GENETIC STRUCTURE OF *LEPTOSPHAERIA MACULANS* POPULATIONS IN NORTH DAKOTA AND IDENTIFICATION OF GENES ASSOCIATED WITH RESISTANCE TO *L. MACULANS* IN *BRASSICA JUNCEA*

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ABSTRACT

Blackleg, caused by *Leptosphaeria maculans* is one of the most devastating diseases of canola (*Brassica napus*) in North Dakota. A study was conducted to characterize prevalence of pathogenicity groups (PG), identify population structure of *L. maculans* and identify sources of resistance among *B. juncea* accessions. Approximately 56% of the isolates belonged to PG-4, 13% to PG-3, 11% to PG-T, 5% to PG-1, and 2% to PG2. The remaining 13% of isolates could not be identified. The 605 single-spore cultures used to study the population genetics of *L. maculans* in ND were grouped according to their county of origin in five regions (NE, NC, NW, WC, and C) and each region was considered a population. These populations were tested for genetic variation at 7 microsatellite, 4 minisatellite, and for mating type loci. High levels of genetic diversity (H = 0.63 to 0.70) and significant gametic disequilibrium ($P \leq 0.001$) was observed in all populations. The ratio of mating type idiomorphs deviated significantly ($P < 0.05$) from 1:1 ratio for four populations. Highly significant ($P \leq 0.001$) G''_{ST} between pairs of populations indicated a strong population differentiation. STRUCTURE analysis identified three distinct genetic populations in which the majority of the isolates from WC and nearly half of the isolates from NC were assigned to subpopulation one. The remaining half of NC isolates were assigned to subpopulation two and rest were assigned to subpopulation three. To identify sources of resistance against PG-2, PG-3, PG-T, and PG-4, a set of 298 *B. juncea* accessions were screened in greenhouse trials. Six accessions were resistant to PG-2 and PG-3, nine accessions were resistant to PG-T and PG-4, and two accessions were resistant to PG-2 and PG-4. DNA extracted from these accessions was screened using 766 DArT markers to identify QTL associated with resistance. Thirteen DArT markers were significantly ($P<0.05$) associated with resistance to PG-2 with variability ranging from 0.9% to 6.4%. Of these, three were also found

significantly ($P<0.05$) associated with resistance to PG-3 with variability of 4.4%. No markers were found to be significantly associated with resistance to PG-T or PG-4 at ($P<0.05$).

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DEDICATION

This dissertation is dedicated to my parents, Mr. Pradeep Kumar Nepal and Mrs. Drupada Nepal, who raised me to be a person I am today; to my beloved husband Mr. Kuldeep KC whose encouragement, support, and understanding have made it possible for me to finish this work; and to my daughter Nirvana KC. Thank you all for your unconditional love that helped me to succeed and instill in me the confidence that I am capable of achieving goals that I sought for.

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CHAPTER 1: GENERAL INTRODUCTION

Leptosphaeria maculans (Desmaz) Ces. & de Not (anamorph = *Phoma lingam* (Tode:Fr.) Desmaz.) is a loculoascomycete that causes blackleg of crucifers. The disease is economically important and has been reported as a serious threat to the oilseed rape/canola industry in major canola growing regions in Europe, Australia and North America (West et al., 2001). The history on prevalence of *L. maculans* dates back to 65 years in Europe (Aubertot et al., 2004), 85 years in Australia (Sivasithamparam et al., 2005), 35 years in Canada (Petrie, 1978) and to around 20 years in the United States of America (Lamey and Hershman, 1993).

Besides taxonomic classification, their virulence profile and ability to produce phytotoxins have been used to characterize and separate different isolates of this pathogen. Broadly, the strains have been classified as virulent (group A) if they cause stem cankers on canola and avirulent (group B) if they do not cause stem cankers on canola (Howlett et al., 2001). Later, these groups were classified as two different species, the aggressive isolates belong to *L. maculans* and the non-aggressive species belong to *L. biglobosa.* Strains of the latter species do not produce toxins, sirodesmins, or pigmentation in liquid media (Shoemaker and Brun, 2001). Strains within each species are sexually compatible with each other but there is no intercrossing between strains of different species (Somda et al., 1997).

The phenotypic reaction of *L. maculans* strains on cotyledons of *B. napus* cultivars ‘Westar’, ‘Glacier’, and ‘Quinta’, helps classify them into pathogenicity groups (PG) 2, 3, and 4 (Koch et al., 1991), and more recently T (Chen and Fernando, 2005; Rimmer, 2006). Isolates that are nonaggressive on these differentials are classified as PG-1.

Pathogens with multiple reproductive systems and high potential for genotype flow pose the greatest risk of overcoming resistance genes (McDonald and Linde, 2002). *L. maculans* has

sexual and asexual reproductive cycles. *L. maculans* is heterothallic and outcrosses prolifically on canola stubbles producing pseudothecia (sexual fruiting bodies) that discharge ascospores (sexual spores). Ascospores are the main sources of infection in most of the disease epidemics and are dispersed with wind currents and rain splashes that normally occur late in spring and coincide with seedling emergence in North Dakota (Markell et al., 2008). Ascospores can be dispersed several miles and infect the seedlings from invasion of the cotyledons and younger leaves via stomata or wounds. After colonization of the tissues it produces masses of pycnidia (asexual fruiting bodies) on dead tissues. Under wet conditions, pycnidia produce pycnidiospores (asexual spores) that are dispersed by rain splash to other leaves and neighboring plants and act as secondary source of inoculum and trigger the disease epidemic. The spores are discharged throughout the growing season or until the residue is completely degraded (Bokor et al., 1975). This reproductive pattern explains the reason for this pathogen to overcome resistance genes in relatively shorter period of time. Thus a study on population genetic structure of *L. maculans* help us understand the evolutionary potential of the pathogen and optimize the screening procedures in plant breeding programs such that the tested lines are exposed to maximum possible variation in pathogen population.

Since the population of *L. maculans* can be very diverse within a field and has a high potential of changing the population structure in a short period of time, breeding for long term resistance to the disease is very challenging. Also the genetic basis of resistance to this disease in *Brassica* species is complex. Previous reports have indicated that inheritance of the resistance trait in the *Brassica* species can be both monogenic or polygenic and dominant or recessive (Cargeeg and Thurling, 1980; Pang and Halloran, 1996; Roy, 1984; Sippell et al., 1991). Qualitative and quantitative trait loci (QTL) conferring resistance to blackleg disease have been

characterized using bi-parental populations of *B. napus* (Mayerhofer et al., 1997; Ferreira et al., 1995; Yu et al., 2008; Kaur et al., 2009) and *B. juncea* (Christianson, 2006). However, the bi-parental mapping technique is cumbersome in terms of time and labor. In addition to this, other limitations such as poor mapping resolution, often missing detection of minor QTL, and detection of only two alleles at any given locus, the technique is becoming less popular (Flint-Garcia et al., 2003). On the other hand, a fairly new technique, association mapping, has been introduced to resolve the limitations of bi-parental mapping. "Association mapping techniques provide an opportunity to detect allele-trait associations in larger sets of genotypes representing a much broader array of the genetic diversity available for a particular trait such as germplasm collections, natural populations, etc." (Snowdon and Friedt, 2004). Association mapping has been successfully used in the detection of major and minor QTL for different agronomic traits in various crop species. It is also used to identify QTL for resistance to various diseases in maize (Kump et al., 2011), wheat (Tommasini et al., 2007; Gurung et al., 2011), barley (Massman et al., 2011; Roy et al., 2010), bean (Shi et al., 2011), and canola (Jestin et al., 2010). To our knowledge, however, no reports have ever been published on detection of QTL for resistance to blackleg among *B. juncea* accessions. It has been reported that *Brassica* species containing B genome; *Brassica nigra* (BB), *Brassica juncea* (AABB), and *Brassica carinata* (BBCC) have high levels of resistance to blackleg (Rimmer and van den Berg, 1992; Dixelius and Wahlberg, 1999). Thus, association mapping technique to detect genes for resistance to blackleg disease by exploring larger sets of *B. juncea* representing a wider array of genetic diversity would be a valuable tool for future breeding strategies.

Conclusively, blackleg is a serious disease of canola in North Dakota. It is very important to understand the population structure of the fungus, identify the sources of resistance to the

disease for future breeding programs, and to detect QTL for resistance to enhance marker assisted selection for efficient canola breeding programs. With regard to these, our study focuses on the following objectives.

I. To identify the pathogenicity groups of *L. maculans* isolates collected from North Dakota in 2004, 2007, and 2009 and study the pathogen diversity among the population.

II. To analyze the population structure of *L. maculans* in North Dakota using minisatellite and microsatellite markers.

III. To identify sources of resistance to blackleg (PG-2, PG-3, PG-4, and PG-T) in *B. juncea* accessions from USDA-ARS National Plant Germplasm System collected from diverse country of origin and identify markers associated with resistance to these PGs using association mapping.

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CHAPTER 2: LITERATURE REVIEW

The Pathogen: *Leptosphaeria maculans*

Leptosphaeria maculans (Desm.) Ces. & de Not [anamorph = *Phoma lingam* (Tode:Fr.) Desm.] is a fungus in the phylum Ascomycota. It is a loculoascomycete that is economically important as it is pathogenic to most cruciferous crops and causes serious yield losses worldwide. Broadly, *L. maculans* is classified as pathotype 'A' or virulent group and pathotype 'B' or avirulent group. Within each group there is different pathogenicity groups that are identified based on host/pathogen interaction phenotypes as well as molecular techniques. Many studies have focused on these two groups and based on symptoms, morphology, toxin production and ITS sequence analysis, the species in pathotype 'A' are named as *L. maculans* and those in pathotype 'B' are named as *L. biglobosa*. These two species are believed to have co-evolved but later differentiated due to host specialization, and geographic and epidemiological isolation. Based on ITS sequence polymorphism, isolates in *L. maculans* are highly polymorphic and are believed to be a younger and currently expanding species; whereas *L. biglobosa* are monomorphic and are fixed species (Berbee et al., 2003).

Taxonomy and nomenclature

The taxonomy and nomenclature associated with this fungus is very confusing since *L. maculans* comprises several distinct species that are morphologically similar. Generally, molecular approaches such as genetic mapping, gene cloning and disruption, and complementary mutants are used to characterize filamentous fungi. For *L. maculans*, they have been characterized based on their virulence profile and chemical analysis of secondary metabolites of both the fungus and its host. Based on these characters, Howlett et al. (2001) broadly classified the strains into two pathotype groups as virulent "A" group that causes stem cankers on canola

and produces the phytotoxin sirodesmin PL and avirulent “B” group that does not cause stem cankers on canola and does not produce the phytotoxin. The strains are sexually compatible if they belong to the same group but incompatible with strains of different groups (Somda et al., 1997). The B pathotype group was designated as *L. biglobosa* sp. nov; whereas the A pathotype was designated as *L. maculans* (Desm.) Ces. & de Not. [anamorph = *Phoma lingam* (Tode:Fr.) Desm.] based on the reproduction barriers between these two groups, a distinct morphological difference on the ascomata, nonaggressive nature of B group, non-production of toxin and absence of pigment in liquid media (Shoemaker and Brun, 2001). Since the A pathotype group produces damaging symptoms on *Brassica* species, this group is economically more important than the B pathotype group.

Classification of *L. maculans*

Kingdom: Fungi

Phylum: Ascomycota

Class: Dothideomycetes

Order: Pleosporales

Family: Leptosphaeriaceae

Genus: *Leptosphaeria*

Species: *maculans* (Desm.) Ces. & De Not.

Leptosphaeria maculans (Desm.) Ces. & De Not. has been placed in the class Ascomycota. This fungus was first described on dead cabbage stems as a saprophytic organism by Tode in 1791 (cited in Williams, 1992) who named it *Sphaeria lingam.* Later in 1849, Desmazieres collected the same fungus from living *Brassica oleracea* plants and transferred it to the genus *Phoma* [*Phoma lingam* (Tode ex Fr.) Desm. (1849)] (Desmaziere, 1849). During the

next 130 years, its name was changed to *Phoma brassicae*, *Phoma oleracea*, *Phoma napobrassicae*, *Plenodomus lingam*, and was also given other generic and specific epithets (Boerema, 1976). In 1957, the sexual stage of *P. lingam* was found in New Zealand and later confirmed as *Leptosphaeria maculans* (Desm.) Ces. & De Not. Currently, the fungus is named as *Leptosphaeria maculans* (Desm.) Ces. & De Not. for the sexual state and *Phoma lingam* (Tode ex Fr.) Desm. for the asexual state.

Morphological features of *L. maculans*

Leptosphaeria maculans produces pseudothecia on the decaying stems and leaves of infected plants. These black, globose fruiting bodies, 300-500 µm in diameter with protruding ostioles, are immersed in the host tissue, and become erumpent when mature (Figure 2.1 A and B). The pseudothecia are composed of hyaline, septate, filiform pseudoparaphyses. They contain cylindrical to clavate asci (Figure 2.1 C). Eight ascospores (35-70 x 5-8 µm) with rounded ends are encapsulated within each bitunicate ascus (80-125 x 15-22 µm) (Figure 2.1 D). The ascospores are biseriate (in two rows) and have a cylindrical to ellipsoidal shape. They are yellow- brown color, with 5 transverse septa, and guttulate (containing small oil droplets). The fungus is heterothallic, i.e. two different mating types must be present for sexual reproduction to occur (Williams, 1992).

Asexual spores are pycnidiospores produced in pycnidia (Figure 2.1 E.). Two types of pycnidia are found in diseased tissues. Type I (sclerotioid form) pycnidia are immersed in the host tissue and become exposed in groups. The pycnidia lack a definite shape, have narrow ostioles, and have walls composed of several layers of thick-walled sclerenchymatous cells. Type II pycnidia are globose and black with a wall composed of several layers of cells. Only the cells in the outer layer are thick-walled. Pycnidiospores (conidia) are unicellular, hyaline (colorless)

spores, cylindrical and straight, although a few may be curved. There is one guttule (oil droplet) at each end of the spore. These asexual spores are produced in pink to pale purple ooze under moist conditions (Figure 2.1 F.) (Williams, 1992).

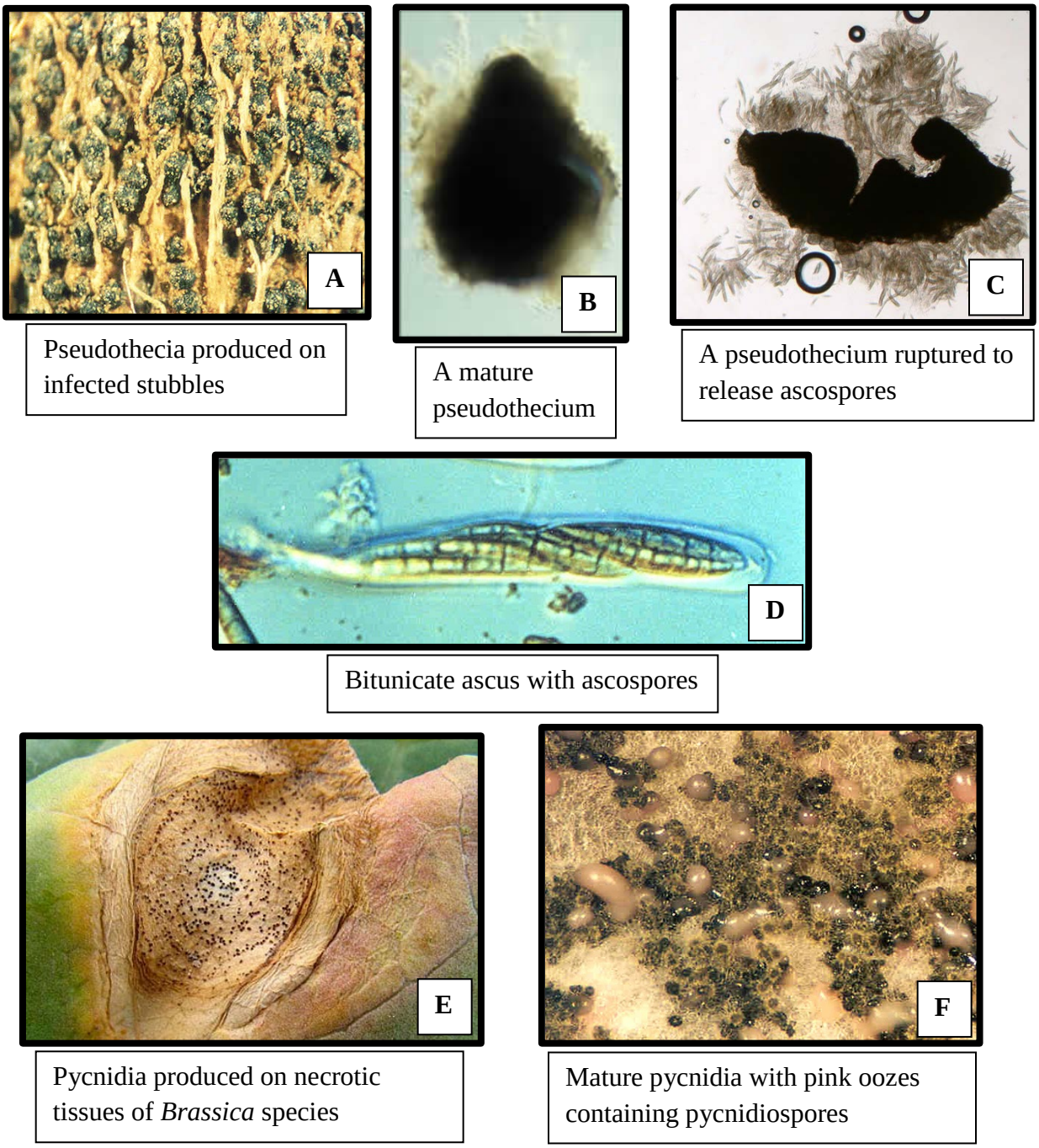

Fig. 2.1. Sexual fruiting bodies (A - C) and spore (D) and asexual fruiting body (E) and spores (F) of *Leptosphaeria maculans.* Figures adapted from Ash, 2000.

Life cycle of *L. maculans*

Both pycnidia and pseudothecia are the survival structures of the fungi. Under suitable conditions of temperature, radiation, and high relative humidity, both conidia and ascospores are released in large numbers. Ascospores**,** the primary source of inoculum in most of the disease epidemics, are produced in pseudothecia on infested canola stubble and are dispersed by wind currents. When they land on seedlings they start infecting the cotyledons and younger leaves via stomata or wounds. Initially the fungus colonizes tissues as a biotroph and the tissues remain symptomless but later the pathogen enters a necrotrophic phase in which it induces cell death and degradation. The fungus then utilizes the resources in necrotic leaf lesions or stems cankers to support the production of pycnidia and produces a large number of pycnidiospores in dead tissues (Hammond and Lewis, 1987; Hammond et al., 1985). Pycnidiospores are the secondary source of inoculum spread by rain splash to neighboring plants and leaves. The fungus descends from aerial tissues to the main stem and finally invades and kills cells of the stem cortex, resulting in a blackened canker that girdles the base of the stem, giving it the appearance of a "blackleg". The severely infected plants cannot stand and lodge without producing seeds. The fungus overwinters on the dead stubble until next favorable conditions are available.

Variation in *L. maculans* strain specialization

As explained earlier, the fungus has been broadly classified under two pathotype groups (virulent “A” group and avirulent “B” group) based on their virulence profile. Isolates of A pathotype were initially classified into pathogenicity groups (PG) 2, 3, and 4 based on interaction phenotypes on cotyledons of *B. napus* differential cultivars ‘Westar’, ‘Glacier’, and ‘Quinta’, (Koch et al., 1991). A new aggressive type PG-T was reported in North American populations of *L. maculans* (Chen and Fernando, 2005; Rimmer, 2006).The nonaggressive isolates on these

three differentials are classified as PG-1. Isolates in the A pathotype are highly diverse in their aggressiveness (Chen and Fernando, 2006) but are genetically similar (Koch et al., 1991). The classification of pathogenicity groups based on the differential interaction has been explained in Table 2.1.

Table 2.1. Phenotypic reaction of *Brassica napus* differential cultivars to inoculation[x] with *Leptosphaeria maculans*.

Pathogenicity Group	Differential cultivars[y]		
	Westar (-)	Glacier (*Rlm2,Rlm3*)	Quinta (*Rlm1, Rlm3*)
1	R	R	R
2	S	R	I
3	S	S	I
T	S	I	S
4	S	S	S

[x] Inoculations made when seedlings are at the cotyledon stage.
[y] Resistance genes present in differentials is included in parentheses.

Isolates of B pathotype are divided into three genetically distinct subgroups, namely NA1, NA2, and NA3. The distinction between these subgroups is supported by isozyme and soluble protein studies (Gall et al., 1995; Somda et al., 1996), analysis of regions of ribosomal DNA including 18S, 5.8S, and the internal transcribed spacers (ITS) (Balesdent et al., 1998), RAPD (Goodwin and Annis, 1991) and AFLP analysis (Purwantara et al., 2000). The AFLP analysis revealed no common bands between these subgroups (Purwantara et al., 2000).

Ecology of *L. maculans*

The survival structures of the fungi such as pseudothecia, ascospores, pycnidia, pycnidiospores, and mycelium occur in crop residues as long as the residues are available in nature which may be up to five years (cited in Hall, 1992). In Western Australia, a study on the survival of the fungus on rapeseed root residues showed that the fungus sporulated on rape root residues located close to the soil surface for up to 18 months and in some other cases the fungus sporulated on roots buried 10 cm for six months but no sporulation was noted on roots buried for

eight months or longer (MacNish, 1979). Similar other evidences have shown that the fungus continues to grow in number and may result in disease epidemics if host crops are planted every year in the same or adjacent fields but tends to decrease if rotated with non-host crops for at least three years (McGee, 1977; Petrie, 1978). Ascospores are the most important primary inoculum. In France, pseudothecia are observed in the field from September onwards and release ascospores intensely in September and October when rape is at an early vegetative stage. The severity of the disease is directly proportional to the number of ascospores discharged from the residue; however, it is inversely proportional to the distance from source of ascospores. Disease incidence and severity declined beyond 2-5 km indicating that aerial dispersal of the ascospores occurs within 5 km (Gladders and Musa, 1980; Hammond and Lewis, 1986; Petrie, 1978). Contaminated seeds may serve as an important inoculum reservoir and are the most common way of introducing the fungus into a new area but are not considered important in an area where the fungus is already present. Some non-host crops in the Compositae, Ongraceae, and Gentianacea families have also been reported as inoculum reservoir of *L. maculans* (Petrie and Vanterpool, 1965).

Ascospores of *L. maculans* are dispersed in the air and may be trapped during several months of the year but peak releases occur in September, October, or November in Europe (Gladders and Musa, 1980), and in June, July, and August in Australia (Bokor et al., 1975; McGee, 1977) and Western Canada (McGee and Petrie, 1979). Temperatures of 8-12^{o} C are favorable for maximum ascospore release (McGee, 1977). In nature, pseudothecia are readily formed on woody remains of infected plants. They begin to appear 1 to 10 months after harvest and occur as long as infested crop residue persists (Petrie, 1986). *In vitro* production of pseudothecia requires special treatments. As *L. maculans* is a heterothallic fungus, strains of

opposite mating types are required to be grown together on V8 juice agar to produce the sexual state under white light at 25^{o} C for 5-15 days. The culture is then covered with a layer of water agar and incubated under near ultraviolet light at 16^{o} C for a further 15-25 days (Xu et al., 1987).

Pycnidia and pycnidiospores of *L. maculans* are produced readily in infected plant tissue in the field and in laboratory. The most favorable conditions for pycnidiospore production are continuous surface wetness, light (500 µmol $m^{-2}s^{-1}$) and temperature of 10-25^{o} C. No spores are produced at 30^{o} C and no pycnidia are developed at 5^{o} C (Vanniasingham and Gilligan, 1989).

Phylogeny of *L. maculans*

Ascomycota is one of the four phyla in the kingdom Fungi. Most phylogenetic studies show that Ascomycota is a monophyletic group that descended from a common ancestral species (Keeling et al., 2000; Liu et al., 1999). Ascomycota and Basidiomycota are the two largest phyla of terrestrial fungi and are one another's closest relatives. Berbee and Taylor (1993) estimated that Ascomycota and Basidiomycota diverged 400 million years ago; whereas Heckman et al., (2001) suggested the divergence was as old as 1.2 billion years ago. Their studies suggest that plants had to compete with fungi ever since their terrestrialization perhaps 460 million years ago and land plants had probably been the main nutrient source for the Ascomycota and Basidiomycota as evidenced by the evolutionary history and predominance of plant saprophytes, pathogens and mycorrhizal species in both phyla (Berbee, 2001).

Within Ascomycota, there are three main subphyla, each including saprobes on plant materials as well as pathogens of plants and animals. The first diverged subphyla was the Taphrinomycotina (Archiascomycetes) that ranged from obligate plant pathogens like *Taphrina* and *Protomyces*, to animal pathogens like *Pneumocystis* and the saprobic fission yeasts *Schizosaccharomyces* (Nishida and Sugiyama, 1994). The next two diverged subphyla were

Saccharomycotina and Pezizomycotina. The Saccharomycotina included over 40 genera of ascomycetous yeasts, only one human pathogen, *Candida*, and only one plant pathogen *Eremothecium*. The Pezizomycotina included more than 3000 genera and are the majority of known ascomycete species. Most of the ascomycetous pathogens and mutualists are included in this subphylum. Most lichens are in the class Lecanoromycetes, and mycorrhizal ascomycetes are concentrated in the class Pezizomycetes (operculate discomycetes). The plant pathogenic fungi are distributed mostly in three classes, Sordariomycetes (Pyrenomycetes), Dothidiomycetes (Loculoascomycetes) and Leotiomycetes (the inoperculate Discomycetes) (Berbee, 2001).

When the correlations between ascomycete classes were analyzed between plant and animal pathogens based on pathogenicity, it was found that gains and losses of animal and plant pathogenicity were concentrated in a few ascomycete classes. The parsimony evaluation on evolution of pathogenicity in these classes showed that the ancestors of *Protomyces* and *Taphrina* were both pathogens; for the species in Sordariomycetes, the ancestor could have been equally a pathogen or a non-pathogen, but for the Dothideomycetes and the Leotiomycetes, the ancestors were not plant pathogens. These patterns of pathogenicity in the classes of ascomycete imply that pathogenicity might have arisen due to class-wide differences in genetic predisposition to pathogenicity. The predisposing characters to each pathogenic class may not be the same for all ascomycetes. For some fungi, the pathogenicity might have resulted from a succession of traits coevolved in response to the evolution of plant defenses. Thus the original traits that unified the pathogens within a class in ascomycetes might have been lost in cost of new characters making it unique to genera and species evolved in a plant/fungus arms race (Berbee, 2001).

Leptosphaeria is one of the largest genera in the order Pleosporales, and class Dothidiomycetes. It comprises of more than 1600 described taxa. *Leptosphaeria maculans* is a species complex, most of which are associated with blackleg of crucifers regardless of geographical and host plant considerations. Rouxel et al. (cited in Mendes-Pereira et al., 2003) have described more than 12 species of *Leptosphaeria* from cruciferous hosts. Camara et al., (2002) established *Leptosphaeria s. str*. as a putative monophyletic clade to include *L. maculans, L. conferta, L. congesta, L. dryadis, L. weimeri* and *L. doliolum*. The clade consists of a series of species pathogenic on dicotyledonous plants. Because of the broad variation in *L. maculans* a number of techniques with resultant terminologies have been utilized to distinguish between components of the *L. maculans* species complex. The terminologies were based on: 1) virulence studies on *Brassica* species; 2) the isolates obtained from differentiating 'Brassica', 'Sysimbrium', 'Thlaspi', 'Erysimum', 'Lepidium' and 'Descurainia' hosts; 3) RFLP studies differentiating between the A and B groups of *L. maculans*; 4) production of toxic secondary metabolites leading to the Tox^{+} / Tox^{0} terminology; and 5) additional RFLP studies supported by soluble protein and isozyme studies leading to the NA1, NA2 and NA3 subgrouping (Mendes-Pereira et al., 2003).

Mendes-Pereira et al., (2003) analyzed the sequences of the entire ITS region, including 5.8S rDNA from 38 isolates that represented the seven subgroups and isolates from different geographic origin and compared them to the closely related *Leptosphaeria* species. When phylogeny between the groups were analyzed using parsimony and distance analysis, a well-supported clade including all the *L. maculans* species complex along with *L. conferta*, a known saprobe of dried crucifer stems, was obtained. Within the clade, two subclades were separated with strong support, with all the Tox^{0} isolates in one clade, and the Tox^{+} and the 'Lepidium'

isolate in the second clade. The first clade corresponded to *L. biglobosa* and the second to *L. maculans s. str*. The analysis further separated groups within each species, which are associated with specific host plants or geographic origin. For example, *L. maculans* 'brassicae' is from cultivated *Brassica*; *L. maculans* ' lepidii ', from *Lepidium* sp.; *L. biglobosa* 'brassicae', from various *Brassica* species; *L. biglobosa* ' thlaspii ' from *Thlaspi arvense*; *L. biglobosa* 'erysimii' from *Erysimum* sp.; and *L. biglobosa* 'canadensis' mostly found in central Canada. The oldest *L. maculans* specimens maintained in international collections clustered with either *L. maculans* 'brassicae', *L. biglobosa* 'brassicae ', or a still different group closely related to *L. biglobosa* ' thlaspii '.

From the study of Mendes-Pereira et al. (2003) it is observed that *L. maculans* 'brassicae' and *L. biglobosa* 'brassicae' were found to be highly specialized on *Brassica* species (*B. juncea*, *B. napus*, and *B. oleracea*), and were world-wide in distribution. They were found in Europe, Canada, and the USA. In contrast to *L. maculans* 'brassicae', *L. biglobosa* 'brassicae' has been reported from China but not from Australia or central Canada. In Ascomycota it has often been speculated that related fungal species frequently infect related host plant species, and that co-evolution leading to host plant specialization was an important factor in fungal reproductive isolation and speciation (Berbee, 2001). In case of these two species, different assumptions have been made for the possible speciation. One assumption suggests that specialization on different crucifer species might have led to speciation within the *L. maculans – L. biglobosa* species complex. One major difference between these two species is ITS sequence polymorphism because *L. biglobosa* 'brassicae' is highly monopnorphic, whereas *L. maculans* 'brassicae' shows a non-negligible ratio of polymorphic residues in its ITS sequence. From this it can be hypothesized that *L. maculans* 'brassicae' is a younger, currently expanding species; whereas

monophyletic *L. biglobosa* 'brassicae' is a fixed species (Berbee et al., 2003). The presence of only one of the two species in few countries also suggests that the geographic isolation could have been the force leading to speciation.

Genetic diversity in *L. maculans* population

The population genetic structure of *L. maculans* has been studied in various geographical scales. Hayden and Howlett (2005) have described genetic structure of the fungus in a disease nursery of *B. napus* in Australia using microsatellite, minisatellite and mating type markers. They reported a very low level of genetic differentiation between ascospores from stubbles and conidia from seedlings, indicating that the isolates of the disease nursery belong to one population. Another study revealed a regional genetic differentiation among the *L. maculans* populations in Australia. Hayden et al. (2007) reported a high level of genetic differentiation of *L. maculans* isolates between Eastern and Western Australia explained by a long arid desert that acted as a natural barrier between the regions; however, the population within the region remained genetically stable over the two consecutive years. Gout et al. (2006) studied the genetic variability and distribution of mating type alleles in field populations of *L. maculans* in France. Interestingly, the population differentiation among the fields in four different regions was low indicating a high degree of gene flow between these populations; however, within each field the level of genetic diversity was high as explained by the equal frequencies of mating type alleles within the field.

The genetic diversity study of *L. maculans* also supports the assumption of genetic differentiation among the *L. maculans* isolates within wide geographic regions. These studies were conducted using Simple Sequence Repeats or microsatellites and minisatellite markers to facilitate gene diversity analyses in *L. maculans.* Minisatellites and microsatellites are tandemly

repeated DNA sequences that are abundantly spread in the genome of all eukaryotic species. The two markers are usually distinguished according to the size of repeats (Debrauwere et al., 1997). Minisatellites have a repeat motif of 10–60 bp resulting in repeated blocks of 0.1–30 kb (Armour et al., 1999), while microsatellites are repeats with base motif of 1–6 bp long, with shorter tandem arrays up to 1 kb or much less (Chambers and MacAvoy, 2000). Various polymorphic minisatellite (Attard et al., 2001; Eckert et al. 2005), microsatellite markers (Hayden et al., 2004) and mating type loci (Cozijnsen and Howlett, 2003) have been identified. These markers can now be applied in genetic diversity studies of *L. maculans*.

The Host: *Brassica spp.*

The genus *Brassica* belongs to Brassicaceae family and contains 37 different species (Gomez-Campo, 1980). The species in this genus provide edible roots, leaves, stems, buds, flowers, and seeds. There are many species that are potential source of oil, condiments and other products. The wild relatives provide nuclear genes that could serve as sources of resistance to certain diseases and pests and also serve as a potential source of cytoplasmic male sterility that could be exploited to develop hybrid seeds in *Brassica* breeding programs (Rakow, 2004).

Economic importance

Different species of *Brassica* are utilized as vegetables and oilseeds. The vegetable *Brassica* are utilized worldwide and are of different forms. The most common *Brassica* species used as vegetable is *B. oleracea.* It includes wide range of cole and cabbage type vegetables such as headed cabbages, Brussels sprouts, cauliflower, broccoli, etc. The other species *B. rapa* var. *pekinensis* (Chinese cabbage) and var. *chinensis* (leaf cabbage), *B. alboglabra* (Chinese kale) are grown extensively in China and some Southeast Asian countries as vegetables. *B. juncea* (leaf mustard) var. *japonica* and *B. juncea* var. *integrifolia* are cultivated as leafy vegetables in China,

India and other Asian countries. *Brassica* vegetables are locally consumed in some countries while in others it represents a big trade. In USA the production is mostly concentrated in California from where it is traded to the other states and to Canada year-round. Similarly, Brittany in France is the production center from where it is marketed to other European countries (Kimber and McGregor, 1995). Breeding of vegetable *Brassica* are conducted by specific government institutions, universities, and private forms with the main objective of crop uniformity and productivity. Development of disease and insect resistance is the next priority for breeding programs (Buzza, 1995).

Brassica oilseeds (rapeseed/canola) are of great economic importance worldwide. It has become one of the most important sources of edible oil after soybean. It is cultivated extensively in India, China, Canada, Europe (France, Germany, and UK), and Australia with an average hectarage of 0.68 to 6.51 million (Food and Agriculture Organization of the United Nations, 1991/1992-2000/2001). In USA it is grown mostly in the northern states in average of 237 thousand ha. Europe is the highest yielding region with annual average yields of about 3 tons/ha. Canada and Australia are the major rapeseed exporting countries while the production in other countries is locally consumed (Kimber and McGregor, 1995).

Canola-quality rapeseeds are of high economic importance in terms of quality of oil and meal. Canola is an acronym for Canadian Oil, Low Acid". However, the Canola Council of Canada has defined it as oilseed rape that produces "oil that must contain less than 2% erucic acid and the solid component of the seed must contain less than 30 micromoles of any one or any mixture of 3-butenyl glucosinolate, 4-pentenyl glucosinolate, 2-hydroxy-3-butenyl glucosinolate, and 2-hydroxy-4-pentenyl glucosinolate per gram of air-dry, oil-free solid." Due to these features, it is also known as double-low rapeseed. Canola was bred originally in Canada in 1970

using traditional plant breeding techniques. Baldur R. Stefansson and Keith Downey identified rapeseed plants with low eicosenoic and erucic acid content from an Argentina *B. napus* accession and developed a cultivar with low erucic acid. Then a second Polish *B. napus* variety with low glucosinolate was identified. These two traits, zero erucic acid and low glucosinolates were combined into one genotype of *B. napus* ('Tower') which was registered for production in Canada in 1974 (Canola Council of Canada, 2011). Canola is now produced worldwide and mostly concentrated in Canada, the European Union, Australia, and USA (Kimber and McGregor, 1995). Canola-quality rapeseed has higher nutritional benefits to humans due to lowest saturated fat content compared to any other vegetable oil. Seed remnants after oil extraction the meal, is a high quality ingredient in animal feed rations due to its low glucosinolate content.

The other *Brassica* species grown as oilseed crops are *B. juncea*, *B. napus*, *B. rapa*, and *B. carinata*. *B. juncea* is cultivated extensively in India and China and make significant contribution to vegetable oil needs. However, the erucic acid content in oil (40-45%) and glucosinolate content in meal (150 µmol/g) is high (Prakash, 1980). Substantial breeding efforts are underway to introgress canola-type traits into adapted *B. juncea* germplasm to improve oil quality. In 2002, Canada registered two canola-quality *B. juncea* oilseed varieties ('Arid' and 'Amulet') that produce oil with no erucic acid and seed meal with <3 µmol/g of glucosinolates (Canola Council of Canada, 2011). *B. rapa* canola are grown in Canada, Sweden and Finland as early maturing oilseed crop in areas where summer is short and does not allow the cultivation of later maturing, higher yielding *B. napus* species. *B. rapa* are also grown as oilseed crop in short season areas in India but with major production problems such as self-incompatibility and susceptibility to diseases. *B. carinata* is cultivated in Ethiopia as oilseed but with limited

acreage. Research is being conducted in Canada to develop canola-quality type which has potential as an oilseed crop for dryland areas (Rakow, 2004).

Brassica oilseeds can be used for many other purposes. Before introducing canola-quality *B. napus*, the oil was used as a lubricant for steam engines and as lamp oil (Downey and Robbelen, 1989). Biodiesel has gained significant interest in fuel market. It could be used as environmentally friendly alternative fuel and lubricant for diesel engines as it has high biodegradability, low toxicity and reduced emissions. There has been subsequent interest in producing biodiesel from canola oil as it has essential fatty acid composition to produce biodiesel. This has become an important fuel component for diesel engine in the European Union which has subsequently increased the production of oilseed crops (Korbitz, 1989).

Origin and taxonomy of *Brassica* species

Brassica nigra (L.) Koch.

B. nigra (L.) Koch is a diploid species containing B genome (BB genome, n=8) (Quiros and Paterson, 2004). The wild relatives of *B. nigra* are found as weed in cultivated fields in the Mediterranean region. It has been found to grow wild near Tangiers, Morocco, and semi-cultivated in Rhodes, Crete, Sicily, Turkey, and Ethiopia. They are cultivated for vegetable in Ethiopia, for spice and often for cooking oil in India (Tsunoda, 1980). *B. nigra* plants are differentiated based on their height of up to 2 m and no vernalization requirement for flower production.

Brassica oleracea L.

B. oleracea L. is also a diploid species with C genome (CC genome, n=9) (Quiros and Paterson, 2004). The wild species of *B. oleracea* are found on the coasts of northern Spain, in western France, and in the southern and southwestern Britain regions in France. It is a perennial

species and develops a strong vegetative stock over years. The leaves are glabrous with grayish surface. The cultivated forms of *B. oleracea* are divided into kales (var. *acephala*), cabbages (var. *capitata*, var. *sabauda*, var. *bullata*), kohlrabi (var. *gongylodes*), inflorescence kales (var. *botrytis*, var. *italica*), branching bush kales (var. *fruticosa*), and Chinese kale. The cultivated groups of *B. oleracea* are believed to be originated from west European wild *B. oleracea* (Snogerup, 1980).

Brassica rapa L**.**

B. rapa L. is a diploid species with A genome (AA genome, n=10) (Quiros and Paterson, 2004). *B. rapa* is believed to be originated from the highlands near the Mediterranean Sea (Tsunoda, 1980). Later it was spread to Eastern Europe and Germany (Nishi, 1980). The cold climate in these regions favored the growth of *B. rapa* which exhibits a short vegetative growth under low-temperature conditions. These are early maturing *Brassica* and are cultivated as oilseed crop in areas with shorter season in India, Sweden, Finland, and Canada.

Brassica carinata A. Braun.

B. carinata is an amphidiploid species with B and C genome (BBCC genome, n= 17) originated from natural crossings between two diploid species, *B. nigra* and *B. oleracea* (Quiros and Paterson, 2004). Wild forms of *B. carinata* have not been reported yet and the cultivated *B. carinata* are concentrated in the Ethiopian plateau. It might have been originated from cross between kale and wild or cultivated *B. nigra* that are grown in the region since ancient times. *B. carinata* are late maturing and the seeds contain mustard oil, the traits that might have inherited from its parent species *B. oleracea* and *B. nigra* respectively. It is cultivated in Ethiopia as leafy vegetables and oilseed crops (Tsunoda, 1980).

Brassica juncea (L.) Czern and Coss.

B. juncea is an amphidiploid species with A and B genome (AABB genome, n= 18) originated from interspecific crosses between the diploid species, *B. nigra* and *B. rapa* (Quiros and Paterson, 2004). The wild forms of *B. juncea* have been reported from near east and southern Iran. It is cultivated extensively in India as an oilseed and as a leaf vegetable and root type (turnip-like) forms var. *napiformis* in China. Since none of the parent species were found as wild species in these countries, they cannot be considered as a center of origin for *B. juncea.* Also, *B. juncea* grown in these two countries have greater differentiation. Chinese *B. juncea* seeds are yellow colored, small sized and contain 2-propenyl (allyl) glucosinolate, whereas Indian *B. juncea* seeds are brown colored, larger sized and contain 3-butenyl glucosinolate (Prakash, 1980). In western countries, especially in western Canada *B. juncea* is grown as condiment mustard.

Brassica napus L.

B. napus is also an amphidiploid species with A and C genome (AACC genome, n= 19) originated from interspecific crosses between the diploid species, *B. rapa* and *B. oleracea* (Quiros and Paterson, 2004). The wild forms of *B. napus* have been found on the beaches of Gothland Sweden, and in the Netherlands and Britain. Wild forms of both parental lines grow on the coastal cliffs of New Zealand and the cultivated form of *B. napus* has also been found in this region. It is believed that *B. napus* has originated from the coast of Northern Europe where the wild forms of both the parents are found. *B. napus* is widely cultivated as oilseeds in many countries (Tsunoda, 1980). It is highly productive *Brassica* oilseed species and is thought to be correlated with high photosynthetic rate per unit leaf area and to chloroplast volume. The genetic

relationship between these *Brassica* species have been defined by U (1935) (Figure 2.2.)

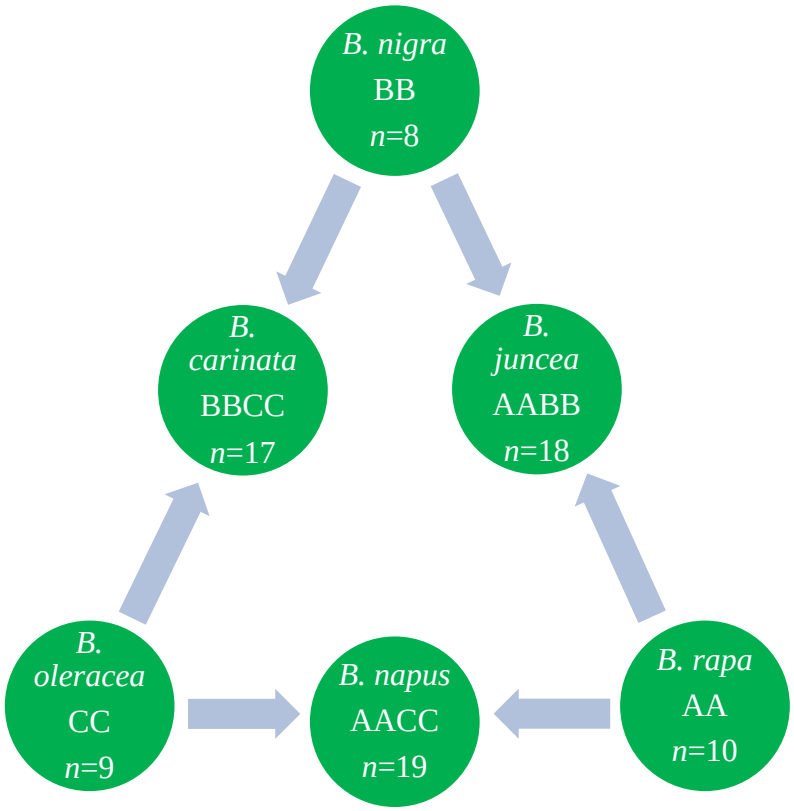

Fig. 2.2. "Triangle of U" showing genetic relationship between six *Brassica* species (U, 1935).

Crop improvement in *Brassica*

The increasing economic importance of *Brassica* crops has led to an increment in research focused on crop breeding to improve traits of interest to the growers, processors, or consumers; including the creation of plants with environmental benefits that provide economic and efficient pest management such as development of insect-resistant, disease resistant, and herbicide resistant genotypes.

Disease resistance

Development of disease resistance is one of the major aspects of *Brassica* breeding programs. Many fungal and bacterial pathogens attack *Brassica* crops and cause serious economic loss to growers. The fungal diseases of economic importance are leaf spot diseases

caused by *Alternaria* species (Verma and Saharan, 1994), stem canker (blackleg) caused by *Leptosphaeria maculans* (Howlett et al., 2001), stem rot (white mold) caused by *Sclerotinia sclerotiorum* (Boland and hall 1994), light leaf spot caused by *Pyrenopeziza brassicae* (Su et al., 1998), verticillium wilt caused by *Verticillium longisporum* (Karapapa et al., 1997), and club root caused by *Plasmodiophora brassicae* (Linnasalmi and Toivianen, 1991). *Xanthomonas campestris* pv. *campestris* is one of the bacterial pathogens of cabbage causing black rot disease. This causes extensive damage to the crop in various geographic areas and is a subject of interest to *Brassica* researchers (Alvarez 2000). Application of disease resistance is the major management strategy to these pathogens whenever available; however other cultural practices and fungicide application are also used to augment the effective disease management.

In North Dakota, blackleg disease caused by *Leptosphaeria maculans* and white mold caused by *Sclerotinia sclerotiorum* are two economically important diseases of canola (*Brassica napus*). Researchers have focused efforts on the identification of effective sources of resistance and breeding for resistance to the disease as well as on the evaluation of cultural practices such as crop rotation and chemical and biological fungicides (del Rio, personal communication).

Resistance to *Leptosphaeria maculans* have been identified in *B. napus* by utilizing *B. napus* gene pool exposed to natural variation in blackleg field trials. In Europe, such selection resulted in the identification of field resistant winter cultivar “Jet Neuf” which has been a major source of resistance since 1970s (Balesdent et al., 2001). Similar strategies have been applied for the identification of resistant spring cultivars in Australia and Canada. However, in light of the high genetic variability and adaptability of this pathogen, one cannot rely on only limited sources of resistance. Thus, exploitation of variability in *Brassica* species helps in identification of promising sources of resistance to the disease. The *Brassica* species carrying B-genome such as

B. nigra, *B. juncea*, and *B. carinata* carry a high degree of resistance in the whole plant (Sacristan and Gerdemann, 1986; Rimmer and van der Berg, 1992). Various researchers have focused on transferring this trait to other *Brassica* species, especially into oilseed rape. Other efforts have been devoted to the identification of resistance source from both closely and distantly related wild species of *B. napus* (Sacristan and Gerdemann, 1986; Roy, 1984). This has widen the pool of potential resistant donors and has provided an opportunity to exploit A, and C genome as possible sources of resistance.

Fourteen major resistance genes (*Rlm1-Rlm9* and *LepR1- LepR4*) and polygenic resistance have been identified against specific races of *Leptosphaeria maculans* in *B. napus* (Delourme et al., 2004; Rimmer, 2006; Yu et al., 2005, 2008). Traces of resistant genes have also been identified in *B. nigra*, *B. rapa,* and *B. juncea* (Chevre et al., 1996; Dusabenyagasani and Fernando, 2008; Saal et al., 2004) and the resistance have been transferred to *B. napus* (Roy, 1984; Crouch et al., 1994; Saal et al., 2004).

The mechanisms of resistance to blackleg disease have been reported to be inherited both qualitatively and quantitatively. The qualitative resistance is controlled by single race-specific genes and is expressed in both seedling and adult plants. The quantitative resistance is controlled by non-race-specific genes and is expressed in the adult plant with minor phenotypic effect (Delourme et al., 2008). Deployment of major qualitative gene provides an effective protection against diseases but monoculture of cultivars with few qualitative genes in a large scale possess a risk of selection pressure to the pathogen leading to resistant breakdown in relatively short period of time (Howlett, 2004; Sprague et al., 2006) as it has been observed in Europe, Australia, and Canada where the registered resistant cultivars became susceptible within four years of release (Easton, 2004; Li et al., 2003; Sprague et al, 2006). On the other hand, quantitative resistance

offers a durable resistance due to the presence of multiple genes that acts against broad spectrum of pathogen isolates and has a minor phenotypic effect thus lowering the selection pressure on the pathogen (Howlett, 2004; Sprague et al., 2006; Delourme et al., 2008). Introgression of both qualitative and quantitative resistances to a cultivar offers both effective and durable resistance against the disease.

The interaction between *Brassica* species and *L. maculans* has been studied to identify the genetics of host-parasite interaction. Both avirulence genes (*AvrLm*) in the pathogen and their corresponding resistance genes in the host (*Rlm*) have been identified in *B. napus* (Ansan-Melaya et al., 1995). Gene-for-gene interactions (*Rlm1 / AvrLm1* in 'Quinta' - PG3; *Rlm2 / AvrLm2* in 'Glacier' - PG2) were demonstrated between *B. napus* and *L. maculans* by using a segregating population of both plant and pathogen (Ansan-Melayah et al., 1995, 1998). Similar interactions have been reported between other *Brassica* species and different isolates of *L. maculans* (Balesdent et al., 2002; Chevre et al., 2003).

The mode of defense in *Brassica* species to *L. maculans* is relatively complex and very limited information is available on this aspect. Some studies have reported deposition of lignin-like compounds and calcium accumulation in stem vessels when *B. napus* were inoculated with *L. maculans* (Hammond and Lewis, 1986). In another case, a hypersensitive response was induced in vascular tissues and a fibrillar-like material occluded the lumen of vessels in HR areas when *B. napus* was inoculated with avirulent isolates of *L. maculans* (Roussel et al., 1999). Rasmussen et al. (1992) and Hennin et al. (2001) reported different accumulation of pathogenesis-related proteins in leaves of resistance and susceptible *Brassica* plants. Additionally, in an incompatible interaction in *B. juncea*, dark-colored material and callose were deposited in leaf tissue when inoculated with *L. maculans* isolates (Chen and Howlett, 1996).

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CHAPTER 3: DISTRIBUTION OF *LEPTOSPHAERIA MACULANS* PATHOGENICITY GROUPS IN NORTH DAKOTA

Abstract

Blackleg, caused by *Leptosphaeria maculans* is one of the most destructive diseases affecting canola (*Brassica napus*) production worldwide. In North Dakota, blackleg was first reported in 1991. The blackleg outbreak of that year was caused by strains belonging to pathogenicity group (PG) 2 of *L. maculans.* Since then little is known about the distribution of PG of *L. maculans* in the state. Blackleg disease prevalence and incidence was assessed annually from 1993 - 2009 in the major canola growing counties in North Dakota. Disease samples from infected stubbles were also collected to determine the distribution of PGs in canola fields. The number of fields with disease incidence greater than 30% increased by six folds in 2009 compared to 2004. Two hundred sixteen isolates of *L. maculans* collected in 2004, 2007, and 2009 were tested for the pathogenicity groups using three sets of *B. napus* differential cultivars, 'Westar', 'Glacier', and 'Quinta'. Out of these isolates 56% were identified as PG4, 13% and 11% as PG3 and PGT respectively. Pathogenicity group 2 was the least frequently isolated pathotype (2%) and PG1 accounted for 5% of the isolates. Remaining 13% of the isolates could not be identified as any of the PG based on the interaction phenotype on three differential canola cultivars. Increasing frequency of more aggressive and unknown pathotypes in the commercial canola fields in North Dakota represents a potential threat to the canola industry.

Introduction

Blackleg, caused by *Leptosphaeria maculans* (Desm.) Ces. & De Not. (anamorph *Phoma lingam* (Tode ex Fr.) Desm.), is one of the most important diseases affecting oilseed rape (*Brassica napus*) production worldwide (West et al., 2001). Recorded prevalence of *L. maculans*

dates back 85 years in Australia, 65 years in Europe, 35 years in Canada, and approximately 20 years in the United States of America (Aubertot et al., 2004; Sivasithamparam et al., 2005; Petrie, 1978; Lamey and Hershman, 1993). The disease caused significant yield losses of € 11.3 million in 1998 and 1999 in Australia (Khangura and Barbetti, 2001) and € 56 million in 2002 and 2003 in United Kingdom (Fitt et al., 2006). In France, the loss due to the disease was estimated to be 5 to 20% of total yield with value ranging from € 36.8 to € 147 million (Allard et al., 2002). In North Dakota, yield losses of up to 45-50% in severely infected fields in canola-growing regions of the state have been reported (del Rio Mendoza et al., 2012; Markell et al., 2008).

Blackleg is reappearing as a serious threat to the canola industry in North Dakota. Canola, oilseed rape genotypes with low erucic acid and glucosinolates contents, was introduced to North Dakota in early 1980. In 1991, a blackleg outbreak was detected in the northeastern corner of the state with incidences that ranged between 8 and 68% (Lamey and Hershman, 1993). *L. maculans* isolates retrieved from affected fields were inoculated on a set of three *B. napus* differential cultivars to identify the pathogenicity groups they belonged to (Mengistu et al., 1991). Only strains belonging to PG-1 and PG-2 were detected with PG-2 being the most prevalent group (Lamey and Hershman, 1993). By 1993, the most popular cultivar, “Westar’, which was very susceptible to blackleg had been abandoned (Lamey and Hershman, 1993). By the end of the 1990s, most commercial genotypes carried genes for resistance against PG-2 (Berglund, 2003) and no new blackleg outbreaks had been reported. In 2003, Bradley et al. (2005) detected the presence of PG-3 and PG-4 strains in canola stems showing typical blackleg cankers. At that time, however, these new PGs accounted for less than 5% and 1%, of all isolates evaluated; the remaining isolates belonged to PG-2 (Bradley et al., 2005). In 2009, several

blackleg outbreaks were detected in various North Dakota counties. The virulence profile of more than one hundred isolates retrieved from infected canola stems collected from these fields indicated that only 3% of them belonged to PG-2 whereas strains of PG-4 and PG-3 accounted for 51% and 25% of the remaining isolates, respectively (del Rio Mendoza et al., 2012). While this information provides strong evidence that a shift in PG prevalence is under way, the extent of the change is still unclear. Thus, the objective of this study was to characterize the changes in PG prevalence that has occurred in ND since 2004.

Materials and Methods

Plant sample and disease data collection

Canola stem samples were collected from 21 North Dakota counties in 2004 and 2007; and from 20 counties in 2009. Samples were collected in one canola field for every 2,025 ha planted to canola in each county in late August to early September when the crop was in the swath and the stubble was freshly cut. Five freshly swathed stems were pulled from ten arbitrarily selected locations to collect 50 stems per field. The crown and the roots of the stem were visually inspected for blackleg symptoms and the incidence of infected plants was recorded. Affected plants were identified by the presence of typical gray to black stem cankers near the soil line that sometimes had pycnidia on them. Blackleg symptomatic tissues were brought to the laboratory to confirm the diagnosis and to isolate the pathogen for further studies.

Isolation of blackleg pathogen and inoculum preparation

A total of 216 isolates were retrieved from samples collected from 12 counties in 2004, 14 counties in 2007, and 11 counties in 2009. Single spore cultures of these isolates were prepared as described in del Rio Mendoza et al. (2012). Briefly, the infected tissues were washed with running tap water to remove the debris from the stem surface and then lightly tapped with

paper towel to remove excess moisture. Infected portions of the stem were cut and surface disinfested by immersing in either 100% commercial bleach (Clorox Regular bleach, The Clorox Company, Oakland, CA) or 30% aqueous solution of the bleach for 10-30 sec depending upon the condition of the sample. Disinfested tissues usually containing pycnidia were scrapped off using a sterile scalpel and suspended in sterile distilled water. About 0.5 ml of the suspension was streaked onto full strength V8 medium amended with streptomycin and penicillin. The medium was prepared with 837 ml distilled water, 163 ml V8 juice (Campbell Soup Co., Camden, NJ), 15 g agar (Bacto-Agar, Becton Dickinson and Co., Sparks, MD), and 3 g $CaCO_3$. The pH of the medium was adjusted to 7.2 by adding 1M NaOH. The medium was sterilized by autoclaving at 120^{o}C and 103.4 kPa for 20 min and then amended with 7.5 ml/L streptomycin (10 μg/ml) and 7.5 ml/L penicillin (10 μg/ml). Inoculated medium was incubated under constant white light for 3 to 4 days at 21^{o} C. Single spore colonies were transferred to new plates containing full strength V8 medium prepared as described but without antibiotic and incubated for two weeks as described. Identity of *Leptosphaeria maculans* isolates was confirmed by colony, pycnidia, and pycnidiospores morphology. Single-spore colonies were stored at -80^{o} C for future use.

Spores from two-week old colonies growing in V8 medium were suspended in 5 ml sterile distilled water by gently scrapping the colony surface with a sterile glass rod. The suspension was collected in a tube and centrifuged at 8000 rpm for five minutes. The supernatant was discarded and the spore pellet was resuspended in 5 ml aqueous solution of glycerol (30% glycerol in sterile distilled water). The concentration of spore suspension was estimated using a hemacytometer and adjusted to 10^7 spores/mL. The suspension was stored at -20^{o} C until needed. The viability of the spores was confirmed whenever the stored suspension was used for

inoculation. For this, a drop of spore suspension was put on a glass slide and left at room temperature for 24 hrs. The germination percentage was then estimated under the microscope.

Identification of pathogenicity groups

Three *Brassica napus* differential cultivars, 'Westar', 'Glacier' and 'Quinta', were used. Seeds of each differential were planted in plastic containers with ten inserts and six wells in each insert. The containers were filled with greenhouse artificial soilless mix (Professional Growing Mix 1 SunGro Horticulture Canada Seba Beach, AB, Canada). Five days after planting, the seedlings were thinned to leave one healthy seedling per well. The seedlings were watered daily and kept in greenhouse at 22/18^{o} C day/night temperatures and 14 h photoperiod. Ten days after planting, each cotyledon was lightly pricked once with a sterile sharp tweezers and a 10 µl aliquot of spore suspension was deposited on each wound. Each isolate was inoculated on triplicate sets of six seedlings per differential. Inoculated seedlings were incubated in mist chambers for the next 24 h and then returned to the greenhouse room. Fourteen days after inoculation, disease severity was scored using the 0-9 scale developed by William and Delwiche (1980) in which a 0-2 reaction was considered resistant (R), 3-6 intermediate (I), and 7-9 susceptible (S).

Data analyses

Fields within each county were divided in three groups using 0%, up to 30%, and >30% incidence as arbitrary thresholds. The percentage of fields in each category was used to calculate the prevalence of fields with no, moderate and severe blackleg incidences. The latter category was also considered a blackleg outbreak. To further characterize the epidemics, the mean percentage of blackleg symptomatic stems and its standard deviation were calculated for the two categories in which blackleg incidence was greater than 0%. The median of disease severity

collected in the inoculation trials was calculated for each isolate using the univariate procedure of SAS (PROC UNIVARIATE, Ver. 9.3, SAS Institute, Inc., Cary, NC). This median was used to classify the isolates into PGs according to the criteria shown in Table 3.1. To obtain a better idea of the changes in *L. maculans* populations in North Dakota in past several years, the information produced in this research was compared to existing literature. Changes in the frequencies of occurrence of each PG over time were used to calculate Simpson's diversity (*D*) and equitability (E_D) indices. The indices were calculated as follow:

$$D = \frac{1}{\sum_{i=1}^{S} p_i^2} \qquad \text{and } E_D = \frac{D}{S}$$

Where, *D* = Simpson's diversity index, *S* = total number of species in the community (richness), p_i = proportion of *S* made up of the *i*th species, and E_D = equitability (evenness) (Simpson, 1949).

Table 3.1. Interaction phenotype of *Brassica napus* differential cultivars to the pathogenicity groups of *Leptosphaeria maculans* and *Leptosphaeria biglobosa.*

Pathogenicity group	Differential cultivars		
	Westar	Glacier	Quinta
1	R	R	R
2	S	R	I
3	S	S	I
T	S	I	S
4	S	S	S

Results

Blackleg prevalence and pathogenicity groups

Table 3.2 summarizes the prevalence of blackleg in 214 canola fields scouted in 2004. Of the 214 fields scouted, approximately 72% of them did not have blackleg symptomatic plants. These fields were located in ten different counties. Approximately 23% of all scouted fields had

blackleg incidences ranging between 1 and 30% with an average incidence across counties of 10%, although two of them, located in Stutsman and Nelson counties, had a mean blackleg incidence of 25%. Approximately 4% of all scouted fields had blackleg outbreaks with incidences greater than 30%. The average blackleg incidence in these fields was 52%. The outbreaks were detected in seven fields located in Cavalier and in two fields located in Towner counties in northeastern North Dakota. Fifty *L. maculans* isolates retrieved from canola residues collected in 2004 from fields in 12 different counties were inoculated on the three differentials. Approximately 50% of all isolates evaluated belonged to PG-4, 22% belonged to PG-3, and 8% belonged to PG-T (Table 3.3). PG-1 isolates were identified in stems from Burke and Cavalier counties. Only one isolate from PG-2 was identified in residues collected in Ramsey County. Six of the 50 isolates tested could not be ascribed to any PG (Table 3.3).

In 2007, 187 fields were surveyed in 21 counties for blackleg prevalence. Table 3.4 summarizes the data on blackleg prevalence observed in 2007. Approximately 37% of the fields scouted did not have blackleg symptomatic plants. These fields were located in Burke, Divide, Foster, Mountrail, Nelson, Sheridan, Stutsman, Wells, and Williams. Approximately 51% of all fields scouted had blackleg incidences between 1% and 30% with an average blackleg incidence across counties of 9.3%. Approximately 12% of the scouted fields had blackleg incidences greater than 30%, a three-fold increment compared to 2004. The average blackleg incidence in these fields was 63% and they were located in Cavalier, Towner, Bottineau, McLean, and Ramsey counties. The first three counties are located in northeastern North Dakota. *L. maculans* was retrieved from 14 different counties in 2007. The three *B. napus* differentials were inoculated with 57 of these isolates. PG-4 was identified as the predominant group accounting for 65% of all isolates evaluated, followed by PG-3 which accounted for 9% of isolates (Table

3.5). Isolates from PG-2 and PG-T accounted for only 2% of isolates each. Isolates from PG-1, retrieved from stems collected in Cavalier, Grand Forks and Williams counties, accounted for 11% of the isolates evaluated. Among the isolates tested, seven could not be ascribed to any particular PG (Table 3.5).

Table 3.2. Blackleg prevalence and incidence in commercial canola fields in North Dakota counties in 2004 [x].

County	Blackleg prevalence				Blackleg incidence			
	Fields with incidence			Total	1-30%		>30%	
	0%	1-30%	>30%	Fields	Mean	Std.dev.	Mean	Std.dev.
Benson	6	9	0	15	7.56	6.54	- [y]	-
Bottineau	22	0	0	22	-	-	-	-
Burke	7	0	0	7	-	-	-	-
Cavalier	5	20	7	32	10.50	6.86	59.71	22.19
Divide	3	0	0	3	-	-	-	-
Eddy	0	1	0	1	6.00	-	-	-
Foster	2	1	0	3	2.00	-	-	-
Hettinger	10	0	0	10	-	-	-	-
McHenry	7	0	0	7	-	-	-	-
McLean	18	0	0	18	-	-	-	-
Mountrail	10	0	0	10	-	-	-	-
Nelson	7	1	0	8	24.00	-	-	-
Ramsey	3	4	0	7	15.50	10.50	-	-
Renville	16	0	0	16	-	-	-	-
Rolette	8	1	0	9	2.00	-	-	-
Sheridan	1	3	0	4	4.00	3.46	-	-
Stutsman	1	1	0	2	26.00	-	-	-
Towner	8	6	2	16	9.33	3.93	45.00	4.24
Ward	17	0	0	17	-	-	-	-
Wells	2	3	0	5	7.33	4.62	-	-
Williams	2	0	0	2	-	-	-	-
Total	155	50	9	214	-	-	-	-

[x] Data generated from a survey conducted by Art Lamey and group to assess the pests in the major canola growing counties in North Dakota. [y] Values not calculated.

Table 3.3. Frequency of pathogenicity groups identified from commercial canola fields in North Dakota counties in 2004.

		Pathogenicity groups					
County	Isolates	1	2	3	4	T	NI[x]
Benson	3	-[y]	-	-	2	-	1
Bottineau	7	-	-	2	4	-	1
Burke	5	1	-	1	3	-	-
Cavalier	8	2	-	-	5	-	1
Hettinger	6	-	-	1	2	1	2
McHenry	5	-	-	1	3	1	-
McLean	4	-	-	1	2	1	-
Ramsey	7	-	1	5	-	-	1
Renville	1	-	-	-	1	-	-
Towner	2	-	-	-	2	-	-
Ward	1	-	-	-	1	-	-
Williams	1	-	-	-	-	1	-
Total	50	3	1	11	25	4	6

[x] Pathogenicity groups not identified. [y] Pathogenicity group not present.

Table 3.4. Blackleg prevalence and incidence in commercial canola fields in North Dakota counties in 2007 [x].

County	Blackleg prevalence				Blackleg incidence			
	Fields with incidence			Total	1-30%		>30%	
	0%	1-30%	>30%	Fields	Mean	Std.dev.	Mean	Std.dev.
Benson	4	5	0	9	10.80	7.16	-[y]	-
Bottineau	2	10	2	14	11.00	8.12	60.00	28.28
Burke	6	0	0	6	-	-	-	-
Cavalier	7	30	8	45	12.80	8.98	51.50	20.14
Divide	2	0	0	2	-	-	-	-
Foster	2	0	0	2	-	-	-	-
Hettinger	2	2	0	4	9.00	7.07	-	-
McHenry	2	3	0	5	11.33	16.17	-	-
McLean	2	5	2	9	8.40	1.67	84.50	20.51
Mountrail	9	0	0	9	-	-	-	-
Nelson	4	0	0	4	-	-	-	-
Pierce	3	4	0	7	10.50	9.57	-	-
Ramsey	0	8	1	9	14.75	8.75	70.00	-
Renville	4	9	0	13	6.44	4.33	-	-
Rolette	0	8	0	8	4.00	2.14	-	-
Sheridan	3	0	0	3	-	-	-	-
Stutsman	1	0	0	1	-	-	-	-
Towner	2	8	9	19	5.50	3.96	50.44	20.02
Ward	12	3	0	15	6.67	4.16	-	-
Wells	2	0	0	2	-	-	-	-
Williams	1	0	0	1	-	-	-	-
Total	70	95	22	187	-	-	-	-

[x] Data was generated from a survey that was conducted in 2007 by late Art Lamey and group to assess the pests in the major canola growing counties in North Dakota. [y] Values not calculated.

Table 3.5. Frequency of pathogenicity groups identified from commercial canola fields in North Dakota counties in 2007.

		Pathogenicity groups					
County	Isolates	1	2	3	4	T	NI[x]
Benson	1	-[y]	-	-	-	-	1
Bottineau	2	-	-	-	2	-	-
Cavalier	11	3	-	-	6	-	2
Foster	1	-	-	1	-	-	-
Grand Forks	4	2	-	-	1	-	1
McHenry	1	-	-	-	1	-	-
McLean	9	-	-	-	8	-	1
Nelson	6	-	-	-	6	-	-
Pembina	2	-	-	2	-	-	-
Rolette	5	-	-	-	5	-	-
Towner	2	-	-	1	1	-	-
Ward	3	-	-	-	3	-	-
Wells	5	-	1	-	3	-	1
Williams	4	1	-	1	1	1	-
unknown	1	-	-	-	-	-	1
Total	57	6	1	5	37	1	7

[x] Pathogenicity groups not identified. [y] Pathogenicity group not detected.

In 2009, 171 fields were surveyed in 20 counties. Table 3.6 summarizes the blackleg prevalence data collected that year. Approximately 26% of all fields scouted did not have blackleg symptomatic plants. These fields were located in Benson, Foster, Hettinger, Sheridan, and Wells counties. Approximately 51% of all fields scouted had blackleg incidences between 1 and 30% with an average incidence across counties of 11%. In this group, McLean County had the highest average with 20%. Approximately 23% of all fields scouted had blackleg incidences greater than 30%. The percentage of fields in this category was almost twice that of 2007 and represented a six-fold increase from the level observed in 2004. These fields were located in ten counties and the average incidence across them was 63%. *L. maculans* was retrieved from infected residues collected from 11 counties in 2009. Of these collections, 109 isolates were tested on the three *B. napus* differentials. Approximately 54% of all isolates tested belonged to PG-4 (Table 3.7). PG-4 isolates were retrieved from nine of the eleven counties from which isolates were tested. Approximately 12 and 17% of all isolates belonged to PG-3 and PG-T,

respectively. These isolates were retrieved from seven and six of the eleven counties represented, respectively. Less than 3% of all isolates tested belonged to PG-2. Fourteen isolates could not be ascribed to any PG.

Table 3.6. Blackleg prevalence and incidence in commercial canola fields in North Dakota counties in 2009 [x].

County	Blackleg prevalence				Blackleg incidence			
	Fields with incidence			Total	1-30%		>30%	
	0%	1 -30%	>30%	Fields	Mean	Std.dev.	Mean	Std.dev.
Benson	4	0	0	4	- [y]	-	-	-
Bottineau	0	8	2	10	13.25	6.92	64.00	5.66
Burke	0	5	2	7	14.00	6.78	68.00	19.80
Cavalier	15	25	0	40	5.12	4.17	-	-
Divide	0	1	0	1	14.00	-	-	-
Foster	1	0	0	1	-	-	-	-
Hettinger	7	0	0	7	-	-	-	-
McHenry	2	1	3	6	2.00	-	50.00	19.70
McLean	0	7	6	13	20.00	6.73	59.83	20.88
Mountrail	0	2	7	9	16.00	5.66	48.43	20.74
Nelson	2	2	0	4	3.00	1.41	-	-
Pierce	0	4	1	5	14.00	12.75	84.00	-
Ramsey	3	4	1	8	11.50	3.42	56.00	-
Renville	0	5	6	11	11.60	7.13	51.67	16.66
Rolette	0	9	0	9	8.44	7.60	-	-
Sheridan	4	0	0	4	-	-	-	-
Towner	5	6	4	15	12.00	9.63	49.50	5.26
Wards	1	7	7	15	12.86	7.10	56.29	5.68
Wells	1	0	0	1	-	-	-	-
Williams	0	1	0	1	2.00	-	-	-
Total	45	87	39	171	-	-	-	-

[x] Data was generated from a survey that was conducted in 2009 by late Art Lamey and group to assess the pests in the major canola growing counties in North Dakota. [y] Values not calculated.

Changes in the number of pathogenicity groups as well as in their relative prevalence over the time covered by this study were reflected in the Simpsons' equitability index. The indices for 2004, 2007, and 2009 were 0.52, 0.37, and 0.47, respectively (Figure 3.1). The index for 2007 was significantly different from the indices for 2004 and 2009 ($p<0.0001$).

Table 3.7. Frequency of pathogenicity groups identified from commercial canola fields in North Dakota counties in 2009.

		Pathogenicity groups					
County	Isolates	1	2	3	4	T	NI[x]
Bottineau	15	1	-	-	9	5	-
Burke	10	-[y]	-	1	5	2	2
Cavalier	13	-	-	1	7	3	2
Divide	3	-	-	2	-	-	1
McHenry	10	-	-	-	8	2	-
McLean	19	-	-	5	10	-	4
Mountrail	3	-	-	-	3	-	-
Pierce	2	-	-	1	-	-	1
Renville	16	-	2	1	8	3	2
Towner	5	-	1	2	2	-	-
Ward	10	-	-	-	4	4	2
unknown	3	-	-	-	3	-	-
Total	109	1	3	13	59	19	14

[x] Pathogenicity groups not identified. [y] Pathogenicity group not detected.

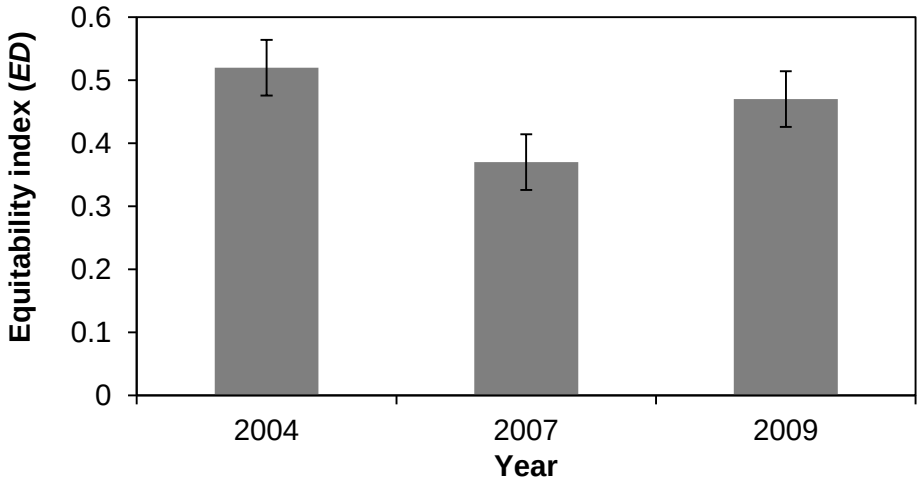

Fig. 3.1. Simpson's equitability index (E_D) for *Leptosphaeria maculans* populations in North Dakota between 2004 and 2009. The index ranges from 0 to 1 with values closer to 0 indicating less diverse/more even population (Simpson, 1949). The bars represent standard error of the equitqbility index.

Discussion

Blackleg is resurging as a major problem for North Dakota canola producers. Results of this study show blackleg prevalence has steadily increased since 2004. Strains of PG-3, 4, and T

are becoming prevalent in more counties. In 2004, almost 72% of fields scouted did not have blackleg symptomatic plants, that number was reduced to 37% in 2007 and to 26% in 2009. At the same time, the proportion of fields experiencing blackleg outbreaks with incidences greater than 30% increased from 4% in 2004 to 23% in 2009. Several factors may have contributed to the rapid expansion of this disease in recent years; perhaps the most important factor is the susceptibility of most commercially available canola cultivars to these strains (Marino, 2011). Another factor is the shortening of crop rotations to increase canola production. This increment has been driven by high demand and good market prices for the commodity. Canola production is concentrated in the northern half of the state (USDA-NASS, 2013) where it competes mainly with wheat for hectarage, thus any increase will have to come either to the expense of wheat area or by shortening the rotations. Since the pathogen survives on crop residues, (Markell et al., 2008) shortening rotations increase the probability that larger amounts of inoculum from the previous season will be available for infection during the next season. Another factor may be the increased seed movement at harvest time as well as seed trade within counties. Infected and/or contaminated seeds can spread the pathogen into new areas (Petrie, 1979; Gabrielson, 1983; Lloyd, 1959; Petrie and Vanterpool, 1974). Infected seed that falls on roadsides may germinate the following summer and serve as sources of inoculum from where spores may be dispersed by the wind into neighboring fields. Airborne spores could be dispersed up to several kilometers away from their source (McGee and Emmett, 1977; Petrie, 1978). A distance of 5-8 km has been considered to be effective to reduce the rate of infection from incoming inoculum (Bokor et al., 1975); however, in northern North Dakota canola production fields are usually located far closer to each other.

At the same time that blackleg prevalence increased, blackleg incidence was also on the rise. Until 2004, most of the fields where disease was present had incidences < 30%. Only nine fields in two counties, Cavalier and Towner had disease incidence greater than 30%. These two counties had an average disease incidence of 60% and 45%, respectively. According to Chen and Fernando (2006), strains of PG-4 were already present in Cavalier County in 2003 whereas strains of PG-3 and PG-T were present in Towner County in 2004. Our data also shows that 63% of the isolates tested from Cavalier County and all the isolates tested from Towner County belong to PG-4. In 2007, 22 fields in three more counties had disease incidence greater than 30% with highest average incidence of 85%. Based on our results from 2004, in Bottineau both PG-3 and PG-4 were present; in McLean PG-3, 4 and T were present; and in Ramsey PG-2 and PG-3 were present. In 2009, 39 fields in six additional counties had disease incidence greater than 30% with highest average incidence of 84%. In all these counties either PG-3, or PG-4, or PG-T or the combination of these were identified in one or the other year. Due to the report that blackleg epidemics in 1991 were caused by strains belonging to PG-2 (Lamey, 1995), most of the canola breeding efforts were concentrated to produce cultivars with resistance to it. These cultivars are most commonly cultivated in the state and until 2002, they were considered either resistant or moderately resistant to PG2 (Berglund, 2003).Thus the appearance of new PGs and unavailability of resistance sources coupled with limited application of fungicides (del Rio Mendoza et al., 2012) might have accounted for increasing disease incidence.

The equitability index calculated for the period between 2004 and 2009 was relatively higher compared to 1984-2001, 2002, and 2003 based on Chen and Fernando's (2006) data on PG groups in ND, indicating more equal distribution of PGs in later years. Isolates from the weakly virulent pathotype PG-1 produce mostly foliar lesions although in some instances may

also produce superficial cankers in stems (Howlett et al., 2001). Since most isolates used in this study were retrieved from stem cankers, it was not surprising to see it as the least prevalent group. The aggressive pathotype PG-2 has been reported as present in North Dakota since 1995 (Lamey, 1995) and was the most common pathotype until 2003 (Chen and Fernando, 2006). The presence of PG-3, PG-4 and PG-T was reported in 2003 in few canola growing counties in North Dakota (del Rio Mendoza et al., 2012; Chen and Fernando, 2006; Bradley et al., 2005). However, the frequency of occurrence of these PGs was very low (Chen and Fernando, 2006). In recent years, the prevalence of PG-2 has significantly decreased. PG-4 has become the predominant pathotype with PG-3 and PG-T being equally prevalent in second place. These changes reflect the history of canola cultivar development in North Dakota. 'Westar' a cultivar with no resistance genes was the most popular genotype planted in the early years when PG-2 was the most prevalent pathotype. The introduction of resistant cultivars started soon after severe blackleg epidemics occurred and by 2000 'Westar' had disappeared from the market (Lamey and Hershman, 1993; Juska et al., 1997). The introduction of resistant cultivars put pressure on the pathogen population to diversify its virulence profile (Balesdent et al., 2005) and by 2004 new pathogenicity groups were detected. Similar results were observed in Canada, France and Australia where the monoculture of canola cultivars with similar source of resistance resulted in collapse of the host resistance. In Australia, *B. napus* cultivars with extremely high degree of resistance to *L. maculans* were developed. The resistance was conferred by single dominant gene, *LepR3* and was derived from *B. rapa* subsp. *sylvestris* (Li et al., 2003; Yu et al., 2004). Several cultivars with this source of resistance were commercially released and were planted widely throughout Australia. However, within three years of cultivation the resistance was overcome and every field with these cultivars was severely affected with blackleg (Sprague et

al., 2006). In France, *B. napus* lines with major gene resistance to *L. maculans* derived from *B. juncea* was overcome in three years and from *B. nigra* was overcome in four years (Brun et al., 2000). Similarly in another study, large scale cultivation of *B. napus* lines with single major gene *Rlm1* resulted in a population shift of *L. maculans* isolates with avirulence gene *AvrLm1* to others within ten years (Rouxel et al., 2003). *Leptosphaeria maculans* are heterothallic fungi and outcross prolifically in canola stubbles under favorable environmental conditions. They have mixed reproduction system with at least one sexual cycle per growing season and multiple asexual cycles producing large number of pycnidiospores (Howlett et al., 2001). These types of pathogen have high potential for genotype flow and pose greater risk of overcoming the resistance genes (McDonald and Linde, 2002). Thus the other possibility for the introduction of new pathotypes could be sexual recombination that produced new pathotypes and shifted the genetic structure to more virulent population.

Not only is the increasing frequency of more virulent pathotype in ND fields a concern for canola growers. There are even new pathotypes which were not identified using the differential cultivars that are widely used for classifying the pathogenicity groups of this pathogen. This indicates a possibility of new pathotypes which are not reported yet. Until 2005, PG-T was detected only in Western Canada and North Dakota (Chen and Fernando, 2005). After that no new PGs have been reported but our results indicate the presence of other PGs in ND fields. Because of the increasing frequency of more aggressive and unknown pathotypes, North Dakota canola production is in vulnerable phase. If the immediate management practices are not employed, may result in a serious disease outbreak in near future. This demands a serious attention to manage this disease either by deploying resistant cultivars with quantitative

resistance or by other managerial practices such as longer crop rotation, better crop residue management and fungicide application.

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CHAPTER 4: GENETIC STRUCTURE OF *LEPTOSPHAERIA MACULANS* POPULATIONS IN COMMERCIAL CANOLA (*BRASSICA NAPUS*) FIELDS IN NORTH DAKOTA

Abstract

Blackleg disease, caused by *Leptosphaeria maculans* is one of the most devastating diseases of canola (*Brassica napus*) in North Dakota. Yield losses due to this disease could amount to 45 to 50% in the ND. In mid-1980's blackleg epidemics in the state were caused by pathogenicity group (PG) 2 of *L. maculans*. Since the identification of PG-2 strains in 2002 until 2009, the *L. maculans* population in North Dakota went through some changes. In 2002 PG-2 was the predominant pathotype while in 2003 and 2004, PG-3, PG-T, and PG-4 were reported for the first time. By 2009, these strains had become predominant while PG-2 was retrieved at very low frequencies. This study was conducted to improve our understanding of the genetic structure of *L. maculans* populations in North Dakota. To fulfill this objective we extracted DNA from 605 single-spore cultures of *L. maculans* isolates collected in North Dakota between 2004 and 2009. These isolates had been collected from commercial canola fields in 23 counties. For the analyses, counties were assigned to five geographic regions (North East, North Central, North West, West Central, and Central) and each region was considered a population. These populations were tested for genetic variation at seven microsatellite and four minisatellite loci and for the frequency of occurrence of two mating type loci. In total 545 multilocus haplotypes were identified and clone-corrected data sets were used for all analyses. The genetic diversity index (*H*) ranged from 0.63 to 0.70 with the West Central and North Eastern populations having the lowest and highest indices, respectively. Significant gametic disequilibrium ($P \leq 0.001$) was observed in all populations indicating that asexual reproduction plays a major role in disease

epidemics in the state. The ratio of mating type idiomorphs was also significantly deviated from 1:1 ratio ($P < 0.05$) in the Central, North Central, North Eastern, and West Central populations. The corrected standardized fixation index (G''_{ST}) between population pairs was highly significant ($P \leq 0.001$) and ranged from 0.05 to 0.27 indicating a strong population differentiation. Recent migration analysis indicated the migration rates between these populations were Low. Analysis of molecular variance indicated that 95% of the genetic variation observed was due to differences between isolates within populations and the rest was explained by variation among populations. *L. maculans* populations were grouped in three genetically distinct subpopulations in which most West Central isolates and nearly half of North Central isolates were assigned to subpopulation one and the remaining North Central isolates were assigned to subpopulation two. Isolates from other regions were assigned to subpopulation three. The low migration rates calculated between the populations also suggest these populations are genetically stable.

Introduction

Canola (*Brassica napus*) was introduced to North Dakota more than 25 years ago. Currently, >90% of the canola produced in the U.S. is grown in North Dakota (NASS, 2012). Blackleg or phoma stem canker, caused by the fungus *Leptosphaeria maculans*, is the most devastating disease of canola worldwide. Yield losses due to the disease have been estimated from $ 18.2 million to $ 236.7 million each season (Khangura and Barbetti, 2001; Allard et al., 2002; Fitt et al., 2006). Blackleg became endemic in North Dakota canola growing areas in mid 1980s (Lamey and Hershman, 1993). This disease is capable of reducing yield in the state by >45% (del Rio Mendoza et al., 2012). During 1991, the virulence profile of *L. maculans* populations in North Dakota was dominated by pathogenicity groups (PG) 1 and 2 only.

However, during 2003 and 2004 isolates from three additional groups, 3, 4, and T, were detected (Chen and Fernando, 2006). A recent report of blackleg outbreaks and their association with these new PGs (del Rio Mendoza et al., 2012) are an indication that *L. maculans* populations in North Dakota may be shifting.

L. maculans is a fungal pathogen with mixed reproductive systems. It is a heterothallic haploid dothideomycetous fungus that outcrosses prolifically on canola stubbles producing pseudothecia (sexual fruiting bodies) that discharge ascospores (sexual spores). Its life cycle includes one sexual recombination per season that produces large amount of ascospores which are considered to be the primary sources of inoculum. This is followed by multiple reproductions of asexual pycnidiospores that are splash dispersed within neighboring plants (Howlett et al., 2001). Four pathogenicity groups (PG-2, PG-3, PG-T, and PG-4) and nine avirulence genes (*AvrLm1 – AvrLm9*) have been identified in *L. maculans* which could theoretically make up to 512 different races (Balesdent et al., 2005). These features predispose *L. maculans* populations to higher evolutionary potential and makes them excellent candidate with ability to shift into more aggressive pathotypes in relatively short periods of time (McDonald and Linde, 2002b). This was evidenced in France and Australia where disease resistance governed by a single major gene (*Rlm1*) was overcome within 3 years of its introduction into *Brassica* cultivars (Li et al., 2003; Rouxel et al., 2003; Sprague et al., 2003). Similar trends have been suspected in North Dakota blackleg populations (del Rio Mendoza et al., 2012; Marino, 2011).

Understanding the genetic variability of a pathogen is very important in predicting the evolutionary potential of the pathogen, optimizing screening procedures in plant breeding programs, and developing more effective management strategies (McDonald and Linde, 2002a). Genetic variations in *L. maculans* have been studied in Australia, a few European countries, and

Canada at various population levels, such as within a disease nursery, within commercial fields, and in inter-continental collections (Pongam et al., 1999; Purwantara et al., 2000; Hayden and Howlett, 2005; Gout et al., 2006; Hayden et al., 2007; Dilmaghani et al., 2012a). The genetic variability has been studied using various types of molecular markers such as restriction fragment length polymorphisms (RFLP), amplified fragment length polymorphisms (AFLP) and random amplified polymorphic DNA (RAPD) (Barrins et al., 2004; Mahuku et al., 1997); and more recently, using microsatellites (or simple sequence repeats) and minisatellites (Hayden and Howlett, 2005; Hayden et al., 2007). The latter two are tandemly repetitive DNA sequences that are abundantly spread in the genome of all eukaryotic species. These two marker types are usually distinguished according to the size of their repeats (Debrauwere et al., 1997). Minisatellites have a repeat motif of 10–60 bp resulting in repeated blocks of 0.1–30 kb (Armoure, 1999), while microsatellites repeats have a base motif that is 1–6 bp long, with shorter tandem arrays up to 1 kb (Chambers and MacAvoy, 2000).

Various polymorphic minisatellite markers (Attard et al., 2001; Eckert et al., 2005; Gout et al., 2006), and microsatellite markers (Hayden et al., 2004), as well as mating type loci (Cozijnsen and Howlett, 2003) have been identified in *L. maculans* that can be used to characterize the genetic diversity, gametic disequilibrium and gene flow between populations. Hayden and Howlett (2005) used these microsatellite, minisatellite and mating type markers and reported a very low level of genetic differentiation between ascospores from stubbles and conidia from seedlings collected in a disease nursery indicating that the isolates of the disease nursery belonged to one population. Similarly, Hayden et al. (2007) reported high levels of genetic differentiation of *L. maculans* isolates between Eastern and Western Australia and explained it to be due to the long arid desert that acted as a natural barrier between these regions; however, the

population within the region remained genetically stable over the two consecutive years of their study. Recently, Dilmaghani et al. (2012b) used 14 minisatellite markers and detected highly clonal *L. maculans* populations in cabbage in Central Mexico. These populations had mating type loci significantly deviated from the 1:1 ratio that is expected in random mating populations. These studies suggest the population structure for this fungus is more complex than previously expected. While the genetic structure of *L. maculans* populations in North Dakota has not been studied, the increment in the frequency and importance of different pathogenicity groups of *L. maculans* (del Rio Mendoza et al., 2012) suggests it has been in state of flux in the past decade. Thus, this study was conducted to characterize the genetic structure of *L. maculans* populations of North Dakota.

Materials and Methods

Sample collection

The *L. maculans* samples used in this study were collected in surveys conducted annually between 1991 and 2009, but not in 1992. In each North Dakota county, one field per every 2,025 ha of canola planted was inspected between late August and early September soon after the crop was swathed and when the stubble was still fresh. In each field, five stems were pulled at ten arbitrarily selected locations and their crown and roots were visually inspected for blackleg symptoms. The disease was identified as gray to black lesions with presence of pycnidia and girdling stem cankers near the soil line. Infected tissues were brought to the laboratory for confirmation as well as isolation of the pathogen for further studies. After confirmation of their identity a reduced number of isolates were placed in long term storage for future use. Among the 605 isolates used in this study two were collected in 2002, 152 in 2004, one in 2005, 430 in 2007, and 20 in 2009.

Isolation of *L. maculans*

All isolates were retrieved as described by del Rio Mendoza et al. (2012). Briefly, the infected tissues were washed with running tap water to remove debris from the stem surface. After lightly tapping the stems with paper towel to remove excess moisture, the visibly infected portions were cut and surface disinfested by immersing in either 100% commercial bleach [Clorox Regular bleach (6% NaOCl), The Clorox Company, Oakland, CA] or 30% aqueous solution of the bleach for 10-30 sec depending upon the condition of the sample. The surfaces of disinfested tissues usually containing pycnidia were scrapped off onto sterile distilled water with a sterile scalpel. About 0.5 ml of the suspension was streaked onto full strength V8 medium amended with streptomycin and penicillin. The medium was prepared with 837 ml distilled water, 163 ml V8 juice (Campbell Soup Co., Camden, NJ), 15 g agar (Bacto-Agar, Becton Dickinson and Co., Sparks, MD), and 3 g $CaCO_3$. The pH of the medium was adjusted to 7.2 with 1M NaOH before autoclaving it at 120^{o} C and 103.4 kPa for 20 min. When the medium was lukewarm it was amended with 7.5 ml streptomycin (10 mg/ml) and penicillin (10 mg/ml). Inoculated medium was incubated under constant white light for 3 to 4 days at 21^{o} C. Single spore colonies were transferred to new plates containing full strength V8 medium prepared as described but without antibiotics and incubated for two weeks under the similar conditions as described. Isolation of *L. maculans* was confirmed by colony, pycnidia, and pycnidiospores morphology. The single spore colony of the pathogen in V8 medium was cut into pieces containing approximately 1 mm^3 of upper surface, kept in two ml plastic vials, labeled and stored at -80^{o} C for future use.

Fungal populations

The isolates used in this study, 605 in total, were collected from 23 counties in North Dakota. The counties were divided into six geographic regions: North East, North Central, North West, West Central, Central, and South West (Figure 4.1), and the isolates collected within each region were considered members of the population of that region. In this way, the North East population consisted of 199 isolates collected from seven counties; the North Central population consisted of 146 isolates from four counties; the North West population consisted of 124 isolates from six counties; the West Central population consisted of 66 isolates from one county; the Central population consisted of 65 isolates from four counties, and the South West population consisted of 5 isolates from one county.

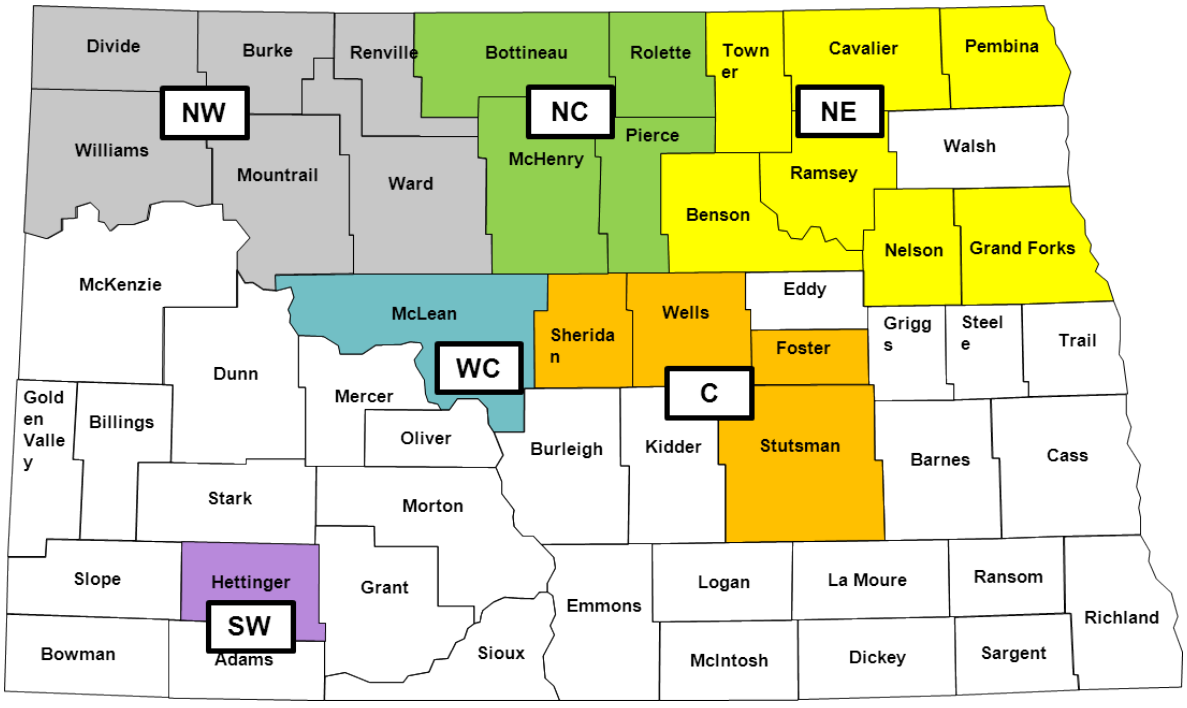

Fig. 4.1. North Dakota geographic regions in which isolates of *Leptosphaeria maculans* were collected from commercial canola (*Brassica napus*) fields. North East = NE, North Centeral = NC, North West = NW, West Centeral = WC, Central = C, and South West = SW.

DNA isolation and quantification

The isolates were cultured in potato dextrose broth and incubated in an orbital shaker at room temperature (21^{o} C) for 4 to 7days. Mycelia were harvested by vacuum filtration and stored at -80^{o} C. The frozen mycelia were lyophilized using VirTis freezer dryer (SP Scientific, Gardiner, NY) for 24 h and ground to fine powder using a high-speed mixer mill (Model MM301, Retsch Inc, Newton, PA). Genomic DNA was extracted from the ground tissue using a cetyltrimethylammonium bromide (CTAB) protocol explained by Murray and Thompson (1980), and later modified by Gurung et al. (2011). Briefly, the ground mycelium was suspended in 750 µL of DNA extraction buffer [1 M Tris HCl (pH 7.5), 5 M NaCl, 0.5 M EDTA (pH 8), and 10% sodium dodecyl sulfate (SDS)]. The suspension was incubated at 65^{o} C for 20 min and then one volume of phenol:chloroform:isomyl alcohol (25:24:1) was added to extract the DNA. The supernatant containing the DNA was pipetted into a separate vial and the extraction procedure repeated once. The aqueous solution containing the DNA was mixed with 1/10 volume of 3M sodium acetate (pH 5.2) and twice the volume of ice-cold absolute ethanol to precipitate DNA. The precipitated DNA was then washed with 70% ethanol, air-dried and suspended in 200 µL of 10 mM Tris buffer [10mM Tris-HCl (pH 8.0), 1 mM EDTA]. The DNA was quantified using a Nano-Drop spectrophotometer (Model ND-1000, Thermo Scientific Inc., Waltham, MA) and the concentration of each DNA sample was adjusted to 10ng/µL for microsatellite and minisatellite analyses.

Microsatellite and minisatellite analyses

In total eleven markers were used for the study. Seven were microsatellite markers *Lema* 4, *Lema* 6, *Lema* 8, *Lema* 12, *Lema* 15, *Lema* 34, and *Lema* 39 designed by Hayden et al. (2004) and the remaining four were minisatellite markers *MinLm4*, *MinLm5*, *Lema* 9, and *Lema*

37). Sequences for minisatellite primers *MinLm4*, and *MinLm5* were described by Eckert et al. (2005); while sequences for *Lema* 9 (GenBank accession AY558893) and *Lema 37* (GenBank accession AY916774) were described by Hayden et al. (2005), and Hayden et al. (2007), respectively. Protocols designed for each primer were followed to amplify the DNA fragments from each isolate. Briefly, 25 µL of mixture was prepared per DNA sample, each contained 15 µL of sterile de-ionized distilled water, 2 µL of 10 ng/µL genomic DNA, 3 µL of 10x *Taq* polymerase buffer, 0.5 µL of 10 mM dNTP mix, 2 µL of 4 µM each reverse and forward primers, and 0.5 µL of *Taq* polymerase (5 units/ µL). PCR was carried out in PTC-100 Peltier Thermal cycler (MJ Research, Inc., Waterman, MA) that was programmed for 5 min at 94^{o} C for initial denaturation, 30 cycles consisting of 30 s at 94^{o} C, 30 s at 58^{o} C, and 1 min at 72^{o} C, followed by final 7 min extension at 72^{o} C. For minisatellite primers the annealing temperature was set to 55 and 60^{o} C for *MinLm4* and *MinLm5*, respectively (Eckert et al., 2005). The amplified fragments were separated in 6% urea non-denaturing polyacrylamide gels within 0.5x TBE buffer (0.09 M Tris-borate and 0.002 M EDTA) stained with 20 µL of ethidium bromide (10 mg/ml). Each 25 µL of amplified sample was stained with 6 µL of 6x loading dye and was loaded in the gel. A 100-bp DNA ladder (Invitrogen Corporation, Carlsbad, CA) was also loaded as a size reference. The gel was run for 3 h at 350 V and pictures were taken using Fluorochem 2200 Image system, (Alpha Innotech Corp., San Leandro, CA).

Mating type determination

Mating types of the 605 isolates were determined using a multiplex PCR assay developed by Cozijnsen and Howlett (2003). Briefly, 25 µL of mixture was prepared per DNA sample, each containing 13 µL of sterile de-ionized distilled water, 2 µL of 10 ng/µL genomic DNA, 3 µL of 10x *Taq* polymerase buffer, 0.5 µL of 10 mM dNTP mix, 2 µL of 4 µM each

primers 9, 10, and 11 as described in Cozijnsen and Howlett (2003), and 0.5 µL of *Taq* polymerase (5 units/ µL). PCR was carried out in PTC-100 Peltier Thermal cycler (MJ Research, Inc., Waterman, MA) that was programmed for 5 min at 94^{o} C for initial denaturation, 30 cycles consisting of 30 s at 94^{o} C, 30 s at 60^{o} C, and 1 min at 72^{o} C, followed by final 7 min extension at 72^{o} C. The amplified fragments were separated in 1% agarose gel within 0.5x TBE buffer (0.09 M Tris-borate and 0.002 M EDTA) stained with 20 µL of ethidium bromide (10 mg/ml). Each 25 µL of amplified sample was stained with 6 µL of 6x loading dye and was loaded in the gel. A 100-bp DNA ladder (Invitrogen Corporation, Carlsbad, CA) was also loaded as a size reference. The gel was run for 2 h at 96 V and pictures were taken using Fluorochem 2200 Image system, (Alpha Innotech Corp., San Leandro, CA). The presence of *MAT1-1* and *MAT1-2* mating type idiomorphs was identified by a single band of 686 and 443 bp, respectively. The distribution of these idiomorphs within a population was analyzed using Fisher's exact χ^2 test.

Data analyses

The bands were manually scored with reference to 100-bp DNA ladder, and the bands with different sizes were considered as different alleles for each marker. The isolates that were not amplified in any of the loci or in more than one or two loci were removed from further analysis. The remaining data were compiled to construct multilocus haplotypes (MLHT). Isolates with identical alleles in all eleven loci were considered clones and only a single representative haplotype (Clone-corrected data) were maintained for each population and used for further analysis. The clonal fraction (CF) was calculated as 1-[(number of different MLHT) / (total number of isolates)] (Dilmaghani et al., 2012a). POPGENE version 1.31 (Yeh et al., 1997) was used to calculate total number of alleles, allele frequencies at each locus, gene diversity in the total sample (H_t), mean gene diversity averaged over five populations (H_s), gene diversity in

individual population (*H*), and Nei's unbiased genetic identity (*I*). Pairwise measures of population differentiation between the population was estimated using a corrected standardized fixation index, G''_{ST} using GenoDive Beta version 2.0 (Meirmans and Tienderen, 2004; Hedrick, 2005; Meirmans and Hedrick, 2011). MULTILOCUS version 1.3 (Agapow and Burt, 2001) was used to calculate multilocus linkage disequilibrium. For this, standardized index of association (*rBarD*) was estimated for each population. The *rBarD* measures multilocus linkage disequilibrium and provides information on whether two different individuals sharing the same allele at one locus also share alleles at different locus (Brown et al., 1980). The *rBarD* ranges from 0 to 1 where 0 is the random association of alleles at different loci, and 1 is the maximum possible linkage disequilibrium among loci. The significance of *rBarD* was tested with 1,000 randomizations of the data and comparing the observed and expected value under the null hypothesis of *rBarD* = 0. Allelic Diversity Analyzer (ADZE) version 1.0 (Szpiech et al., 2008) was used to compute the number of alleles corrected for sample size (allelic richness, A_R) and the number of alleles unique to a single population or group of populations (private allelic richness, PA_R). ADZE is useful while analyzing this dataset as it adjusts differences in sample size across the populations and implements rarefaction procedure to count alleles specific to the population (Szpiech et al., 2008).

Analysis of molecular variance (AMOVA) was conducted using GenoDive Beta version 2.0 (Meirmans and Tienderen, 2004) to determine the degree of genetic differentiation within and among five regional groups of populations in North Dakota. The significance ($P \leq 0.001$) of variance components and F_{ST} values were tested by 1000 permutations of haplotypes among the population.

The software STRUCTURE version 2.3 (Pritchard et al., 2010) was used to affiliate individual isolates of *L. maculans* from sampled geographical locations to specific genetic clusters and to test the possibility of admixture. For this analysis, five independent runs were performed for each value of *K* ranging from 1 to 10. It consisted of a burn-in period of 10,000 iterations and a Markov Chain Monte Carlo (MCMC) sampling of 100,000 iterations. The STRUCTURE outputs were processed with STRUCTURE HARVESTER (Earl and vanHoldt, 2012) to determine the effecting number of population.

The software BayesAss version 3 (Rannala and Yang, 2003; Wilson and Rannala, 2003) was used to estimate recent migration rates between sampled *L. maculans* populations from North Dakota state. The analysis uses the Bayesian approach to detect the number of shared genotypes in a population that had migrated over last few generations. While running the MCMC, the following mixing rates for parameter changes were used: migration rates = 0.2; allele frequencies = 0.3; and inbreeding coefficients = 0.4 as suggested by Rannala and Yang (2003). We used 1,000,000 iterations to perform MCMC with a burn-in length of 10,000,000 iterations. Convergence was assessed based on the results of several runs started with different random number seeds as suggested by Rannala and Yang (2003).

Results

Genetic diversity within populations

All markers used in this study were polymorphic. A total of 56 alleles were detected and the number of alleles per locus ranged from 4 to 6. The isolates that did not amplify in any of the loci (n=43) and five others from the South West population were removed from the dataset. Of the remaining 557 isolates, 545 were multilocus haplotypes. Clones were found in every sampling region (Table 4.1). A total of eleven clones were identified, three in samples from

Bottineau County, two from Wells County, and one each from Ward, McLean, and Cavalier counties. The remaining three clones were shared among different counties; one between Bottineau and Rolette counties, one between Cavalier and McLean counties, and the other in Bottineau and McLean counties. Except for the clone in Ward County, all others were identified among the samples collected in 2007. The clonal fraction value across populations ranged from 0.006 in the North East population to 0.046 in the West Central population with a mean value of 0.025 (Table 4.1). The clone corrected data was used for further analysis. The Nei's measure of gene diversity (*H*) was relatively high in all populations ranging from 0.628 to 0.692. The North East and West Central populations had the highest and lowest indices, respectively (Table 4.1). LD measured by standardized index of association (*rBarD*) ranged from 0.01 to 0.07 and were significantly different from zero ($P \leq 0.001$) in all tested populations. Central and North East populations had the highest and lowest LD, respectively (Table 4.1). The calculation of allelic richness (A_R) revealed non-significant differences in allelic richness among populations. However, the highest allelic richness (4.77) was found in North East and lowest (4.415) was found in North West population. The private allele richness (PA_R) shows number of unique alleles assigned to each single population and this was highest (0.119) for North East population and lowest (0.00) for the Central and North West populations, respectively (Table 4.1).

Genetic diversity at each locus across all populations was also determined. The genetic diversity detected for each locus varied with a range of 0.499 to 0.778 (H_t) and 0.477 to 0.729 (*Hs*). The highest diversity was observed at the *Lema 34* locus and the lowest at *Lema 37*. While comparing the genetic diversity contributed by each locus to the populations within each region, *Lema 37* had the smallest indices in the Central, North East, and North West regions and was second smallest in the other two regions. *Lema 4* was the second less variable marker. Markers

Table 4.1. Genetic diversity for microsatellite and minisatellite markers and mating type of populations of *Leptosphaeria maculans* sampled from five canola growing regions in North Dakota.

ID	Regions	n^a	$n_g{}^b$	CF[c]	*rBarD*[d]	H^e	$A_R{}^f$	$PA_R{}^g$	Mating types[h]	
									Mat1-1	Mat1-2
C	Center	61	59	0.033	0.077^{**}	0.631	4.511 ± 0.247	0.000 ± 0.000	21	38^{*}
NC	North Center	146	141	0.034	0.059^{**}	0.664	4.516 ± 0.140	0.002 ± 0.002	55	86^{**}
NE	North East	162	161	0.006	0.010^{**}	0.692	4.770 ± 0.204	0.119 ± 0.081	57	104^{***}
NW	North West	123	122	0.008	0.039^{**}	0.676	4.415 ± 0.240	0.000 ± 0.000	56	66^{ns}
WC	West Center	65	62	0.046	0.063^{**}	0.628	4.648 ± 0.240	0.061 ± 0.061	21	41^{*}
Total		557	545	….[i]	….	….	….	….	210	335^{***}

[a] Number of isolates used for this study.
[b] Multilocus haplotypes (Clone-corrected sample size).
[c] Clonal fraction.
[d] Standardized index of association. The null hypothesis of linkage equilibrium was rejected if $P \leq 0.001^{**}$.
[e] Genetic diversity averaged across seven microsatellite and four minisatellite loci.
[f] Allelic richness averaged across loci (± standard error).
[g] Private allelic richness averaged across loci (± standard error).
[h] Number of isolates carrying mating type 1 (*MAT1-1*) or 2 (*MAT1-2*); null hypothesis of mating types being at 1:1 ratio not rejected (ns) or rejected at $P = 0.05^{*}$, $P = 0.01^{**}$, $P = 0.001^{***}$.
[i] Values not calculated.

Lema 34 and *Lema 39* yielded the highest G''_{ST} values with 0.285 and 0.283, respectively, while *Lema 9* yielded the lowest G''_{ST} (0.054) across all populations (Table 4.2).

Distribution of mating types

A single amplicon that matched either *MAT1-1* or *MAT1-2* was detected from the PCR amplification of the mating type idiomorphs in all isolates. The distribution of mating type idiomorphs among clone-corrected data from the entire population differed significantly ($P \leq 0.001$) from the 1:1 expected ratio (Table 4.1). Of the 545 isolates analyzed, 39% were *MAT1-1* and 61% were *MAT1-2*. When the ratio was calculated for each region, the frequency of the mating type idiomorphs differed significantly from 1:1 ratio for the Central, North Central, North East, and West Central ($P \leq 0.05$) populations. The recovery ratio for *MAT1-2* was between 1.6 and 1.9 times that of *MAT1-1* isolates in these regions and approximately 1.6 times that of the population across regions (Table 4.1).

Population differentiation (G''_{ST}) and genetic identity (I) between populations

The level of genetic differentiation (G''_{ST}) was moderate to high in all pair-wise comparisons between five populations. It ranged from 0.045 to 0.266 and the values of genetic differentiation between all population pairs were highly significant ($P \leq 0.001$). The highest genetic differentiation was observed between populations from the North West and West Central regions whereas the lowest was observed between populations from the North Central and West Central regions (Table 4.3).The level of genetic identity (I) between all possible pairwise comparisons ranged from 0.799 to 0.960. The highest I value was observed between populations from the North Central and West Central regions and the lowest between populations from the North West and West Central regions (Table 4.3).

Table 4.2. Summary of genetic variation statistics for seven microsatellite and four minisatellite loci in *Leptosphaeria maculans* populations sampled from five canola growing regions in North Dakota[a].

Loci	Repeat Motifs	No. of alleles[b]	H_t [c]	H_s [d]	G''_{ST} [e]	Gene diversity [f]				
						C	NC	NE	NW	WC
Lema 4	$(CA)_{14}$	4	0.622	0.597	0.122	0.619	0.561	0.630	0.641	0.499
Lema 6	$(GT)_{11}$	5	0.722	0.676	0.242	0.564	0.681	0.695	0.700	0.702
Lema 8	$(GA)_7AA(GA)_7$	5	0.708	0.661	0.240	0.622	0.673	0.650	0.673	0.652
Lema 12	$(TGC)_5TAC(TGC)_7$	6	0.692	0.680	0.069	0.627	0.700	0.695	0.719	0.623
Lema 15	$CG(CA)_4\ CG(CA)_6\ CG(CA)_4$	5	0.748	0.721	0.163	0.786	0.607	0.734	0.738	0.699
Lema 34	$(ATG)_4N_{22}(TGG)_8$	6	0.778	0.729	0.283	0.619	0.731	0.780	0.701	0.775
Lema 39	$(GTACA)_3$	5	0.742	0.688	0.285	0.707	0.655	0.771	0.711	0.557
MinLm4	(ACAACTTCAATCCGCCT GCTGATTATCCCTATCGA TATCAGACCCAGCAAG)$_4$	5	0.736	0.698	0.207	0.749	0.726	0.723	0.652	0.603
MinLm5	$(AACCGCCCAGCCACTC)_7$	6	0.737	0.694	0.238	0.739	0.684	0.749	0.659	0.598
Lema 9	(AATCCCCACAATGCACA CCTATACGAAACACTGC ACACCC)	5	0.710	0.700	0.054	0.693	0.708	0.674	0.713	0.673
Lema 37	$(GGATTGGGT)_2N_{21}$(GGAT TGGGT)$_2$	4	0.499	0.477	0.108	0.219	0.580	0.508	0.528	0.521
Mean	....	....	0.699	0.666	0.183	0.631	0.664	0.692	0.676	0.627

[a] Repeat motif of the seven microsatellite loci from Hayden et al., 2004.
[b] Number of alleles in the total sample over the five populations.
[c] H_t = Gene diversity in the total sample over all populations.
[d] H_s = Mean gene diversity averaged over five populations.
[e] G''_{ST} = Corrected standardized fixation index over all populations per locus.
[f] Nei's gene diversity for individual population.

The hierarchical analysis of molecular variance (AMOVA) conducted on all isolates indicated that 95% of the total variance was distributed within populations and 5% was distributed among populations. The overall F_{ST} for the population was 0.05 at $P \leq 0.001$ (Table 4.4).

Table 4.3. Estimates of population differentiation (G''_{ST}) (above diagonal) and genetic identity (I) (below diagonal) between pairs of populations of *Leptosphaeria maculans* in five canola growing regions in North Dakota.[a]

	North Dakota regions[b]				
Populations	C	NC	NE	NW	WC
C	….	0.253**	0.124**	0.223**	0.248**
NC	0.809	….	0.152**	0.203**	0.045**
NE	0.905	0.885	…..	0.124**	0.127**
NW	0.831	0.847	0.904	…..	0.266**
WC	0.813	0.960	0.904	0.799	…..

[a] $^{**}G''_{ST}$ = corrected standardized fixation index significant at $P \leq 0.001$.
[b] C = Central; NC = North Central; NE = North East; NW = North West; WC = West Central.

Table 4.4. Analysis of molecular variance (AMOVA) of multilocus haplotypes in *Leptosphaeria maculans* population from five canola growing regions in North Dakota.

Sources of variation	Df [a]	Estimated variance	Variation (%)	F_{st} [b]	P value
Among whole population	4	0.21	5		
Within population	540	3.70	95	0.05	0.001

[a] Df= degrees of freedom.
[b] F_{st} = fixation index; significance determined by 1000 random permutations.

Migration scenario

Recent migration events were estimated using BayesAss. The analysis indicated very low gene flow between regional populations. However, there was a significant (P = 0.05) bi-directional gene flow between the populations from the North Central and West Central regions.

The recent migration rate from North Central into West Central populations was 12.8 % and from West Central into North Central populations was 8.8 % (Table 4.5).

Table 4.5. Migration rate estimates between *Leptosphaeria maculans* populations from five canola growing regions in North Dakota estimated using BayesAss.

Source of migrants	Destination of migrants				
	C[a]	NC	NE	NW	WC
C	93.30 (0.022)[b]	2.30 (0.012)	1.20 (0.011)	2.50 (0.016)	0.70 (0.007)
NC	0.50 (0.004)	85.70 (0.044)	0.50 (0.005)	0.50 (0.004)	12.80 (0.044)
NE	0.44 (0.005)	0.52 (0.004)	97.90 (0.009)	0.47 (0.004)	0.70 (0.005)
NW	0.30 (0.003)	0.50 (0.005)	0.40 (0.004)	98.50 (0.007)	0.40 (0.003)
WC	1.00 (0.009)	8.80 (0.024)	3.70 (0.019)	1.07 (0.009)	85.50 (0.031)

[a] C = Central; NC = North Central; NE = North East; NW = North West; WC = West Central
[b] Percentage of population composed of recent immigrants and (± values of 95% confidence intervals).

Population structure

The number of clusters present in the entire set as a whole was calculated as *K=3* (Figure 4.2). We set the membership value (*q*) for individual isolate at $q > 0.8$. Based on this criterion, most individuals exhibited solid membership to only one of these 3 clusters; admixed individuals were primarily observed in every sampled population (Table 4.6). Of the 59 isolates collected from the Central region, 20 isolates did not belong to any of the clusters and were classified as admixed. From the 39 remaining isolates, 69% were assigned to cluster 3 while the remaining isolates were equally shared between clusters 1 and 2 (Table 4.6). Among the 141 isolates collected from the North Central region, 38 were identified as admixed. From the 103 remaining isolates, 56% were ascribed to cluster 1 and 42% to cluster 2. A negligible number of isolates (2%) were ascribed to cluster 3 (Table 4.6). Among 161 isolates from North East region, 69

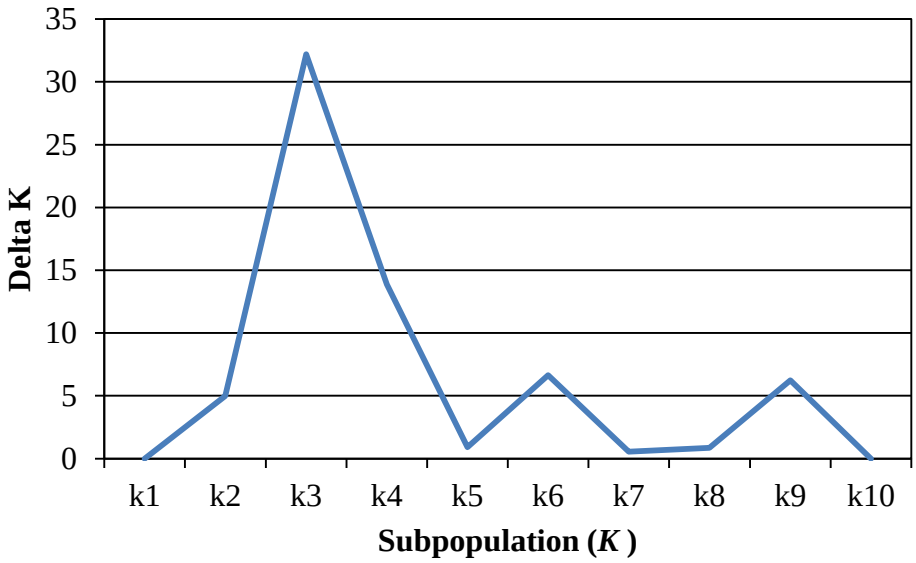

Fig. 4.2. Number of genetic subpopulations of *Leptosphaeria maculans* populations collected from five canola growing regions in North Dakota as estimated by Delta *K* values.

Table 4.6. Population structure of *Leptosphaeria maculans* based on individual's ancestry as determined by STRUCTURE analysis.

	Clusters				
Regions	1	2	3	Admixed	Total
Central	6	6	27	20	59
North Central	58	43	2	38	141
North East	20	21	51	69	161
North West	9	14	49	50	122
West Central	27	13	5	17	62
Total	120	97	134	194	545

isolates were classified as admixed. From the 92 remaining isolates, 55 % were ascribed to cluster 3, 22 % to cluster 1 and 23% to cluster 2 (Table 4.6). Among the 122 isolates from the North West population, 50 were classified as admixed. From the 72 remaining isolates, 68% were assigned to cluster 3 while 13 and 19% were assigned to cluster 1 and 2, respectively (Table 4.6). Among the 62 isolates from the West Central population, 17 were classified as admixed. From the remaining 45 isolates, 60% were assigned to cluster 1, 29 % to cluster 2 and

11% to cluster 3 (Table 4.6). Altogether, 351 (64%) isolates were assigned to either of the clusters; however, 194 (36%) could not be assigned to any cluster at $q > 0.8$.

Discussion

We used a collection of *L. maculans* isolates from commercial canola (*Brassica napus*) fields in North Dakota to infer the population genetic structure of this pathogen in the state using seven microsatellite and four minisatellite markers previously used by other researchers (Hayden et al., 2004, 2005, 2007; Eckert et al., 2005). The scrutiny revealed similar levels of high genetic diversity among populations from five canola-producing regions in North Dakota. This level of variability reflects in part the history of the expansion of canola cultivation in North Dakota. The canola production area in the state changed from 671 thousand acres in 1997 to 1.7 million acres in 2012 (NASS, 2012). The increased seed trade, movement of seeds during harvest, as well as transfer of plant materials from field to field during routine production practices might have increased the spread of *L. maculans* resulting in recurrent introduction of the pathogen as well as in maintaining the genetic variability of its populations as suggested by Dilmaghani et al. (2012a). Further, the increments in area planted to canola were accompanied by changes in sensitivity of canola genotypes to *L. maculans* populations and subsequent selection pressure. The adoption of materials with no resistance genes during the early 1980s led to blackleg epidemics in the mid 1980's (Lamey and Hershman, 1993); these were followed by the rather quick replacement of susceptible materials with either moderately resistant or resistant genotypes (Berglund, 2003). In 2003, strains with previously unreported virulence profiles were detected for the first time (Bradley et al., 2005). In 2009, blackleg outbreaks caused by these new strains were observed (del Rio Mendoza et al., 2012). Similar cases have been repeatedly reported from different canola growing regions worldwide (Li et al., 2003; Easton, 2004; Sprague et al., 2006).

The existence of active sexual recombination within the population has been used to explain the high levels of genetic diversity in other parts of the world; after all, in North America ascospores are considered the primary source of inoculum for almost all blackleg epidemics in those regions (Petrie, 1995; Mahuku et al., 1997; West et al, 2001). In North America, however, production of sexual spores is very limited (Dilmaghani et al., 2012a). Ghanbarnia et al. (2011) reported a high density of pycnidia and low density of pseudothecia on stubble pieces and absence of ascospores on spore traps while a very high level of disease incidence and disease severity on infected plants during two years in Western Canada. They suggested pycnidiospores were the main source of inoculum for disease incidence in those two years. Other studies have also shown delayed pseudothecial maturation due to extreme weather conditions that result in low ascospores production (Petrie, 1994; West et al., 2001; Toscano-Underwood, 2003). Longer freezing periods that could interrupt ascocarp development have also been pointed to explain this in Canada (Petrie, 1994). North Dakota experiences somewhat similar weather conditions, remaining under freezing condition for nearly five months with temperatures as low as -48 ^{o} F during the coldest months (NDAWN, 2012). Results from our study support the notion that ascospores may not be as important in the region as they are in other parts of the world. The North Dakota populations evaluated in this study departed significantly from the 1:1 ratio for mating types and were all considered to be in linkage disequilibrium, a condition favoring asexually reproducing populations. The latter two lead us to believe *L. maculans* populations in ND are mostly asexually reproducing and pycnidiospores may be the primary source of inoculum for blackleg epidemics. However, the low clonal fraction observed in all populations indicates the role of sexual reproduction cannot be overlooked.

Distribution of mating type idiomorphs significantly deviated from the 1:1 expected ratio in four of the five North Dakota populations evaluated. These results contradict findings by researchers in other parts of the world (Hayden and Howlett, 2005; Gout et al., 2006). There could be several possible explanations for the results obtained in our study. One possible explanation is these results are an artifact resulting from the sampling method used. In this study, efforts were made to sample one field for every 2,025 ha of canola planted in each county; in this way in any given year some counties would have up to 40 fields scouted whereas others would not have a single field scouted. In each field 50 canola stems were inspected for blackleg symptoms and a few symptomatic stems were taken to the lab for isolation. Isolates retrieved from these stems that were stored constituted the population used in this study. Waalwijk et al. (2002) suggested the departure from a 1:1 ratio on mating types of some of the populations of *M. graminicola* was due to the relatively small size of the samples processed, which is at least six times smaller than the samples we used. Also the frequency of *MAT1-2* allele is consistently higher in all populations. From this result, one can hypothesize that there are some driving factors that favor the reproduction of *MAT1-2* resulting in significant gametic disequilibrium and thus limiting the sexual reproduction of this fungus in North American environment. However the fitness of these mating types in different environmental conditions needs to be evaluated.

The Hierarchical analysis of molecular variance (AMOVA) revealed a significantly (P<0.001) low genetic variation (5%) due to the difference in isolates from different *L. maculans* population, whereas the isolates from each individual population was responsible for the high genetic variation (95%). The genetic differentiation (G''_{ST}) among most of the pairwise comparisons between individual population were relatively higher and statistically significant. These results indicate that *L. maculans* populations in North Dakota are not part of a single,

panmictic population. However, a low yet significant level of differentiation was observed between the North Central and West Central populations. These populations also had higher levels of genetic identity indicating a possible genetic homogenization. A population study conducted in France revealed that *L. maculans* isolates collected from four locations separated by more than 500 km had very low levels of genetic differentiation indicating a coevolving panmictic population (Gout et al., 2006). However, in that case the occurrence of ascospores was common and was airborne which enabled the genetic homogenization of the population. Contrary to this scenario, *L. maculans* populations in North Dakota seem to be asexually reproducing. The production and dispersal of ascospores are limited. Pycnidiospores are splash dispersed and are not means of long distance dispersal. This might have restricted the migration and potential gene flow among the population. This is also supported by the recent migration analysis in our study. The recent migration rates between the different populations of *L. maculans* were very low which indicated constrained gene flow consequentially resulting into significant population differentiation between regions in North Dakota. Only the populations from the North Central and West Central regions had relatively higher rate of recent bidirectional migration, yet the rate is not high enough for genetic homogenization as indicated by significant genetic differentiation between these populations. This result indicates that the process of homogenization, however, may be in progress.

The evidence for genetic differentiation between *L. maculans* populations in North Dakota is also supported by STRUCTURE analysis. The maintenance of two genetically differentiated populations in the North Central region of North Dakota indicates the isolates in these regions have diverse ancestry and the introductions of the pathogen is relatively recent for homogenization to take place (Hayden et al., 2007; Dilmaghani et al., 2012a). Most of the West

Central and half of the North Central populations share a common ancestry which could be explained by the geographical location of the isolates. Among the North Central isolates, those belonging to the northern most counties (i.e. Bottineau and Rolette) were assigned to subpopulation 1 whereas isolates from McHenry County further in the south were assigned to subpopulation two. The membership of isolates from three geographically distinct regions (Central, North East, and North West) in a single genetic population is harder to explain. A recent study on migration patterns and population biology of *L. maculans* on worldwide collection of isolates suggested USA as origin of *L. maculans* and from here independent introductions into Eastern Canada (Ontario), Europe and Australia (Dilmaghani et al., 2012a). However, Dilmaghani et al (2012a) used isolates collected from Georgia but not from North Dakota in USA. Pongam et al. (1999) studied the genetic variation among *L. maculans* isolates collected from North Dakota, Georgia, Ontario, Western Canada, the United Kingdom, France, Germany, and Australia. Interestingly, the isolates from North Dakota, Western Canada, Georgia, and the UK formed one cluster and the rest formed the other. Pongam et al. (1999) indicated that Georgia and North Dakota isolates could share a common ancestry. Based on the similarity of isolates between Western Canada and North Dakota, they also suggested that *L. maculans* was introduced into North Dakota from Western Canada, a result contradicting the conclusion of Dilmaghani et al. (2012a). However, the conclusions reached by Pongam et al. are limited by the low number of samples used to represent a population.

Our study provides a good insight into genetically differentiated population of *L. maculans* in North Dakota and possible reproduction pattern. To our knowledge this is the first report that explains the genetic structure of this fungus in a large geographical scale in North Dakota. After validating the results from this study will help direct the canola breeding program

by screening the breeding lines with diverse population of *L. maculans.* At the same time, it helps in better disease management practices by limiting the movement of infected plant materials within and across the counties.

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CHAPTER 5: IDENTIFICATION OF *BRASSICA JUNCEA* SOURCES OF RESISTANCE AGAINST ISOLATES OF *LEPTOSPHAERIA MACULANS* AND OF MARKERS ASSOCIATED WITH RESITANCE TO THEM

Abstract

A set of 298 *Brassica juncea* accessions maintained by the National Plant Germplasm System USDA-ARS at its North Central Regional Plant Introduction Station in Ames, IA were screened for resistance to PG-2, PG-3, PG-T, and PG-4 strains of *L. maculans* in greenhouse conditions. Two week old seedlings were inoculated with a mixture of spores from five isolates of every PG at a concentration of 1 x 10^7 pycnidiospores ml^{-1}. The phenotypic reaction to inoculation was assessed using 0 to 9 scale two weeks later. Non-parametric analysis of the phenotypic reaction allowed the identification of six accessions resistant to PG-2 and PG-3, nine accessions resistant to PG-T and PG-4, and two accessions resistant to PG-2 and PG-4. DNA extracted from 279 accessions was screened using 766 polymorphic DArT markers. Association mapping was used to identify quantitative trait loci associated with resistance to these PGs. Regression-based association analysis was performed using multiple linear models for marker-trait association. Thirteen DArT markers significantly associated with resistance to PG2 were identified using a mixed-linear model with structure and kinship. Among these markers, three were also found to be significantly associated with resistance to PG3 using a linear model with kinship ($P<0.05$). None of the markers were found to be significantly associated with resistance to PGT and PG4 at $P<0.05$. From this study we identified the markers associated with resistance to PG2 and PG3. An increased number of markers would increase the discovery of QTL significantly associated with resistance to PGT and PG4 isolates.

Introduction

Blackleg disease caused by *Leptosphaeria maculans* (Desmaz) Ces. & de Not (anamorph = *Phoma lingam* (Tode:Fr.) Desmaz.) is one of the major constrains of canola production worldwide (Gugel and Petrie, 1992; Howlett et al., 2001; Howlett, 2004). The fungus *L. maculans* is haploid and outcrosses prolifically on canola stubbles. It can produce both sexual (ascospores) and asexual (pycnidiospores) spores in the same growing season. Ascospores released from pseudothecia on infested stubbles serve as primary source of inoculum (Gugel and Petrie, 1992; Hammond et al., 1985). Pycnidiospores act as secondary sources of inoculum and are splash-dispersed from pycnidia produced on canola residues onto leaves and neighboring plants. Once spores germinate, they penetrate plant tissues directly, through microscopic wounds or natural openings (Howlett et al., 2001). Hyphae then move down to the stem via vascular tissues of the petiole and eventually to the crown where it produces stem cankers (Hammond et al., 1985; Hammond and Lewis, 1987). Cankers that girdle the stem cause plant lodging, premature death and yield losses (Howlett et al., 2001).

The use of resistant cultivars whenever available and implementation of cultural practices such as crop rotation, use of disease-free seeds, fungicide seed treatment, and foliar fungicide applications are recommended to combat this disease (Markell et al., 2008). Cultural practices alone are not always reliable and sufficient to control the disease. Thus, effective genetic resistance is always the most economical and environmentally safe strategy to manage the disease (Salisbury et al., 1995; Sprague et al., 2006).

In North Dakota most commercial canola cultivars are hybrids that carry genes for resistance against pathogenicity group (PG) 2 strains of *L. maculans*. Resistance gene *Rlm2* confers good levels of protection against PG-2 strains whereas genes *Rlm1*and *Rlm3* confer

limited levels of protection but strains of PG-4 defeat all three genes (Mengistu et al. 1991). The detection of PG-4 and other PGs in the region represent a serious threat to the canola industry because most commercial canola cultivars are highly susceptible to strains of PG-4 and PG-3 (Marino, 2011). Further, blackleg outbreaks detected in 2009 were found to be caused mostly by strains of PG-4 (del Rio Mendoza et al. 2012). Identification of good sources of resistance against these and other PGs is urgent.

The mechanisms of resistance to blackleg have been reported to be inherited both qualitatively and quantitatively (Delourme et al., 2008; Sprague et al., 2006). The qualitative resistance is controlled by single race-specific genes and is expressed in both seedling and adult plants. Quantitative resistance is controlled by non-race-specific genes and is expressed in the adult plant with minor phenotypic effect (Delourme et al., 2008). Qualitative resistance provides effective protection against the disease but at the same time exerts a significant selection pressure on the pathogen that could lead to breakdown of the resistance (Howlett, 2004; Sprague et al., 2006). An extreme example of this occurred in Australia with a synthetic line developed by Crouch et al. (1994) from a cross between *B. oleracea* subsp. *alboglabra* and a *B. rapa* subsp. *sylvestris*. Complete protection against the disease was conferred by a single dominant gene, *LepR3*, which inhibied spore germination and plant colonization (Li and Cowling, 2003; Yu et al., 2004; Sosnowski et al., 2004). In 2000, several *B. napus* lines carrying this gene were released in Australia. In 2002, Easton (2004) observed canker symptoms on lines carrying the *LepR3* gene in southeastern Australia. Li et al. (2003) observed a similar situation in Western Australia. In 2003, severe epidemics were observed in every canola field sown with canola cultivars carrying this gene (Sprague et al., 2006). On the other hand, quantitative resistance offers a more durable resistance due to the presence of multiple genes that as a group have a

broader spectrum of action although provide lower levels of resistance; the activity of these genes produces a lower amount of selection pressure on the pathogen (Howlett, 2004; Sprague et al., 2006; Delourme et al., 2008). Since the effect of these genes on the phenotype of the plant is less evident and more difficult to observe than the effect of genes conferring qualitative protection, researchers resort to the identification of quantitative trait loci (QTL) that are associated with resistance to the disease (Delourme et al., 2008). Introgression of qualitative and quantitative types of resistance offers both effective and durable resistance against the disease. In order to achieve this goal, availability of genetically diverse germplasm and identification of broader range of resistant sources is important.

Various efforts have been put forth to identify and deploy blackleg resistance genes into canola breeding lines. These genes have been identified in *B. rapa*, *B. napus*, *B. juncea*, and *B. nigra* (Ferreira et al., 1995; Mayerhofer et al., 1997; Chevre et al., 2003; Christianson et al., 2006; Chevre et al., 1996). The latter two species and *B. carinata* are believed to have high levels of resistance to *L. maculans* (Rimmer and van den Berg, 1992). *B. napus* (AACC), *B. juncea* (AABB), and *B. carinata* (BBCC) are amphidiploid species derived from natural crosses between three diploid species, *B. rapa* (AA), *B. nigra* (BB), and *B. oleracea* (CC) (U, 1935). Since all *Brassica* species share similar genome, resistance loci identified in one species can be introgressed into *B. napus* cultivars (U, 1935). Hybrids generated by Plieske et al. (1998) from crosses between *B. napus* and *Brassica* species containing B genome segregated for a single dominant gene conferring resistance to the disease. In a separate study, crosses made between *B. nigra* and *B. juncea* with *B. napus*, produced asymmetric somatic hybrids and three different resistance loci were identified, each locus being co-segregated with the B genome allele of different RFLP markers (Dixelius, 1999; Dixelius and Wahlberg, 1999). Christianson et al.

(2006) also identified two genes *LMJR1* and *LMJR2* from a cross between two *B. juncea* lines that conferred resistance to *L. maculans*. These genes were mapped to linkage groups J13 and J18 respectively and were positioned in the B genome.

The objective of this study is to identify sources of resistance to PG-2, PG-3, PG-T, and PG-4 strains of *L. maculans* among a collection of *B. juncea* plant introductions maintained by the USDA-ARS National Plant Germplasm System and to identify markers associated with resistance to these PGs.

Materials and Methods

Preparation of plant materials

A total of 298 accessions of *Brassica juncea* were screened in greenhouse conditions for this study. 'Westar' a *Brassica napus* cultivar was used as a susceptible control for all experiments. Seeds for all plant materials were obtained from the collection curated by the USDA-ARS National Plant Germplasm System at the North Central Regional Plant Introduction Station in Ames, IA (http://www.ars-grin.gov/ars/MidWest/Ames/). The information on geographic origin of these accessions is provided in Appendices A. Seeds were planted in plastic containers with ten inserts and six wells in each insert. The containers were filled with artificial soilless mix (Professional Growing Mix 1, SunGro Horticulture Canada, Seba Beach, AB, Canada). Three seeds of each accession were planted at a depth of five mm in each well. Five days later, the seedling populations were thinned down to one healthy seedling per well. A total of three seedlings were left per accession per PG. The seedlings were watered daily and kept in greenhouse at 22/18^{o} C day/night temperatures and 14 h photoperiod. Ten days after planting, each cotyledon was lightly wounded once with sterile sharp tweezers and an aliquot of inoculum deposited on each wound.

Accessions were planted in five batches and the batches were planted at an interval of less than a week. Each batch contained 59 accessions and Westar as susceptible control. Each batch was replicated three times and each accession had three plants per replication per PG. The entire experiment was repeated three times (trials).

Inoculum preparation and storage

Leptosphaeria maculans isolates collected from North Dakota fields and belonging to PG-2, 3, T, and 4 were used for the study. Single spore cultures of these isolates had been classified into pathogenicity groups as part of a previous study (Chapter 3) and were stored as frozen cultures at -80^{o} C. Inoculum for each pathogenicity group was prepared by combining equal amounts of pycnidiospore suspensions of five isolates. In this way, PG-2 isolates BL942, BL951, BL1017, BL735, and BL1856; PG-3 isolates BL729, BL730, BL731, BL1855, and BL1807; PG-T isolates BL1068, BL1094, BL1044, BL737, and BL1821; and PG-4 isolates BL736, BL1025, BL1811, BL1015, and BL1878 were used for the study. While preparing inoculum, each piece of the frozen culture was inoculated on V8 medium. The medium was prepared with 837 ml distilled water, 163 ml V8 juice (Campbell Soup Co., Camden, NJ), 15 g agar (Bacto-Agar, Becton Dickinson and Co., Sparks, MD), and 3 g $CaCO_3$. The pH of the medium was adjusted to 7.2 by adding 1M NaOH. The medium was sterilized by autoclaving at 120^{o} C and 103.4 kPa for 20 min. The inoculated medium was incubated at 21^{o} C under constant white light for two weeks. When the pinkish mass of pycnidiospores became visible, colony surface was scrapped gently with 5 ml sterile distilled water using sterile glass rod to release the spores. The spore suspension was collected on a tube and centrifuged at 8000 rpm for 5 min. The supernatant was discarded and the spore pellet was re-suspended in 5 ml aqueous solution of glycerol (30% v/v glycerol and sterile distilled water). The concentration of the spore suspension

was determined using a hemacytometer and adjusted to a concentration of 1 x 10^7spores/mL. After adjusting the concentrations, the suspensions of five isolates from each PG were mixed in equal parts and were labeled as respective PG for inoculation. Inocula prepared this way were stored at -20^o C and used throughout the study. The viability of the spores was confirmed whenever the stored suspension was used for inoculation. For this, a drop of spore suspension was put on a glass slide and left at room temperature for 24 hrs. The germination percentage was then estimated under the microscope.

Inoculation and incubation

Ten days old seedlings were inoculated at the cotyledon stage with the spore suspensions. Each cotyledon was lightly pricked once with sterile sharp tweezers and a 10 µl aliquot of spore suspension was deposited on it. The inoculated seedlings were incubated in mist chambers for 24 h to facilitate infection. The seedlings were then returned to the greenhouse room and incubated at 22/18^o C day/night temperatures and 14 h photoperiod for 14 days. During this incubation period the true leaves were pinched off to delay senescence of cotyledons and to facilitate disease assessment.

Data collection and analyses

Fourteen days after inoculation, the disease severity was scored on each cotyledon using a 0-9 scale developed by William and Delwiche (1980) in such a way that for each seedling two estimations of severity were made. Data from the three trials were merged into a single set that had nine replications. Since the scale used to measure severity was categorical, the median of ratings were calculated for each replication within an accession. The median that was calculated using the univariate procedure (PROC UNIVARIATE) of SAS (Ver. 9.3, SAS Institute, Inc., Cary, NC) represented 54 severity readings and was used to ascribe the accessions into different

reaction categories. In this way, a 0-2 reaction was considered resistant (R), 2-3.5 was considered resistant to moderately resistant (R-MR), 3.5-5 was considered moderately resistant (MR), 5-7 was considered moderately resistant to susceptible (MR-S), and 7-9 susceptible (S). The number of accessions that fell in each disease category was calculated using the frequency procedure (PROC FREQ) of SAS. Non-parametric tests were used to analyze the severity data. Briefly, the rank procedure (PROC RANK) of SAS was used to rank the median rating of each experimental unit. The ranks were then analyzed using ANOVA-type statistics in the mixed procedure (PROC MIXED). This analysis also calculates the least square means (lsmeans) of the treatments that are used to calculate relative treatment effects. Relative treatment effects and its 95% confidence interval were also calculated using LD_CI.SAS macro. The confidence intervals were used to discriminate among treatments. These analyses were performed for every PG. Smaller relative treatment effects were associated with higher resistance and thus, the 5% accessions with the smallest relative effects were identified as promising sources of resistance.

Association analysis

Phenotypic data

Association mapping was used to identify markers significantly associated with resistance to PG-2, PG-3, PG-T, and PG-4 isolates of *L. maculans.* The overall median value for each accession was used as phenotypic variable for all marker trait associations.

Genotypic data

DNA extraction. Approximately five seeds of each of the 279 *B. juncea* accessions were planted in artificial soilless mix (Professional Growing Mix 1 SunGro Horticulture Canada Seba Beach, AB, Canada). Freshly cut cotyledon leaves of 10 day old plants were flash frozen in liquid nitrogen and then stored at -80 ^{o}C for 24 hours. The frozen tissues were lyophilized using

VirTis freezer dryer (SP Scientific, Gardiner, NY) for 24 h and were ground to fine powder using a high-speed mixer mill (Model MM301, Retsch Inc, Newton, PA). Approximately 200 g of ground tissue were used to extract genomic DNA using the CTAB protocol explained by Murray and Thompson (1980), and modified by Gurung et al. (2011a). The concentration of DNA was quantified using a Nano-Drop spectrophotometer (Model ND-1000, Thermo Scientific Inc., Waltham, MA) and the concentration of each DNA sample was adjusted to100 ng/µL. A 100 µL aliquot of each sample was sent for DArT marker analysis to Diversity Arrays Technology Pty. Ltd. (Yarralumla, Australia; http://www.diversityarrays.com). A total of 766 DArT markers were used to screen the 279 *B. juncea* accessions.

Association mapping

FastPHASE 1.2 (Scheet and Stephens, 2006) was used to impute missing data from DArT analysis. Minor allele frequencies (MAF) < 0.01 were removed from further analysis. MAF are frequencies at which less common alleles of the DArT markers occur in a given population and their contribution to the identification of significant markers associated with a trait is almost of no importance (Liu and Muse, 2005).

The Linkage disequilibrium (LD) statistic was calculated as the square value of correlation coefficient (r^2) between DArT markers using the correlation procedure (PROC CORR) of SAS. Markers with LD < 0.5 with every other marker were used to estimate Structure (Q), Principal Component (PCA) and Kinship (K) matrices. Six models were used to test the association between DArT markers and resistance to four different PGs of *L. maculans.* The models were tested to correct the structure and co-ancestry relatedness in the entire population and are summarized in Table 5.1 as described by Stich et al. (2008), and Mamidi et al. (2011). The linear model used to construct other models was:

$$Y = X\alpha + Q\beta + I\gamma + \varepsilon$$

Where Y is a vector of phenotypic values, *X* is the vector of genotypes at the candidate marker, α is the fixed effect for the candidate marker, *Q* is a population structure matrix that can also be replaced by "*PCA*" which is a matrix of the significant principal component vectors that explain 50% of variation, β is a vector of fixed effects regarding population structure, *I* is an identity matrix, γ is a vector of the random effects that belong to co-ancestry, and ε is a vector of residuals. The variances of the random effects are assumed to be $Var(\gamma) = 2KV_g$ and $Var(e) = IV_R$ where *K* is the kinship matrix, V_g the genetic variance, and V_R the residual variance (Yu et al., 2006).

Table 5.1. Models used for association mapping and their respective statistics.

Models	Statistical model	Description
Naïve	$Y = X\alpha + \varepsilon$	Y is related to Marker without correction factors.
PCA	$Y = X\alpha + P\beta + \varepsilon$	Y is related to Marker with correction factor for PCA.
Structure (Q)	$Y = X\alpha + Q\beta + \varepsilon$	Y is related to Marker with correction factor for Structure.
Kinship (K)	$Y = X\alpha + K\gamma + \varepsilon$	Y is related to Marker with correction factor for Kinship.
Q + K	$Y = X\alpha + Q\beta + K\gamma + \varepsilon$	Y is related to Marker with correction factor for structure and Kinship.
PCA + K	$Y = X\alpha + P\beta + K\gamma + \varepsilon$	Y is related to Marker with correction factor for PCA and Kinship.

The software STRUCTURE version 2.3 (Pritchard et al., 2010) was used to determine population structure. For this analysis, five independent runs were performed for each value of *K* ranging from 1 to 10. Each run consisted of a burn-in period of 20,000 iterations and a Markov Chain Monte Carlo (MCMC) sampling of 100,000 iterations. The output is a matrix with a

number of columns equal to the number of estimated subpopulations and the probability of membership of each individual to a subpopulation. The output was processed with Structure Harvester (Earl and vanHoldt, 2011) to determine the optimum number of subpopulations using Delta K approach (Evanno et al., 2005). Structure matrix (*Q* matrix) with one column less than the number of subpopulations was used in mixed model to achieve linear independence (Stich et al., 2008). The principal components (PC) that explained at least 25% of the variation were estimated using the principal component procedure (PROC PRINCOMP) of SAS 9.3. PCA matrix (*P* matrix) was used in the mixed model instead of *Q* matrix. Kinship matrix (*K*) was calculated as shared allele percent using the Distance procedure (PROC DISTANCE) of SAS 9.3.

The best model was selected based on lowest mean square difference (MSD). To calculate MSD, an average of the squared differences between observed and expected *P* values from a model was obtained (Mamidi et al., 2011). The expected *P* values were calculated by dividing the rank of an observed *P* value by the total number of markers (Stich et al., 2008). MSD was calculated for every model and every PG.

Significant markers were identified based on the false positive discovery rate (*pFDR*). The multiple-testing procedure (PROC MULTTEST) of SAS 9.3 was used to calculate *pFDR* for the selected models in each PG. A cutoff criterion of $pFDR < 0.05$ was used to identify markers significantly associated with resistance to individual PG of *Leptosphaeria maculans*. The phenotypic variations (R^2) explained by individual significant markers were calculated using a simple linear regression and the variation explained by combination of markers was calculated using stepwise regression in SAS 9.3.

Results

Phenotypic analysis

Evaluation of *Brassica juncea* accessions for their reaction to PG-2 isolates of *L. maculans*

Response of the various *B. juncea* accessions to PG-2 isolates varied significantly ($P < 0.001$). The severity ratings on the accessions ranged from 2 to 9. Out of the 298 accessions evaluated, four were considered resistant (R); seven were considered resistant to moderately resistant (R-MR); 17 were considered moderately resistant (MR); 24 were considered moderately resistant to susceptible (MR-S), and 246 accessions were considered susceptible (S) to PG-2 (Figure 5.1).

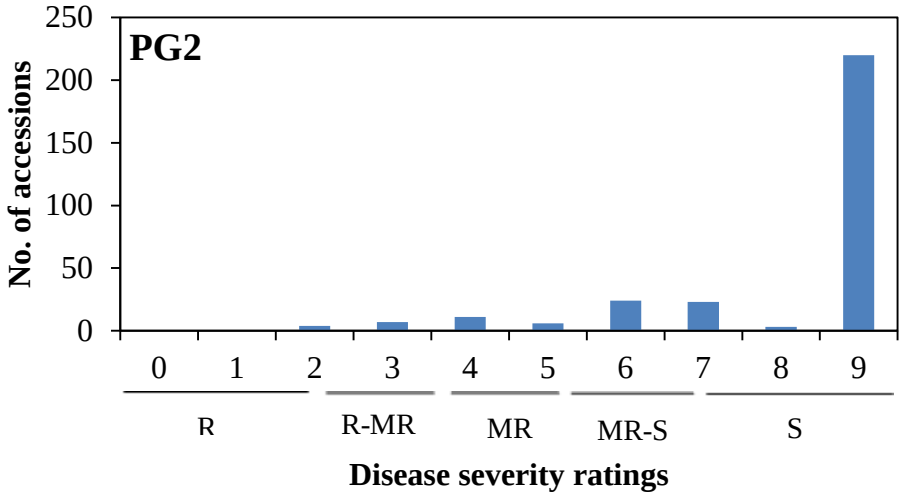

Fig. 5.1. Frequency distribution of the reaction of 298 *Brassica juncea* accessions to inoculation with a mixture of five PG-2 isolates of *Leptosphaeria maculans.* A 0-2 reaction was considered resistant (R), 2-3.5 was considered resistant to moderately resistant (R-MR), 3.5-5 was considered moderately resistant (MR), 5-7 was considered moderately resistant to susceptible (MR-S), and 7-9 susceptible (S). Accessions were inoculated at the cotyledon stage using a suspension of 10^7 spores ml^{-1}.

Fourteen *B. juncea* accessions (5% of all accessions evaluated) were identified as the most resistant (Figure 5.2). Accessions within this elite group had relative treatment effects

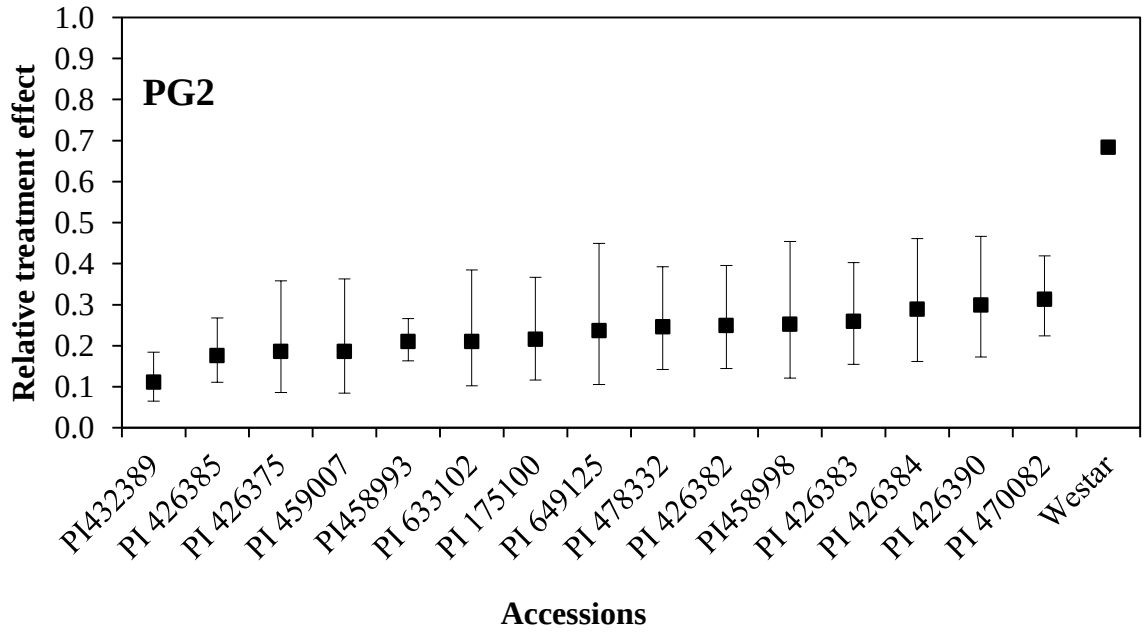

Fig. 5.2. Relative treatment effects and 95% confidence intervals (vertical bars) for the reaction of elite *Brassica juncea* accessions to inoculation with PG-2 isolates of *L. maculans.* Relative treatment effects calculated on 9 observations per accession.

below 0.3, although 95% confidence interval of relative treatment effects in some accessions run up to 0.4. Of this elite group, approximately 47% of the accessions were collected from Pakistan, 27% from India, and 7% from Bangladesh, China, South Korea, and USA (Table 5.2). The median disease severity of these accessions ranged from 2 to 5, the mean rank ranged from 299.56 to 805.8, and the relative treatment effect ranged from 0.11 to 0.30 (Table 5.2). The relative treatment effect of the 14 selected accessions were not significantly different from each other based on confidence interval at $P = 0.05$ (Figure 5.2). Plant introductions 432389, 426385, and 458993 had the smallest confidence intervals indicating less variation of phenotypic reaction within the accessions. The relative treatment effect of the remaining 95% accessions is presented in Appendix, Table 1.

Table 5.2. Median, mean rank, and relative treatment effects for blackleg severity ratings of elite *B. juncea* accessions inoculated with PG-2 isolates of *Leptosphaeria maculans* at the cotyledon stage.

		Disease severity[a]		Relative treatment effect	
Accession	Origin	Median	Mean rank	Mean	95% confidence interval
PI432389	Bangladesh	2	299.6	0.11	0.06 – 0.18
PI 426385	Pakistan	4	473.6	0.18	0.11 – 0.27
PI 426375	Pakistan	2	501.1	0.19	0.09 – 0.36
PI 459007	India	3	501.2	0.19	0.08 – 0.36
PI458993	India	4.4	566.1	0.21	0.16 – 0.27
PI 633102	Pakistan	2.5	566.4	0.21	0.10 – 0.38
PI 175100	India	4	582.0	0.22	0.12 – 0.37
PI 649125	U.S.A.	2	637.3	0.24	0.11 – 0.45
PI 478332	China	3.5	662.8	0.25	0.14 – 0.39
PI 426382	Pakistan	4	671.8	0.25	0.14 – 0.40
PI458998	India	4	680.1	0.25	0.12 – 0.45
PI 426383	Pakistan	5	698.9	0.26	0.15 – 0.40
PI 426384	Pakistan	5	777.6	0.29	0.16 – 0.46
PI 426390	Pakistan	4	805.8	0.30	0.17 – 0.47
PI 470082	South Korea	6	843.4	0.31	0.22 – 0.42
Westar		9	1841.0	0.68	0.68 – 0.69

[a] Disease severity based on 0-9 scale of Williams and Delwiche (1980).

Evaluation of *Brassica juncea* accessions for their reaction to PG-3 isolates of *L. maculans*

Responses of *B. juncea* accessions to PG-3 isolates varied significantly ($P < 0.001$). The severity ratings on the accessions ranged from 2.5 to 9. Out of the 298 accessions evaluated, 3, 16, 13, and 266 were rated as R-MR, MR, MR-S and S, respectively (Figure 5.3). Disease reaction of the 13 most resistant accessions, the top 5% of accessions, is presented in Table 5.3. Approximately 60% of the selected accessions were collected in Pakistan, 20% in India, 13% in China, and 7 % from Bangladesh (Table 5.3). The median disease severity of these elite accessions ranged from 2.5 to 5.5, the mean rank ranged from 287.2 to 743.6, and the relative treatment effect ranged from 0.11 to 0.28 (Table 5.3). The relative treatment effect of the 13 selected accessions were not significantly different from each other based on confidence interval

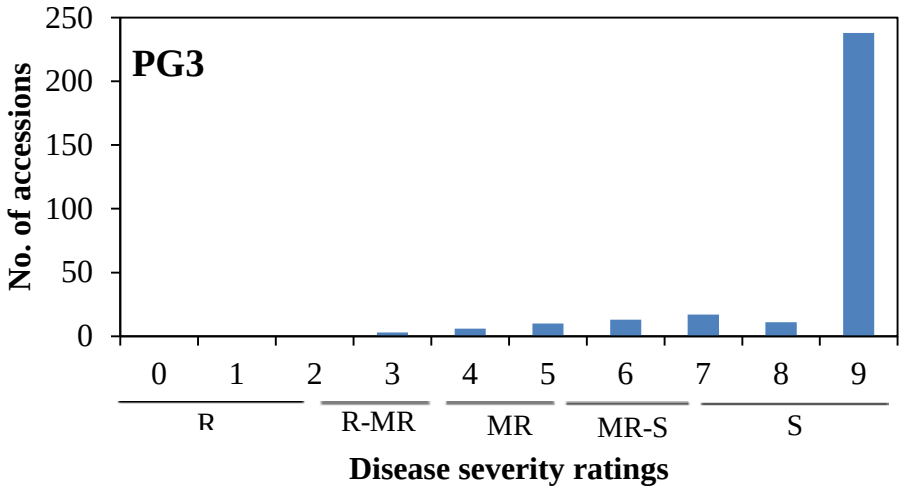

Fig. 5.3. Frequency distribution of the reaction of 298 *Brassica juncea* accessions to inoculation with a mixture of five PG-3 isolates of *Leptosphaeria maculans.* A 0-2 reaction was considered resistant (R), 2-3.5 was considered resistant to moderately resistant (R-MR), 3.5-5 was considered moderately resistant (MR), 5-7 was considered moderately resistant to susceptible (MR-S), and 7-9 susceptible (S). Accessions were inoculated at the cotyledon stage using a suspension of 10^7 spores ml^{-1}.

Table 5.3. Median, mean rank, and relative treatment effects for blackleg severity ratings of elite *Brassica juncea* accessions inoculated with PG-3 isolates of *Leptosphaeria maculans* at the cotyledon stage.

		Disease severity[a]		Relative treatment effect	
Accession	Origin	Median	Mean rank	Mean	95% confidence interval
PI 459007	India	2.5	287.2	0.11	0.07 – 0.17
PI 426385	Pakistan	3.5	374.5	0.14	0.09 – 0.22
PI 426379	Pakistan	3	437.4	0.16	0.07 – 0.34
PI 426384	Pakistan	3.5	462.2	0.17	0.08 – 0.34
PI432389	Bangladesh	2.5	464.4	0.17	0.08 – 0.34
PI 426371	Pakistan	4	520.4	0.19	0.10 – 0.34
PI 426408	Pakistan	5	596.8	0.22	0.12 – 0.36
PI 426375	Pakistan	4.5	623.7	0.23	0.11 – 0.43
PI 478334	China	5.5	696.0	0.26	0.13 – 0.44
PI 181017	Pakistan	5	700.5	0.26	0.14 – 0.44
PI 426380	Pakistan	5.5	714.1	0.27	0.14 – 0.44
PI 426377	Pakistan	5	728.8	0.27	0.15 – 0.44
PI 478326	China	3.5	743.6	0.28	0.13 – 0.50
PI 175100	India	6	815.9	0.30	0.18 – 0.46
PI 649110	India	7	925.9	0.34	0.23 – 0.47

[a] Disease severity based on 0-9 scale of Williams and Delwiche (1980).

at P = 0.05 (Figure 5.4). Plant introductions 459007 and 426385 had the numerically smallest relative treatment effects and the former also had the smallest confidence interval of the group. The relative treatment effect of remaining 95% accessions is presented in Appendix, Table 1.

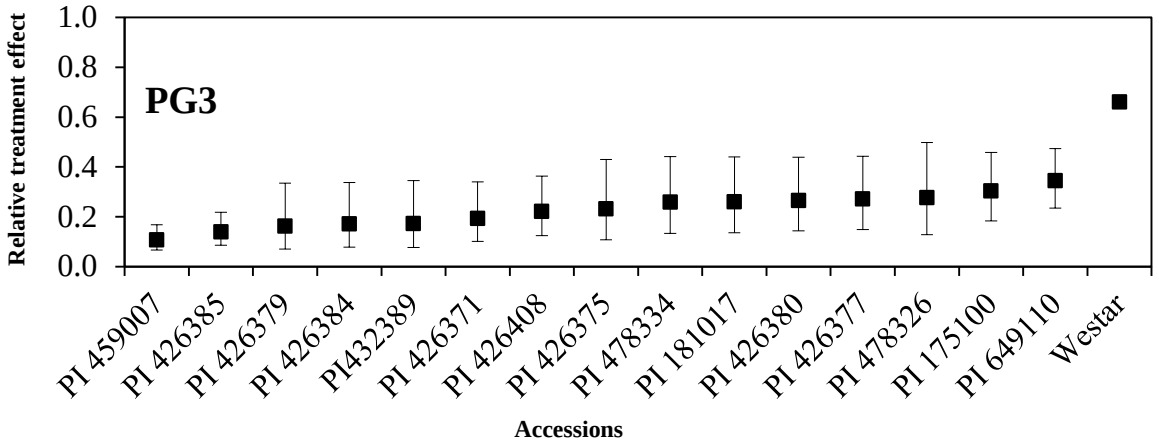

Fig. 5.4. Relative treatment effects and 95% confidence intervals (vertical bars) for the reaction of elite *Brassica juncea* accessions to inoculation with PG-3 isolates of *L. maculans.* Relative treatment effects calculated on 9 observations per accession.

Evaluation of *Brassica juncea* accessions for their reaction to PG-T isolates of *L. maculans*

The response of *B. juncea* accessions to PG-T isolates varied significantly (P < 0.001). The severity ratings ranged from 1.5 to 9. Out of the 298 accessions evaluated, 11, 62, 60, 31, and 134 were rated as R, R-MR, MR, MR-S and S, respectively (Figure 5.5). Disease reaction of the 15 most resistant accessions is presented in Table 5.4. Among these elite accessions approximately 47% originated from India, 33% from Pakistan, 13% from USA, and 7 % from Poland (Table 5.4). The median disease severity for these elite accessions ranged from 1.8 to 3, mean rank ranged from 370.5 to 741.2, and relative treatment effect ranged from 0.14 to 0.28 (Table 5.4). The relative treatment effects of these elite accessions were not significantly different from each other based on their 95% confidence interval (Figure 5.6). Plant introductions 179858, 649112, and 426320 had numerically smallest relative treatment effects. The relative treatment effect of the rest accessions is presented in Appendix, Table 2.

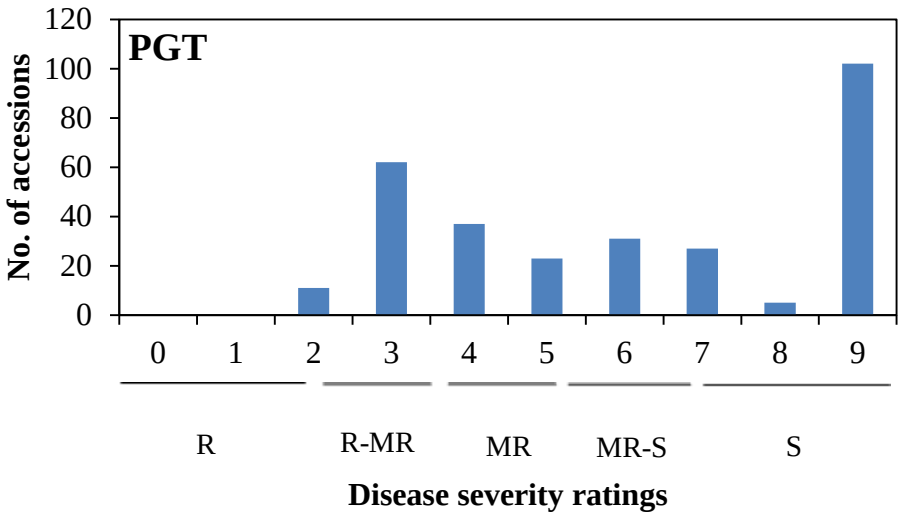

Fig. 5.5. Frequency distribution of the reaction of 298 *Brassica juncea* accessions to inoculation with a mixture of five PG-T isolates of *Leptosphaeria maculans.* A 0-2 reaction was considered resistant (R), 2-3.5 was considered resistant to moderately resistant (R-MR), 3.5-5 was considered moderately resistant (MR), 5-7 was considered moderately resistant to susceptible (MR-S), and 7-9 susceptible (S). Accessions were inoculated at the cotyledon stage using a suspension of 10^7 spores ml^{-1}.

Table 5.4. Median, mean rank, and relative treatment effects for blackleg severity ratings of elite *Brassica juncea* accessions inoculated with PG-T isolates of *Leptosphaeria maculans* at the cotyledon stage.

		Disease severity[a]		Relative treatment effect	
Accession	Origin	Median	Mean rank	Mean	95% confidence interval
PI 311726	Poland	1.8	370.5	0.14	0.07 – 0.26
PI 179858	India	2.2	500.9	0.19	0.15 – 0.24
PI 649113	India	1.5	523.4	0.19	0.11 – 0.32
PI 426320	Pakistan	2	532.1	0.20	0.13 – 0.29
PI 426304	Pakistan	2	556.3	0.21	0.13 – 0.32
PI 426330	Pakistan	2.5	583.2	0.22	0.14 – 0.32
PI 649109	India	2.5	644.4	0.24	0.14 – 0.38
PI 649105	India	2.5	649.4	0.24	0.15 – 0.37
PI 649122	U.S.A.	3	664.4	0.25	0.14 – 0.40
Ames 9914	India	3	686.1	0.25	0.14 – 0.41
PI 649123	U.S.A.	2	691.4	0.26	0.16 – 0.39
PI 426316	Pakistan	2.5	692.2	0.26	0.13 – 0.44
PI 649112	India	3	723.4	0.27	0.19 – 0.36
PI 347619	India	2.5	731.3	0.27	0.19 – 0.37
PI 426340	Pakistan	2	741.2	0.28	0.15 – 0.44

[a] Disease severity based on 0-9 scale of Williams and Delwiche (1980).

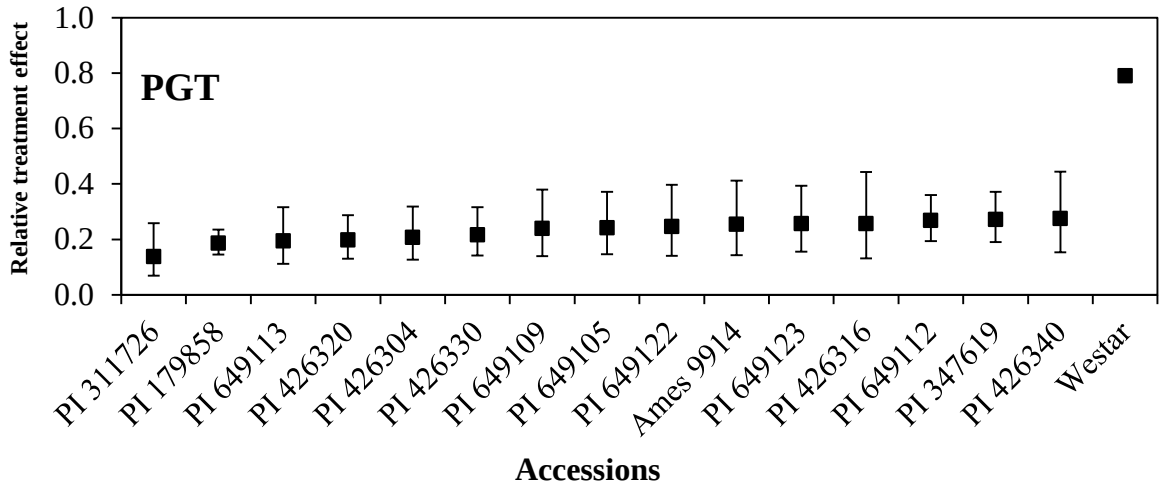

Fig. 5.6. Relative treatment effects and 95% confidence intervals (vertical bars) for the reaction of elite *Brassica juncea* accessions to inoculation with PG-T isolates of *L. maculans.* Relative treatment effects calculated on 9 observations per accession.

Evaluation of *Brassica juncea* accessions for their reaction to PG-4 isolates of *L. maculans*

The response of *B. juncea* accessions to PG-4 isolates of *L. maculans* varied significantly ($P < 0.001$). The severity ratings ranged from 1.5 to 9. Out of the 298 accessions evaluated, 8, 39, 63, 31, and 157 were rated as R, R-MR, MR, MR-S and S, respectively (Figure 5.7).

Disease reaction of the 15 most resistant accessions is presented in Table 5.5. Among these elite accessions 47% were collected in Pakistan, 33% in India, and 7% in Nepal, Poland, and USA (Table 5.5). The median disease severity of these accessions ranged from 2 to 4, the mean rank ranged from 333.1 to 746, and the relative treatment effect ranged from 0.12 to 0.28 (Table 5.5). The relative treatment effect of these elite accessions were not significantly different from each other based on their confidence interval at $P = 0.05$ (Figure 5.8). The confidence interval of the relative treatment effects of plant introductions 426320, 179858, and 426306 were small indicating less variation of phenotypic reaction within these accessions. The relative treatment effect of the remaining 95% accessions is presented in Appendix, Table 2.

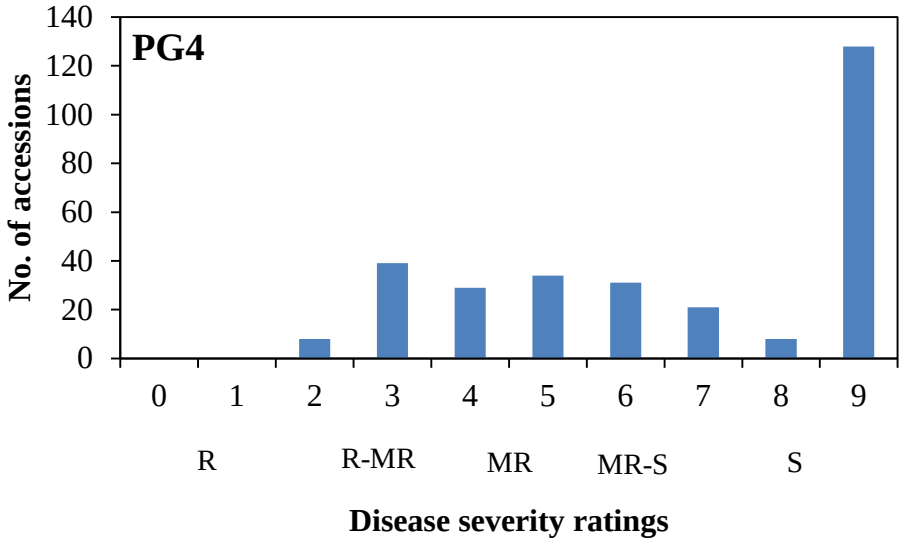

Fig. 5.7. Frequency distribution of the reaction of 298 *Brassica juncea* accessions to inoculation with a mixture of five PG-4 isolates of *Leptosphaeria maculans.* A 0-2 reaction was considered resistant (R), 2-3.5 was considered resistant to moderately resistant (R-MR), 3.5-5 was considered moderately resistant (MR), 5-7 was considered moderately resistant to susceptible (MR-S), and 7-9 susceptible (S). Accessions were inoculated at the cotyledon stage using a suspension of 10^7 spores ml^{-1}.

Table 5.5. Median, mean rank, and relative treatment effects for blackleg severity ratings of elite *B. juncea* accessions inoculated with PG-4 isolates of *Leptosphaeria maculans* at the cotyledon stage.

		Disease severity[a]		Relative treatment effect	
Accession	Origin	Median	Mean rank	Mean	95% confidence interval
PI 426320	Pakistan	2	333.1	0.12	0.08 – 0.19
PI 426299	Pakistan	2	497.1	0.18	0.10 – 0.32
Ames 9914	India	2	510.1	0.19	0.10 – 0.33
PI 179858	India	2.8	542.4	0.20	0.18 – 0.22
PI 426330	Pakistan	2.5	544.7	0.20	0.10 – 0.36
PI 649123	U.S.A.	2	556.0	0.21	0.09 – 0.42
PI 286417	Nepal	3	590.7	0.22	0.13 – 0.34
PI 251239	Pakistan	3	595.1	0.22	0.12 – 0.37
PI 426306	Pakistan	3	625.8	0.23	0.16 – 0.32
PI 311726	Poland	3.3	693.8	0.26	0.14 – 0.43
PI 426316	Pakistan	3	702.3	0.26	0.17 – 0.37
PI 649109	India	3	716.4	0.27	0.15 – 0.43
PI 649113	India	3	719.2	0.27	0.15 – 0.43
PI 633102	Pakistan	4	733.2	0.27	0.18 – 0.39
PI458993	India	3.6	746.0	0.28	0.19 – 0.38

[a] Disease severity based on 0-9 scale of Williams and Delwiche (1980).

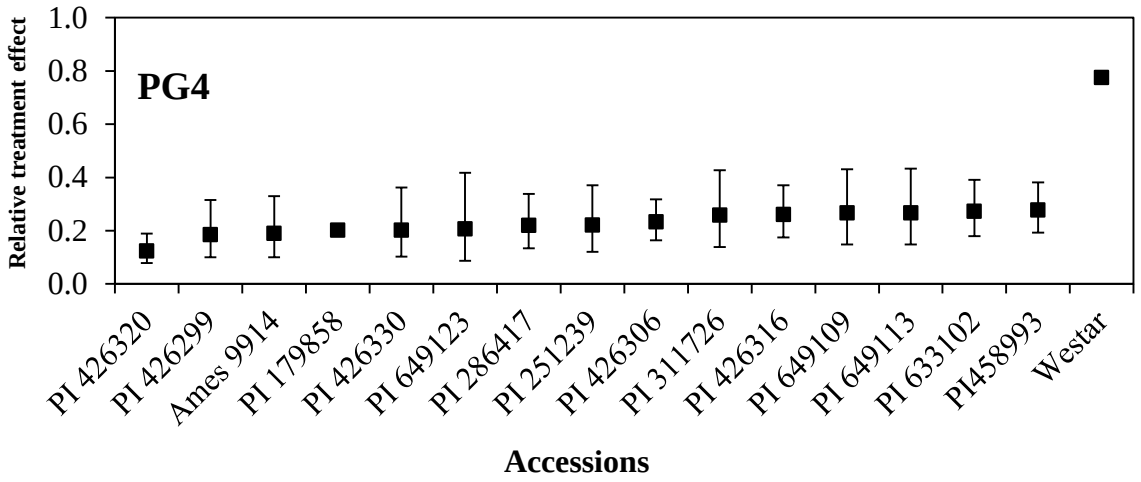

Fig. 5.8. Relative treatment effects and 95% confidence intervals (vertical bars) for the reaction of elite *Brassica juncea* accessions to inoculation with PG-4 isolates of *L. maculans.* Relative treatment effects calculated on 9 observations per accession.

Plant introductions 426375, 426384, 426385, 459007, 175100, and 432389 were resistant to both PG-2 and PG-3. None of the accessions that were resistant to PG-T were also resistant to PG-2 or PG-3 and none of the accessions that were resistant to PG-4 were also resistant to PG-3. Plant introductions 633102, and 458993 were resistant to PG-2 and PG-4; while 179858, 311726, 426316, 649109, 649113, 649123, Ames 9914, 426320, and 426330 were resistant to PG-T and PG-4 (Table 5.6).

Association analysis

Nearly 1.2% (n=9) of the DArT markers used to characterize the genome of each accession had MAF < 0.01 and were removed from further analysis. Nearly 85% of the remaining markers had LD > 0.5 with every other marker and thus only 115 markers which had LD < 0.5 were used to estimate Structure (Q), Principal Component (PCA) and Kinship (K) matrices. The number of subpopulations present in *Brassica juncea* accessions was calculated as *K*=4 supported by a high ΔK value (Figure 5.9). For the entire dataset a total of two PCs explained ~ 25% of variability and were used for mixed model analysis.

Table 5.6. Accessions with resistance to multiple PGs of *Leptosphaeria maculans*.

	Disease reaction [x]			
Accessions	PG2	PG3	PGT	PG4
PI 175100	**4**	**6**	3.5	4
PI 426375	**2**	**4.5**	3.5	4
PI 426384	**5**	**3.5**	9	3
PI 426385	**4**	**3.5**	3	4.5
PI 459007	**3**	**2.5**	3	3.5
PI 633102	**2.5**	**3.5**	3.5	4
PI 432389	**2**	**2.5**	3.5	4.5
Ames 9914	3	9	**3**	**2**
PI 179858	7.3	7	**2.2**	**2.8**
PI 311726	9	9	**1.8**	**3.3**
PI 426316	9	9	**2.5**	**3**
PI 426320	6.8	5	**2**	**2**
PI 426330	6.5	9	**2.5**	**2.5**
PI 649109	9	9	**2.5**	**3**
PI 649113	5.5	9	**1.5**	**3**
PI 649123	9	9	**2**	**2**

[x] Disease reaction based on 0-9 severity scale in which 0-2 = resistant (R), 2-3.5= resistant to moderately resistant (R-MR), 3.5-5 = moderately resistant (MR), 5-7 = moderately resistant to susceptible (MR-S), and 7-9 = susceptible (S).

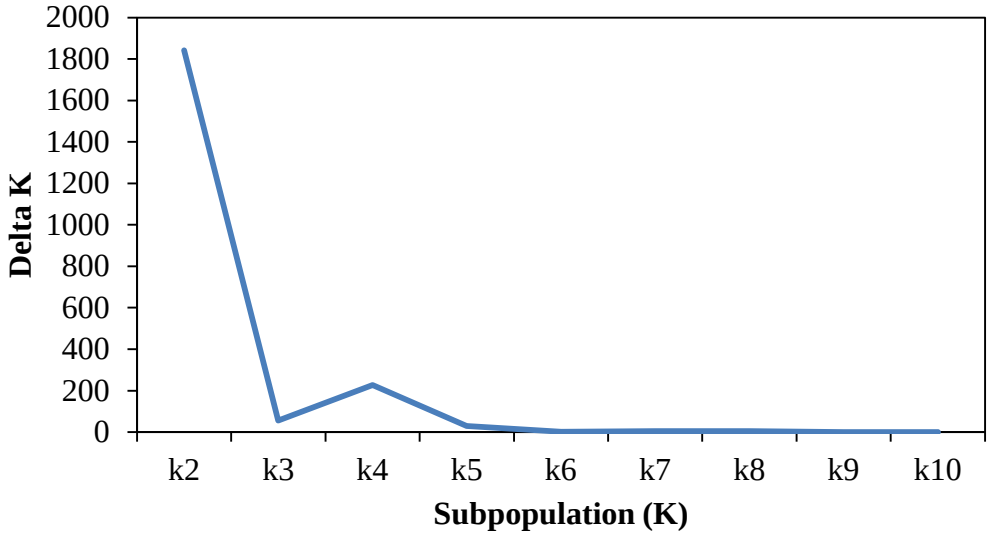

Fig. 5.9. Δk plots for detecting the number of subpopulations.

Association mapping of QTL for resistance to *L. maculans*

Resistance to PG-2: The mean square difference (MSD) between observed and expected probability values for all six models ranged from 0.002 to 0.005 (Table 5.7). Models Q + K had the smallest MSD values and were selected to identify markers significantly associated with resistance to PG-2. Based on Q + K mixed model, thirteen markers were found to be significantly associated with resistance to PG-2 (Table 5.8) when multiple testing correction (*pFDR*<0.05) was applied. The phenotypic variability explained by these markers ranged from 0.94 to 5.94%. Stepwise regression analysis identified markers brPb_809966, brPb_809516, brPb_807813, brPb_657781, and brPb_660465 as the most important and estimated their combined effect explained 15.3% of the phenotypic variability observed.

Resistance to PG-3: The MSD between observed and expected *P*-values for all six models ranged from 0.002 to 0.005 (Table 5.7). The K model had the least MSD of 0.002 and was selected to identify significant markers associated with resistance to PG3 isolates of *L. maculans*. Based on K model, three markers, brPb_807978, brPb_808436, and brPb_809966, were significantly associated with resistance to PG-3 (Table 5.9) when multiple testing correction (*pFDR*<0.05) was applied. The combined phenotypic variability explained by these markers was 4.36%. These three markers were also significantly associated with resistance to PG-2.

Resistance to PG-T: The MSD between observed and expected *P*-values for all six models tested ranged from 0.002 to 0.09 (Table 5.7). The Q had the smallest MSD of 0.002; however, it could not detect any marker that was significantly associated with resistance to PG-T.

Table 5.7. Test statistics for six models used to detect marker-trait associations for four different PGs of *Leptosphaeria maculans.*

PG2		PG3		PGT		PG4	
Model	MSD[a]	Model	MSD[a]	Model	MSD[a]	Model	MSD[a]
Q +K	0.002	K	0.002	Q	0.002	K	0.001
Q	0.002	Naive	0.002	K	0.002	PCA	0.001
Naïve	0.004	PCA	0.003	Q + K	0.003	Q + K	0.002
K	0.004	PCA + K	0.004	PCA	0.012	Q	0.003
PCA	0.004	Q	0.004	Naïve	0.07	PCA + K	0.004
PCA + K	0.005	Q + K	0.005	PCA + K	0.09	Naïve	0.074

[a] Mean square difference.

Table 5.8. Significant markers associated with resistance to PG-2 isolates of *L. maculans.*

Markers	*P*- value	*pFDR*	R^2
brPb_807842	<0.0001	0.019	0.94
brPb_807978	0.0001	0.019	2.74
brPb_808436	0.0001	0.019	2.74
brPb_809966	0.0001	0.019	2.74
brPb_809516	0.0005	0.041	2.34
brPb_807813	0.0006	0.041	2.11
brPb_808071	0.0006	0.041	1.14
brPb_660465	0.0007	0.041	5.94
brPb_659520	0.0007	0.041	3.26
brPb_660505	0.0007	0.041	3.26
brPb_660716	0.0007	0.041	3.26
brPb_809224	0.0007	0.041	3.26
brPb_657781	0.0007	0.041	3.26

Table 5.9. Significant markers associated with resistance to PG-3 isolates of *L. maculans.*

Markers	*P*- value	*pFDR*	R^2
brPb_807978	0.0001	0.021	4.36
brPb_808436	0.0001	0.021	4.36
brPb_809966	0.0001	0.021	4.36

Resistance to PG-4: The MSD between observed and expected *P*-values for all six models tested ranged from 0.001 to 0.074 (Table 5.7). Model K had the least MSD at 0.001; however, it could not detect any marker that was significantly associated with resistance to PG-4.

Discussion

In this study, *Brassica juncea* accessions were screened for their reaction to inoculation with spores of PG-2, PG-3, PG-T, and PG-4 isolates of *Leptosphaeria maculans*. A few potential sources of resistance to all PGs were identified. *B. juncea* was produced by the natural cross between *B. rapa* (A genome) and *B. nigra* (B genome) (U, 1935). The B genome is known to harbor resistance genes against *L. maculans* and have been repeatedly exploited to transfer them into *B. napus* (Roy, 1978; Sacristan and Gerdemann, 1986; Zhu et al., 1993; Somda et al., 1999). The resistant accessions identified in this study could be used to develop resistant lines by introgression into agronomically suitable *B. napus* cultivars.

The phenotypic reactions of all accessions to all PGs were assessed at the seedling stage and all screening procedures were conducted in greenhouse conditions. Field validations, while highly desirable were not conducted because PG-3, PG-T, and PG-4 still have limited geographic distribution in the state. However, the evaluation of the reaction of elite accessions as adult plants is under way in greenhouse conditions. Nevertheless, these two types of resistance seem to be controlled by the same genes in *B. juncea*. Keri et al. (1997) suggested the same pattern of disease reaction on plants while evaluating the reaction in a cross between resistant and susceptible accessions of *B. juncea* with both cotyledon and stem inoculation. Based on these reactions they concluded that both the seedling and adult plant resistance to *L. maculans* in *B. juncea* is controlled by two nuclear genes with dominant recessive epistatic gene action. Similarly, Dixelius and Wahlberg (1999) also identified genes for both seedling and adult resistance to *L. maculans* in three conserved regions of the B genome of *B. juncea.* Thus, it is possible that the accessions identified in this study as resistant in the seedling stages may also harbor adult plant resistance.

Our study shows that most accessions carrying resistance to PG-2 also carry resistance to PG-3, while those resistant to PG-T also were resistant to PG-4 and vice versa. These associations suggest that resistance to each pair, PG-2/ PG-3 and PG-T/ PG-4, may be controlled by the same QTL. While this may be true on the plant side, on the pathogen side, little is known about the genetic relatedness between these groups. In 1991, Koch et al. reported partial differences in the phylogeny of PG-2, PG-3, and PG-4 based on RFLP data. The isolates belonging to the latter two groups were separated in two different clusters; however, the limited number of isolates used in their study prevented them from reaching a clear conclusion regarding the association between pathogenicity groups and genetic background of the isolates. Pongam et al. (1999) also assessed genetic variation among *L. maculans* isolates and its relation to pathogenicity data but their results were inconclusive as well. In their study, almost all PG-2 isolates were clustered together, but most of them had been collected from North Dakota between 1995 and 1996. Among isolates collected from geographically distant regions, like European countries, Australia, and Western and Eastern Canada, isolates from PG-2 and PG-3 showed greater levels of similarity between each other than that between PG-4 and PG-2 or PG-3. A more detailed study on genetic relatedness among these PGs would help us understand the mechanism of host pathogen interaction and consequentially the mechanism of host resistance.

In our study, we have identified sources of resistance to four major PGs of economic importance in North Dakota. To our knowledge, this is the first report of the screening of a large number of plant introduction materials using multiple pathogenicity groups. Results from our study indicatethat resistance to PG-2 and PG-3 may be linked and that resistance to PG-4 and PG-T may also be linked; however, most materials with resistance against PG-2 were susceptible to PG-4. Results of this study seem to corroborate observations made by del Rio Mendoza et al.

(2012) who indicated that the 2009 blackleg outbreaks detected in North Dakota were being caused mostly by isolates from PG-4. The canola genotypes planted in North Dakota had been bred for resistance against PG-2 (Berglund, 2003), thus genotypes which are resistant to PG-2 may also be resistant to PG-3 but not to PG-4. Further, work conducted as part of this dissertation (chapter 3) indicates that isolates from PG-4 are becoming more prevalent in North Dakota than PG-3. Crosses between appropriate accessions identified in this study could lead to the development of lines that are resistant to multiple PGs resulting in more durable, long term protection against this disease. To facilitate the transfer of resistance genes into agronomically-acceptable cultivars the resistance present in these materials needs to be characterized. Once this is achieved, the process of transferring the resistance could be accelerated by efficient phenotypic screening, marker assisted selection, and/or tissue culture techniques (Roy, 1984; Su, 2009).

Association mapping was used to identify markers associated with resistance to individual PGs. In this way, 13 DArT markers were associated with resistance to PG-2; these markers explained between 0.9 to 6% of the phenotypic variability. Three of them were also associated with resistance to PG-3 where each explained 4.3% of the phenotypic variability. Having the same markers associated with resistance to two pathogenicity groups suggests resistance against these two groups may be governed by the same genes or by genes closely linked to each other. At this time, we can't say whether these markers are associated with QTL already described by other researchers (Pilet et al., 1998; Pilet et al., 2001) and Delourme et al., 2004). However, a good number of them were mapped to linkage group (LG) 16 of the genetic map of Lombard and Delourme (2001) and the rest were mapped to an LG that corresponds to N7 group of Parkin et al. (1995).Plants that carry resistance genes expressed at the seedling stage

also are, resistant at the adult stage. Some of these markers have been mapped to the reference group N7 of *B. napus* (Parking et al., 1995; Dion et al., 1995; Ferreira et al., 1995; Mayerhofer et al., 1997; Rimmer, 2006; Zhu and Rimmer, 2003). While we identified markers significantly associated with resistance to PG-2 and PG-3 isolates of *L. maculans* in *B. juncea* population, we could not assign them to a genomic location due to the unavailability of information about these markers on the *B. juncea* genome. BLAST analysis of these markers' sequences allowed us to locate some of these resistance genes in the *Arabidopsis thaliana* genome. DArT markers brPb_807978, brPb_808436, and brPb_809966 that are associated with resistance to both PG-2 and PG-3 and marker brPb_807813 associated with resistance to PG-2 corresponded to *HMGR1* and *HMGR2* genes in *A. thaliana* that encode for hydroxyl methylglutaryl CoA reductase 1 and 3-hydroxy-3-methylglutaryl-CoA reductase 2, respectively. The former is an enzyme that catalyzes the first step in the phenylpropanoid and terpenoid pathways that produce phytoalexins in response to a variety of stresses including response to a pathogen attack (Hammerschmidt, 1999). Phytoalexins are plant secondary metabolites and are important in plant disease resistance as they can directly affect the integrity of pathogen's structures (cell membranes, DNA) or functions (oxidative phosphorylation) (Hadrami et al., 2009). *HMG* has also been shown to be involved in resistance to several other diseases. Choi et al. (1992) showed that *HMG* encoding 3-hydroxy-3-methylglutaryl-CoA reductase is essential for the biosynthesis of sesquiterpene phytoalexins in potato that are involved in resistance to late blight disease in potato (Sakuma and Tomiyama, 1967; Yoshioka et al., 1996). Fung et al. (2008) also evidenced the up-regulation of HMG CoA reductase in powdery mildew (*Erysiphe necator*) and resistant grapevine (*Vitis vinifera*) interaction. Wang et al. (2012) reported that up-regulation of 3-hydroxy-3-methylglutaryl-CoA reductase, among other enzymes resulted in increased resistance to *Botrytis*

cinerea in *A. thaliana*. The other two DArT markers, brPb_809516 and brPb_808071, which were associated with resistance to PG-2 corresponded to *A. thaliana* genes *bHLH* and *GRF*, which encode for basic helix-loop-helix DNA-binding superfamily protein and general regulatory factor, respectively. These proteins have also been reported to be involved in disease resistance in many plant disease patho-systems (Glazebrook et al., 1997; Dixon, 2001; Belkhadir et al., 2004; DeLille et al., 2001; Fujita et al., 2006; Finnie et al., 1999).

We could not identify any marker significantly associated with resistance to PG-T and PG-4 isolates of *L. maculans*. One possibility for this result could be low density markers. Based on the results from phenotypic analysis of the accessions against PG-T and PG-4 isolates of *L. maculans*, we have identified promising accessions that are consistently resistant to these PGs; however we could not identify the markers that could associate this resistance while using only 766 DArT markers. The size of the *Brassica* genome ranges from 529 to 696 Mb for diploids and 1068-1284 Mb for polyploids (Johnston et al., 2005). Thus it is possible that the number of markers that we used may not be enough to identify the QTL associated with resistance to these PGs and there are other QTL for resistance to these PGs in the genome of these accessions. Application of high density markers will improve the possibility of identification of significantly associated markers.

In this study we have reported *Brassica juncea* accessions that are resistant to moderately resistant to multiple pathogenicity groups of *Leptosphaeria maculans*. These materials could become an important resource to canola breeding programs. With a planned breeding program, the resistance identified in these accessions can be introgressed into agronomically adapted canola cultivars. We have also identified markers associated with resistance to PG2 and PG3 isolates of *L. maculans* which belong to gene families in *Arabidopsis thaliana* that are reported

to be directly or indirectly involved in resistance to several diseases. These markers can be used in MAS to accelerate the canola breeding for disease resistance.

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APPENDIX

Table A.1. Median, mean rank, and relative treatment effects along with 95% confidence intervals for blackleg severity ratings in relation to *B. juncea* accessions and PG2 and PG3 isolates of *Leptosphaeria maculans.*

		PG2					PG3				
Accessions	**Origin**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**
PI432389	Bangladesh	2	299.6	0.11	0.06	0.18	2.5	464.4	0.17	0.08	0.34
PI 426385	Pakistan	4	473.6	0.18	0.11	0.27	3.5	374.5	0.14	0.09	0.22
PI 426375	Pakistan	2	501.1	0.19	0.09	0.36	4.5	623.7	0.23	0.11	0.43
PI 459007	India	3	501.2	0.19	0.08	0.36	2.5	287.2	0.11	0.07	0.17
PI 633102	Pakistan	2.5	566.4	0.21	0.1	0.38	3.5	757.2	0.28	0.13	0.5
PI458993	India	4.4	566.1	0.21	0.16	0.27	6	873.9	0.32	0.18	0.51
PI 175100	India, Uttar Pradesh	4	582	0.22	0.12	0.37	6	815.9	0.3	0.18	0.46
PI 649125	US, Iowa	2	637.3	0.24	0.11	0.45	9	1619.6	0.6	0.48	0.71
PI 478332	China	3.5	662.8	0.25	0.14	0.39	4.5	760.1	0.28	0.13	0.5
PI 426382	Pakistan	4	671.8	0.25	0.14	0.4	7	937.4	0.35	0.21	0.52
PI458998	India	4	680.1	0.25	0.12	0.45	7	829.3	0.31	0.16	0.51
PI 426383	Pakistan	5	698.9	0.26	0.15	0.4	9	1458.6	0.54	0.39	0.69
PI633092	Russian, Federation	2	765.2	0.28	0.13	0.52	9	1021.5	0.38	0.2	0.61
PI 426384	Pakistan	5	777.6	0.29	0.16	0.46	3.5	462.2	0.17	0.08	0.34
PI 426379	Pakistan	3	817.7	0.3	0.15	0.53	3	437.4	0.16	0.07	0.34
PI 649101	US, Minnesota	3.5	797.1	0.3	0.17	0.47	7.5	1105.1	0.41	0.26	0.58
PI 426390	Pakistan	4	805.8	0.3	0.17	0.47	7	1068.8	0.4	0.24	0.57
PI 426377	Pakistan	4.5	808.6	0.3	0.17	0.47	5	728.8	0.27	0.15	0.44
PI 633106	Pakistan	5.5	808.3	0.3	0.17	0.47	6	822.3	0.31	0.16	0.51
PI 649119	India	2.5	839.5	0.31	0.16	0.53	5.5	1062.1	0.39	0.25	0.57
PI 280637	Ethiopia	3	823.7	0.31	0.15	0.52	5	816.6	0.3	0.16	0.5
PI 470082	Korea, South	6	843.4	0.31	0.22	0.42	8	1000.7	0.37	0.27	0.49
Ames 9914	India	3	869.7	0.32	0.17	0.53	9	1483.5	0.55	0.41	0.69

(continued)

Table A.1. Median, mean rank, and relative treatment effects along with 95% confidence intervals for blackleg severity ratings in relation to *B. juncea* accessions and PG2 and PG3 isolates of *Leptosphaeria maculans.* (continued)

		PG2					PG3				
Accessions	**Origin**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**
PI 426336	Pakistan	5.5	852.7	0.32	0.19	0.47	9	1291.7	0.48	0.34	0.62
PI 288724	India, Gujarat	5.5	848.3	0.32	0.19	0.48	9	1396.9	0.52	0.38	0.65
PI603013	Pakistan, Punjab	6.5	874.8	0.32	0.17	0.53	6	972.2	0.36	0.2	0.56
PI 633104	Pakistan	4	885.4	0.33	0.18	0.53	6	879.8	0.33	0.19	0.51
PI 426408	Pakistan	5	881.9	0.33	0.2	0.48	5	596.8	0.22	0.12	0.36
PI 649107	India	3	906.6	0.34	0.19	0.53	6	864.9	0.32	0.18	0.51
PI 426360	Pakistan	5	905.6	0.34	0.19	0.53	8	1073.2	0.4	0.25	0.57
PI 271442	India, Gujarat	5.5	912.2	0.34	0.19	0.54	9	1528.6	0.57	0.45	0.68
PI 426340	Pakistan	5.5	921.3	0.34	0.19	0.53	9	1482.3	0.55	0.41	0.69
PI449438	Pakistan	5.5	921.6	0.34	0.19	0.53	9	1114.7	0.41	0.24	0.61
PI 649112	India	5.5	935.3	0.35	0.2	0.53	9	1169.4	0.43	0.27	0.62
PI 432383	Bangladesh	5.7	951.6	0.35	0.21	0.54	9	1282.7	0.48	0.33	0.62
PI 181033	Pakistan, Punjab	5.4	971.9	0.36	0.19	0.58	9	1213.6	0.45	0.3	0.61
PI 426366	Pakistan	5.8	977.1	0.36	0.22	0.53	7	1126.4	0.42	0.28	0.57
PI 478331	China	6	976.5	0.36	0.22	0.54	8	1192.6	0.44	0.31	0.58
PI 426299	Pakistan	6	975.1	0.36	0.21	0.54	9	1358.8	0.5	0.35	0.65
PI 180266	India, Gujarat	6.4	964.3	0.36	0.24	0.49	7	1071.8	0.4	0.28	0.53
PI 426306	Pakistan	7	973.8	0.36	0.19	0.58	6	997.5	0.37	0.21	0.56
PI 426291	Pakistan	4	1007.8	0.37	0.21	0.58	9	1778	0.66	0.65	0.67
PI 181017	Pakistan, Sind	5.5	993.6	0.37	0.2	0.58	5	700.5	0.26	0.14	0.44
PI 426330	Pakistan	6.5	1003.7	0.37	0.23	0.55	9	1316	0.49	0.33	0.65
PI 176884	Turkey	6.7	1003.4	0.37	0.26	0.5	8	1022.4	0.38	0.28	0.49
PI 426318	Pakistan	4	1035.6	0.38	0.22	0.58	9	1487.1	0.55	0.41	0.69
PI 649113	India	5.5	1030.2	0.38	0.22	0.58	9	1138.3	0.42	0.25	0.61
PI 459002	India	5.5	1037.7	0.39	0.25	0.54	9	1367.9	0.51	0.36	0.65

(continued)

Table A.1. Median, mean rank, and relative treatment effects along with 95% confidence intervals for blackleg severity ratings in relation to *B. juncea* accessions and PG2 and PG3 isolates of *Leptosphaeria maculans.* (continued)

		PG2					PG3				
Accessions	**Origin**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**
PI 426371	Pakistan	7	1041.6	0.39	0.22	0.59	4	520.4	0.19	0.1	0.34
PI 458978	US, Oregon	5.5	1086.5	0.4	0.25	0.58	7	1046.1	0.39	0.23	0.57
PI537018	Pakistan	6.5	1074.9	0.4	0.27	0.55	6.5	1063.8	0.4	0.25	0.57
PI 426320	Pakistan	6.8	1084.3	0.4	0.27	0.56	5	798.7	0.3	0.15	0.5
PI 426363	Pakistan	7	1071	0.4	0.23	0.59	9	1302.6	0.48	0.32	0.65
PI 426346	Pakistan	9	1086.7	0.4	0.21	0.63	9	1190.8	0.44	0.25	0.65
PI 633115	Mongolia	6	1098.8	0.41	0.25	0.59	7	1089.3	0.4	0.26	0.57
PI 426335	Pakistan	6	1095.6	0.41	0.25	0.59	9	1375.8	0.51	0.37	0.65
PI 633097	China, Xizang	6	1104.1	0.41	0.25	0.59	9	1402.7	0.52	0.39	0.65
PI 426369	Pakistan	6.5	1094.3	0.41	0.25	0.59	4.5	837.1	0.31	0.17	0.51
PI 449437	Pakistan	6.5	1115	0.41	0.26	0.59	7	1122.4	0.42	0.28	0.57
PI 426332	Pakistan	7	1091.8	0.41	0.24	0.59	9	1243.2	0.46	0.28	0.65
PI 633110	Germany	7	1114.9	0.41	0.26	0.59	9	1193.8	0.44	0.28	0.62
PI 426359	Pakistan	6	1143.9	0.42	0.27	0.59	7	1088.6	0.4	0.26	0.57
PI 649121	Nepal	6	1139.9	0.42	0.27	0.59	9	1117.5	0.42	0.24	0.61
PI 426367	Pakistan	6.4	1126.4	0.42	0.26	0.59	6.5	986.1	0.37	0.24	0.52
PI 426337	Pakistan	6.5	1129.8	0.42	0.27	0.59	9	1301.9	0.48	0.32	0.65
PI 478337	China	6.5	1126.5	0.42	0.26	0.59	4	889.9	0.33	0.16	0.56
PI 633103	Pakistan	7	1123.2	0.42	0.29	0.55	9	1188.3	0.44	0.28	0.61
PI 250130	Pakistan, North-West Frontier	8.5	1130.2	0.42	0.26	0.59	9	1334.5	0.5	0.34	0.66
PI 426349	Pakistan	9	1126.1	0.42	0.24	0.63	9	1221.7	0.45	0.27	0.65
PI 426356	Pakistan	9	1132.9	0.42	0.24	0.63	9	1070.2	0.4	0.22	0.61
PI 251239	Pakistan, Punjab	9	1138.4	0.42	0.24	0.63	6	1056.7	0.39	0.24	0.57
PI 426325	Pakistan	9	1124.8	0.42	0.23	0.63	8.5	1119.2	0.42	0.27	0.58

(continued)

Table A.1. Median, mean rank, and relative treatment effects along with 95% confidence intervals for blackleg severity ratings in relation to *B. juncea* accessions and PG2 and PG3 isolates of *Leptosphaeria maculans.* (continued)

Accessions	Origin	PG2					PG3				
		Median	Mean rank	RE	Lower	Upper	Median	Mean rank	RE	Lower	Upper
PI 478329	China	6	1153.4	0.43	0.28	0.59	7	1017.2	0.38	0.22	0.57
PI 426334	Pakistan	7	1149.2	0.43	0.27	0.6	9	1308.2	0.49	0.32	0.65
PI 426357	Pakistan	7	1160.2	0.43	0.28	0.6	9	1119.8	0.42	0.24	0.61
PI 179858	India, Rajasthan	7.3	1167.9	0.43	0.31	0.56	7	982.6	0.36	0.23	0.53
Ames 21754	Pakistan	9	1164.9	0.43	0.25	0.64	9	1239.4	0.46	0.28	0.65
PI 649123	US, Iowa	9	1165.3	0.43	0.26	0.63	9	1107.4	0.41	0.24	0.61
PI 340213	India	9	1170.5	0.43	0.26	0.63	9	1301.1	0.48	0.32	0.65
PI 426380	Pakistan	6.5	1173.8	0.44	0.29	0.6	5.5	714.1	0.27	0.14	0.44
PI 180264	India, Rajasthan	6.8	1179	0.44	0.29	0.6	9	1291.7	0.48	0.34	0.62
PI 323270	Pakistan	6.8	1172.4	0.44	0.28	0.6	9	1275.6	0.47	0.33	0.62
PI 426305	Pakistan	7	1172.4	0.44	0.29	0.6	9	1496.7	0.56	0.42	0.68
PI 633091	Russian, Federation	8	1179.2	0.44	0.29	0.6	9	1332	0.49	0.34	0.65
PI 478327	China	8.5	1195.4	0.44	0.3	0.6	9	1332	0.49	0.33	0.66
PI 175602	Turkey, Kayseri	9	1187.4	0.44	0.27	0.63	9	1446	0.54	0.38	0.69
PI 458928	Canada, Saskatchewan	9	1189.2	0.44	0.27	0.63	9	1269.6	0.47	0.3	0.65
PI 458942	Germany	9	1175.6	0.44	0.26	0.63	9	1132.3	0.42	0.25	0.61
PI 458996	India	9	1173.2	0.44	0.26	0.63	5	843	0.31	0.17	0.51
PI 649124	US, Iowa	9	1185	0.44	0.26	0.63	8	1013.4	0.38	0.22	0.57
PI 179653	India, Gujarat	9	1179.2	0.44	0.26	0.63	9	1355.3	0.5	0.35	0.65
PI 179640	Pakistan, Punjab	7	1200.8	0.45	0.3	0.6	8	1107.6	0.41	0.3	0.54
PI 173857	India, Uttar Pradesh	9	1208.4	0.45	0.28	0.63	9	1342.4	0.5	0.34	0.66
PI 179192	Turkey, Edirne	9	1224.2	0.45	0.29	0.63	9	1474.4	0.55	0.4	0.69
PI 180420	India, Rajasthan	9	1208.9	0.45	0.27	0.64	9	1248.4	0.46	0.28	0.65
PI 426341	Pakistan	9	1204.5	0.45	0.27	0.64	9	1393.8	0.52	0.34	0.69
PI 426342	Pakistan	9	1201.9	0.45	0.28	0.63	9	1098	0.41	0.23	0.61

(continued)

Table A.1. Median, mean rank, and relative treatment effects along with 95% confidence intervals for blackleg severity ratings in relation to *B. juncea* accessions and PG2 and PG3 isolates of *Leptosphaeria maculans.* (continued)

		PG2					PG3				
Accessions	**Origin**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**
PI 426344	Pakistan	9	1223.1	0.45	0.28	0.64	9	1447.6	0.54	0.38	0.69
PI 478326	China	9	1214.6	0.45	0.28	0.63	3.5	743.6	0.28	0.13	0.5
PI 478334	China	9	1216.5	0.45	0.28	0.63	5.5	696	0.26	0.13	0.44
PI 426296	Pakistan	9	1224.9	0.45	0.29	0.63	9	1541.1	0.57	0.46	0.68
PI 633094	Russian, Federation	9	1202	0.45	0.27	0.64	8	1073.9	0.4	0.25	0.57
PI 537021	Korea, South	8	1236.4	0.46	0.32	0.6	9	1375.8	0.51	0.37	0.65
PI 173872	India	9	1239.7	0.46	0.29	0.64	9	1209.1	0.45	0.29	0.62
PI 179855	India, Rajasthan	9	1249.1	0.46	0.3	0.64	9	1308.2	0.49	0.32	0.65
PI 426348	Pakistan	9	1230.3	0.46	0.29	0.64	9	1293.5	0.48	0.31	0.66
PI 458997	India	9	1230.3	0.46	0.29	0.63	9	1198.6	0.45	0.29	0.62
PI 633089	Russian, Federation	9	1245.7	0.46	0.3	0.63	9	1778	0.66	0.65	0.67
PI 426301	Pakistan	9	1236.7	0.46	0.29	0.63	9	1296.4	0.48	0.32	0.65
PI 633105	Pakistan	9	1236	0.46	0.29	0.63	9	1551.6	0.58	0.47	0.68
PI 633107	Pakistan	9	1227.9	0.46	0.29	0.64	9	1115.1	0.41	0.24	0.61
PI 174801	India, Uttar Pradesh	9	1254.9	0.47	0.27	0.67	9	1287.6	0.48	0.31	0.65
PI 179183	Turkey, Tekirdag	9	1269.7	0.47	0.31	0.64	9	1534.2	0.57	0.45	0.68
PI 250140	Pakistan, Punjab	9	1265.1	0.47	0.3	0.64	9	1482.6	0.55	0.4	0.69
PI 254361	India, Delhi	9	1265.4	0.47	0.28	0.67	9	1534.1	0.57	0.45	0.68
PI 358591	Ethiopia	9	1265.4	0.47	0.28	0.67	9	1263.6	0.47	0.29	0.65
PI 426339	Pakistan	9	1257.1	0.47	0.3	0.64	9	1209.3	0.45	0.26	0.65
PI 426350	Pakistan	9	1266.6	0.47	0.28	0.67	9	1402.2	0.52	0.35	0.69
PI 426298	Pakistan	9	1273.2	0.47	0.31	0.64	9	1483.5	0.55	0.41	0.69
PI 426315	Pakistan	9	1270.2	0.47	0.32	0.63	9	1309.6	0.49	0.32	0.65
PI 633098	India, Punjab	9	1264.6	0.47	0.31	0.64	9	1325.1	0.49	0.33	0.65
PI 633109	Germany	9	1274.8	0.47	0.32	0.64	9	1526.7	0.57	0.44	0.68

(continued)

Table A.1. Median, mean rank, and relative treatment effects along with 95% confidence intervals for blackleg severity ratings in relation to *B. juncea* accessions and PG2 and PG3 isolates of *Leptosphaeria maculans.* (continued)

		PG2					PG3				
Accessions	**Origin**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**
PI 649108	India	9	1259.4	0.47	0.31	0.63	9	1301.2	0.48	0.32	0.65
PI 649117	India	9	1274.6	0.47	0.31	0.64	9	1465.4	0.54	0.39	0.69
PI 470241	Indonesia	7.8	1285.6	0.48	0.35	0.61	9	1778	0.66	0.65	0.67
PI 173874	India, Delhi	9	1287.6	0.48	0.32	0.64	9	1249.3	0.46	0.29	0.65
PI 180269	India, Gujarat	9	1292.8	0.48	0.29	0.67	9	1154.9	0.43	0.27	0.61
PI 217516	Pakistan, Punjab	9	1295.9	0.48	0.3	0.67	9	1263.6	0.47	0.29	0.66
PI 370745	India	9	1281.6	0.48	0.29	0.67	9	1234.7	0.46	0.28	0.65
PI 390141	Pakistan	9	1284.4	0.48	0.29	0.67	9	1458.3	0.54	0.39	0.69
PI 426358	Pakistan	9	1292	0.48	0.32	0.64	9	1223.3	0.45	0.3	0.62
PI 633099	US, California	9	1292.4	0.48	0.32	0.64	9	1088.5	0.4	0.23	0.61
PI 426294	Pakistan	9	1293.3	0.48	0.32	0.64	9	1300.1	0.48	0.32	0.65
PI 179635	India, Rajasthan	9	1325.2	0.49	0.31	0.67	9	1595.6	0.59	0.46	0.72
PI 179644	India, Rajasthan	9	1314.6	0.49	0.31	0.67	9	1334.6	0.5	0.34	0.65
PI 426355	Pakistan	9	1309.3	0.49	0.3	0.67	9	1216.4	0.45	0.27	0.65
PI 426361	Pakistan	9	1318.6	0.49	0.33	0.65	9	1133.7	0.42	0.25	0.61
PI 458927	Canada, Saskatchewan	9	1328.8	0.49	0.35	0.64	9	1307.4	0.49	0.32	0.65
PI 633113	Italy, Calabria	9	1330.5	0.49	0.35	0.64	9	1640.7	0.61	0.51	0.7
PI 649109	India	9	1311.4	0.49	0.33	0.64	9	1463.6	0.54	0.39	0.69
PI 426304	Pakistan	9	1307.5	0.49	0.3	0.67	9	1483.5	0.55	0.41	0.69
PI633078	Canada	9	1326.7	0.49	0.31	0.68	9	1469.3	0.55	0.39	0.69
PI 633111	Germany	9	1333.2	0.5	0.32	0.67	9	1313.7	0.49	0.32	0.66
PI 250137	Pakistan, North-West Frontier	9	1359	0.5	0.33	0.67	9	1295.8	0.48	0.31	0.66
PI 370744	India	9	1336.1	0.5	0.32	0.67	9	1279.6	0.48	0.3	0.66
PI 426253	Pakistan	9	1358.6	0.5	0.33	0.68	9	1330.2	0.49	0.33	0.66

(continued)

Table A.1. Median, mean rank, and relative treatment effects along with 95% confidence intervals for blackleg severity ratings in relation to *B. juncea* accessions and PG2 and PG3 isolates of *Leptosphaeria maculans.* (continued)

		PG2					PG3				
Accessions	**Origin**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**
PI 458943	Germany	9	1344.8	0.5	0.33	0.67	9	1305.4	0.48	0.32	0.65
PI 458994	India	9	1344.5	0.5	0.33	0.67	9	1331.1	0.49	0.33	0.66
PI 478328	China	9	1338	0.5	0.36	0.64	5.5	1005.6	0.37	0.22	0.56
PI 633083	US, California	9	1358.2	0.5	0.36	0.65	8	1171.4	0.44	0.3	0.58
PI 633093	Russian, Federation	9	1355.6	0.5	0.33	0.68	9	1303.5	0.48	0.32	0.65
PI 179857	India, Rajasthan	9	1347	0.5	0.33	0.67	9	1484.9	0.55	0.41	0.69
PI 120923	Turkey, Izmir	9	1368.7	0.51	0.34	0.67	9	1140.3	0.42	0.26	0.61
PI 432381	Bangladesh	9	1367.8	0.51	0.37	0.65	9	1778	0.66	0.65	0.67
PI 347616	India	9	1386.1	0.51	0.35	0.68	9	1253.9	0.47	0.29	0.65
PI 390136	Pakistan	9	1373.4	0.51	0.34	0.67	9	1282.9	0.48	0.34	0.62
PI 426338	Pakistan	9	1359.7	0.51	0.33	0.68	9	1351.9	0.5	0.35	0.65
PI 426347	Pakistan	9	1384.2	0.51	0.34	0.68	9	1436.9	0.53	0.37	0.69
PI 432390	Bangladesh	9	1362.8	0.51	0.34	0.67	9	1667.2	0.62	0.54	0.7
PI 478333	China	9	1367.8	0.51	0.37	0.65	8	1183.4	0.44	0.31	0.58
PI 500675	Zambia	9	1372.2	0.51	0.34	0.67	9	1347.7	0.5	0.35	0.65
PI 633096	Russian, Federation	9	1365.8	0.51	0.34	0.67	9	1611.7	0.6	0.47	0.71
PI 180421	India, Rajasthan	9	1365.2	0.51	0.34	0.68	9	1611.7	0.6	0.47	0.71
PI 347619	India	9	1366.9	0.51	0.33	0.68	9	1376.2	0.51	0.37	0.65
PI478330	China	9	1366.6	0.51	0.34	0.67	9	1554.4	0.58	0.47	0.68
PI 169077	Turkey	9	1388.8	0.52	0.35	0.68	9	1177.4	0.44	0.27	0.62
PI 179636	India, Rajasthan	9	1387.7	0.52	0.35	0.67	7	1047.1	0.39	0.24	0.57
PI 458992	India	9	1395.7	0.52	0.36	0.67	9	1191.9	0.44	0.29	0.61
PI 633090	Russian, Federation	9	1409.4	0.52	0.36	0.68	9	1640.7	0.61	0.51	0.7
PI 649122	US, Iowa	9	1392.8	0.52	0.36	0.68	9	1302.9	0.48	0.32	0.66
PI 113310	China, Nei Mongol	9	1394.7	0.52	0.36	0.68	9	1458.6	0.54	0.39	0.69

(continued)

Table A.1. Median, mean rank, and relative treatment effects along with 95% confidence intervals for blackleg severity ratings in relation to *B. juncea* accessions and PG2 and PG3 isolates of *Leptosphaeria maculans.* (continued)

		PG2					PG3				
Accessions	**Origin**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**
PI458934	Sweden	9	1403.6	0.52	0.36	0.68	9	1778	0.66	0.65	0.67
PI633087	Russian, Federation	9	1400.2	0.52	0.36	0.68	9	1215.9	0.45	0.3	0.62
PI 169085	Turkey, Tekirdag	9	1428.4	0.53	0.38	0.68	9	1102.6	0.41	0.24	0.61
PI 432386	Bangladesh	9	1434.9	0.53	0.38	0.68	9	1778	0.66	0.65	0.67
PI 426362	Pakistan	9	1429.2	0.53	0.38	0.67	9	1356.2	0.5	0.35	0.66
PI 426405	Pakistan	9	1416.8	0.53	0.37	0.68	9	1188.3	0.44	0.28	0.61
PI 458995	India	9	1436.3	0.53	0.38	0.68	9	1338.3	0.5	0.34	0.65
PI 633086	Russian, Federation	9	1414.7	0.53	0.37	0.68	9	1474.3	0.55	0.4	0.69
PI 649114	India	9	1437.3	0.53	0.39	0.68	6	1014.9	0.38	0.22	0.56
PI478325	China	9	1414.7	0.53	0.37	0.67	4.5	812.3	0.3	0.15	0.51
PI 181040	Pakistan, Sind	9	1458.7	0.54	0.4	0.68	9	1446.7	0.54	0.38	0.69
PI 269448	Pakistan	9	1459.1	0.54	0.4	0.67	9	1478.2	0.55	0.4	0.69
PI 286417	Nepal	9	1463.6	0.54	0.36	0.71	9	1398.6	0.52	0.34	0.69
PI 288727	India	9	1453.1	0.54	0.36	0.71	9	1496	0.56	0.41	0.69
PI 432367	Bangladesh	9	1448.7	0.54	0.39	0.68	9	1467.6	0.55	0.4	0.69
PI 432377	Bangladesh	9	1463.2	0.54	0.4	0.68	9	1427.6	0.53	0.4	0.65
PI 458929	Canada, Saskatchewan	9	1466.3	0.54	0.41	0.68	9	1329.4	0.49	0.33	0.66
PI 426293	Pakistan	9	1454.8	0.54	0.4	0.68	9	1415	0.53	0.39	0.65
PI 426302	Pakistan	9	1448.7	0.54	0.39	0.68	9	1534.2	0.57	0.45	0.68
PI 426316	Pakistan	9	1447.5	0.54	0.4	0.67	9	1375.8	0.51	0.37	0.65
PI 649120	Russian, Federation	9	1462.8	0.54	0.4	0.68	9	1261.8	0.47	0.33	0.62
PI531272	Hungary	9	1465.4	0.54	0.4	0.68	9	1633.9	0.61	0.5	0.71
PI 175082	India, Uttar Pradesh	9	1479.8	0.55	0.38	0.71	9	1778	0.66	0.65	0.67
PI 347617	India	9	1485.4	0.55	0.38	0.71	9	1629.1	0.61	0.49	0.71
PI 370746	India	9	1493.8	0.55	0.39	0.71	9	1286.5	0.48	0.31	0.65

(continued)

Table A.1. Median, mean rank, and relative treatment effects along with 95% confidence intervals for blackleg severity ratings in relation to *B. juncea* accessions and PG2 and PG3 isolates of *Leptosphaeria maculans.* (continued)

		PG2					PG3				
Accessions	**Origin**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**
PI 390138	Pakistan	9	1482.9	0.55	0.42	0.68	9	1778	0.66	0.65	0.67
PI 432379	Bangladesh	9	1472	0.55	0.37	0.71	9	1342.7	0.5	0.34	0.66
PI 500651	Zambia	9	1483.6	0.55	0.38	0.71	9	1465.4	0.54	0.39	0.69
PI 649104	India	9	1485.4	0.55	0.38	0.71	9	1431.7	0.53	0.37	0.69
PI 179191	Turkey, kirklareli	9	1485.4	0.55	0.38	0.71	9	1778	0.66	0.65	0.67
PI 175607	Turkey, Tekirdag	9	1504.4	0.56	0.4	0.71	9	1238.6	0.46	0.28	0.65
PI 426324	Pakistan	9	1502.3	0.56	0.39	0.71	9	1778	0.66	0.65	0.67
PI 426354	Pakistan	9	1516.4	0.56	0.4	0.71	9	1286.6	0.48	0.31	0.65
PI 426364	Pakistan	9	1515.9	0.56	0.41	0.71	9	1374.7	0.51	0.36	0.66
PI 426406	Pakistan	9	1501.9	0.56	0.39	0.71	7	1030.4	0.38	0.23	0.57
PI 633108	Pakistan	9	1515.9	0.56	0.41	0.71	9	1543.2	0.57	0.46	0.68
PI531267	China	9	1518	0.56	0.41	0.71	9	1456.7	0.54	0.38	0.69
PI 175068	India, Uttar Pradesh	9	1526.2	0.57	0.41	0.71	9	1413.2	0.52	0.35	0.69
PI 181041	Pakistan, Sind	9	1526.2	0.57	0.41	0.71	9	1611.7	0.6	0.47	0.71
PI 257240	China	9	1537.8	0.57	0.41	0.71	9	1633.9	0.61	0.5	0.71
PI 346876	India	9	1542.3	0.57	0.43	0.71	9	1314.3	0.49	0.32	0.65
PI 426343	Pakistan	9	1542.2	0.57	0.42	0.71	9	1414.8	0.53	0.35	0.69
PI 340220	India	9	1556.7	0.58	0.43	0.71	9	1778	0.66	0.65	0.67
PI 426321	Pakistan	9	1571.3	0.58	0.45	0.71	9	1662.4	0.62	0.53	0.7
PI 426297	Pakistan	9	1573.7	0.58	0.45	0.71	9	1500.9	0.56	0.42	0.68
Ames 15645	US California	9	1568.3	0.58	0.44	0.71	9	1440.8	0.54	0.37	0.69
PI 426407	Pakistan	9	1568.3	0.58	0.44	0.71	9	1352.6	0.5	0.35	0.65
PI 249555	Thailand	9	1577.6	0.59	0.46	0.71	9	1611.7	0.6	0.47	0.71
PI 436559	China	9	1596.1	0.59	0.47	0.71	9	1431.2	0.53	0.41	0.65
PI 426303	Pakistan	9	1578.6	0.59	0.45	0.71	9	1201.6	0.45	0.29	0.62

(continued)

Table A.1. Median, mean rank, and relative treatment effects along with 95% confidence intervals for blackleg severity ratings in relation to *B. juncea* accessions and PG2 and PG3 isolates of *Leptosphaeria maculans.* (continued)

		PG2					PG3				
Accessions	**Origin**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**
PI 426319	Pakistan	9	1578.6	0.59	0.45	0.71	9	1611.7	0.6	0.47	0.71
PI 181042	Pakistan, Sind	9	1620.8	0.6	0.49	0.7	9	1260.2	0.47	0.29	0.65
PI 390135	Pakistan	9	1625.8	0.6	0.5	0.7	9	1662.4	0.62	0.53	0.7
PI 390137	Pakistan	9	1601.8	0.6	0.48	0.7	9	1778	0.66	0.65	0.67
PI 390140	Pakistan	9	1625.8	0.6	0.5	0.7	9	1649.8	0.61	0.52	0.7
PI 426292	Pakistan	9	1625.8	0.6	0.5	0.7	9	1656.7	0.62	0.52	0.7
PI 426323	Pakistan	9	1617.1	0.6	0.49	0.7	9	1334.1	0.5	0.33	0.66
PI 426327	Pakistan	9	1603.9	0.6	0.48	0.7	9	1611.7	0.6	0.47	0.71
PI 426295	Pakistan	9	1620.8	0.6	0.49	0.7	9	1649.8	0.61	0.52	0.7
PI 426300	Pakistan	9	1601.8	0.6	0.48	0.7	9	1534.2	0.57	0.45	0.68
PI 212594	Afghanistan, Nangarhar	9	1642.8	0.61	0.46	0.74	9	1447.6	0.54	0.38	0.69
PI 271455	India, Himachal Pradesh	9	1638.1	0.61	0.46	0.74	9	1778	0.66	0.65	0.67
PI 311726	Poland	9	1642.8	0.61	0.46	0.74	9	1778	0.66	0.65	0.67
PI 432378	Bangladesh	9	1651.2	0.61	0.47	0.74	9	1193.1	0.44	0.29	0.61
PI 603012	Germany	9	1635.4	0.61	0.51	0.7	9	1778	0.66	0.65	0.67
PI 633095	Russian, Federation	9	1630.9	0.61	0.5	0.7	9	1368.5	0.51	0.36	0.65
PI 426178	Afghanistan	9	1642.8	0.61	0.46	0.74	9	1778	0.66	0.65	0.67
PI478335	China	9	1645	0.61	0.51	0.7	9	1649.8	0.61	0.52	0.7
PI 181035	Pakistan, Punjab	9	1673.3	0.62	0.49	0.73	9	1547.5	0.57	0.46	0.68
PI 208734	Cuba	9	1661.8	0.62	0.48	0.74	9	1633.9	0.61	0.5	0.71
PI 426322	Pakistan	9	1661.8	0.62	0.48	0.74	9	1649.8	0.61	0.52	0.7
PI 426329	Pakistan	9	1673.3	0.62	0.49	0.73	9	1611.7	0.6	0.47	0.71
PI 633085	US, California	9	1661.8	0.62	0.48	0.74	9	1649.8	0.61	0.52	0.7
PI 649105	India	9	1673.3	0.62	0.49	0.73	7	996	0.37	0.21	0.56
PI 215636	India, Haryana	9	1661.8	0.62	0.48	0.74	9	1778	0.66	0.65	0.67

(continued)

Table A.1. Median, mean rank, and relative treatment effects along with 95% confidence intervals for blackleg severity ratings in relation to *B. juncea* accessions and PG2 and PG3 isolates of *Leptosphaeria maculans.* (continued)

		PG2					PG3				
Accessions	**Origin**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**
PI 432380	Bangladesh	9	1706.2	0.63	0.53	0.73	9	1519.4	0.56	0.44	0.68
PI 183115	India, Gujarat	9	1691.7	0.63	0.51	0.73	9	1656.7	0.62	0.52	0.7
PI 183117	India, Gujarat	9	1683.6	0.63	0.51	0.73	9	1640.7	0.61	0.51	0.7
PI 209021	Cuba	9	1683.6	0.63	0.51	0.73	9	1611.7	0.6	0.47	0.71
PI 311734	Poland	9	1691.7	0.63	0.51	0.73	9	1624.8	0.6	0.49	0.71
PI 379103	Former Serbia & Montenegro	9	1683.6	0.63	0.51	0.73	9	1778	0.66	0.65	0.67
PI 426328	Pakistan	9	1706.2	0.63	0.53	0.73	9	1429.3	0.53	0.37	0.69
PI 426365	Pakistan	9	1683.6	0.63	0.51	0.73	9	1292.2	0.48	0.31	0.65
PI 426402	Pakistan	9	1706.2	0.63	0.53	0.73	9	1304.8	0.48	0.35	0.62
PI 633116	India, Delhi	9	1683.6	0.63	0.51	0.73	9	1507.8	0.56	0.43	0.68
PI 649102	US, Minnesota	9	1683.6	0.63	0.51	0.73	9	1611.7	0.6	0.47	0.71
PI 633112	Cuba, Santiago de Cuba	9	1683.6	0.63	0.51	0.73	9	1356.2	0.5	0.35	0.66
PI 195553	Ethiopia, Welo	9	1728.7	0.64	0.56	0.72	9	1619.6	0.6	0.48	0.71
PI 271453	India, Gujarat	9	1735.9	0.64	0.57	0.72	9	1640.7	0.61	0.51	0.7
PI 340221	India	9	1712.7	0.64	0.54	0.72	9	1778	0.66	0.65	0.67
PI 390139	Pakistan	9	1728.7	0.64	0.56	0.72	9	1444.4	0.54	0.37	0.69
PI 426331	Pakistan	9	1721.4	0.64	0.55	0.72	9	1595.6	0.59	0.46	0.72
PI 426404	Pakistan	9	1712.7	0.64	0.54	0.72	9	1543.2	0.57	0.46	0.68
PI 426312	Pakistan	9	1721.4	0.64	0.55	0.72	9	1328.1	0.49	0.33	0.65
PI 426314	Pakistan	9	1721.4	0.64	0.55	0.72	9	1205.7	0.45	0.29	0.62
PI 340211	India	9	1740.4	0.65	0.57	0.72	9	1778	0.66	0.65	0.67
PI 633077	Canada	9	1745.4	0.65	0.58	0.71	9	1778	0.66	0.65	0.67
PI 192936	China	9	1841	0.68	0.68	0.69	9	1778	0.66	0.65	0.67
PI 211000	Afghanistan, Badakhshan	9	1841	0.68	0.68	0.69	9	1778	0.66	0.65	0.67

(continued)

Table A.1. Median, mean rank, and relative treatment effects along with 95% confidence intervals for blackleg severity ratings in relation to *B. juncea* accessions and PG2 and PG3 isolates of *Leptosphaeria maculans.* (continued)

		PG2					PG3				
Accessions	**Origin**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**
PI 212082	Afghanistan, Kondoz	9	1841	0.68	0.68	0.69	9	1778	0.66	0.65	0.67
PI 340207	India	9	1841	0.68	0.68	0.69	9	1778	0.66	0.65	0.67
PI 340212	India	9	1841	0.68	0.68	0.69	9	1778	0.66	0.65	0.67
PI 340214	India	9	1841	0.68	0.68	0.69	9	1778	0.66	0.65	0.67
PI 340215	India	9	1841	0.68	0.68	0.69	9	1778	0.66	0.65	0.67
PI 340218	India	9	1841	0.68	0.68	0.69	9	1778	0.66	0.65	0.67
PI 340219	India	9	1841	0.68	0.68	0.69	9	1633.9	0.61	0.5	0.71
PI 340223	India	9	1841	0.68	0.68	0.69	9	1580.9	0.59	0.44	0.72
PI 347615	India	9	1841	0.68	0.68	0.69	9	1402.5	0.52	0.35	0.69
PI 347618	India	9	1841	0.68	0.68	0.69	9	1778	0.66	0.65	0.67
PI 387819	Thailand	9	1841	0.68	0.68	0.69	9	1778	0.66	0.65	0.67
PI 426326	Pakistan	9	1841	0.68	0.68	0.69	9	1496.1	0.56	0.42	0.69
PI 432387	Bangladesh	9	1841	0.68	0.68	0.69	9	1778	0.66	0.65	0.67
PI 432388	Bangladesh	9	1841	0.68	0.68	0.69	9	1778	0.66	0.65	0.67
PI 531268	China	9	1841	0.68	0.68	0.69	9	1778	0.66	0.65	0.67
PI 531269	China	9	1841	0.68	0.68	0.69	9	1778	0.66	0.65	0.67
PI 531270	Former Soviet Union	9	1841	0.68	0.68	0.69	9	1778	0.66	0.65	0.67
PI 531271	Hungary	9	1841	0.68	0.68	0.69	9	1778	0.66	0.65	0.67
PI 603011	India, Uttar Pradesh	9	1841	0.68	0.68	0.69	9	1778	0.66	0.65	0.67
PI 633084	US, California	9	1841	0.68	0.68	0.69	9	1662.4	0.62	0.53	0.7
PI 633114	Korea, North	9	1841	0.68	0.68	0.69	9	1534.2	0.57	0.45	0.68
PI 649110	India	9	1841	0.68	0.68	0.69	7	925.9	0.34	0.23	0.47
Ames 15649	US California	9	1841	0.68	0.68	0.69	9	1778	0.66	0.65	0.67
Ames 21749	Pakistan	9	1841	0.68	0.68	0.69	9	1778	0.66	0.65	0.67
PI 209781	Germany	9	1841	0.68	0.68	0.69	9	1584.9	0.59	0.44	0.72

(continued)

Table A.1. Median, mean rank, and relative treatment effects along with 95% confidence intervals for blackleg severity ratings in relation to *B. juncea* accessions and PG2 and PG3 isolates of *Leptosphaeria maculans*. (continued)

		PG2					PG3				
Accessions	**Origin**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**
PI 432384	Bangladesh	9	1841	0.68	0.68	0.69	9	1778	0.66	0.65	0.67
PI 649111	India	9	1841	0.68	0.68	0.69	9	1543.2	0.57	0.46	0.68
PI169078	Turkey	9	1841	0.68	0.68	0.69	8	1009.8	0.38	0.28	0.49
Westar		9	1841	0.68	0.68	0.69	9	1778	0.66	0.65	0.67

Table A.2. Median, mean rank, and relative treatment effects along with 95% confidence intervals for blackleg severity ratings in relation to *B. juncea* accessions and PGT and PG4 isolates of *Leptosphaeria maculans.*

		PGT					PG4				
Accession	**Origin**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**
PI 311726	Poland	1.8	370.5	0.14	0.07	0.26	3.3	693.8	0.26	0.14	0.43
PI 179858	India, Rajasthan	2.2	500.9	0.19	0.15	0.24	2.8	542.4	0.2	0.18	0.22
PI 649113	India	1.5	523.4	0.19	0.11	0.32	3	719.2	0.27	0.15	0.43
PI 426320	Pakistan	2	532.1	0.2	0.13	0.29	2	333.1	0.12	0.08	0.19
PI 426304	Pakistan	2	556.3	0.21	0.13	0.32	3	757.2	0.28	0.16	0.45
PI 426330	Pakistan	2.5	583.2	0.22	0.14	0.32	2.5	544.7	0.2	0.1	0.36
PI 649105	India	2.5	649.4	0.24	0.15	0.37	4	884.7	0.33	0.2	0.49
PI 649109	India	2.5	644.4	0.24	0.14	0.38	3	716.4	0.27	0.15	0.43
PI 649122	US, Iowa	3	664.4	0.25	0.14	0.4	4	983.7	0.37	0.2	0.56
Ames 9914	India	3	686.1	0.25	0.14	0.41	2	510.1	0.19	0.1	0.33
PI 426316	Pakistan	2.5	692.2	0.26	0.13	0.44	3	702.3	0.26	0.17	0.37
PI 649123	US, Iowa	2	691.4	0.26	0.16	0.39	2	556	0.21	0.09	0.42
PI 649112	India	3	723.4	0.27	0.19	0.36	4	902.6	0.34	0.28	0.39
PI 347619	India	2.5	731.3	0.27	0.19	0.37	3	960.6	0.36	0.21	0.54
PI 346876	India	9	2047.7	0.76	0.7	0.81	9	2085	0.77	0.77	0.78
PI 633093	Russian, Federation	9	2047.7	0.76	0.7	0.81	9	1765.9	0.66	0.53	0.76
PI 633095	Russian, Federation	9	2054.7	0.76	0.71	0.81	9	1869.1	0.69	0.52	0.83
PI 176884	Turkey	9	2127.5	0.79	0.78	0.8	7.6	1617.4	0.6	0.47	0.72
PI 432381	Bangladesh	9	2127.5	0.79	0.78	0.8	8.5	1580.4	0.59	0.51	0.66
PI 211000	Afghanistan, Badakhshan	9	2127.5	0.79	0.78	0.8	9	2085	0.77	0.77	0.78
PI 340207	India	9	2127.5	0.79	0.78	0.8	9	2085	0.77	0.77	0.78
PI 470082	Korea, South	9	2127.5	0.79	0.78	0.8	9	1646.1	0.61	0.44	0.76
PI 531268	China	9	2127.5	0.79	0.78	0.8	9	2085	0.77	0.77	0.78
PI 633105	Pakistan	9	2127.5	0.79	0.78	0.8	9	2085	0.77	0.77	0.78
Ames 21749	Pakistan	9	2127.5	0.79	0.78	0.8	9	2012.2	0.75	0.69	0.8

(continued)

Table A.2. Median, mean rank, and relative treatment effects along with 95% confidence intervals for blackleg severity ratings in relation to *B. juncea* accessions and PGT and PG4 isolates of *Leptosphaeria maculans.*(continued)

		PGT					PG4				
Accession	**Origin**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**
PI 426340	Pakistan	2	741.2	0.28	0.15	0.44	2	689.7	0.26	0.13	0.44
Ames 15649	US California	2	801.9	0.3	0.14	0.53	1.5	761	0.28	0.11	0.56
PI 175100	India, Uttar Pradesh	3.5	804.2	0.3	0.18	0.46	4	978.4	0.36	0.18	0.59
PI 179653	India, Gujarat	3	809.7	0.3	0.17	0.47	3.5	862.3	0.32	0.19	0.49
PI 288724	India, Gujarat	2.5	812.3	0.3	0.16	0.49	4	1023.4	0.38	0.2	0.6
PI 390140	Pakistan	3	822.1	0.31	0.18	0.47	5.5	1372.8	0.51	0.34	0.68
PI 633106	Pakistan	3	839.6	0.31	0.19	0.47	3.5	1053.7	0.39	0.22	0.59
PI 649114	India	2.5	832.5	0.31	0.18	0.48	3.5	882.2	0.33	0.2	0.49
PI 271442	India, Gujarat	2.5	836.1	0.31	0.19	0.47	2.5	737.4	0.27	0.15	0.45
PI 426407	Pakistan	2.5	835.2	0.31	0.17	0.5	3	831.1	0.31	0.18	0.48
PI 649107	India	3	863.6	0.32	0.23	0.43	4	917.8	0.34	0.23	0.47
PI 426299	Pakistan	3	872.3	0.32	0.19	0.5	2	497.1	0.18	0.1	0.32
Ames 21754	Pakistan	2	901.7	0.33	0.15	0.59	2	707.6	0.26	0.11	0.5
PI 426306	Pakistan	3	886.3	0.33	0.2	0.48	3	625.8	0.23	0.16	0.32
PI 173857	India, Uttar Pradesh	3	912.9	0.34	0.18	0.54	7.5	1283.3	0.48	0.28	0.68
PI 250130	Pakistan, North-West Frontier	2.5	903.9	0.34	0.18	0.53	3	803.4	0.3	0.15	0.51
PI 390136	Pakistan	3	927.2	0.34	0.18	0.56	6	1352.6	0.5	0.32	0.68
PI 426354	Pakistan	2.5	905.2	0.34	0.15	0.59	9	1512.5	0.56	0.38	0.73
PI 426297	Pakistan	2.5	914.2	0.34	0.2	0.51	3.5	1068.4	0.4	0.25	0.56
PI 649119	India	2.5	904.2	0.34	0.2	0.51	3	808.6	0.3	0.15	0.51
PI 649124	US, Iowa	2.5	923.8	0.34	0.19	0.54	3	852	0.32	0.16	0.54
PI 251239	Pakistan, Punjab	3.5	922.4	0.34	0.22	0.48	3	595.1	0.22	0.12	0.37
PI458998	India	2.5	919.3	0.34	0.19	0.53	3	760.3	0.28	0.15	0.46
PI 217516	Pakistan, Punjab	2	929.7	0.35	0.18	0.56	3	1062.2	0.39	0.22	0.6

(continued)

Table A.2. Median, mean rank, and relative treatment effects along with 95% confidence intervals for blackleg severity ratings in relation to *B. juncea* accessions and PGT and PG4 isolates of *Leptosphaeria maculans.*(continued)

		PGT					PG4				
Accession	**Origin**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**
PI 113310	China, Nei Mongol	4	954.7	0.35	0.22	0.51	4.5	1072.6	0.4	0.23	0.6
PI 649121	Nepal	2.5	942.3	0.35	0.19	0.55	4.5	993.8	0.37	0.2	0.57
PI 426253	Pakistan	2.5	974.5	0.36	0.18	0.59	9	1458.4	0.54	0.36	0.72
PI 426385	Pakistan	3	980.9	0.36	0.21	0.55	4.5	1245.7	0.46	0.3	0.63
PI 633104	Pakistan	3	968.9	0.36	0.21	0.54	3.5	848.8	0.32	0.19	0.47
PI 633109	Germany	3	972.3	0.36	0.2	0.56	4.5	1167	0.43	0.27	0.61
PI 426325	Pakistan	3.5	958.2	0.36	0.22	0.52	3	707.9	0.26	0.14	0.44
PI 426335	Pakistan	3.5	977.6	0.36	0.23	0.52	4.5	1012.8	0.38	0.23	0.55
PI 633102	Pakistan	3.5	972	0.36	0.27	0.46	4	733.2	0.27	0.18	0.39
PI 633107	Pakistan	3	959.6	0.36	0.24	0.49	6	1110	0.41	0.25	0.6
PI 254361	India, Delhi	2.5	998.8	0.37	0.19	0.59	2.5	945	0.35	0.18	0.58
PI 286417	Nepal	4	984.7	0.37	0.21	0.56	3	590.7	0.22	0.13	0.34
PI 426346	Pakistan	3	1004.9	0.37	0.21	0.57	4.5	1139.6	0.42	0.29	0.57
PI 649125	US, Iowa	4.5	985	0.37	0.23	0.53	3	916.8	0.34	0.19	0.54
PI 180269	India, Gujarat	3	1011.9	0.38	0.2	0.6	2.5	833.4	0.31	0.16	0.51
PI 347617	India	2.5	1029.7	0.38	0.2	0.6	4	1300.5	0.48	0.34	0.63
PI 370746	India	3	1023.5	0.38	0.2	0.61	9	1529.7	0.57	0.4	0.72
PI 426349	Pakistan	3	1030	0.38	0.2	0.6	4	1178.8	0.44	0.28	0.61
PI 426355	Pakistan	3.5	1033.3	0.38	0.2	0.61	9	1375.2	0.51	0.3	0.72
Ames 15645	US California	3	1027.4	0.38	0.22	0.57	5.5	1125.1	0.42	0.24	0.62
PI 179640	Pakistan, Punjab	3	1018.4	0.38	0.25	0.53	4.5	902.7	0.34	0.2	0.5
PI 649101	US, Minnesota	3	1033.3	0.38	0.23	0.56	2	762.4	0.28	0.13	0.51
PI 426347	Pakistan	3	1062.4	0.39	0.21	0.61	9	1759.2	0.65	0.53	0.76
PI 500651	Zambia	2	1050.7	0.39	0.18	0.65	6.5	1291.9	0.48	0.28	0.68
PI 633108	Pakistan	2.5	1051.4	0.39	0.23	0.58	9	1526.7	0.57	0.39	0.73

(continued)

Table A.2. Median, mean rank, and relative treatment effects along with 95% confidence intervals for blackleg severity ratings in relation to *B. juncea* accessions and PGT and PG4 isolates of *Leptosphaeria maculans.*(continued)

		PGT					PG4				
Accession	**Origin**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**
PI478330	China	3	1052.3	0.39	0.21	0.61	6	1015.2	0.38	0.21	0.58
PI603013	Pakistan, Punjab	4	1062.3	0.39	0.29	0.51	2.5	728.6	0.27	0.14	0.45
PI 173872	India	3	1068.9	0.4	0.21	0.62	3	996.4	0.37	0.19	0.59
PI 288727	India	3.5	1078.6	0.4	0.25	0.58	3	883.7	0.33	0.18	0.52
PI 459007	India	3	1065.3	0.4	0.24	0.57	3.5	991.3	0.37	0.22	0.55
PI 426303	Pakistan	3	1066.8	0.4	0.24	0.58	3.5	986.8	0.37	0.21	0.56
PI 649102	US, Minnesota	3	1070.3	0.4	0.26	0.56	4.5	1134.4	0.42	0.28	0.58
PI633078	Canada	4	1064.4	0.4	0.3	0.5	9	1534.2	0.57	0.39	0.73
PI 269448	Pakistan	6	1097.1	0.41	0.22	0.63	2.5	992.7	0.37	0.19	0.59
PI 426342	Pakistan	4.6	1094.7	0.41	0.25	0.59	5	1051.5	0.39	0.23	0.58
PI 426382	Pakistan	3.5	1091.3	0.41	0.27	0.56	9	1525.2	0.57	0.4	0.72
PI 458997	India	3.5	1114.2	0.41	0.31	0.52	6	1361.2	0.51	0.35	0.66
PI 426295	Pakistan	3	1112.8	0.41	0.27	0.58	5	1219.1	0.45	0.29	0.62
PI 179857	India, Rajasthan	4	1097.9	0.41	0.29	0.53	3	932.3	0.35	0.21	0.51
PI531267	China	3.5	1099.7	0.41	0.26	0.58	5	1147	0.43	0.26	0.61
PI537018	Pakistan	3	1108.9	0.41	0.23	0.62	9	1506.1	0.56	0.38	0.73
PI 179635	India, Rajasthan	3	1131.6	0.42	0.25	0.61	5.5	1293.6	0.48	0.3	0.67
PI 181041	Pakistan, Sind	4	1121.4	0.42	0.24	0.62	4	1232.4	0.46	0.27	0.66
PI 323270	Pakistan	4.5	1142	0.42	0.24	0.63	3	1001.6	0.37	0.2	0.59
PI 347616	India	3	1143.1	0.42	0.25	0.62	5.5	1307.7	0.49	0.34	0.64
PI 426341	Pakistan	3	1120.6	0.42	0.24	0.61	9	1425.5	0.53	0.33	0.72
PI 426350	Pakistan	3	1141.3	0.42	0.24	0.63	5	1188.1	0.44	0.32	0.57
PI 432384	Bangladesh	4.8	1139.4	0.42	0.27	0.6	6.6	1398.4	0.52	0.41	0.63
PI 358591	Ethiopia	3	1151.8	0.43	0.22	0.66	9	1607.2	0.6	0.45	0.73
PI 426327	Pakistan	4	1144.9	0.43	0.26	0.6	6	1445.5	0.54	0.39	0.68

(continued)

Table A.2. Median, mean rank, and relative treatment effects along with 95% confidence intervals for blackleg severity ratings in relation to *B. juncea* accessions and PGT and PG4 isolates of *Leptosphaeria maculans.*(continued)

		PGT					PG4				
Accession	**Origin**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**
PI 426338	Pakistan	3.5	1146.4	0.43	0.25	0.63	5	998.6	0.37	0.25	0.51
PI 426344	Pakistan	5	1148.6	0.43	0.27	0.6	5	1231.3	0.46	0.29	0.64
PI 426357	Pakistan	4	1160.1	0.43	0.25	0.63	5	1309.5	0.49	0.35	0.63
PI 649117	India	4	1166.7	0.43	0.26	0.63	9	1497.3	0.56	0.37	0.73
PI 432383	Bangladesh	5	1172.6	0.44	0.29	0.6	5.4	1181	0.44	0.31	0.58
PI 180264	India, Rajasthan	5	1196.1	0.44	0.33	0.57	6.7	1389.5	0.52	0.39	0.64
PI 181040	Pakistan, Sind	3	1192.4	0.44	0.28	0.62	3	968.2	0.36	0.19	0.58
PI 426375	Pakistan	4	1192.1	0.44	0.28	0.62	4.5	1128	0.42	0.25	0.61
PI 426312	Pakistan	4	1184.2	0.44	0.34	0.54	3.5	1040.3	0.39	0.24	0.55
PI 649104	India	5.5	1176.4	0.44	0.28	0.6	9	1498.4	0.56	0.38	0.72
PI 340213	India	4	1179.2	0.44	0.32	0.56	4	923.7	0.34	0.23	0.48
PI 426178	Afghanistan	5	1195.2	0.44	0.3	0.6	4.8	1018.8	0.38	0.22	0.57
PI432389	Bangladesh	3.5	1172.4	0.44	0.27	0.62	4.5	1132.6	0.42	0.28	0.58
PI 173874	India, Delhi	6	1204.9	0.45	0.24	0.67	6.5	1326.6	0.49	0.31	0.68
PI 183115	India, Gujarat	3	1222.9	0.45	0.26	0.66	9	1362.1	0.51	0.3	0.71
PI 212594	Afghanistan, Nangarhar	5	1204.2	0.45	0.25	0.67	3	987.8	0.37	0.19	0.59
PI 249555	Thailand	3	1204.7	0.45	0.28	0.63	6	1245.8	0.46	0.27	0.67
PI 280637	Ethiopia	3	1212.8	0.45	0.26	0.66	3	1014.6	0.38	0.23	0.55
PI 370745	India	3	1199.8	0.45	0.25	0.66	9	1552.5	0.58	0.42	0.72
PI 390137	Pakistan	5.5	1205.1	0.45	0.27	0.64	5.5	1358.8	0.5	0.33	0.68
PI 531271	Hungary	5.1	1211.1	0.45	0.31	0.6	4.8	1021.5	0.38	0.26	0.51
PI 426315	Pakistan	4.5	1219.6	0.45	0.28	0.64	6	1386.9	0.52	0.34	0.69
PI 633116	India, Delhi	5.5	1206.8	0.45	0.27	0.64	7	1393	0.52	0.37	0.66
PI 179855	India, Rajasthan	5.5	1242.1	0.46	0.29	0.65	5.5	1329.5	0.49	0.32	0.67
PI 181035	Pakistan, Punjab	6	1241.4	0.46	0.25	0.68	6	1127.2	0.42	0.24	0.62

(continued)

Table A.2. Median, mean rank, and relative treatment effects along with 95% confidence intervals for blackleg severity ratings in relation to *B. juncea* accessions and PGT and PG4 isolates of *Leptosphaeria maculans.*(continued)

		PGT					PG4				
Accession	**Origin**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**
PI 311734	Poland	3.5	1237.8	0.46	0.27	0.67	9	1800.6	0.67	0.51	0.79
PI 340214	India	4.5	1241	0.46	0.26	0.68	7.5	1385.8	0.51	0.35	0.68
PI 347615	India	3.5	1227	0.46	0.26	0.67	9	1547.7	0.57	0.39	0.74
PI 379103	Former Serbia & Montenegro	3	1241.1	0.46	0.27	0.67	9	1666.7	0.62	0.49	0.74
PI 390139	Pakistan	6	1235.4	0.46	0.26	0.67	9	1509.4	0.56	0.38	0.72
PI 459002	India	5.5	1231.6	0.46	0.31	0.61	9	1681.1	0.62	0.5	0.73
PI 181033	Pakistan, Punjab	9	1274.8	0.47	0.25	0.71	3.5	1105.7	0.41	0.24	0.61
PI 426291	Pakistan	9	1275.7	0.47	0.25	0.71	9	1469.8	0.55	0.37	0.71
PI 426326	Pakistan	4.5	1277.9	0.47	0.3	0.65	7.5	1326.6	0.49	0.34	0.65
PI 426332	Pakistan	3.5	1274.3	0.47	0.32	0.64	9	1513.2	0.56	0.39	0.72
PI 426339	Pakistan	4.5	1268.9	0.47	0.28	0.67	6.5	1387.6	0.52	0.35	0.68
PI 426367	Pakistan	4	1260.2	0.47	0.31	0.63	5	1333.1	0.5	0.32	0.67
PI 470241	Indonesia	4	1276.8	0.47	0.28	0.67	9	1548.4	0.58	0.37	0.76
PI 426314	Pakistan	5.5	1256.4	0.47	0.3	0.64	3	1019.3	0.38	0.21	0.59
PI 649108	India	4.5	1267.1	0.47	0.31	0.64	3.5	882.8	0.33	0.21	0.47
PI633087	Russian, Federation	6	1274.7	0.47	0.32	0.64	3	743.8	0.28	0.16	0.43
PI633092	Russian, Federation	5.5	1276.8	0.47	0.37	0.59	4.5	1119.4	0.42	0.28	0.57
PI 181042	Pakistan, Sind	4.5	1288.4	0.48	0.28	0.68	9	1415.1	0.53	0.32	0.72
PI 250140	Pakistan, Punjab	4	1297.9	0.48	0.29	0.68	2.5	1018.8	0.38	0.2	0.6
PI 340211	India	4.5	1282.2	0.48	0.3	0.66	5.5	1409.1	0.52	0.39	0.65
PI 426343	Pakistan	6.5	1301.8	0.48	0.31	0.66	6	1377	0.51	0.35	0.67
PI 478327	China	3.5	1295.9	0.48	0.3	0.67	9	1583.4	0.59	0.4	0.76
PI 633084	US, California	5.4	1301.4	0.48	0.36	0.61	6	1294.4	0.48	0.36	0.6
PI458934	Sweden	9	1287.8	0.48	0.26	0.71	9	1202.3	0.45	0.22	0.69

(continued)

Table A.2. Median, mean rank, and relative treatment effects along with 95% confidence intervals for blackleg severity ratings in relation to *B. juncea* accessions and PGT and PG4 isolates of *Leptosphaeria maculans.*(continued)

		PGT					PG4				
Accession	**Origin**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**
PI 181017	Pakistan, Sind	6	1310.4	0.49	0.29	0.69	9	1374.3	0.51	0.31	0.71
PI 257240	China	4	1331.7	0.49	0.32	0.68	6	1286.6	0.48	0.3	0.67
PI 271453	India, Gujarat	8.5	1320.6	0.49	0.29	0.7	5.5	1234	0.46	0.27	0.66
PI 390135	Pakistan	9	1313.1	0.49	0.27	0.71	9	1622.5	0.6	0.45	0.73
PI 390141	Pakistan	9	1315.4	0.49	0.27	0.71	9	1523.4	0.57	0.4	0.72
PI 426334	Pakistan	5	1324.2	0.49	0.32	0.66	5	1307.6	0.49	0.34	0.63
PI 426337	Pakistan	7	1326.1	0.49	0.32	0.66	4.5	1227	0.46	0.3	0.62
PI 426371	Pakistan	6	1307.7	0.49	0.31	0.66	4	1124.1	0.42	0.28	0.57
PI 426408	Pakistan	3	1323.9	0.49	0.32	0.67	9	1860.5	0.69	0.57	0.79
PI 478332	China	4.5	1310.8	0.49	0.3	0.68	9	1542.4	0.57	0.37	0.75
PI 426296	Pakistan	5.5	1320.3	0.49	0.32	0.66	8	1492.6	0.55	0.4	0.7
PI 633103	Pakistan	3.5	1321.5	0.49	0.31	0.67	9	1664.1	0.62	0.46	0.76
PI 180266	India, Gujarat	6	1312.1	0.49	0.3	0.68	5.5	1377.7	0.51	0.34	0.68
PI 180420	India, Rajasthan	6	1355.9	0.5	0.32	0.69	2.5	992.7	0.37	0.19	0.59
PI 426356	Pakistan	7	1333.8	0.5	0.3	0.69	9	1647.7	0.61	0.47	0.74
PI 426362	Pakistan	6	1339.9	0.5	0.34	0.66	6	1202.5	0.45	0.28	0.63
PI 426377	Pakistan	6	1345.7	0.5	0.34	0.66	7.5	1415.9	0.53	0.36	0.68
PI 458996	India	4	1346.8	0.5	0.34	0.66	4.5	1215.3	0.45	0.29	0.63
PI 500675	Zambia	5.5	1341.2	0.5	0.31	0.68	9	1290.6	0.48	0.27	0.7
PI 537021	Korea, South	3.5	1344.6	0.5	0.32	0.68	6	1173.4	0.44	0.26	0.63
PI 175068	India, Uttar Pradesh	7	1379.3	0.51	0.32	0.7	9	1606.8	0.6	0.4	0.77
PI 250137	Pakistan, North-West Frontier	7	1371.2	0.51	0.32	0.7	9	1576.9	0.59	0.41	0.74
PI 370744	India	9	1367.7	0.51	0.29	0.72	7.5	1593.9	0.59	0.47	0.71
PI 426322	Pakistan	5	1370.2	0.51	0.33	0.68	5	1167.5	0.43	0.3	0.58

(continued)

Table A.2. Median, mean rank, and relative treatment effects along with 95% confidence intervals for blackleg severity ratings in relation to *B. juncea* accessions and PGT and PG4 isolates of *Leptosphaeria maculans.*(continued)

		PGT					PG4				
Accession	**Origin**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**
PI 426331	Pakistan	4	1379.3	0.51	0.37	0.65	5	1352.9	0.5	0.33	0.67
PI 426336	Pakistan	8.5	1360.7	0.51	0.31	0.7	5.5	1332.6	0.5	0.32	0.67
PI 531269	China	4	1379.1	0.51	0.34	0.68	4	1321.1	0.49	0.32	0.67
PI 633077	Canada	9	1376.4	0.51	0.31	0.71	9	1419.2	0.53	0.33	0.71
PI 426300	Pakistan	6.5	1376.4	0.51	0.34	0.68	9	1660.3	0.62	0.44	0.76
PI 179191	Turkey, kirklareli	6.5	1359.6	0.51	0.31	0.7	3	833.6	0.31	0.17	0.5
PI 180421	India, Rajasthan	7.5	1380.4	0.51	0.33	0.69	6.5	1480.3	0.55	0.38	0.71
PI458993	India	6.2	1371	0.51	0.37	0.65	3.6	746	0.28	0.19	0.38
PI 426324	Pakistan	6	1394.2	0.52	0.38	0.66	5.5	1317.3	0.49	0.34	0.64
PI 426361	Pakistan	5.5	1390.2	0.52	0.37	0.66	9	1533.8	0.57	0.39	0.73
PI 478329	China	9	1406.3	0.52	0.32	0.72	9	1637.1	0.61	0.43	0.76
PI 426294	Pakistan	7	1397.1	0.52	0.34	0.7	3.5	1082.7	0.4	0.23	0.6
PI 175082	India, Uttar Pradesh	9	1422.6	0.53	0.33	0.72	9	1710.8	0.64	0.44	0.8
PI 179192	Turkey, Edirne	5.5	1420.4	0.53	0.36	0.69	7	1257.4	0.47	0.27	0.67
PI 390138	Pakistan	6	1427.7	0.53	0.36	0.69	6.8	1505.3	0.56	0.41	0.7
PI 426323	Pakistan	7	1437.1	0.53	0.36	0.7	6	1383.7	0.51	0.35	0.68
PI 426329	Pakistan	7.5	1434.3	0.53	0.36	0.7	5.5	1378.8	0.51	0.35	0.67
PI 426390	Pakistan	6	1426.4	0.53	0.36	0.69	9	1526.1	0.57	0.4	0.72
PI 426405	Pakistan	7	1417.3	0.53	0.34	0.7	4.5	1193.9	0.44	0.28	0.62
PI 432388	Bangladesh	9	1435.6	0.53	0.33	0.73	9	1388.3	0.52	0.31	0.72
PI 633089	Russian, Federation	7	1429.7	0.53	0.35	0.7	7	1239.1	0.46	0.27	0.67
PI 633097	China, Xizang	5.5	1436.9	0.53	0.4	0.66	4	761.6	0.28	0.15	0.48
PI 169085	Turkey, Tekirdag	9	1465.4	0.54	0.31	0.76	9	1286.5	0.48	0.26	0.7
PI 426321	Pakistan	4.5	1442.7	0.54	0.38	0.69	5.5	1243.5	0.46	0.31	0.62
PI 426328	Pakistan	7	1462.7	0.54	0.4	0.68	6	1455.8	0.54	0.39	0.68

(continued)

Table A.2. Median, mean rank, and relative treatment effects along with 95% confidence intervals for blackleg severity ratings in relation to *B. juncea* accessions and PGT and PG4 isolates of *Leptosphaeria maculans.*(continued)

		PGT					PG4				
Accession	**Origin**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**
PI 426366	Pakistan	5.5	1461.9	0.54	0.42	0.66	9	1425.7	0.53	0.34	0.71
PI 531270	Former Soviet Union	7	1467	0.54	0.38	0.7	9	1298.9	0.48	0.27	0.7
PI 633086	Russian, Federation	9	1466.4	0.54	0.35	0.72	4	1181.4	0.44	0.27	0.62
PI 426298	Pakistan	6	1450.8	0.54	0.38	0.69	5	1221	0.45	0.28	0.64
PI449438	Pakistan	7.5	1451.3	0.54	0.36	0.71	3	924.8	0.34	0.19	0.54
PI 179644	India, Rajasthan	9	1489.4	0.55	0.36	0.73	9	1812.3	0.67	0.53	0.79
PI 340223	India	9	1491.3	0.55	0.35	0.74	7	1431.6	0.53	0.37	0.69
PI 387819	Thailand	9	1468.3	0.55	0.31	0.76	9	2016.7	0.75	0.7	0.8
PI 426364	Pakistan	6	1475.1	0.55	0.39	0.69	9	1642	0.61	0.46	0.74
PI 426369	Pakistan	5	1479.3	0.55	0.39	0.7	6.5	1345.2	0.5	0.35	0.65
PI 426380	Pakistan	7	1468.5	0.55	0.41	0.67	5	1420.5	0.53	0.37	0.68
PI 449437	Pakistan	6.5	1481	0.55	0.38	0.71	9	1475.9	0.55	0.36	0.72
PI 458942	Germany	6.5	1486.7	0.55	0.39	0.7	9	1600.4	0.59	0.4	0.77
PI 633090	Russian, Federation	9	1485.8	0.55	0.32	0.76	9	1503.4	0.56	0.35	0.75
PI 426293	Pakistan	9	1483.9	0.55	0.35	0.74	6.5	1408.3	0.52	0.36	0.68
PI 120923	Turkey, Izmir	9	1498.8	0.56	0.33	0.76	9	1446.2	0.54	0.34	0.72
PI 175602	Turkey, Kayseri	7	1515.9	0.56	0.4	0.72	9	1467.9	0.55	0.36	0.72
PI 179183	Turkey, Tekirdag	9	1510.1	0.56	0.35	0.75	9	1525.9	0.57	0.39	0.73
PI 426406	Pakistan	9	1508.9	0.56	0.38	0.73	9	1716.8	0.64	0.44	0.8
PI 458995	India	7	1496.4	0.56	0.42	0.68	4.5	1107.5	0.41	0.24	0.61
PI 603012	Germany	9	1502.1	0.56	0.37	0.73	9	1706.2	0.63	0.48	0.77
PI 426302	Pakistan	9	1515.4	0.56	0.36	0.74	6	1174.3	0.44	0.26	0.63
PI 633115	Mongolia	9	1516.6	0.56	0.37	0.74	5	1269.7	0.47	0.32	0.63
PI478325	China	9	1512.7	0.56	0.37	0.73	3.5	1050.9	0.39	0.21	0.61
PI 432386	Bangladesh	7	1537.9	0.57	0.43	0.71	6.7	1437	0.53	0.36	0.7

(continued

Table A.2. Median, mean rank, and relative treatment effects along with 95% confidence intervals for blackleg severity ratings in relation to *B. juncea* accessions and PGT and PG4 isolates of *Leptosphaeria maculans.*(continued)

		PGT					PG4				
Accession	**Origin**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**
PI 340218	India	8	1527.8	0.57	0.39	0.73	9	1678.5	0.62	0.49	0.74
PI 426363	Pakistan	9	1540.9	0.57	0.39	0.74	5	1192.2	0.44	0.25	0.65
PI 603011	India, Uttar Pradesh	6.5	1527.7	0.57	0.42	0.7	9	1643.6	0.61	0.47	0.74
PI 633098	India, Punjab	6.5	1540.8	0.57	0.41	0.72	9	2085	0.77	0.77	0.78
PI 649110	India	6.8	1537	0.57	0.44	0.69	6.8	1460.7	0.54	0.41	0.67
PI 649111	India	7	1543.6	0.57	0.42	0.71	9	1480.6	0.55	0.36	0.73
PI 179636	India, Rajasthan	9	1568.9	0.58	0.4	0.75	6.5	1240.7	0.46	0.26	0.67
PI 340220	India	9	1565.8	0.58	0.36	0.77	9	1701.3	0.63	0.48	0.76
PI 426365	Pakistan	6	1557.4	0.58	0.44	0.71	6.5	1247.5	0.46	0.3	0.64
PI 426383	Pakistan	6.5	1560.8	0.58	0.44	0.71	9	1532.1	0.57	0.4	0.72
PI 478326	China	9	1562.5	0.58	0.41	0.73	9	1664.7	0.62	0.45	0.76
PI 175607	Turkey, Tekirdag	9	1578.6	0.59	0.4	0.75	9	1567.9	0.58	0.41	0.74
PI 340212	India	9	1586.9	0.59	0.38	0.77	9	1682.9	0.63	0.47	0.76
PI 340219	India	9	1600.1	0.59	0.42	0.75	9	1664.1	0.62	0.46	0.76
PI 340221	India	9	1591.3	0.59	0.38	0.77	5.5	1508	0.56	0.42	0.69
PI 426292	Pakistan	8.5	1594.8	0.59	0.44	0.73	9	1581.4	0.59	0.4	0.75
PI 426359	Pakistan	9	1578.7	0.59	0.42	0.74	9	1578	0.59	0.39	0.76
PI 426360	Pakistan	6.5	1576.3	0.59	0.45	0.71	9	1507.1	0.56	0.39	0.72
PI 426384	Pakistan	9	1592.1	0.59	0.42	0.74	3	1031.1	0.38	0.21	0.59
PI 426404	Pakistan	9	1581.9	0.59	0.43	0.73	9	1854.4	0.69	0.57	0.79
PI 458943	Germany	9	1580.4	0.59	0.42	0.73	9	1717.6	0.64	0.49	0.76
PI 478337	China	9	1593.1	0.59	0.39	0.76	9	1625.1	0.6	0.43	0.76
PI 209781	Germany	9	1599.4	0.59	0.42	0.74	9	1765.4	0.66	0.53	0.76
PI 215636	India, Haryana	9	1588.4	0.59	0.42	0.74	9	1509.6	0.56	0.38	0.72
PI 426379	Pakistan	9	1606.9	0.6	0.43	0.75	2.5	960.2	0.36	0.18	0.58

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Table A.2. Median, mean rank, and relative treatment effects along with 95% confidence intervals for blackleg severity ratings in relation to *B. juncea* accessions and PGT and PG4 isolates of *Leptosphaeria maculans.*(continued)

		PGT					PG4				
Accession	**Origin**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**
PI 478333	China	9	1623.8	0.6	0.44	0.74	9	1679.7	0.62	0.47	0.76
PI 633083	US, California	9	1601.8	0.6	0.41	0.76	7	1363.2	0.51	0.33	0.68
PI 183117	India, Gujarat	9	1635.6	0.61	0.43	0.76	9	1381.2	0.51	0.32	0.7
PI 458978	US, Oregon	9	1635.3	0.61	0.45	0.74	9	1557.4	0.58	0.4	0.74
PI 478331	China	9	1654.4	0.61	0.47	0.74	9	1817.2	0.68	0.52	0.8
PI 633112	Cuba, Santiago de Cuba	9	1655.3	0.61	0.47	0.74	9	1612.1	0.6	0.42	0.76
PI 432380	Bangladesh	7.4	1663.5	0.62	0.49	0.73	9	2085	0.77	0.77	0.78
PI 208734	Cuba	9	1672.6	0.62	0.44	0.77	9	1812.8	0.67	0.53	0.79
PI 340215	India	8	1655.7	0.62	0.55	0.68	9	2085	0.77	0.77	0.78
PI 426402	Pakistan	9	1672.6	0.62	0.44	0.77	9	1725.7	0.64	0.5	0.76
PI 458994	India	9	1672.7	0.62	0.48	0.75	6.5	1468.7	0.55	0.39	0.69
PI 426358	Pakistan	9	1698.9	0.63	0.49	0.75	9	1868.6	0.69	0.58	0.79
PI 432377	Bangladesh	9	1691.3	0.63	0.49	0.75	9	1403.9	0.52	0.33	0.71
PI 432378	Bangladesh	9	1698.3	0.63	0.5	0.75	9	1485	0.55	0.37	0.72
PI 649120	Russian, Federation	9	1702.2	0.63	0.49	0.75	6	1237.6	0.46	0.33	0.6
PI478335	China	9	1682.9	0.63	0.44	0.78	4	1141.8	0.42	0.23	0.65
PI 432379	Bangladesh	7	1727.3	0.64	0.54	0.73	9	1703.7	0.63	0.47	0.77
PI 432390	Bangladesh	9	1714.3	0.64	0.5	0.75	9	1478.2	0.55	0.33	0.75
PI 458992	India	9	1715.1	0.64	0.46	0.78	9	1603.1	0.6	0.44	0.73
PI 478328	China	9	1727.7	0.64	0.51	0.75	9	1702.9	0.63	0.51	0.74
PI 426305	Pakistan	9	1709.9	0.64	0.46	0.78	7	1380.5	0.51	0.33	0.69
PI531272	Hungary	9	1712.8	0.64	0.48	0.77	9	1629.7	0.61	0.46	0.74
PI 633111	Germany	9	1757.3	0.65	0.46	0.81	9	1860.5	0.69	0.57	0.79
PI 432387	Bangladesh	9	1745.5	0.65	0.49	0.78	9	1703.8	0.63	0.47	0.77
PI 478334	China	9	1762.5	0.65	0.5	0.78	9	1628.2	0.6	0.45	0.74

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Table A.2. Median, mean rank, and relative treatment effects along with 95% confidence intervals for blackleg severity ratings in relation to *B. juncea* accessions and PGT and PG4 isolates of *Leptosphaeria maculans.*(continued)

		PGT					PG4				
Accession	**Origin**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**
PI 633085	US, California	9	1738.2	0.65	0.48	0.78	9	1502.9	0.56	0.38	0.72
PI 426301	Pakistan	9	1745.6	0.65	0.49	0.78	9	1801.2	0.67	0.56	0.76
PI 426348	Pakistan	9	1767.2	0.66	0.48	0.8	8	1571.8	0.58	0.44	0.71
PI 169077	Turkey	9	1808.8	0.67	0.49	0.81	9	1859.6	0.69	0.51	0.83
PI 192936	China	9	1803.3	0.67	0.52	0.79	9	1755.6	0.65	0.55	0.74
PI 426319	Pakistan	9	1799.3	0.67	0.53	0.78	9	1817.3	0.68	0.57	0.76
PI 633094	Russian, Federation	9	1810.9	0.67	0.51	0.8	9	2007.1	0.75	0.68	0.8
PI 209021	Cuba	9	1829.1	0.68	0.51	0.81	9	1709.8	0.64	0.52	0.74
PI 347618	India	9	1830.4	0.68	0.59	0.76	9	1672.8	0.62	0.47	0.75
PI 432367	Bangladesh	9	1827.1	0.68	0.51	0.81	9	1855.5	0.69	0.56	0.8
PI 458927	Canada, Saskatchewan	9	1823.8	0.68	0.56	0.78	9	1565.1	0.58	0.38	0.76
PI 458929	Canada, Saskatchewan	9	1859.2	0.69	0.61	0.76	9	2085	0.77	0.77	0.78
PI 426318	Pakistan	9	1866.1	0.69	0.55	0.8	6.5	1345.4	0.5	0.32	0.68
PI 633099	US, California	9	1845.8	0.69	0.57	0.78	9	1923.8	0.71	0.58	0.82
PI 633114	Korea, North	9	1852.6	0.69	0.58	0.78	9	1852	0.69	0.57	0.79
PI 212082	Afghanistan, Kondoz	9	1901.6	0.71	0.58	0.81	5	1312.8	0.49	0.34	0.64
PI 633096	Russian, Federation	9	1908.6	0.71	0.59	0.81	9	1965.4	0.73	0.63	0.81
PI 633110	Germany	9	1913.4	0.71	0.6	0.8	9	1670.6	0.62	0.45	0.77
PI 436559	China	9	1932.4	0.72	0.61	0.8	9	1955.1	0.73	0.62	0.81
PI 458928	Canada, Saskatchewan	9	1928.1	0.72	0.61	0.81	9	1923.8	0.71	0.58	0.82
PI 174801	India, Uttar Pradesh	9	1957.1	0.73	0.59	0.83	9	2085	0.77	0.77	0.78
PI 195553	Ethiopia, Welo	9	1981.4	0.74	0.62	0.83	9	1781.7	0.66	0.48	0.8
PI 271455	India, Himachal Pradesh	9	2026.4	0.75	0.67	0.82	9	2085	0.77	0.77	0.78
PI 633091	Russian, Federation	9	2026.4	0.75	0.67	0.82	9	1776.5	0.66	0.54	0.76
PI 633113	Italy, Calabria	9	2020.6	0.75	0.66	0.82	9	1990.4	0.74	0.66	0.8

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Table A.2. Median, mean rank, and relative treatment effects along with 95% confidence intervals for blackleg severity ratings in relation to *B. juncea* accessions and PGT and PG4 isolates of *Leptosphaeria maculans.*(continued)

		PGT					PG4				
Accession	**Origin**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**	**Median**	**Mean rank**	**RE**	**Lower**	**Upper**
PI169078	Turkey	9	2127.5	0.79	0.78	0.8	9	1465.2	0.54	0.35	0.73
Westar		9	2127.5	0.79	0.78	0.8	9	2085	0.77	0.77	0.78