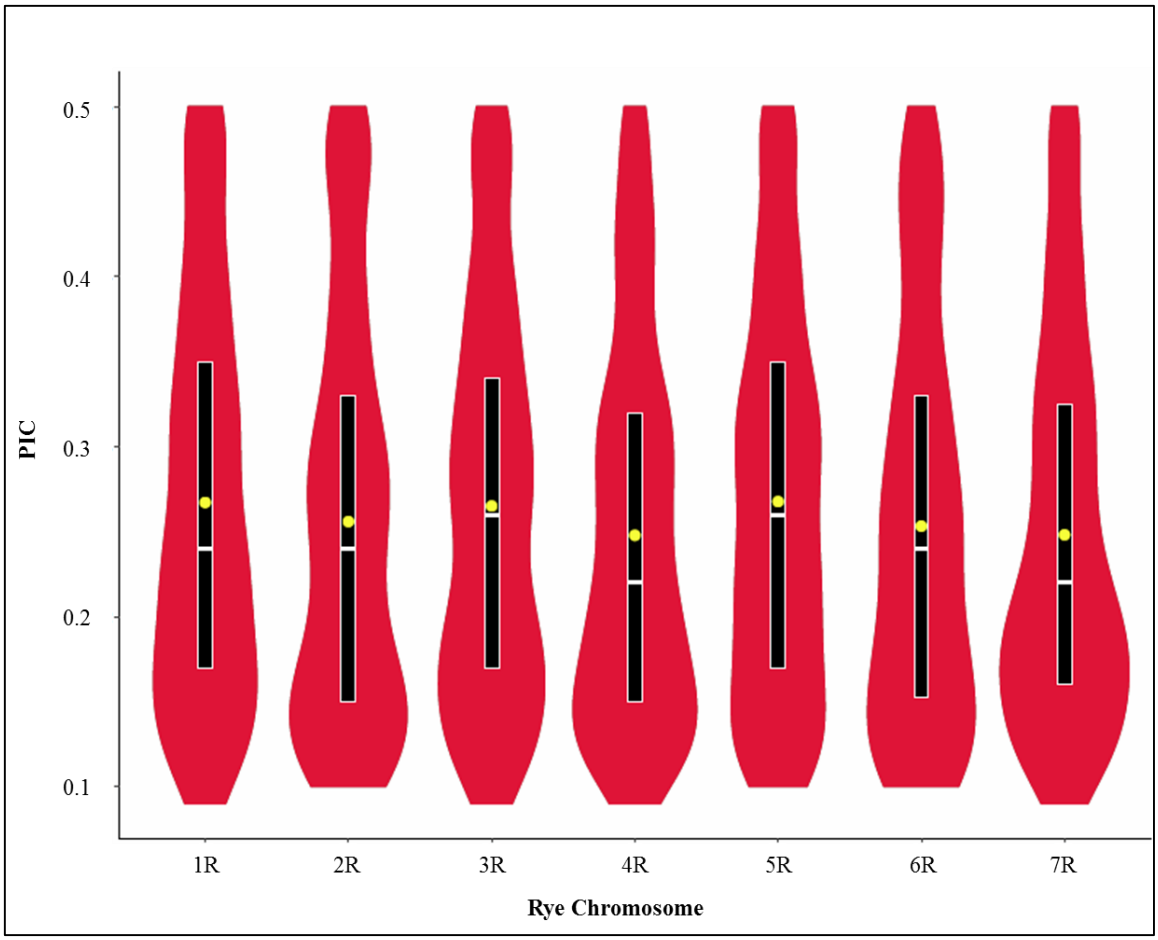

Figure 4-2: Distribution of PIC values for SNPs (160 *Secale cereale* subsp. *cereale* accessions) corresponding to each chromosome of rye. X-axis: PIC value and Y-axis rye chromosomes. Violin plots show the density distribution of SNPs for the chromosome corresponding PIC values. Box plots represent first and third quartiles. Horizontal white bars are corresponding median PIC value and yellow dot stands for average PIC value.

The average percentage dissimilarity (GD) among the entire set of *S. cereale* subsp. *cereale* was 0.48, and it ranged from 0.26 to 0.63. Lowest GD (0.26) was found between two accessions namely SD_Sc150 and SD_Sc148. Highest GD (0.63) was found between SD_Sc195 and SD_Sc186. Average GD for individual chromosomes ranged from 0.46

to 0.49 (Figure 4-3). The average GD among wild species (18 accessions) was 0.51, and it ranged from 0.15 to 0.66. Among the wild species, SD_Sc330 (*S. sylvestre*) and SD_Sc322 (*S. vavilovii*) were the most diverse accessions, and SD_Sc330 (*S. sylvestre*) and SD_Sc331 (*S. sylvestre*) were the most similar accessions with 0.66 and 0.15 GD respectively. GD matrix based farthest Neighbor-joining phylogenetic tree (Figure 4-4) accurately clustered each of the three species namely *S. cereale*, *S. strictum*, and *S. sylvestre* into different clusters, except for SD_Sc323, the only spring type accession of *S. strictum*, which falls in a cluster of *S. cereale*. On the contrary, *S. vavilovii* clades were found scattered within the clusters of *S. cereale*. Spring type accession of *S. vavilovi* (SD_Sc322) was found in the same cluster as spring type accession of *S. strictum*. *S. sylvestre* and *S. strictum* were found to be closely related to each as compared to *S. cereale*.

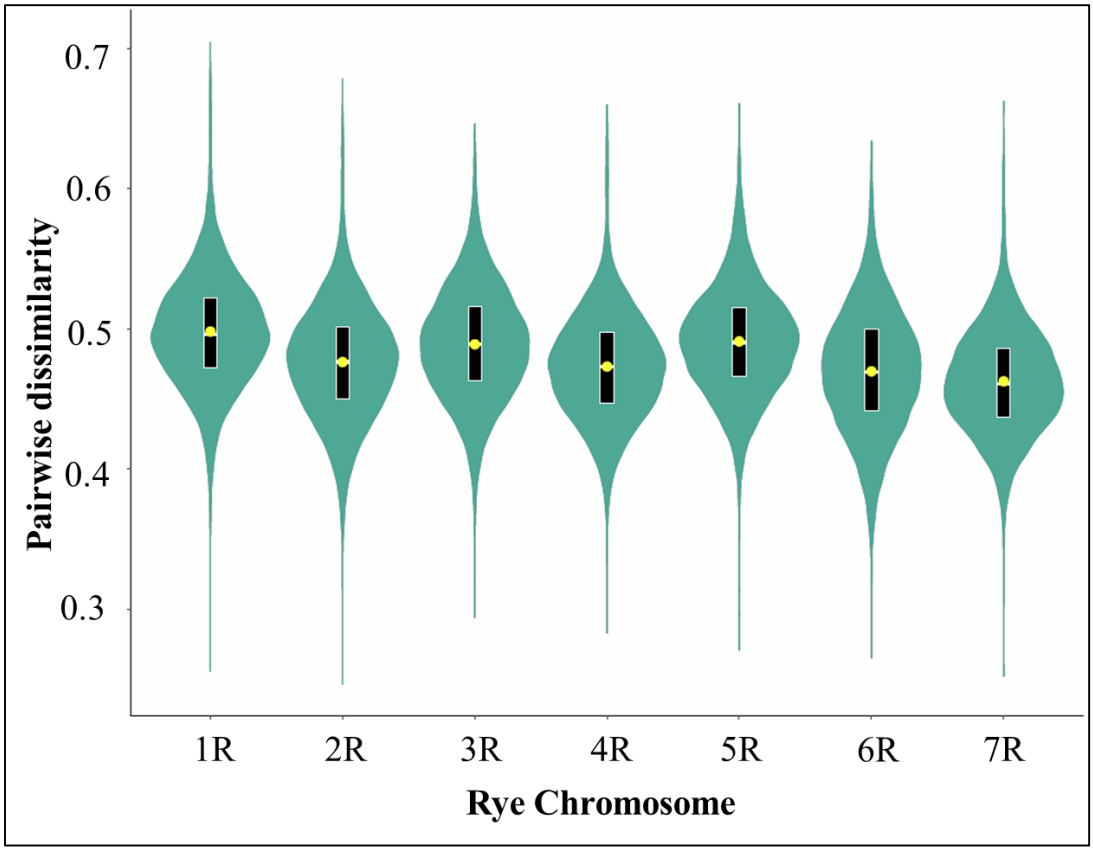

Figure 4-3: Distribution of pairwise dissimilarity values among *Secale cereale* subsp. *cereale* for the total number SNPs corresponding to each chromosome of rye. X-axis: pairwise dissimilarity (percentage) and Y-axis rye chromosomes. Violin plots show the density distribution of pairwise dissimilarities values. Box plots represent first and third quartiles. Horizontal white bars are corresponding median pairwise dissimilarity and yellow dot stands for average pairwise dissimilarity corresponding to each chromosome.

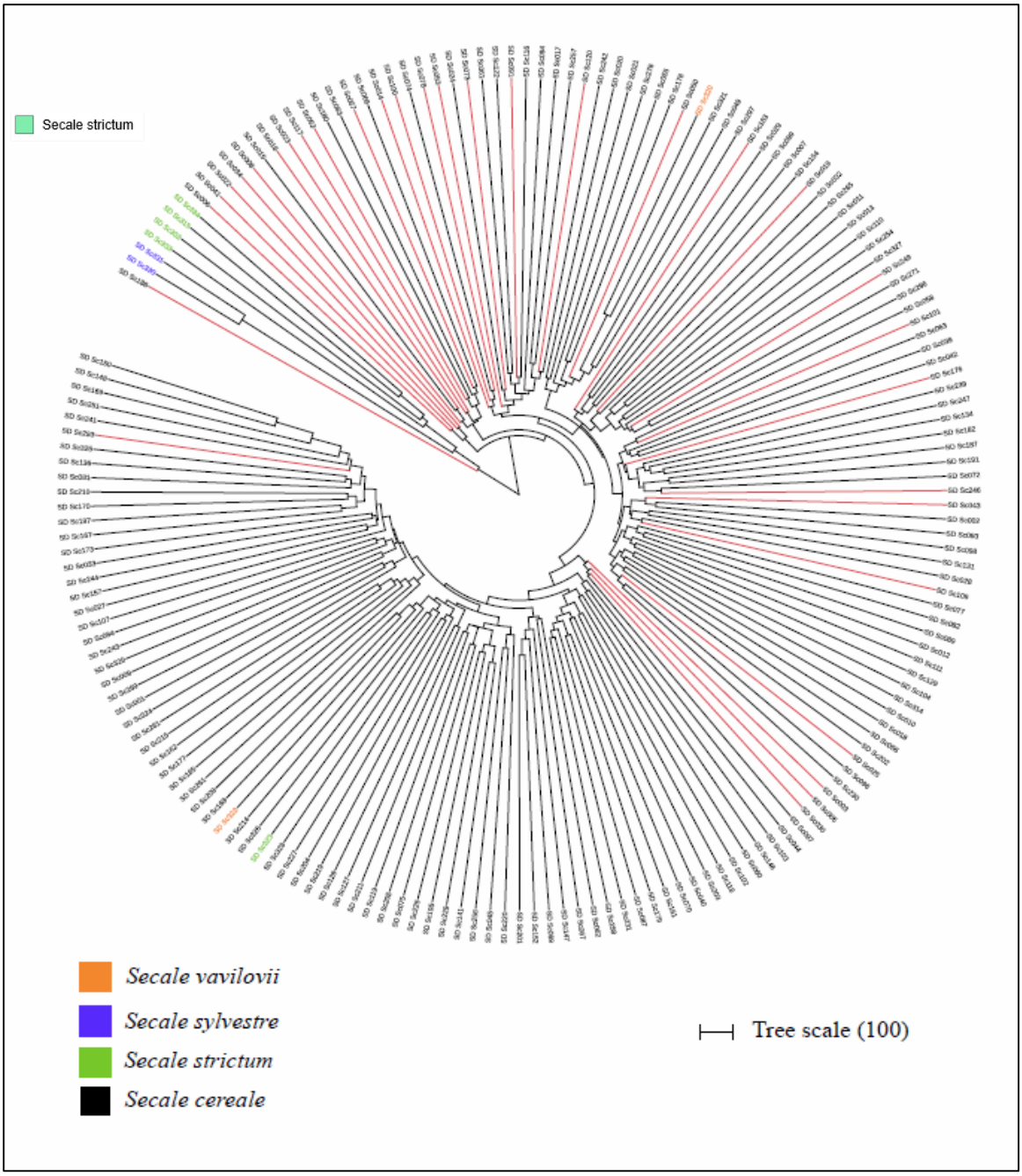

Figure 4-4: Pairwise dissimilarity based neighbor-joining tree. Mini core set (doted clades) representing all the major clusters of *Secale cereale* subsp. *cereale*. *S. strictum* and *S. sylvestre* clearly fall into different clusters. Accessions of *S. vavilovii* are present among the *S. cereale* cluster.

4.4.3 Population structure and principal component analysis (PCA)

Bayesian clustering (STRUCTURE) analysis was performed on the 178 *Secale* sp. accessions and the estimated likelihood [LnP (D)] was found to be greatest at K = 3, suggesting three major populations that explain a significant genetic variation. (Figure 4-5). Among all accessions, 67% (120) belongs to one of the three populations with more than 70% ancestry contributed by any one population. The three populations namely P1, P2, and P3 consisted of 66, 51, and three accessions respectively. 32% (58) of the accessions were admixtures, sharing ancestry (<20%) with two of the three populations. Among admixtures, P12 contains 55 accessions which have shared ancestry (P12) among P1 and P2, only P13 has three accessions sharing ancestry from P1 and P3. No accession shared significant ancestry (above 20%) between P2 and P3. Accessions of *S. cereale* subsp. were majorly found in P1, P2, and P12, whereas, P3 and P13 consisted of wild accessions of *Secale strictum* and *Secale sylvestre*.

The GD based PCA results were relatively consistent with the model-based population structuring (Figure 4-6A). First and second PCA explained 40% and 3% of the genetic diversity respectively. Main populations (P1, P2, and P3) are clearly separated in the diversity space. Admixtures, namely P12 and P23 lie between the corresponding populations with which they share ancestry. P3 mostly consist of wild species of *S. strictum* and *S. sylvestre* and is separated from rest of the evaluated accessions (Figure 4-6B). One accession of *S. strictum* was found in the population of *S. cereale* subsp.. Interestingly, this accession is the only spring type accession of *S. strictum*. We also found some *S. vavilovii* accessions in the *S. cereale* diversity space. Relationship of genetic clustering with growth habit (spring vs winter) and geographic origin was also

accessed. No strong association between genetic clustering and growth habit was observed as accessions from both types overlapped in the diversity space (Figure 4-6C). Similarly, no correlation was found between genetic clusters and the geographic regions (Fig 4-6D). Geographic regions were divided according to Bolibok- Bragoszewska *et al*., dividing Europe into 5 regions: east, west, south, north and central; and combining other countries into corresponding broad geographic regions like Middle East, Asia, South America, North America, Australia, and Russia [124].

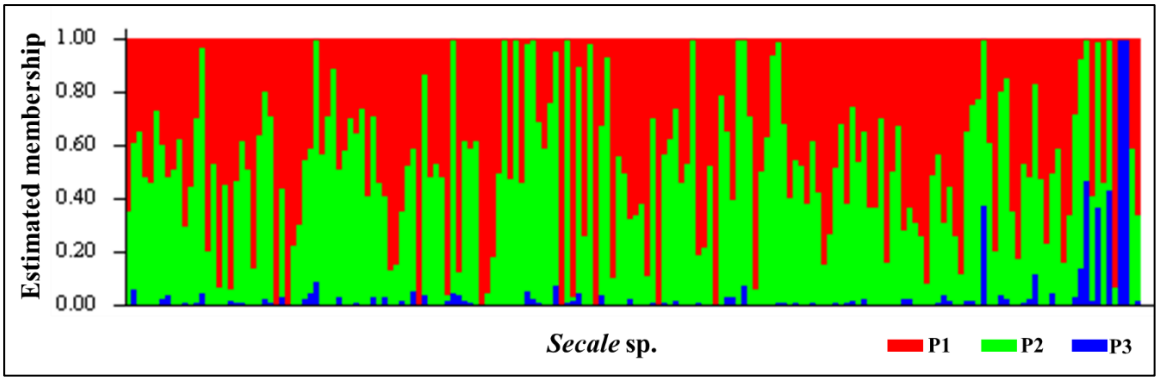

Figure 4-5: Model-based structure results (K=3) for 178 *Secale* sp. accessions presented as a barplot. Y-axis represents the estimated membership of individuals from populations. Each bar on X-axis represents one individual. Accessions are ordered according to the species and order is given in Appendix table 2.

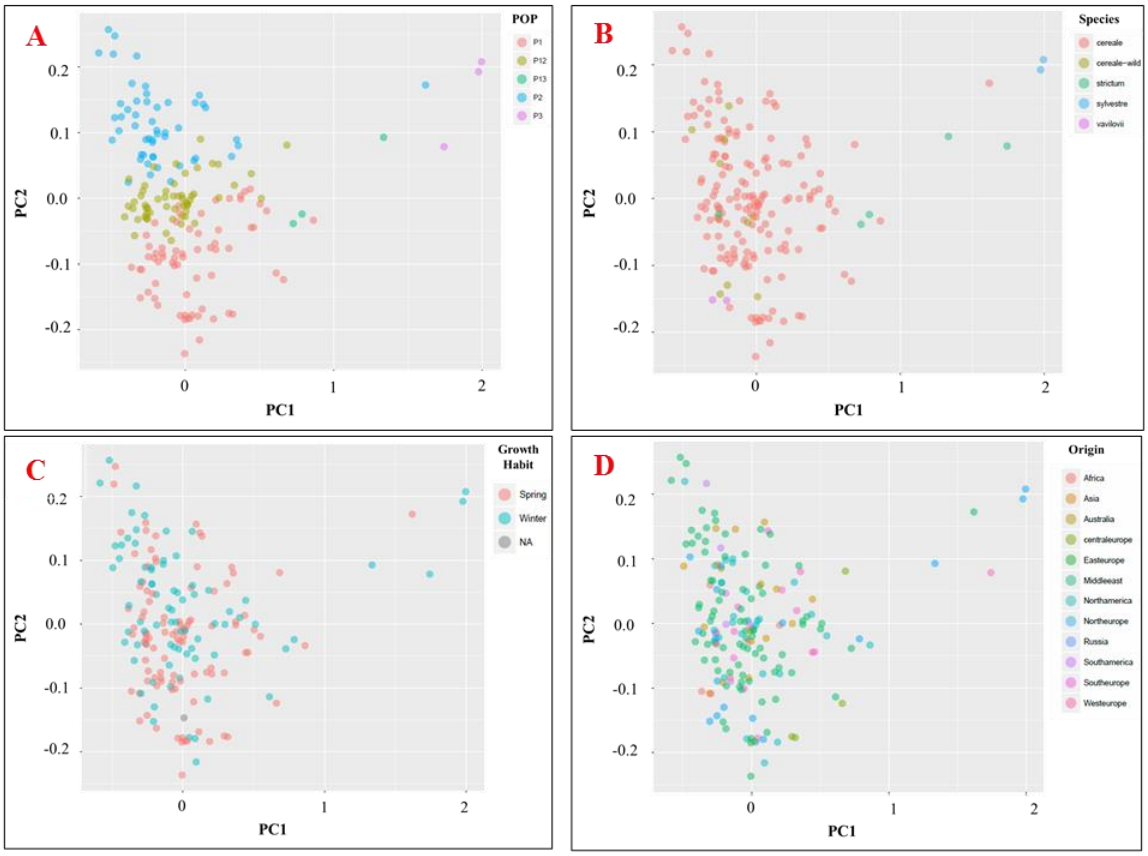

Figure 4-6: Pairwise dissimilarity based PCA. First PCA (PC1) explains 40% of the genetic diversity and the second PCA explains 3%. A) Individuals are colored according to the populations determined by model-based structure results. B) Individuals are colored for corresponding *Secale* sp. C) Individuals are colored according to spring or winter type habit. D) Individuals are colored according to the geographic origin.

4.4.4 Mini core of rye

A mini core set of 32 accessions was extracted from 160 accessions of *S. cereale* subsp. *cereale* (PIC = 0.2518). Though the mini core size is only 20% of the entire set, it covered 99% of the allelic diversity of the entire set. We ensured accessions of mini core set covers all the main clusters, with a minimum of one accession from each cluster (Figure 4-4). Mini core also captured a large portion of the geographic diversity (27

countries) of the global collection (70 countries) by representing major geographic regions (Figure 4-1). The average PIC value and I-index of mini core set are not significantly ($p < 0.01$) different from the entire set (Table 4-2). Average GD is significantly ($p < 0.01$) higher among mini core accessions as compared to the global set (Table 4-2). Based on all the aforementioned results we eliminated the redundant accessions and established a core set by keeping only the diverse ones.

Table 4-2: Comparison of mini core set and global set of *Secale cereale* subsp. *cereale* for the diversity indices.

	Size	Average PIC	Average I-index†	Average GD‡
Global Set	160	0.26	0.60	0.48
Mini core set	32	0.25	0.59	0.51
T-test (p-value)		0.02	0.11	$1.90e^{-90}$*

†Shannon's diversity index ‡Pairwise genetic dissimilarity *Significant at $\alpha < 0.01$.

4.4.5 Reaction to *Pyrenophora tritici repentis* race 5 (*PTR* race 5)

All 178 accessions of *S. cereale* were evaluated for resistance to tan spot (*PTR* race 5), however, we performed GWAS analysis only on *S. cereale* subsp. *cereale* (160 accessions). We observed a variety of response to *PTR* race 5 inoculations with 31.8% (51) accessions being resistant (R -category 1), 26.9% (43) accessions moderately resistance (MR - category 2), 24.4% (39) moderately susceptible (MS - category 3) and another 16.8% (27) being susceptible (S) falling in category 4 and 5. As expected resistant check (Salamouni) showed resistant (Score - 1) response and the susceptible

check (6B662) produced chlorosis reaction with a score of 4 to 5. All these results were consistent in both experiments.

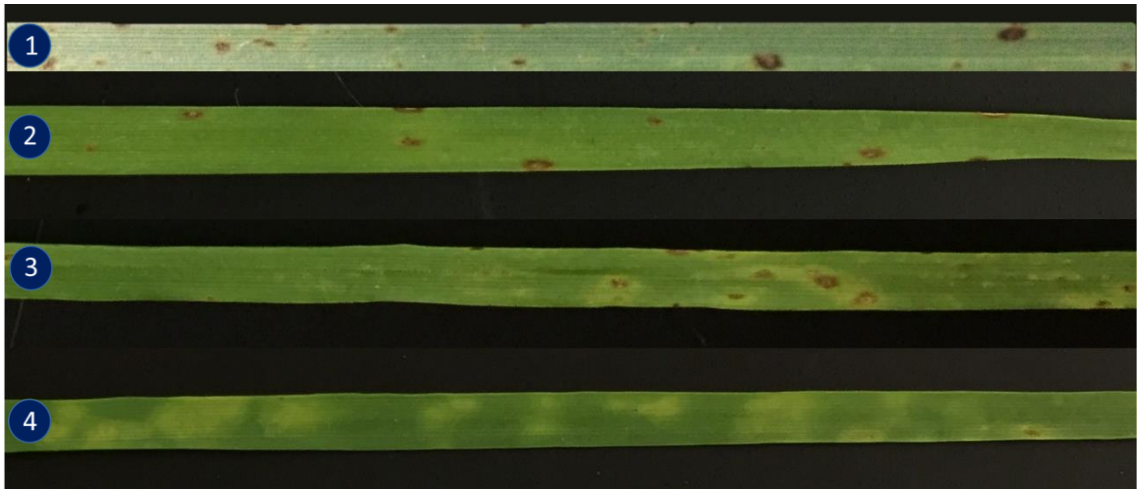

Figure 4-7: Tan spot lesions scoring, based on the 1 to 5 scale (Lamari and Bernier 1989). 1 – Resistant wheat *Salamouni* (check), 2 – Resistant rye, 3 – Moderately susceptible rye, 4 – Susceptible rye.

4.4.6 Marker-trait association (MTA) for tan spot (*PTR* race 5) resistance in rye

Out of the tested linear models, we focused on MLM, since individuals have a kinship as well as there is a population structure. The decision for statistically significant associated SNPs was based on a threshold of $< 1.0 \times 10^{-3}$ p-value. Following this criterion, we identified one region on chromosome 2R (*QTs.sdsu-2R*) and other on 5R (*QTs.sdsu-5R*) associated with resistance against *PTR* race 5. The two SNPs "*S5R_16433036"* ($p=1.4 \times 10^{-4}$) on chromosome 5R and "*S2R_6856816"* ($p=4.5 \times 10^{-4}$) on chromosome 2R explained 13.11 % and 11.62 % of the variation respectively (Figure 4-8). We further evaluated the consistency of our results by repeating the analysis with GLM, and CMLM (Figure 4-8) and the QTLs identified with the MLM algorithm showed significant associations with all other algorithms. Finally, we also validated the candidate SNPs

using 5K jackknife approach. Both significant markers, *S5R_16433036 and S2R_6856816* were consistent in the five repetitions of 5K jackknife with a p-value = < 1.0×10^{-3}.

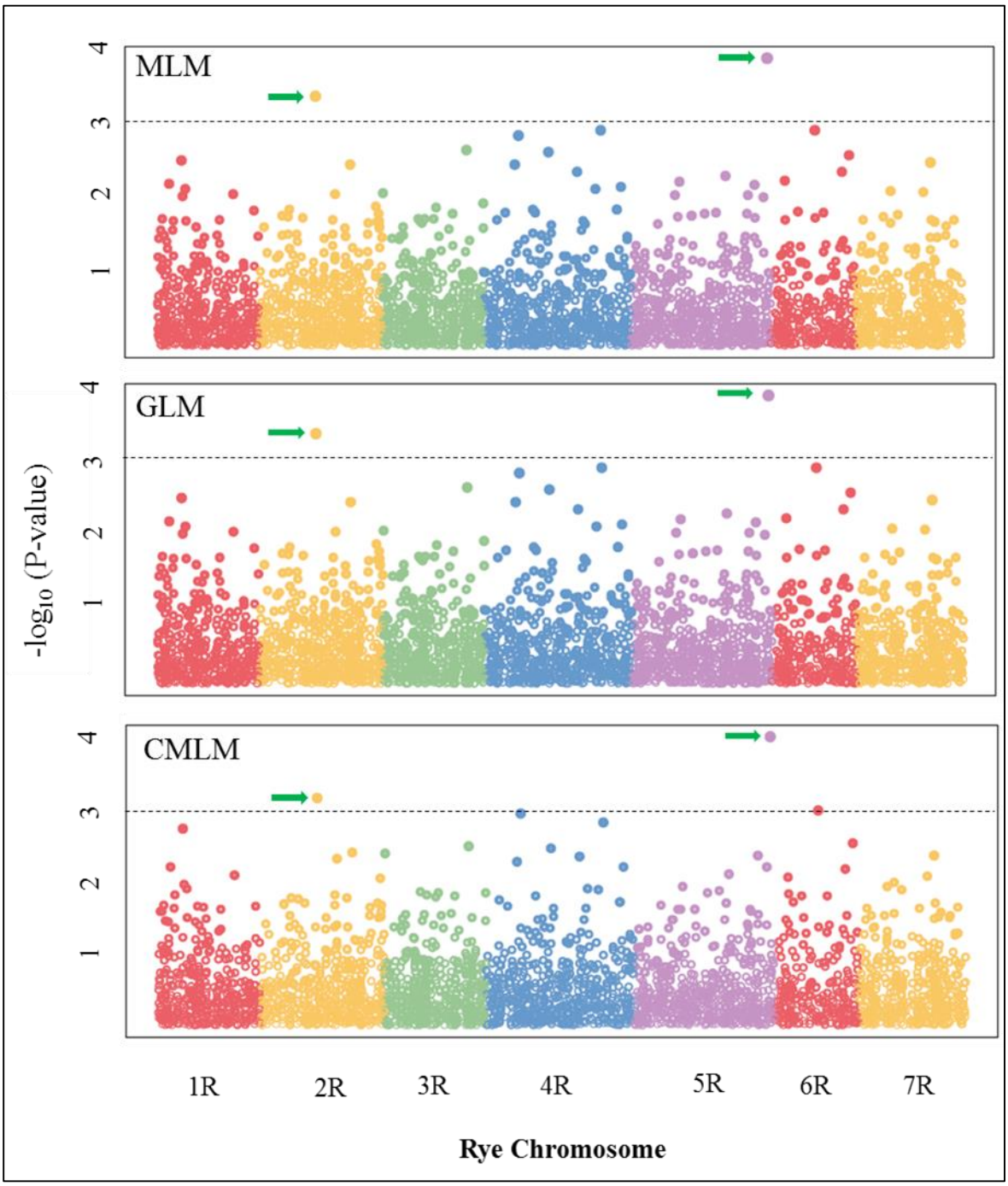

Figure 4-8: Genome-wide association scan for tan spot (*PTR* race 5) resistance in rye. Three different model based Manhattan plots representing $-\log_{10}$ (p-value) for SNPs distributed across all of the 7 chromosomes of Rye. Y-axis: $-\log_{10}$ (p-value) and x-axis: Rye chromosomes. The dashed line stands as a threshold for significant markers with $-\log_{10}$ (p-value) of > 3 which correspond to a p-value of $<1 \times 10^{-3}$. Two reported SNPs of this study are pointed with arrows. SNP of 5R (*S5R_16433036*) and SNP of 2R (*S2R_6856816*) come significant in all of the tested models.

4.4.7 Comparative analysis with wheat

Syntenic analysis with wheat was mainly focused on comparing the QTLs identified in our study. The *QTs.sdsu-2R* mapped on rye chromosome 2R had a hit on a corresponding region of wheat group 2. A tan spot (*PTR* race 5) insensitivity gene (*tsc2*) has been reported in wheat on chromosome 2B. We could not perform a precise syntenic analysis due to unavailability of complete ordered assembly of rye genome. The chromosome 5R region (*QTs.sdsu-5R, S5R_16433036*) showed hit on the 4B chromosome of wheat although 5R has a great deal of syntenic with group 5 of wheat. However, no QTL or gene for tan spot resistance/insensitivity has been reported on 4B in wheat.

Overall, chromosomes 1, 2, and 5 were highly syntenic with corresponding wheat homeologous groups whereas other chromosomes of rye showed rearrangements as expected (Figure 4-9). Based on general syntenic analysis of all seven chromosomes of rye with seven homeologous groups (21 chromosomes) of wheat, broader pictures of synteny between the wheat genome and the rye genome was observed (Figure 4-9). Majority of chromosome 1R, 2R, 3R and 5R is syntenic to wheat homeologous group 1, 2, 3, and 5 chromosomes respectively. However, blocks of rye chromosome 4R showed

synteny with wheat group 4, 6 and 7. Chromosome 6R is also syntenic to wheat group 6 and 3, though due to fewer markers for 6R synteny in not very clear. Chromosome 7R shared syntenic blocks with wheat group 5, 4, and 7.

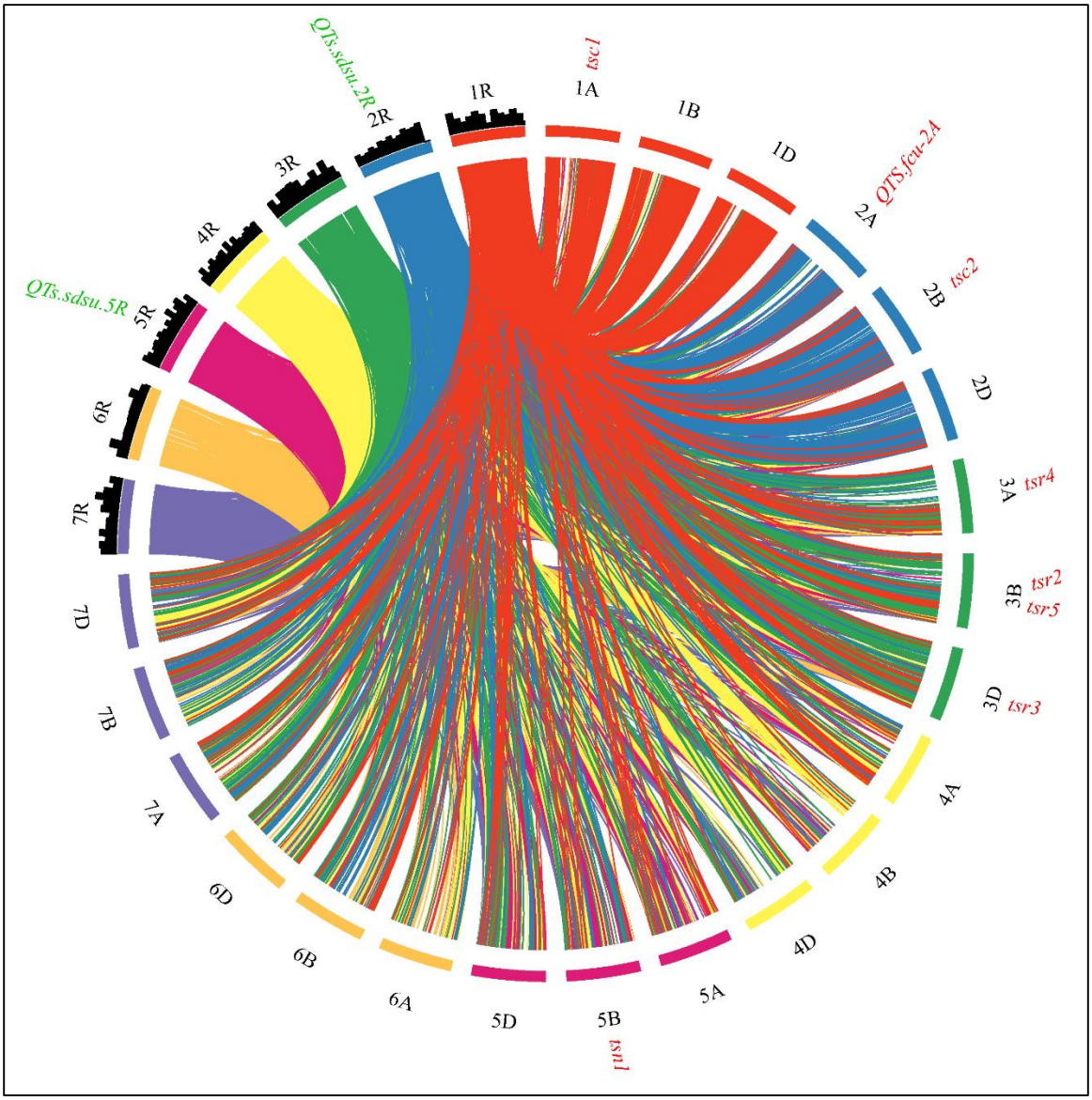

Figure 4-9: Synteny between wheat genome (IWGSC RefSeq v1.0) and rye genome (1000bp flanking sequence of 4,037 SNPs). Black bars on rye chromosomes denotes SNP density. *QTs.sdsu-5R* and *QTs.sdsu*-2R are presented adjacent to their corresponding rye chromosomes. Red italics denotes the tan spot insensitivity genes (*tsn1*, *tsc1*, and *tsc2*)

and resistance genes (*tsr2*, *tsr3*, *tsr4*, and *tsr5*) adjacent to their corresponding wheat chromosomes.

4.5 Discussion

4.5.1 Genome coverage by SNPs

Assessing the genetic diversity in germplasm resources in addition to the morphological or physiological observations can help in better exploitation of germplasm for crop improvement. In rye, several diversity studies have been conducted using DNA based markers [94,118–123,242]. But due to laborious genotyping methods and technological limitations these studies were based on only limited number of markers such as 11 PCR-RFLPs [118]; 14 allozyme and 3 SSR [119]; 15 SSR [120], 24 SSR [94]; 20 isozyme loci, 14 ISSR, and 38 SSR [121]; 242 ISSRs and 169 RAPDs [122]; 779 AFLP [123], 576 SNPs [242]. Furthermore, the chromosomal position of these markers was not reported. To address this issue of anonymous and less number of markers, so far a single study has been conducted by Bolibok-Brągoszewska *et al*. [124]. Authors used 1,054 DArT markers, more or less equally distributed on all seven chromosomes of rye and concluded that these DArT markers provide a better picture of genetic diversity in the rye gene pool. This achievement can be attributed to the comparatively high number of markers used in this study as well as the distribution of markers on all the chromosomes of rye. In the present study, we employed genotype by sequencing (GBS) approach for even better coverage of the genome.

To our knowledge, this is the first report of double enzyme digestion-based GBS in rye. GBS being a next-generation sequencing based method along with large number SNPs, it also has its own advantages for high diversity species like rye. We discovered ~ 4000

Genome wide distributed polymorphic SNPs that covered a significant portion of the rye genome. All chromosomes have more or less equal number of SNPs except chromosome 6R, which has 358 SNPs, significantly lower than the average 576 SNPs identified per chromosome. For GBS, the discovery of markers is directly related to the genetic diversity of the genomic region, more diversity corresponds to a larger number of markers [304]. Based on this fact, it can be concluded that chromosome number 6R was likely less diverse as compared to the other rye chromosomes. This finding is in line with several previous studies that have concluded chromosome 6R to be genetically less diverse among rye germplasm [80,81,124,241]. Seeing the GBS advantage in analyzing diversity and GWAS, it's reasonable to state that once rye whole genome is sequenced, the NGS based genotyping methods such as GBS may yield even better coverage of the rye genome [80].

4.5.2 Diversity analysis

Diversity panel consisted of 160 accessions of *S. cereale* subsp. *cereale* and 18 accessions of wild relatives. We mainly focused on *S. cereale* subsp. *cereale* because it is the only cultivated species of rye. The average PIC value for all SNPs based on *Secale cereale* subsp. *cereale* is 0.26 with a range from 0.09 to 0.5. There are only few SNPs based genetic diversity studies in rye which leaves a narrow scope for comparison. Nevertheless, Varshney *et al* [305] identified 96 SNPs in rye based on eSNPs in barley and reported the average PIC value to be 0.32. As those markers were carefully and deliberately selected hence slightly high PIC value in that case as compared to our study. In comparison to genetic diversity studies based on DArT markers, PIC value in our study (0.26) is lower than the reported average of 0.38 [124]. Average PIC values rank

even higher in SSR marker-based studies 0.67 for 16 SSR markers [306] and 0.57 for 22 SSR markers [307]. This higher average value can be credited to multi-allelic fashion of the SSR markers as PIC for multi-allelic markers ranges from 0 to 1 but for bi-allelic markers such as SNPs, it ranges from 0 to 0.5 only. However, lower PIC values of SNPs can be overweighed by their enormous number and genome wide distribution thus giving a similar picture about the diversity. The PIC value for individual chromosomes was almost same with a range from 0.25 to 0.27. This indicates that the selected SNPs were not in bias with any of the chromosomes and polymorphic SNPs were evenly distributed on all of the seven chromosomes of rye.

Average GD values among *Secale cereale* subsp. *cereale* was 0.48 with a range from 0.26 to 0.63 and is comparable with other studies in rye. Shang *et al*. analyzed 30 wild and 47 cultivated accessions and found average GD to be 0.36 [94], whereas, Ma *et al*. reported dissimilarity among 42 rye accessions ranged from 0.036 to 0.565 [308]. DArT marker based study comparing different 378 accessions, reported the average GD to be 0.39 [124]. It is noteworthy to mention SD_Sc195 and SD_Sc186 accessions with highest dissimilarity index of 0.63. As these are the most diverse accessions, these may be of future interest for exploiting heterosis. Among wild species, the average GD is 0.51, higher as compared to cultivated species and it ranged from 0.15 to 0.66. This higher GD in wild species is in accordance with the expectation that wild species conserve larger diversity [124]. Therefore, wild species can further be exploited to infuse diversity into cultivated germplasm. In particular, SD_Sc330 (*Secale sylvestre*) and SD_Sc322 (*Secale vavilovii*) were the most diverse accessions.

Three clustering approaches, namely Bayesian clustering, PCA and Neighbor-Joining clustering, were tested to group individuals based on 4,037 SNPs. Results among all three methods were consistent. Bayesian clustering predicted 3 populations: P1, P2, and P3. P1 and P2 both consisted of *S. cereale* subsp. and *S. vavilovii* accessions; P3 consisted of *S. sylvestre* and *S. strictum* accessions. These clusters were apparent on PCA too. Different clustering of *S. sylvestre* and *S. strictum* from other species have been reported in most of the previous studies [241,308]. Genome composition of *Secale sylvestre* was 100% from the P3 population, whereas, *S. strictum* had about 10 to 20% from P1. Sharing of ancestry among some accessions of *S. strictum* and *S. cereale* subsp. group (P1) suggests the compatibility among *S. strictum* and cultivated species. This sharing of ancestry also supports the proposed idea that *S. strictum* is the potential ancestor of *Secale cereale* [87–90]. Unlike other wild *Secale* sp. *S. vavilovii* accessions were found among the clusters of *S. cereale* subsp. which is in accordance with previous reports [241,308], suggesting its classification needs to be revisited. Wild species of *S. cereale* cannot be separated out of the clusters of the *S. cereale* subsp. *cereale* in our study similar to previous studies [124], suggesting an active gene transfer among these species. After comparing geographic origin with genetic clusters, we found no correlation between them. Similarly, many studies based on different marker systems have also reported that geographic diversity does not correspond to the genetic clustering of the individuals [94,118,123,124]. This may be due to sharing of the common genetic background among the accessions being analyzed in each study as it is also observed by Bolibok-Brągoszewska *et al.* in 2014 [124].

In many studies on different crop species such as rye [308], triticale [309] and wheat [310] it has been reported that vernalization requirement can lead to population divergence. After comparing genetic clustering with growth habit (winter vs spring), we did not identify any substantial association between growth habit and vernalization requirement. The germplasm being tested had some facultative genotypes, reported to behave as winter or spring type but that was not demonstrated by the genetic clustering. In conclusion, we did not observe any strong association of genetic clustering with geographic origin or growth habit. With the available data, the P3 population was clearly explained as wild-type *S. strictum* and *S. sylvestre* but P1 and P2 are clusters within the S. *cereale* subsp., these two clusters were not linked to any of the physiological or geographical data available.

4.5.3 Mini Core representing the global set

Most of the plant genetic resources are preserved as accessions in the form of gene banks [68]. Number of accessions for particular species may go up to thousands. Owing to the large number of accessions, management in gene banks and utilization by breeders has always been a challenge [68]. One of the strategies to handle such large number of accessions is a mini core collection (MC). The concept of mini core collections implies to keep as few diverse accessions as possible from the full collection which can represent the genetic diversity of full set to the best [69,70,75]. Based on that concept there are mini core collections for number of crops including wheat [71,72], rice [73], maize [74], soybean [68], and rye [307]. Adding one more collection to that list, we identified a mini core set of 32 accessions representing genetic (99% alleles) and geographic diversity (all major regions) of 160 accessions of *S. cereale* subsp. *cereale*. PIC value and Shanon's

diversity index of mini core is comparable to the total set while average GD is significantly higher than the total set. Thus, the mini core consists of very diverse accessions carrying similar information as the whole set. This mini core set can be easily and efficiently exploited for rye or wheat improvement. X. *Triticosecale* novel accessions developed based on these 32 accessions can make a very diverse set, which can be used for gene mining and mobilizing genes into wheat germplasm. Also, out of the 160 accessions analyzed in this study, preservation of 32 accessions in, mini core set could reduce the conservation cost still retaining 99% of the allelic diversity.

4.5.4 Identification of potential genomic regions conferring tan spot (*PTR* race 5) resistance

Rye is known for its resilience to the abiotic and biotic stress tolerance [307] and it has contributed number important genes into wheat germplasm [104,106,107,311]. For the improvement of rye germplasm and for efficient gene transfer to other crops like wheat, characterization, and mapping of the important genes is a most critical step. In this study, we performed GWAS using 160 accessions of *Secale cereale* subsp. *cereale* to demonstrate the utility of the rye collection and the genotyping information obtained from GBS. Using this panel two potential loci conferring resistance to *PTR* race 5 were mapped. The two SNPs (*S2R_6856816"* on chromosome 2 and "*S5R_16433036"* on chromosome 5) collectively explained 24.73% of the phenotypic variation using MLM and were consistent using other two models (GLM and CMLM). Though in our earlier study [282] we reported that rye carries good resistance to tan spot, however, no QTLs for tan spot resistance have been earlier reported in rye.

Syntenic analysis of rye and wheat revealed that the significant marker linked to tan spot resistance on chromosome 2R is homologous to chromosome group 2 of wheat. On wheat chromosome 2B, major insensitivity gene *tsc2* has been located by Friesen and Faris [183]. In the same study they found several minor *PTR* race 5 related QTLs such as *QTS.fcu-2A* (*PTR* race 5) on chromosome 2A [170,183] and in a recent GWAS analysis for *PTR* race 1 Juliana *et al.* mapped QTL on chromosome 2A [312]. Thus these reports suggest that wheat group 2 chromosomes harbor *PTR* resistance related genes, and it's possible that our QTL *QTs.sdsu*-2R in rye may be a homologous counterpart of *tsc2* or other tan spot resistance QTLs discovered on chromosome group 2 of wheat. However, the precise syntenic analysis was limited by the incomplete genome assembly of rye. QTL *QTs.sdsu-5R* had a most significant hit on chromosome 4B of wheat. Though most of the chromosome 5R of rye is syntenic to chromosome group 5 of wheat, a small segment also hits a region on chromosome 4B which also includes our candidate SNP. So far no QTL/gene related to tan spot resistance or insensitivity has been reported on chromosome 4B of wheat. Thus, *QTs.sdsu-5R* may harbor novel genes for *PTR* race 5 resistance. The QTLs identified in our study can be easily transferred using linked SNPs into wheat and triticale for improving tan spot resistance in these crops. Using similar approach genes/QTLs controlling agronomic; biotic and abiotic stress tolerance can be mapped in rye and mobilized for triticale and wheat.

4.6 Conclusions

Our study reports the first genetic diversity analysis in rye which is based on more than 4,000 genome-wide distributed markers. We developed a mini core set of 32 accessions that retains ~99% of the allelic diversity. These accessions can be used for triticale and

wheat improvement. Genetic clustering was neither linked with geographic origins and nor with growth habit, suggesting individuals shared a common genetic background due to germplasm exchange and no major genomic changes happened due to vernalization requirements. Further, demonstrating the use of GWAS in rye we identified two genomic regions conferring resistance to tan spot (*PTR* race 5) in rye and the linked SNPs *S5R_16433036* (*QTs.sdsu-5R*) *and S2R_6856816* (*QTs.sdsu-2R*) can be utilized for marker-assisted breeding for tan spot resistance genes.

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APPENDIX

Table 1: Number of accessions of each Secale subsp. in the diversity set of 178 lines. These lines represent 56 different countries around the globe.

Sr.no.	Genera	Species	Subspecies	No. of lines
1	*Secale*	*cereale*	*cereale*	160
2	*Secale*	*cereale*	*tetraploidum*	1
3	*Secale*	*cereale*	*afghanicum*	1
4	*Secale*	*cereale*	*dighoricum*	1
5	*Secale*	*cereale*	*segetale*	2
6	*Secale*	*cereale*	*Unranked rigidum*	1
7	*Secale*	*cereale*	*ancestrale*	3
8	*Secale*	*vavilovi*	-	2
9	*Secale*	*strictum*	*anatolicum*	1
10	*Secale*	*strictum*	*strictum*	1
11	*Secale*	*strictum*	*siliatoglume*	1
12	*Secale*	*strictum*	*kupriganovi*	1
13	*Secale*	*strictum*	*africanum*	1
14	*Secale*	*sylvestre*	-	2
Total				**178**

Table 2: Detailed description about the Secale cereale accessions used in this study. Populations are based on structure results and reaction against *P. tritici repentis* (race 5) is also presented.

SD_code	**Country**	**PI No.**	***Genera***	***species***	**subsp.**	**Population**	*PTR* race 5
SD_Sc001	Sweden	Cise 1	*Secale*	*cereale*	*cereale*	P2	1.83
SD_Sc002	Sweden	Cise 20	*Secale*	*cereale*	*cereale*	P12	3.83
SD_Sc003	United States	Cise 28	*Secale*	*cereale*	*cereale*	P1	1.00
SD_Sc005	United States	Cise 38	*Secale*	*cereale*	*cereale*	P1	1.50
SD_Sc006	Australia	Cise 79	*Secale*	*cereale*	*cereale*	P1	2.00
SD_Sc007	France	Cise 84	*Secale*	*cereale*	*cereale*	P12	1.00
SD_Sc008	Bosnia and Herzegovina	PI 349919	*Secale*	*cereale*	*cereale*	P1	3.00
SD_Sc009	Ireland	Cise 106	*Secale*	*cereale*	*cereale*	P12	1.17
SD_Sc011	Japan	Cise 108	*Secale*	*cereale*	*cereale*	P12	1.00
SD_Sc012	Japan	Cise 109	*Secale*	*cereale*	*cereale*	P12	3.00
SD_Sc013	Korea, South	Cise 110	*Secale*	*cereale*	*cereale*	P1	3.00
SD_Sc014	United States	Cise 174	*Secale*	*cereale*	*cereale*	P1	4.00

SD_Sc015	United States	Cise 176	*Secale*	*cereale*	*cereale*	P2	3.00
SD_Sc016	Canada	Cise 183	*Secale*	*cereale*	*cereale*	P12	3.00
SD_Sc017	United States	Cise 521	*Secale*	*cereale*	*cereale*	P2	3.00
SD_Sc018	Israel	PI 201991	*Secale*	*cereale*	*cereale*	P12	1.00
SD_Sc019	Pakistan	PI 218110	*Secale*	*cereale*	*cereale*	P1	1.00
SD_Sc020	Pakistan	PI 219740	*Secale*	*cereale*	*cereale*	P1	1.00
SD_Sc021	Pakistan	PI 219741	*Secale*	*cereale*	*cereale*	P1	1.00
SD_Sc022	Afghanistan	PI 223896	*Secale*	*cereale*	*cereale*	P1	1.00
SD_Sc023	Iran	PI 227870	*Secale*	*cereale*	*cereale*	P12	1.00
SD_Sc024	Kazakhstan	PI 234655	*Secale*	*cereale*	*cereale*	P1	1.00
SD_Sc025	Kazakhstan	PI 234656	*Secale*	*cereale*	*cereale*	P12	1.00
SD_Sc027	France	PI 235536	*Secale*	*cereale*	*cereale*	P2	2.67
SD_Sc028	Brazil	PI 239580	*Secale*	*cereale*	*cereale*	P1	2.83
SD_Sc029	Argentina	PI 240676	*Secale*	*cereale*	*cereale*	P1	1.00
SD_Sc030	Brazil	PI 241578	*Secale*	*cereale*	*cereale*	P1	2.67

SD_Sc032	Iran	PI 243741	*Secale*	*cereale*	*cereale*	P1	1.00
SD_Sc033	Greece	PI 249936	*Secale*	*cereale*	*cereale*	P2	2.60
SD_Sc034	Iran	PI 250744	*Secale*	*cereale*	*cereale*	P1	3.80
SD_Sc039	Austria	PI 254810	*Secale*	*cereale*	*cereale*	P1	3.80
SD_Sc040	Spain	PI 256026	*Secale*	*cereale*	*cereale*	P2	3.83
SD_Sc041	Switzerland	PI 263561	*Secale*	*cereale*	*cereale*	P12	1.00
SD_Sc042	Estonia	PI 265471	*Secale*	*cereale*	*cereale*	P1	1.00
SD_Sc043	Finland	PI 265473	*Secale*	*cereale*	*cereale*	P12	1.00
SD_Sc044	Turkey	PI 266975	*Secale*	*cereale*	*cereale*	P12	1.00
SD_Sc045	Latvia	PI 267098	*Secale*	*cereale*	*cereale*	P12	1.00
SD_Sc049	Hungary	PI 272333	*Secale*	*cereale*	*cereale*	P1	1.00
SD_Sc050	Afghanistan	PI 275356	*Secale*	*cereale*	*cereale*	P1	2.83
SD_Sc052	Russian Federation	PI 280838	*Secale*	*cereale*	*cereale*	P12	1.00
SD_Sc053	Russian Federation	PI 280841	*Secale*	*cereale*	*cereale*	P1	1.00
SD_Sc055	Iran	PI 289814	*Secale*	*cereale*	*cereale*	P1	2.67

SD_Sc056	Pakistan	PI 289827	*Secale*	*cereale*	*cereale*	P12	2.00
SD_Sc057	Slovakia	PI 290423	*Secale*	*cereale*	*cereale*	P12	2.80
SD_Sc058	Netherlands	PI 290425	*Secale*	*cereale*	*cereale*	P1	1.00
SD_Sc060	Germany	PI 290435	*Secale*	*cereale*	*cereale*	P12	2.80
SD_Sc061	Hungary	PI 290436	*Secale*	*cereale*	*cereale*	P12	3.00
SD_Sc062	Ukraine	PI 290439	*Secale*	*cereale*	*cereale*	P12	4.67
SD_Sc063	Finland	PI 290440	*Secale*	*cereale*	*cereale*	P12	3.80
SD_Sc066	Bulgaria	PI 294794	*Secale*	*cereale*	*cereale*	P12	1.20
SD_Sc067	Bulgaria	PI 294795	*Secale*	*cereale*	*cereale*	P12	4.00
SD_Sc069	Romania	PI 306487	*Secale*	*cereale*	*cereale*	P12	1.20
SD_Sc070	Romania	PI 306495	*Secale*	*cereale*	*cereale*	P12	3.00
SD_Sc072	Brazil	PI 314964	*Secale*	*cereale*	*cereale*	P1	3.00
SD_Sc073	France	PI 315957	*Secale*	*cereale*	*cereale*	P12	3.20
SD_Sc074	Netherlands	PI 315962	*Secale*	*cereale*	*cereale*	P1	1.83
SD_Sc075	Canada	PI 323363	*Secale*	*cereale*	*cereale*	P12	2.00

SD_Sc077	United States	PI 323377	*Secale*	*cereale*	*cereale*	P2	1.67
SD_Sc078	Spain	PI 323383	*Secale*	*cereale*	*cereale*	P1	1.33
SD_Sc081	Poland	PI 323449	*Secale*	*cereale*	*cereale*	P2	1.67
SD_Sc082	Poland	PI 323454	*Secale*	*cereale*	*cereale*	P2	2.00
SD_Sc083	Austria	PI 326407	*Secale*	*cereale*	*cereale*	P2	4.00
SD_Sc084	South Africa	PI 330413	*Secale*	*cereale*	*cereale*	P2	2.17
SD_Sc087	Germany	PI 330424	*Secale*	*cereale*	*cereale*	P12	4.00
SD_Sc089	South Africa	PI 330431	*Secale*	*cereale*	*cereale*	P12	2.40
SD_Sc091	Sweden	PI 330439	*Secale*	*cereale*	*cereale*	P2	3.00
SD_Sc093	Netherlands	PI 330445	*Secale*	*cereale*	*cereale*	P12	2.17
SD_Sc094	United Kingdom	PI 330526	*Secale*	*cereale*	*cereale*	P2	1.33
SD_Sc096	Poland	PI 338383	*Secale*	*cereale*	*cereale*	P2	1.00
SD_Sc097	Montenegro	PI 344980	*Secale*	*cereale*	*cereale*	P1	3.00
SD_Sc098	Macedonia	PI 344991	*Secale*	*cereale*	*cereale*	P1	3.17
SD_Sc099	Macedonia	PI 344998	*Secale*	*cereale*	*cereale*	P1	1.20

SD_Sc100	Serbia	PI 345000	*Secale*	*cereale*	*cereale*	P1	2.17
SD_Sc101	United Kingdom	PI 345531	*Secale*	*cereale*	*cereale*	P12	2.17
SD_Sc102	Australia	PI 345739	*Secale*	*cereale*	*cereale*	P2	2.17
SD_Sc103	Australia	PI 345740	*Secale*	*cereale*	*cereale*	P12	2.17
SD_Sc104	Australia	PI 346416	*Secale*	*cereale*	*cereale*	P12	2.17
SD_Sc107	Montenegro	PI 349912	*Secale*	*cereale*	*cereale*	P2	2.33
SD_Sc109	Bosnia and Herzegovina	PI 349923	*Secale*	*cereale*	*cereale*	P12	2.17
SD_Sc110	Turkey	PI 357067	*Secale*	*cereale*	*cereale*	P1	1.80
SD_Sc111	Croatia	PI 362391	*Secale*	*cereale*	*cereale*	P1	1.00
SD_Sc116	Afghanistan	PI 366503	*Secale*	*cereale*	*cereale*	P2	3.00
SD_Sc117	Sweden	PI 368157	*Secale*	*cereale*	*cereale*	P12	3.67
SD_Sc118	Estonia	PI 372114	*Secale*	*cereale*	*cereale*	P2	4.67
SD_Sc119	Ukraine	PI 372115	*Secale*	*cereale*	*cereale*	P12	2.67
SD_Sc120	Belarus	PI 372116	*Secale*	*cereale*	*cereale*	P12	2.83
SD_Sc122	Belarus	PI 372119	*Secale*	*cereale*	*cereale*	P2	3.67

SD_Sc127	Serbia	PI 378230	*Secale*	*cereale*	*cereale*	P1	2.17
SD_Sc128	Serbia	PI 378231	*Secale*	*cereale*	*cereale*	P1	2.00
SD_Sc129	Macedonia	PI 378233	*Secale*	*cereale*	*cereale*	P1	1.33
SD_Sc131	Macedonia	PI 378239	*Secale*	*cereale*	*cereale*	P12	1.00
SD_Sc134	Germany	PI 392069	*Secale*	*cereale*	*cereale*	P12	4.00
SD_Sc136	Lithuania	PI 404227	*Secale*	*cereale*	*cereale*	P2	2.17
SD_Sc141	United Kingdom	PI 414080	*Secale*	*cereale*	*cereale*	P12	1.50
SD_Sc146	India	PI 430004	*Secale*	*cereale*	*cereale*	P1	1.33
SD_Sc147	Chile	PI 436165	*Secale*	*cereale*	*cereale*	P12	3.40
SD_Sc148	Chile	PI 436171	*Secale*	*cereale*	*cereale*	P2	3.67
SD_Sc150	Chile	PI 436192	*Secale*	*cereale*	*cereale*	P2	4.00
SD_Sc152	Israel	PI 445980	*Secale*	*cereale*	*cereale*	P2	1.20
SD_Sc154	Canada	PI 445984	*Secale*	*cereale*	*cereale*	P1	3.17
SD_Sc157	Canada	PI 445998	*Secale*	*cereale*	*cereale*	P2	3.17
SD_Sc161	Japan	PI 446020	*Secale*	*cereale*	*cereale*	P2	3.17

SD_Sc162	Mexico	PI 446058	*Secale*	*cereale*	*cereale*	P12	1.40
SD_Sc163	Lithuania	PI 446123	*Secale*	*cereale*	*cereale*	P1	4.67
SD_Sc167	Greece	PI 446151	*Secale*	*cereale*	*cereale*	P2	2.00
SD_Sc168	Poland	PI 446177	*Secale*	*cereale*	*cereale*	P12	2.17
SD_Sc169	Latvia	PI 446181	*Secale*	*cereale*	*cereale*	P2	1.33
SD_Sc170	Portugal	PI 446195	*Secale*	*cereale*	*cereale*	P2	3.00
SD_Sc173	Romania	PI 446245	*Secale*	*cereale*	*cereale*	P2	2.83
SD_Sc176	Estonia	PI 446514	*Secale*	*cereale*	*cereale*	P1	3.50
SD_Sc177	China	PI 447337	*Secale*	*cereale*	*cereale*	P2	3.00
SD_Sc178	China	PI 452132	*Secale*	*cereale*	*cereale*	P1	1.67
SD_Sc179	China	PI 452133	*Secale*	*cereale*	*cereale*	P1	3.67
SD_Sc180	United States	PI 464583	*Secale*	*cereale*	*cereale*	P1	2.17
SD_Sc182	United States	PI 491395	*Secale*	*cereale*	*cereale*	P1	3.00
SD_Sc185	United States	PI 522185	*Secale*	*cereale*	*cereale*	P2	1.00
SD_Sc186	Morocco	PI 525203	*Secale*	*cereale*	*cereale*	P2	2.40

SD_Sc187	Morocco	PI 525205	*Secale*	*cereale*	*cereale*	P1	3.67
SD_Sc191	Italy	PI 534929	*Secale*	*cereale*	*cereale*	P1	2.67
SD_Sc195	Romania	PI 534943	*Secale*	*cereale*	*cereale*	P12	3.00
SD_Sc197	Ukraine	PI 534948	*Secale*	*cereale*	*cereale*	P2	2.17
SD_Sc201	United States	PI 534954	*Secale*	*cereale*	*cereale*	P2	3.00
SD_Sc202	Czechoslovakia	PI 534956	*Secale*	*cereale*	*cereale*	P12	1.50
SD_Sc203	Austria	PI 534960	*Secale*	*cereale*	*cereale*	P2	3.33
SD_Sc204	United States	PI 534961	*Secale*	*cereale*	*cereale*	P2	1.83
SD_Sc205	United States	PI 534962	*Secale*	*cereale*	*cereale*	P1	1.67
SD_Sc209	Belgium	PI 534970	*Secale*	*cereale*	*cereale*	P12	2.80
SD_Sc210	Argentina	PI 534987	*Secale*	*cereale*	*cereale*	P2	1.00
SD_Sc211	Argentina	PI 534988	*Secale*	*cereale*	*cereale*	P12	1.00
SD_Sc214	Kenya	PI 535006	*Secale*	*cereale*	*cereale*	P12	1.83
SD_Sc215	Austria	PI 535007	*Secale*	*cereale*	*cereale*	P2	1.00
SD_Sc219	Portugal	PI 535083	*Secale*	*cereale*	*cereale*	P1	1.00

SD_Sc220	Portugal	PI 535094	*Secale*	*cereale*	*cereale*	P1	2.75
SD_Sc225	France	PI 535144	*Secale*	*cereale*	*cereale*	P1	1.17
SD_Sc227	United States	PI 535154	*Secale*	*cereale*	*cereale*	P12	1.83
SD_Sc229	United States	PI 535159	*Secale*	*cereale*	*cereale*	P1	1.50
SD_Sc230	Romania	PI 535163	*Secale*	*cereale*	*cereale*	P12	1.83
SD_Sc231	Uruguay	PI 535174	*Secale*	*cereale*	*cereale*	P2	2.00
SD_Sc239	Poland	PI 535192	*Secale*	*cereale*	*cereale*	P12	2.83
SD_Sc241	United States	PI 535199	*Secale*	*cereale*	*cereale*	P2	2.33
SD_Sc242	Mexico	PI 542467	*Secale*	*cereale*	*cereale*	P12	1.33
SD_Sc243	United States	PI 542469	*Secale*	*cereale*	*cereale*	P2	2.00
SD_Sc244	Brazil	PI 542470	*Secale*	*cereale*	*cereale*	P2	1.00
SD_Sc246	Argentina	PI 543398	*Secale*	*cereale*	*cereale*	P1	4.33
SD_Sc247	Turkey	PI 543408	*Secale*	*cereale*	*cereale*	P1	1.83
SD_Sc249	Turkey	PI 543593	*Secale*	*cereale*	*cereale*	P1	3.00
SD_Sc251	Turkey	PI 543664	*Secale*	*cereale*	*cereale*	P2	2.50

SD_Sc254	United States	PI 543729	*Secale*	*cereale*	*cereale*	P1	2.17
SD_Sc256	United States	PI 552973	*Secale*	*cereale*	*cereale*	P1	2.20
SD_Sc257	United States	PI 559980	*Secale*	*cereale*	*cereale*	P2	4.00
SD_Sc258	United States	PI 559981	*Secale*	*cereale*	*cereale*	P12	3.40
SD_Sc261	Turkey	PI 560572	*Secale*	*cereale*	*cereale*	P2	1.00
SD_Sc265	Sweden	PI 561674	*Secale*	*cereale*	*cereale*	P1	3.00
SD_Sc271	Turkey	PI 568106	*Secale*	*cereale*	*cereale*	P1	1.00
SD_Sc278	Pakistan	PI 578092	*Secale*	*cereale*	*cereale*	P1	1.00
SD_Sc281	Canada	PI 590948	*Secale*	*cereale*	*cereale*	P2	3.00
SD_Sc293	United States	PI 628642	*Secale*	*cereale*	*cereale*	P2	1.67
SD_Sc296	Tajikistan	PI 639328	*Secale*	*cereale*	*cereale*	P1	1.00
SD_Sc297	Tajikistan	PI 639336	*Secale*	*cereale*	*cereale*	P1	1.00
SD_Sc269	Pakistan	PI 561809	*Secale*	*cereale*	*cereale*	P2	1.33
SD_Sc326	Armenia	PI 618662	*Secale*	*cereale*	*afghanicum*	P12	2.33
SD_Sc010	Japan	Cise 107	*Secale*	*cereale*	*ancestrale*	P12	-

SD_Sc324	Soviet Union	PI 445975	*Secale*	*cereale*	*ancestrale*	P2	3.00
SD_Sc327	Turkey	PI 618663	*Secale*	*cereale*	*ancestrale*	P1	3.00
SD_Sc329	Turkey	PI 618669	*Secale*	*cereale*	*tetraploidum*	P2	2.00
SD_Sc332	South Africa	PI 630963	*Secale*	*strictum*	*africanum*	P13	-
SD_Sc323	United States	PI 445973	*Secale*	*strictum*	*anatolicum*	P12	2.66
SD_Sc333	Poland	PI 630967	*Secale*	*strictum*	*ciliatoglume*	P3	2.50
SD_Sc315	Armenia	PI 592292	*Secale*	*strictum*	*kuprijanovii*	P13	3.00
SD_Sc334	Poland	PI 630971	*Secale*	*strictum*	*strictum*	P13	1.50
SD_Sc330	Ukraine	PI 618674	*Secale*	*sylvestre*	-	P3	3.00
SD_Sc331	Bulgaria	PI 618675	*Secale*	*sylvestre*	-	P3	2.50
SD_Sc320	Afghanistan	PI 253957	*Secale*	*vavilovii*	-	P1	-
SD_Sc322	Hungary	PI 284842	*Secale*	*vavilovii*	-	P1	-

Table 3: Detailed description about the *T. turgidum* subsp. mini core accessions. Results for the screening for Fusarium head blight (FHB), leaf rust greenhouse screening (LR-GH), leaf rust field screening (LR-field) and tan spot (*PTR* race 5) screening are also presented. Number beside categories denote the average rating score for the corresponding disease. Green color highlights the resistant accessions.

Accession	***T. turgidum* *subsp.***	**Origin**	**FHB**	**LR - GH**	**LR-field**	**Tan spot**
PI341800	*carthlicum*	Russian Federation	-	S - 3.4	MR50	MS - 3.3
MG4330-66	*diccocoides*	-	-	S - 4	0	MR - 2
MG4343	*diccocoides*	-	S - 82.3	-	S80	-
PI352323	*dicoccoides*	Asia minor	S - 96.6	S - 4	0	MR - 2
PI428021	*dicoccoides*	Turkey	MS - 77.4	S - 4	S80	MR - 2
PI428054	*dicoccoides*	Turkey	S - 98.7	S - 3.1	S60	MS - 3
PI428057	*dicoccoides*	Turkey	MS - 61.1	S - 4	S50	MR - 2.2
PI428080	*dicoccoides*	Turkey	S - 93.3	S - 3.8	S80	MS - 3.2
PI428095	*dicoccoides*	Israel	S - 85.9	MS - 3	MR20	MS - 3.8

PI428105	*dicoccoides*	Israel	MR - 29.0	S - 3.5	MS90		R - 1
PI428143	*dicoccoides*	Lebanon	MS - 45.1	MR - 2	S80		R - 1
PI470944	*dicoccoides*	Syria	S - 100	MS - 3	S80		-
PI538657	*dicoccoides*	Turkey	-	S - 3.3	-		MR - 2.6
PI538672	*dicoccoides*	Israel	MS - 33.3	S - 4	S80		R - 1.5
PI538709	*dicoccoides*	Lebanon	S - 100	-	-		R - 1
PI538719	*dicoccoides*	Israel	S - 98.3	-	S80		-
Cltr4013	*dicoccon*	India	MR - 30.0	MS - 3	MR10		MS - 3.5
PI94667	*dicoccon*	Russian Federation	MS - 63.3	MS - 3	MR10		MS - 3.6
PI352369	*dicoccon*	Czech Republic	MS - 47.1	MS - 3	MR20		MS - 3.5
PI355497	*dicoccon*	Former Soviet Union	MR - 26.0	S - 3.2	MR20		R - 1.6
PI434993	*dicoccon*	Montenegro	-	S - 4	-		MS - 3.3
MG5293-1	*dicoccon*	Italy	R - 14.68	MR - 2		0	S - 4.16
MG5416-1	*dicoccon*	-	R - 15	S - 3.8		0	S - 4.6

MG5473	*dicoccon*	Spain	MS - 51.7	S - 4	S20	S - 4.8
Cltr1471	*durum*	Algeria	-	R - 1	5R	MS - 3.5
Cltr6870	*durum*	Tunisia	-	MS - 2.1	10R	S - 4
PI8898	*durum*	India	-	R - 1	-	MS - 3.5
PI47889	*durum*	Spain	-	MR - 2	MR20	MS - 3.3
PI60741	*durum*	Egypt	-	R - 0.6	S20	MS - 3.3
PI185233	*durum*	United Kingdom	-	R - 1	10R	S - 4.2
PI192843	*durum*	Portugal	-	S - 3.5	-	MR - 2.7
PI204050	*durum*	Portugal	-	MR - 2	0	S - 4
PI244061	*durum*	Yemen	-	HR - ;	5R	S - 4.1
PI265010	*durum*	Bosinia and Herzegovina	-	S - 4	S80	MR - 2.1
PI352459	*durum*	France	-	MS - 2.6	-	-
PI621771	*durum*	Iran	-	S - 4	S90	MS - 3.6
PI627550	*durum*	Iran	-	MS - 3	-	MS - 4

PI286547	*polonicum*	Ecuador	-	S - 4	MR20	MS - 3.8
PI289606	*polonicum*	United Kingdom	-	MS - 2.6	MR20	MS - 4
					S10/S80/S8	
PI306549	*polonicum*	Romania	-	S - 4	0	MS - 3.6
PI67343	*turanicum*	Australia	-	S - 4	-	S - 4.2
PI68287	*turanicum*	Azerbaijan	-	S - 4	MR20	S - 4.6
PI352514	*turanicum*	Azerbaijan	-	S - 4	-	MS - 3.4
PI134951	*turgidum*	Portugal	-	MS - 2.2	R10	R - 1.7
PI542679	*turgidum*	Algeria	S - 100	MR - 1.5	S80	MR - 2.8
PI56263	*turgidum*	Portugal	-	MS - 2.6	-	S - 4.2
PI191104	*turgidum*	Spain	-	MS - 2.3	MS40	R - 1.8
KU7348	*abyssinicum*	Ethiopia	-	MS - 3	10R	-
KU138	*carthlicum*	-	-	-	-	MS - 4
KU14468	*dicoccoides*	Israel	-	MR - 2	S60	-
KU15917	*dicoccoides*	Israel	S - 100	MR - 2	S80	MR - 2.8

KU108-1	*dicoccoides*		-	MS - 50.3	MS - 2.6	S80	MS - 3.2
KU109	*dicoccoides*	Israel		-	S - 3.6	-	MR - 2
KU8941	*dicoccoides*	Iran		S - 100	S - 3.6	-	R - 1.1
KU14456	*dicoccoides*	Israel		S - 94.7	MS - 3	MS20	R - 1
KU14508	*dicoccoides*	Israel		MS - 32.2	MS - 3	S90	-
KU8821A	*dicoccoides*	Iraq		S - 90.5	MS - 3	S80	R - 1.1
KU108-3	*dicoccoides*	-		MS - 66.3	S - 4	S80	MR - 2
KU108-4	*dicoccoides*	Syria		S - 99.8	-	-	MS - 3.5
KU195	*dicoccoides*	Israel		MS - 69.9	S - 4	S80	MR - 2
KU1921	*dicoccoides*	Turkey		S - 100	S - 4	S80	R - 1.8
KU1974	*dicoccoides*	Turkey		-	S - 4	-	R - 1
KU8805	*dicoccoides*	Iraq		-	S - 4	S80	MR - 2
KU14493	*dicoccoides*	Israel		S - 86.6	S - 3.6	S80	S - 4
KU15808	*dicoccoides*	Turkey		S - 97.5	S - 4	S50	MS - 3.1
KU15819	*dicoccoides*	Turkey		S - 100	S - 4	S80	MS - 3.3

KU13451	*dicoccoides*	Israel	MS - 47.6	-	MS60	MS - 3.6
KU117	*dicoccon*	-	-	MS - 3	MS80	MS - 3.8
KU124	*dicoccon*	-	MR - 20.5	-	MS80	R - 1.7
KU1058	*dicoccon*	Spain	R - 14.2	-	0	S - 5
KU15549	*dicoccon*	Russian Federation	MS - 61	-	MR50	-
KU111	*dicoccon*	-	MS - 50.5	S - 4	5R	S - 4
KU114	*dicoccon*	-	-	S - 4	-	-
KU15626	*durum*	Yemen	-	MR - 2	0/0	S - 4.1
KU3679	*durum*	Syria	-	-	-	-
KU11701	*durum*	Greece	-	-	-	-
KU3701	*durum*	Turkey	-	R - 1	MS40	S - 4
KU15591	*durum*	Egypt	-	R - 0.3	S20	S - 4
KU128-1	*durum*	China	-	S - 4	S10	-
KU129-1	*durum*		-	S - 4	-	-
KU1359	*durum*	Greece	-	S - 4	-	S - 4

KU1369	*durum*	Greece	-	S - 4		0	MS - 3.8
KU1522	*durum*	Russian Federation	-	S - 4	S90		MS - 4
KU3732	*durum*	Turkey	-	S - 4	S80/S80		S - 4.1
KU11752	*durum*	Greece	-	S - 4		0	MR - 2.5
KU11805	*durum*	Greece	-	S - 4	S40		MS - 3.6
KU11830	*durum*	Greece	-	HR - ;	-		MR - 2
KU15681	*durum*	Iran	-	S - 4	S80		S - 4
KU137	*turanicum*	-	-	S - 4	MR20		MS - 3.6
KU190-2	*paleocolchicum*	USSR	-	S - 4	-		-
KU141	*polonicum*	-	-	S - 4	-		S - 4.2
KU146	*pyramidale*	-	-	S - 4	0/0		S - 4.4
KU15774	*turgidum*	Portugal	-	S - 4	MR10		S - 4
KU15787	*turgidum*	Algeria	-	S - 4	S100		S - 4
KU149	*turgidum*	-	-	MR - 1.1	MR80		S - 4