Introgression of resistance to *Leptosphaeria maculans* from *Brassica juncea* into *B. napus* and analysis of blackleg resistance in synthetic hexaploid *Brassica* species

by

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ABSTRACT

Kang-Choi, Minkyung, M. Sc., The University of Manitoba, August, 2016. Introgression of resistance to *Leptosphaeria maculans* from *Brassica juncea* into *B. napus* and analysis of blackleg resistance in synthetic hexaploid *Brassica* species. Major professor: Dr. Genyi Li.

Canola (*B. napus* L.), one of the most valuable oilseed crops in the world, has been reported with significant crop losses up to 100% due to blackleg disease caused by *Leptosphaeria maculans*. Genetic resistance is a primary method to control blackleg, and the highest levels of resistance can be introduced from *Brassica* species containing the B-genome through interspecific hybridization. With successful introgression of resistance, the BC_3 recombinant lines, derived between resistant *B. juncea* UM lines and susceptible *B. napus* L. cv. Westar, showed high levels of resistance to two *L. maculans* isolates 03-15-03 and PG4-1M at the seedling stage. In the analysis of blackleg resistance in synthetic hexaploid *Brassica* lines using susceptible *B. juncea* UM3086, the resistance against *L. maculans* isolate 03-15-03 in F_2, BC_1, BC_2 and BC_2F_2 populations indicated that two genes are inherited in backcross populations and each gene can confer the same level of blackleg resistance. The high number of resistant phenotypes and compatibility of interspecific crosses in hexaploids crossed with tetraploid *Brassica* imply that synthetic lines are a feasible tool for developing blackleg resistance.

FORWORD

This thesis is written in manuscript style. A general introduction and review of literature precedes manuscripts that comprise the main part of the thesis. Each manuscript consists of an abstract, introduction, materials and methods, results and discussion. A general discussion and conclusions, a list of references and appendices follow the manuscripts.

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CHAPTER 1. GENERAL INTRODUCTION

1.0 GENERAL INTRODUCTION

Brassica napus L. is an oilseed rape and belongs to the Cruciferae family, also known as mustard family. The rapeseed *B. napus*, an allotetraploid, occurs in nature, but can also be reconstituted by interspecific crosses between two wild species, *B. rapa* and *B. oleracea* (Tsunoda 1980) (Figure 1.1). There are two other allotetraploid species, *B. juncea* and *B. carinata*, which also occur in nature or from the crosses between *B. rapa* and *B. nigra* and between *B. nigra* and *B. oleracea* respectively (Figure 1.1). Among six crop species of *Brassica*, canola (*B. napus* L.) has been cultivated extensively because of its outstanding values for vegetable oil, animal feed and bioenergy. In 1975, a variety Tower was registered as the first canola-quality *B. napus* (Stefansson and Kondra 1975), and nowadays, most *B. napus* is grown as canola quality, zero erucic acid (less than 1%) and low glucosinolate content (15 micromoles per gram of seed) (Rakow 2004).

Canola is an economically important crop in Canada and *B. napus* L. is primarily grown in western Canada (Alberta, Saskatchewan and Manitoba) as it is a cool-season crop. According to 2013 statistical reports, Canada was the top canola producing country in the world (Statista 2015). In the last decade, total canola production has been increased due to increasing world demand. In Canada, Manitoba contributes an average 18% annually (Figure 1.2). Moreover, canola has brought many economic benefits resulting in an average 19 billion dollars including direct and indirect impacts during the period between 2009 and 2011, and its annual contribution has more than doubled in the last decade (Canola Council of Canada 2016).

However, blackleg, a commonly known disease in cruciferous crops caused by the fungal pathogen *L. maculans* (Desm.) Ces. Et de Not., became epidemic since 1960s, and serious disease incidence and severe yield losses have been reported worldwide. In Canada, yield losses due to the highly virulent *L. maculans* was first reported in Saskatchewan in 1970s and afterwards, blackleg disease spread to neighboring provinces (Gugel and Petrie 1992). The disease was more serious in 1980s (Fernando et al. 2007) and during the time, the estimated yield loss was estimated to be 500 million dollars (Canola Council of Canada 2016). Additionally, a new blackleg yield loss model estimates that 17 to 24% yield loss is expected as each 20% increase in blackleg severity (Hwang et al. 2016).

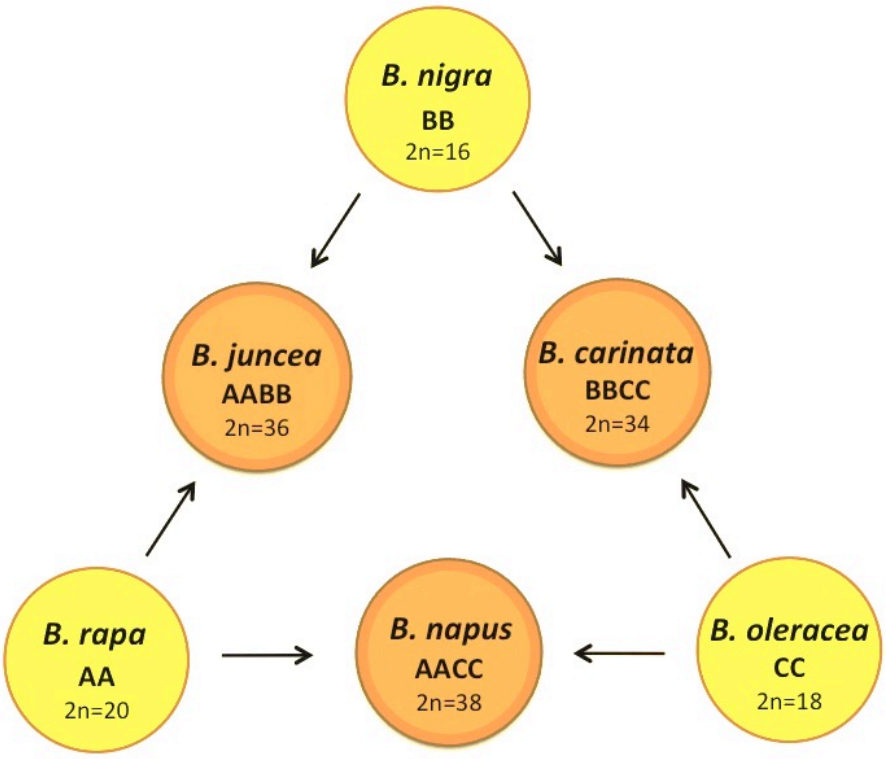

Figure 1.1. Genomic relationships among six *Brassica* species (U, 1935).

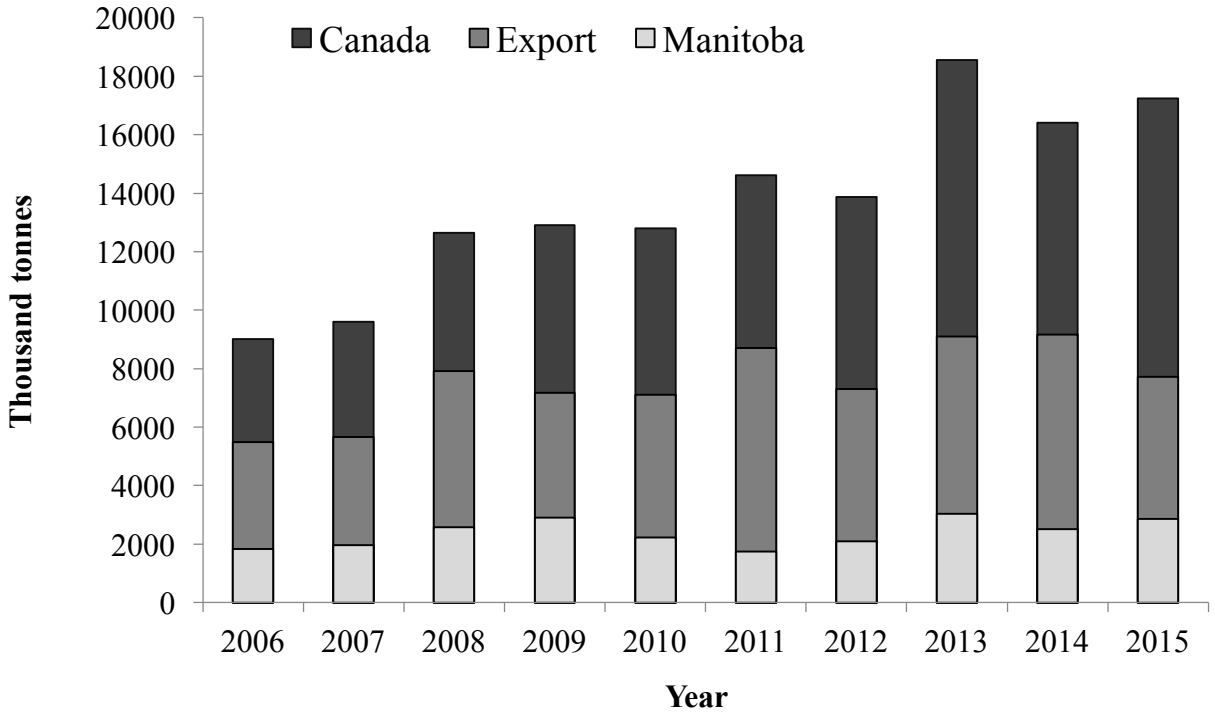

Figure 1.2. Historical data of canola production in Canada and in the province of Manitoba. Total Canada exports data of canola grains to all destinations. (Statistics Canada 2016 CANSIM Table 001-0010 (Crop production data) and CANSIM Table 001-0015 (Export data).

In general, various control methods such as cultural practices (e.g. crop rotation and stubble management), chemical treatments (e.g. fungicide application and seed treatment) and genetic resistance (e.g. resistant cultivars) are available for blackleg disease (Gugel and Petrie 1992). Among these, however, genetic resistance is the most cost-effective and environmental friendly management strategy. In western Canada, blackleg management has been successful with the combination of genetic resistance and four-year crop rotation (Peng et al. 2015). Therefore, efforts at developing resistant canola cultivars have been continued.

Genetic resistance in the plants - pathogen system is based on gene-for-gene concept that resistant occurs when the host plant resistance gene (R-gene) is present with the corresponding

avirulence gene (Avr-gene) in the virulent pathogen or vice versa as previously explained by Flor (1971). In the interaction between *B. napus* and *L. maculans*, two types of resistance exist (Ansan-Melayah et al. 1998). One is qualitative resistance (race-specific) and the other is quantitative resistance (race-nonspecific). Qualitative resistance is highly effective particularly at the seedling stages, but subsequent use of same gene resistance causes selection pressure on pathogen population. Various levels of effectiveness with quantitative resistance have been reported although it has been considered to be more durable (Jestin et al. 2015)

In western Canada, the commonly observed avirulence genes in 87 *L. maculans* isolates were *AvrLm2*, *AvrLm6*, *AvrLm10*, *AvrLepR3* from 1997 to 2000 and 2003 to 2005 (Kutcher et al. 2010a). A recent study also showed that most *L. maculans* isolates carry four or five avirulence alleles and four Avr-genes (*AvrLm2*, *AvrLm4*, *AvrLm6* and *AvrLm7*) were found mostly in 674 isolates (Liban et al. 2015). Two avirulence genes (*AvrLm2* and *AvrLm6*) seem to have appeared in the last two decades. Because variations exist in different field locations, high frequency of the other Avr-gene *AvrLm3* was reported by Balesdent et al. (2005) and Dilmaghani et al. (2009).

At this writing, at least 15 major resistance genes are known in *Brassica* species. Twelve of them (*Rlm1*, *Rlm2*, *Rlm3*, *Rlm5*, *Rlm7*, *Rlm8*, *Rlm9*, *Rlm11*, *LepR1*, *LepR2*, *LepR3* and *LepR4*) were identified in the *Brassica* A genome (Balesdent et al. 2002, 2013; Van de Wouw et al. 2009; Yu et al. 2005, 2007, 2008; Delourme et al. 2006; Rimmer 2006; Zhu and Rimmer 2003; Ansan-Melayah et al. 1998; Mayerhofer et al. 1997; Ferreira et al. 1995), and three resistance genes (*Rlm5*, *Rlm6* and *Rlm10*) were mapped in *Brassica* B genome (Chèvre et al. 1996, 1997;

Balesdent et al. 2002). Among these, only two R genes, *LepR3* and *Rlm2*, have been cloned recently (Larkan et al. 2013, 2015).

Nonetheless, a limited number of R genes in Canadian canola varieties and germplasm have been reported by Zhang et al. (2015). With a known R gene profile, a total of seven different R genes (*Rlm1*, *Rlm2*, *Rlm3*, *Rlm4*, *Rlm9*, *LepR1* and *LepR2*) were found in 104 Canadian *B. napus* accessions. Almost 56% of these varieties carry the same resistance gene, *Rlm3*. The study also reported that each of the six Avr genes (*AvrLm2*, *AvrLm4*, *AvrLm5/AvrLmJ1*, *AvrLm6*, *AvrLm7* and *AvrLm11*) were showed at greater than 60% of frequency in 300 *L. maculans* isolates collected in Manitoba in 2012 (Zhang et al. 2015).

Thus, new resistance genes are needed in the canola industry. Additionally, a field study showed the positive effect of rotation of resistant canola cultivars (Marcroft et al. 2012). This will become a more important management strategy for blackleg which can prevent single gene breakdown occurred previously in Australia and France (Rouxel et al. 2003; West et al. 2001).

Many studies have reported that high levels of blackleg resistance are found in two *Brassica* species containing the B-genome, *B. nigra* (BB) and *B. juncea* (AABB) (Chen et al. 2011; Li et al. 2004; Roy 1978, 1984). Yet, no R genes in *Brassica* B-genome have been cloned and no known R genes have been reported in *B. carinata* (BBCC). Since high frequency of two Avr-genes *AvrLm5*/*AvrLmJ1* and *AvrLm6* have appeared to be in Canadian canola fields, developing new blackleg resistant canola lines carrying the corresponding R genes such *Rlm5* and *Rlm6*, that are previously mapped in *B. juncea*, would be an important strategy for canola breeders.

To introduce blackleg resistance from the *Brassica* B-genome into canola, interspecific hybridization is sometimes used as a conventional breeding method. Hexaploid *Brassica* lines synthesized from the crosses between *B. rapa* and *B. carinata* can be used in the interspecific hybridization with *B. napus*. Previously, synthetic hexaploid lines have been used to introduce agronomically important traits such as seed coat color, high yields and oil contents (Zou et al. 2010; Chen et al. 2010; Li et al. 2005; Rahman 2001; Meng et al. 1998). To date, no reports on blackleg resistance in hexaploid *Brassica* lines have been published.

CHAPTER 2. LITERATURE REVIEW

2.0 LITERATURE REVIEW

2.1 *Brassica* species

2.1.1 Canola description

Canola (*Brassica napus* L.) is one of the most valuable oilseeds in the world as its final products provide benefits to human and animals. In Canada, canola is mainly produced for vegetable oil, animal feed as well as biofuel feedstock (Canola Council of Canada 2016). According to Canadian Grain Commission (2016), the oil content in canola seed is at least double as high (44.2%) than in soybean (21.5%), which is also used for cooking oil (Figure 2.1). However, protein content is about a half in canola seeds (20.9 g per kg of dry matter) compared to soybean (39.6 g per kg of dry matter). No significant differences in oil and protein contents were found between canola and flaxseed.

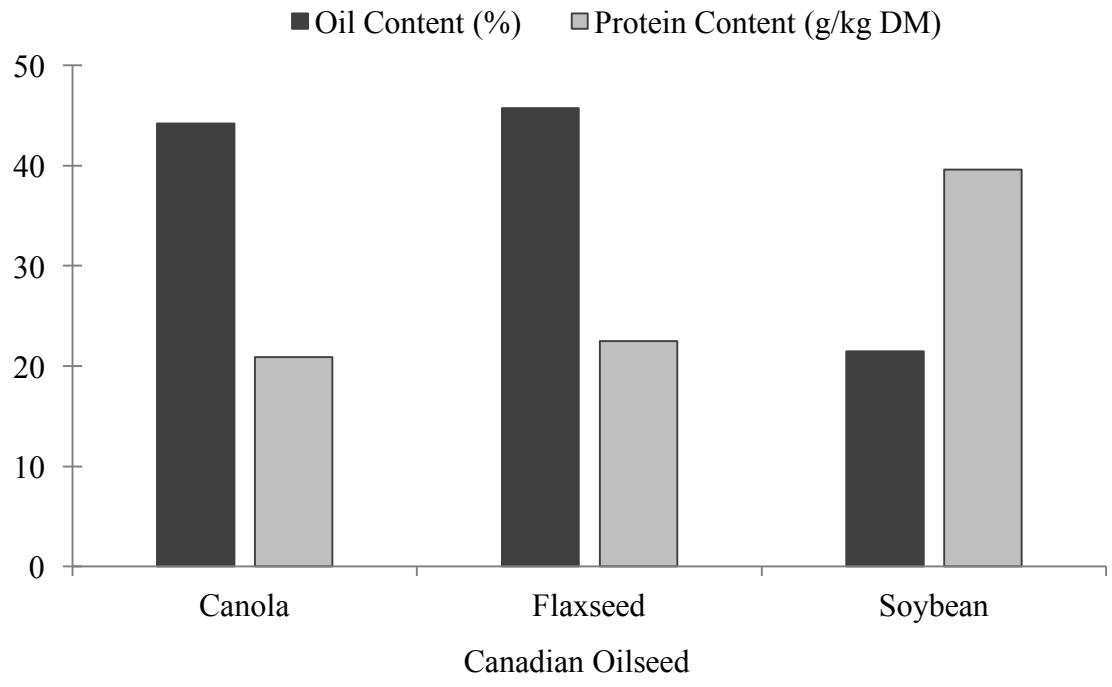

Figure 2.1. Mean Oil and protein contents in Canadian oilseeds harvested in 2015. Canadian Grain Commission (2016) – Harvest and export quality reports on Canadian grain

In regards to nutrition, canola is known as the healthiest vegetable oil among oilseeds. Canola oil has the lowest saturated fatty acid content (7 g per 100 g fat) and contains an ideal ratio of omega-6/omega-3 essential fatty acids. (Figure 2.2). Previously, unbalanced 15/1 ratio of omega-6/omega-3 in western diets was reported, and series of clinical trials showed the 3/1 ratio is recommended (Simopoulos 2002). Another study about the effect of omega-6/omega-3 ratio in different dietary oil consumption (sunflower oil vs. soybean oil) showed that the imbalanced ratio can negatively affect hormone secretion (Oliva et al. 2014). Moreover, most plant oils have either excessive amount of omega-6 and deficient in omega-3 or high in saturated fatty acids (Figure 2.2).

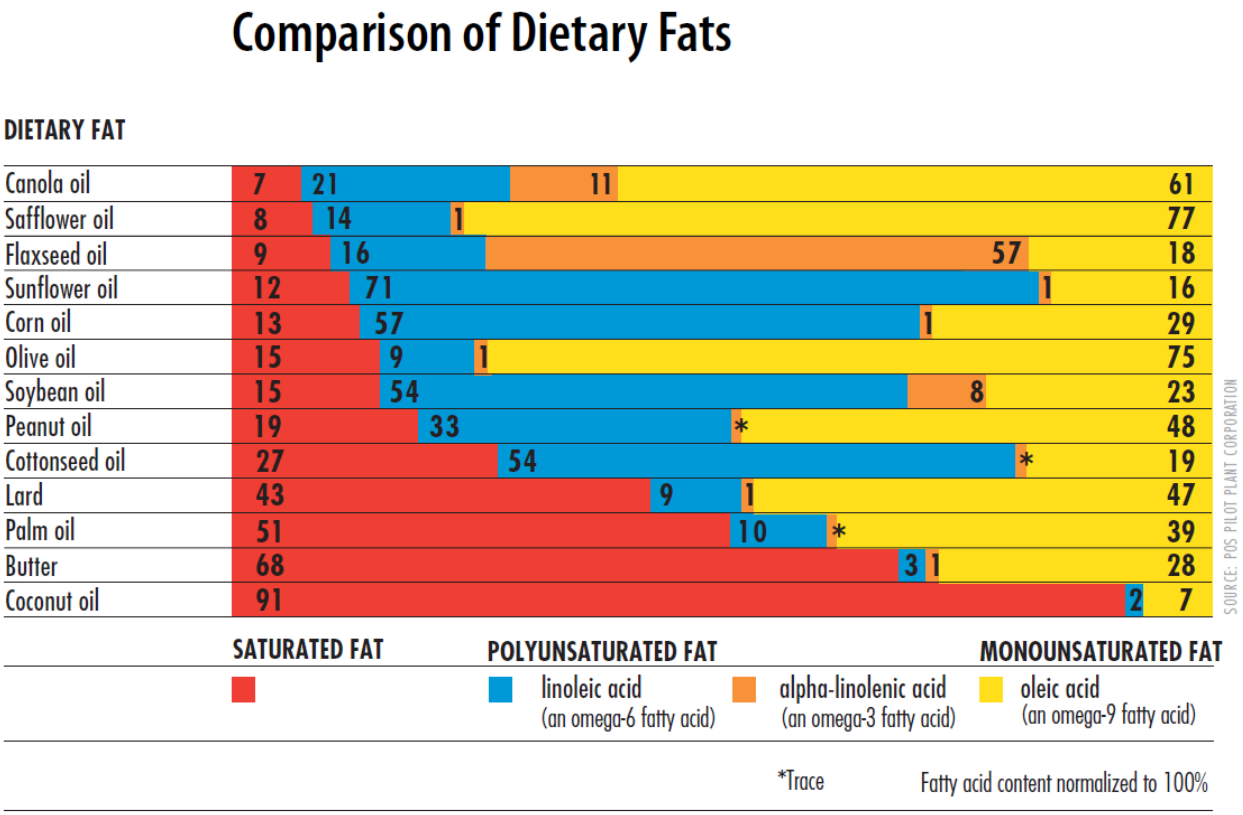

Figure 2.2 Comparison of dietary fatty acids. Canola Council of Canada (2016).

In recent years, canola seed after oil extraction is used as a great source of protein for animals (Canola Council of Canada 2016). Additionally, a demand of canola quality rapeseed from European countries has been increased for use of biodiesel that is environmentally friendly. Also, canola is suitable as a biofuel because it has a low level of saturated fatty acids under cool temperatures (Rakow 2004).

In general, canola is named after the development of a variety that contains a low erucic acids concentration (Downey and Röbbelen 1989). Two types of canola, *B. rapa* (Polish type) and *B. napus* (Argentine type), have been widely grown in western Canada (Downey and Röbbelen 1989). Before, rapeseed was grown and produced mainly for industrial uses such as lubricants and lamp oil (Downey and Röbbelen 1989). The outstanding features of rapeseed for industrial purposes were later found to be due to the high level of erucic acids in the oil (Cook and McMaster 2002). Some Asian countries used rapeseed as cooking oils and condiments; some *Brassica* species were also grown as vegetable crops. Rapeseed has winter types and spring types, and winter type rapeseeds are widely grown in Europe because of its adaptability to cold temperature (Kimber and McGregor 1995). In contrast, spring types are mostly grown in western Canada because winter types did not grow well during Winter growing season (Downey and Röbbelen 1989).

2.1.2. *Other Brassica* species

In *Brassica* crop species, there are three diploid (*B. rapa*, *B. nigra* and *B. oleracea*) and three allotetraploid species (*B. juncea*, *B. napus* and *B. carinata*). Within diploid species, *B. rapa* L.

(AA genome, n=10, previously known as *B. campestris* L.) is a vegetable crop, in which three forms existed: Polish oilseed rape, Chinese cabbage, and turnip (Prakash and Hinata 1980). The most distinctive character of *B. rapa* is its adaptability to extreme cold temperature and short growing season (Tsunoda 1980). Because of this, *B. rapa* has been adapted rapidly in longer Winter regions such as Europe and spread to Asian countries (Nishi 1980). Pakash and Hinata (1980) state that *B. rapa* cultivars of different geographical origins may have different genotypes.

B. nigra (L.) Koch (BB genome, n=8) is a wild species, a weed (Tsunoda 1980). On the other hand, another wild species, *B. oleracea* L. (CC genome, n=9) is a leafy vegetable crop, which has fleshy, hairless leaves and is defined as a woody plant. There are six major types of varieties that are commonly known as kales, cabbages, kohlrabi, inflorescence kales such as broccolis, branching bush kales, and Chinese kale (Snogerup 1980).

In allotetraploid *Brassica* species, the term allotetraploid means that the existing species or cultivar is derived from two different parental species having a complete diploid set of chromosomes from each parent (Kellogg 2003). Another term for allotetraploid is amphidiploid. On the other hand, autotetraploids have a complete diploid chromosome set from the same species (Comai 2005).

An example of an allotetraploid, *B. napus* L. (AACC genome, n=19) is derived from crosses between *B. rapa* and *B. oleracea*, and have been cultivated for high yielding oilseeds worldwide (Tsunoda 1980). While canola quality *B. napus* is mainly produced in Canada, High Erucic Acid

Rapeseed (HEAR) varieties are also grown under contract production for industrial uses (Rakow 2004). Another allotetraploid *Brassica juncea* (L.) Czern & Coss (AABB genome, n=18) is derived from crosses between *B. rapa* and *B. nigra*, and there are yellow seed-type (yellow mustard) and brown-seed type (Indian mustard). In western Canada, Indian mustard is primarily grown, and both seed types are high in erucic acid (greater than 30%) and in glucosinolates (150 micromoles per g seed). *Brassica juncea* has various useful traits such as heat and drought tolerance, and shattering and disease resistance (Rimmer and van den Berg 1992; Downey and Röbbelen 1989). In addition, yellow-seeded *B. juncea* has low fiber content, which provides high percentage of oil and protein (Edwards et al. 2007).

Brassica carinata A. Braun (BBCC genome, n=17) is developed from a cross between *B. nigra* and *B. oleracea*. It is also called Ethiopian mustard and only cultivated forms are known (Rakow 2004). *Brassica carinata* contains high erucic acid (35 to 44%) and has a longer growing season. However, it produces high yields and has the adaptability to moist and cool temperate conditions (Cargeeg and Thurling 1980). Other various traits such as resistance to disease, insect pest and shattering are also reported in *B. carinata* (Alemayehu and Becker 2001)

2.1.3 Development of Synthetic Brassica lines

For various purposes, allohexaploid *Brassica* accessions have been developed through interspecific hybridization (Chen et al. 2011; Zou et al. 2010; Chen et al. 2010; Li et al. 2005; Rahman 2001; Meng et al. 1998). The goal has been to increase genetic diversity particularly for oilseed *B. napus*, and to improve seed quality for various end uses and utilize in *Brassica* breeding programs. Previously, synthetic hexaploid lines (AABBCC) have been developed in

two crossing strategies (Malek et al. 2012; Pradhan et al. 2010; Tian et al. 2010; Li et al. 2005). Since *B. rapa* and *B. napus* are more extensively cultivated for oilseeds than other species, one way is by crossing between *B. rapa* (AA) and *B. carinata* (BBCC) and another way is through hybridization between *B. napus* (AACC) and *B. nigra* (BB).

Polyploidization has been successful in various important crops such as wheat, cotton and sugar beets (Sleper and Poehlman 2006; Liu et al. 2001; Guzy et al. 1989). Generally, hybrid vigor is shown in polyploidy plants (Zou et al. 2010; Comai 2005; Guzy et al. 1989), and an increase in plant height, fruit length, silique number and seed size was observed in hexaploid *Brassica* lines (Malek et al. 2012; Howard 1942).

Synthetic hexaploid materials are used not only to improve certain traits, but also can be used importantly in various genetic studies. For instance, use of hexaploid *Brassica* lines can facilitate the studies about genome relationship, genetic compatibility and trait introgression in interspecific hybridization with lower ploidy *Brassica* species (Cui et al. 2012; Mason et al. 2010; Schelfhout et al. 2006; Chèvre et al. 1996; Roy 1978). To date, no hexaploid lines are available for agricultural purposes, but development of hexaploid *Brassica* as a new cultivar may be possible in the near future with current technologies (Chen et al. 2011).

2.2 Blackleg disease

2.2.1 Disease description

To describe a disease, the 'disease triangle' was stated by Stevens (1960). It occurs when the following three factors are encountered: a favorable environment, a susceptible host and a

virulent pathogen (Stevens 1960). However, other variables can also impact on disease incidence and severity such as environmental variations, foreign pathogens and insect vectors (Grulke 2011).

The Virulent Pathogen

Blackleg is a fungal disease caused by the pathogen *Leptosphaeria* species, *L. biglobosa* and *L. maculans* (West et al. 2001; Sjödin and Glimelius 1988; Cargeeg and Thurling 1980). In previously described pathogen populations, pathogenicity groups (PG) PG1, PG2, PG3, PG4 and PGT were found in western Canada (West et al. 2001; Mengistu et al. 1991). *Leptosphaeria biglobosa* is classified as PG1 and described as weakly virulent pathotype (Shoemaker and Brun 2001) although it showed increased virulence in certain canola fields in Europe (Jedryczka et al. 2002) as well as under highly humid conditions (Hadrami et al. 2010). On the other hand, *L. maculans* is sub-classified into four PGs (PG2 - 4 and PGT) and described as highly virulent pathotypes (McGee and Petrie 1978). This classification was based on plant – pathogen interactions on the three *B. napus* cultivars, Westar, Glacier and Quinta (Koch et al. 1991; Mengistu et al. 1991). Within *L. maculans* pathotypes, PG2 was predominant in western Canada in 1980s, and two pathogen groups PG3 and PGT were reported as new pathotypes in the late 1990s. Later, another new pathotype PG4 was reported by Chen and Fernando (2006).

The Favorable Environment

Despite the fact that defining favorable environmental conditions for a disease is difficult due to its variations, widely known conditions for blackleg disease would be described the same as in the experiments for *Brassica – L. maculans* interaction studies. The general controlled-

environment for developing symptoms of blackleg is the temperature range between 15 °C and 18 °C during the night, and 20 °C and 24 °C during the day based on greenhouse setting (Zhang et al. 2015; Larkan et al. 2013; Kutcher et al. 2010a; Chen and Fernando 2006; Yu et al. 2005; Delourme et al. 2004; Sjödin and Glimelius 1988). Additionally, the field conditions in which *B. napus* was highly infected by blackleg were reported as relatively warm temperatures (19 °C – 20 °C) and 250 – 750 millimeters of rainfall in western Canada (West et al. 2001). Although relative humidity (RH) is not specified for controlled setting, a certain level of humidity is required for spore germination and infection process (Hadrami et al. 2010; West et al. 2001; Juska et al. 1997). Previous studies also showed that the level of humidity can affect the virulence of *L. maculans* (Hadrami et al. 2010; Ghanbarnia et al. 2009). Under field conditions, RH level and soil temperature can also affect the persistence of fungal spores on crop residues (West et al. 2001).

The Susceptible Host

Blackleg disease has been found in most cruciferous family species over 100 years (Henderson 1918), and its severe epidemics in oilseed *Brassicas* were reported worldwide in 1950s (France), 1970s (Germany and Austrailia) and 1980s (western Canada) (Gugel and Petrie 1992). Among these countries, different types of rapeseed are grown due to the growing weather conditions. For instance, winter type rapeseed is widely grown in parts of Europe including Germany, England and France; spring types are mainly grown in Australia and Canada (Gugel and Petrie 1992). Blackleg has been reported as a devastating disease in rapeseed and canola (Kutcher et al. 2010a; Fernando et al. 2007).

2.2.2 Disease cycle

Blackleg disease is initiated from the infected crop residues in which asocospores are produced or from the secondary inoculum, called pycnodiospores (Guo and Fernando 2005; Howlett et al. 2001). Infected seeds can result in blackleg infection at seedling stages (Hall 1992). However, Wood and Barbetti (1977) showed a significantly low rate of infection caused by infected seeds, and less than 5% of frequency in seed infection was reported by Hall (1996). In the sexual reproduction cycle, *Leptosphaeria maculans* produces a sexual fruiting body called a psudothesium, which produces ascospores. During asexual reproduction, pycnidiospores are released from asexual fruiting body called pycnidia. These spores can be present on the stubble prior to seeding or several months after harvesting (Petrie 1995). Ascospores are spread by wind and to distant areas whereas asexual spores, pycnidiospores, are spread by rain-splash and to relatively short distance (Figure 2.3) (Fernando et al. 2007). Seedlings infected at earlier stages were showed more severe symptoms and losses (West et al. 1999).

Both asexual and sexual spores can infect plants as an initial inoculum. However, in western Canada, pycnidiospores are the primary source of inoculum as they are found in field stubbles (Guo and Fernando 2005). This might be resulted in less severe crop losses compared to the growing regions primarily infected by ascospores. *Leptophaeria maculans* overwinters on crop residues and can survive for several years as four-year crop rotation is recommended (Peng et al. 2015; West et al. 2001).

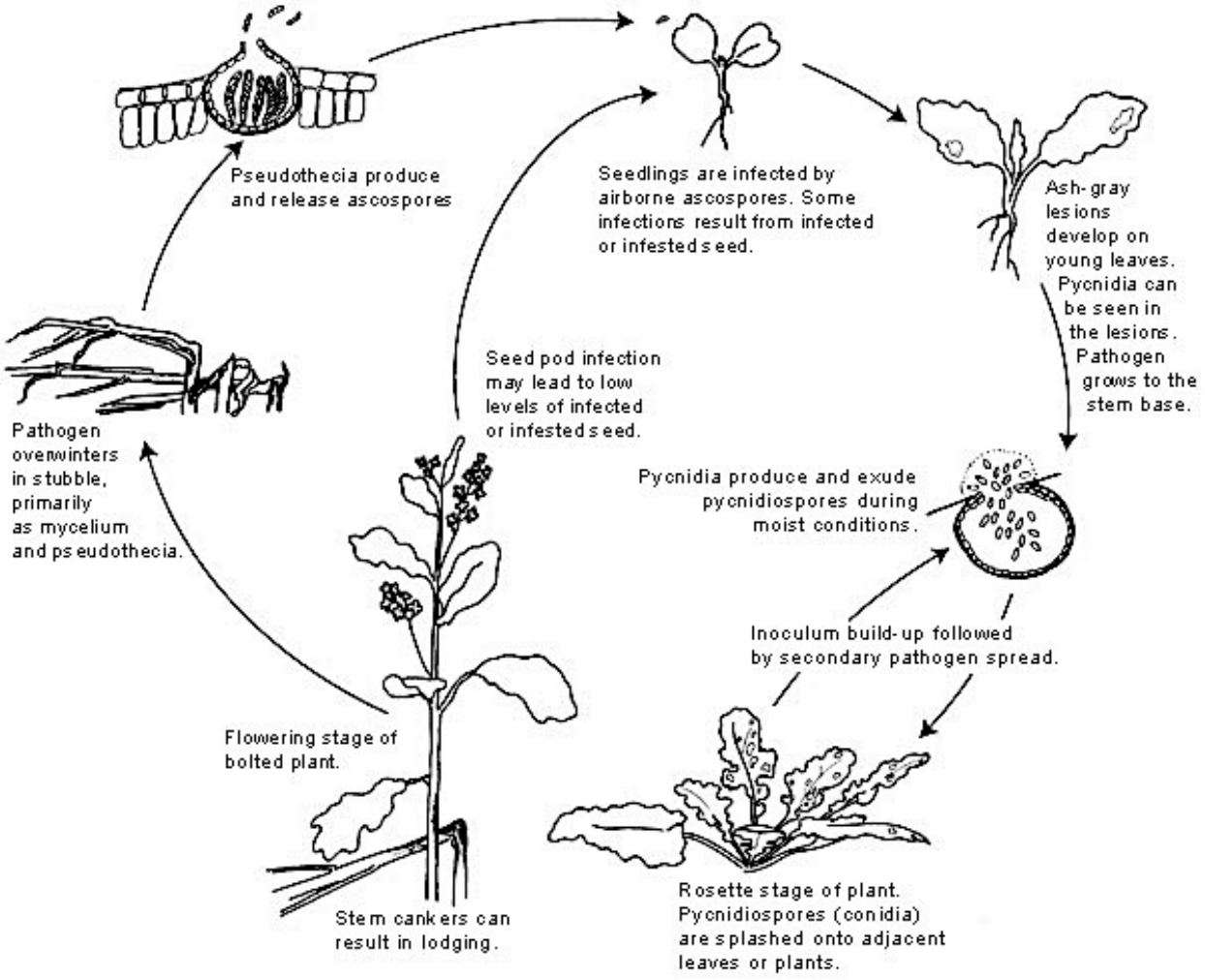

Figure 2.3 Blackleg disease cycle. *Source: Ash (2000)*.

2.2.3 Symptoms of Blackleg

General indications of blackleg disease are cotyledon and leaf lesions at the early stages, upper or basal stem canker in the later growing season (Fernando et al. 2007; Guo et al. 2006; West and Fitt 2005; Gugel and Petrie 1992). However, with the presence of fungal pathogen, all parts of plants can be infected throughout a growing season (Guo and Fernando 2005). Other symptoms such as damping-off, root rot and pod lesion are also observed. On the lesions, black-dotted

fruiting body pycnidia may be present. Pseudothecia, sexual fruiting body, may also be present in the later growing season or can survive on stubbles (Gugel and Petrie 1992). The most damaging symptoms of blackleg are stem canker, which leads to significantly poor seed yields or plant death (West et al. 2001; Duczek 1997).

2.2.4 Significance of Blackleg

Blackleg is an economically important disease in *Brassica* species worldwide. In 1950s, up to 80% disease incidence and 40% yield loss were reported in some fields in France. The epidemics of blackleg causing severe damages were observed in the late 1960s. Similarly, up to 100% blackleg incidence and 50% yield loss were observed in some fields of European countries in 1970s. Similar outbreaks of blackleg also occurred in Australia (Gugel and Petrie 1992). In western Canada, blackleg infection caused by weakly virulent pathotypes was first reported in Saskatchewan in 1961. No significant yield losses (mostly less than 1%) were observed during that time. In the 1980s, some fields had yield losses of up to 56%, although the average was only 6% within Saskatchewan. During this time, disease incidence and yield loss were observed as high as 90% and 50% in Manitoba respectively. It was also reported that winter type rapeseed showed higher disease incidence than spring types (Assabgui and Hall 1989, 1990). In recent years, Mendoza et al. (2012) reported that an average 61% of blackleg incidence was observed in about 45% of 88 fields in North Dakota. Blackleg has been described as one of the devastating diseases in canola, and a recent study about blackleg yield loss model showed that each 20% increase in blackleg severity would result in a 17% to 24% yield loss (Hwang et al. 2016).

2.3 Blackleg disease management

No single management strategy can completely control a disease. Rather, integrated management systems are more effective and last longer (Gugel and Petrie 1992). Generally, four major control methods have been discussed: cultural practices, chemical applications, biological control and genetic resistance. In most growing fields, seeding with resistant cultivars has been effective along with chemical and cultural practices. In western Canada, blackleg management has been successful with genetic resistance and four-year of crop rotation in the last two decades (Peng et al. 2015).

2.3.1 Cultural Practices

Crop rotation and tillage can effectively reduce blackleg incidence and disease severity (Kharbanda 1999; Gugel and Petrie 1992). Crop rotation is an effective control method as it can alter the pathogen population by depriving a virulent pathogen of a susceptible host. Gugel and Petrie (1992) report that blackleg in rapeseed fields can be managed through crop rotation. Many researchers also recommend a four-year rotation for rapeseed growers worldwide including western Canada, Europe and Australia (Peng et al. 2015; Fernando et al. 2007; Gugel and Petrie 1992). A four-year field study that proved the benefits of crop rotation, rotation length has minimal impact on blackleg incidence (Guo et al. 2005). Similarly, Morrall et al. (1999) reported that no significant effect of crop rotation length on blackleg incidence was found. In a third study conducted in one location, Carman Manitoba from 1999 to 2002, significant reduction of blackleg incidence (49% to 21%) was observed in the fields with at least one-year break of canola crop compared to the fields with no rotation (Guo et al. 2005).

As a farmer's perspective, choice of tillage system is an important factor in terms of input cost. Gulden and Entz (2005) compared the energy use in conventional tillage and zero tillage system, and showed a 14% reduction of physical input cost in zero tillage system. Minimum or zero tillage is also beneficial to environment by retaining soil temperature, soil moisture and organic matter, and reducing soil erosion compared to conventional tillage (Mathew et al. 2012). Previously, the effect of a minimum or zero tillage system, used to minimize the spread of disease inoculum, was proved in wheat fields (Dill-Macky and Jones 2000). Unfortunately, however, 37% to 71% higher in blackleg incidence was observed in the fields with no rotation or one-year rotation and zero tillage compared to conventional tillage. In three-year crop rotations, for instance, canola-wheat-flax-canola, both tillage systems showed low percentage of blackleg incidence (Guo et al. 2005). Conventional tillage is also effective as burying to manage blackleg stubbles (West et al. 2001).

2.3.2 Chemical Control

Fungicide application as a chemical control method has different effects based on yield potential, inoculum level and cultivar resistance level (Fernando et al. 2007). Application of fungicide at early stages (2 – 4 leaf growth stage) resulted in an increase of 4 bushels per acre. However, this method showed almost no effects on crop yields under low disease pressure, but was most effective under moderate or high disease level. Despite the effectiveness of fungicide application in the presence of blackleg (West et al. 2001; Gugel and Pietrie 1992), other factors should also be considered such as yield potential and cultivar susceptibility (West et al. 2001). However, continuous use of fungicides showing same modes of action would result of *L. maculans* becoming resistant (Gossen et al. 2014). Rotation of fungicides with different mode of action

would be more effective in its application although chemical application is only recommended when disease pressure is moderate to high or cultivars show susceptibility (Gossen et al. 2014).

2.3.3 Biological Control

Biological control methods for blackleg disease are less well-known than other control methods. According to previous studies, this control method can be used in at least two ways. First, weakly-virulent pathotypes of *L. maculans* species can be used as competitors with virulent *L. maculans* for host plants (Petrie 1982). Secondly, use of other microorganisms can reduce disease pressure due to the *L. maculans* in oilseed rape (Kharbanda et al. 1999; Tewari et al. 1997; Kharbanda and Dahiya 1990). A fungus species *Penicillium verrucosum* was found to have toxic effect to *L. maculans*. Another species *Cyathus striatus* and *C. olla* were known to use plant stubble as their substrates and also inhibit *L. maculans*. A most recent study showed that several soil bacteria produce compounds toxic to *L. maculans* and are effective to suppress blackleg disease (Ramarathnaln and Fernando 2006).

2.3.4 Genetic Resistance

Among management strategies for blackleg, use of genetic resistance has been described as the most cost-effective, environmental friendly and sustainable management approach (Fernando et al. 2007; West et al. 2001; Gugel and Petrie 1992). Previously, a field study showed the effect of cultivar resistance, as zero percent yield loss was observed and only 10% loss in the field sown with moderate resistant cultivar. However, significantly reduced effect on blackleg control was observed in the field with no rotations and even seeding with the same resistant cultivar in the following year resulted in 65% to 100% yield loss (West et al. 2001). Because of the possibility

of pathogen evolution, development of resistant cultivars from various source of resistance has been emphasized to prevent blackleg outbreaks due to the selection pressure (West et al. 2001; Gugel and Petrie 1992).

2.4 The gene-for-gene concept

Blackleg resistance in *Brassica* can be described as two main types: qualitative resistance confirming at the seedling stages and quantitative resistance occurring at adult plant stages (Ansan-Melayah et al. 1998; Pang and Halloran 1996). Determination of qualitative resistance is based on 'gene-for-gene concept' that is previously described in flax (*Linum usitatissimum* L.) and the flax rust pathogen (*Melampsora lini*) studies (Flor 1942, 1955, 1971). The study found interaction between each individual resistance gene in plants and in pathogen. Thus, the theory in *Brassica – L. maculans* has been utilized to identify plant resistance (R genes) and pathogen avirulence genes (Avr gene) by phenotyping at the seedling stage.

In the earlier stages of description of the gene-for-gene concept, pathogen races were classified into five different groups (PG1 – 4 and PGT) using three *B. napus* cultivars Westar, Glacier and Quinta (Mengistu et al. 1991). These three cultivars differentiate pathogen races by different interaction phenotypes (Table 2.1). For instance, all three differential cultivars showed resistance response to less virulence *L. maculans* PG1. The cultivar Westar is susceptible to all other virulent *L. maculans* pathotypes PG2 – 4 and PGT. The cultivar Glacier shows resistance response to two PGs, PG2 and PGT while it is susceptible to PG3 and PG4. For the cultivar Quinta, it shows resistance response to PG2 and PG3 while it is susceptible to PG4 and PGT. Within five pathogen groups, two major *Leptosphareria* species that are virulent pathotypes

(PG2 – 4 and PGT) and weakly virulent pathotype PG1 have been distinguished by identifying morphological differences and using genetic and molecular markers (Gall et al. 1995; Johnson and Lewis 1990; Petrie 1988).

Table 2.1. Interaction phenotype on differential *B. napus* L. cultivars Westar, Glacier and Quinta against *Leptosphaeria maculans* pathogenicity groups.

	Interaction Phenotype[a]		
	B. napus L. cultivar		
Pathogenicity Group	Westar	Glacier	Quinta
PG1	R	R	R
PG2	S	R	R
PG3	S	S	R
PG4	S	S	S
PGT	S	R	S

R: Resistant response at seedling stage
S: Susceptible response at seedling stage
[a]: Mengistu et al. 1991

2.4.1 Qualitative Resistance

Plant resistance genes (R-genes) would lead to a disease resistance response by producing proteins (R proteins), which was triggered through direct or indirect recognition by pathogen effector proteins (Avr proteins) produced at the infection sites (Jones and Dangl 2006). The process of recognition of Avr proteins and R proteins lead four different ways of defense mechanisms (Belkhadir et al. 2004). One of them is plant's hypersensitive response, also known as programmed cell death. Some other mechanisms are increased ion fluxes, extracellular oxidative burst and transcriptional responses at and around infection sites.

To date, there are at least fifteen qualitative genes known in *Brassica* species. The R genes *Rlm1*, *Rlm2*, *Rlm3*, *Rlm4*, *Rlm7* and *Rlm9* (Delourme et al. 2004; Ansan-Melayah et al. 1998) have been identified in *B. napus* and located in the two linkage groups in the A-genome. Two resistance genes *Rlm8* and *Rlm11* (Balesdent et al. 2002, 2013) were identified in *B. rapa*. Four additional resistance genes, *LepR1*, *LepR2*, *LepR3* and *LepR4* were introgressed from *B. rapa* subsp. *sylvestris* into *B. napus,* and mapped in different linkage groups: *LepR1* in A2; *LepR2-3* in A10; *LepR4* in A6. In contrast, a few resistance genes were mapped in the B-genome *Brassica* species, *B. nigra* (*Rlm10*) and *B. juncea* (*Rlm5* and *Rlm6*) (Balesdent et al. 2002; Chèvre et al. 1996, 1997).

2.4.2 Avirulence gene

Avirulence genes (Avr genes) are effector proteins, which initiate plant's defense mechanism and function by either altering plant cell structure or cell functions, producing toxins or serve as elicitors (Thakur and Sohal 2013; Dodds and Thrall 2009). In *Leptosphaeria* species, markers which are closely linked to avirulence genes in *L. maculans* have been found on genetic maps (Balesdent et al. 2002). Unlike the resistance gene in *Brassica,* several Avr genes *AvrLm1* (Gout et al. 2006), *AvrLm4 – 7* (Parlange et al. 2009), *AvrLm6* (Fudal et al. 2007), *AvrLm11* (Balesdent et al. 2013), and *AvrLmJ1* (Van de Wouw et al. 2014) have been cloned.

With several R genes identified, corresponding avirulence genes have also been described (Kutcher et al. 2010b). For resistance gene *Rlm1*, for instance, the corresponding avirulence gene is *Avrlm1* (Table 2.2). In the same manner, resistance gene and the corresponding avirulence

gene can be described as *Rlm2 – AvrLm2*, *Rlm3 – AvrLm3*, *Rlm4 – AvrLm4*, *Rlm5 – AvrLm5*, *Rlm6 – AvrLm6*, *Rlm7 – AvrLm7*, *Rlm8 – AvrLm8*, *Rlm9 – AvrLm9*, *Rlm10 – AvrLm10* and *LepR3 – AvrLepR3*.

Table 2.2. Known major resistance genes in *Brassica* species and its corresponding avirulence genes in *Leptosphaeria maculans*.

Resistance gene[a]	Source	Location on chromosome	Corresponding Avirulence gene[b]
Rlm1	*B. napus*	A7	*Avrlm1*
Rlm2	*B. napus*	A7	*Avrlm2*
Rlm3	*B. napus*	A7	*Avrlm3*
Rlm4	*B. napus*	A7	*Avrlm4*
Rlm7	*B. napus*	A7	*Avrlm7*
Rlm9	*B. napus*	A7	*Avrlm9*
Rlm5	*B. juncea*	-	*Avrlm5*
Rlm6	*B. juncea*	B8	*Avrlm6*
Rlm10	*B. nigra*	B4	*Avrlm10*
Rlm8	*B. rapa*	-	*Avrlm8*
Rlm11	*B. rapa*	-	-
LepR1	*B. rapa* spp. *sylvestris*	A2	-
LepR2	*B. rapa* spp. *sylvestris*	A10	-
LepR3	*B. rapa* spp. *sylvestris*	A10	*AvrLepR3*
LepR4	*B. rapa* spp. *sylvestris*	A6	-

[a]: Raman et al. 2013
[b]: Kutcher et al. 2010b

2.4.3 Quantitative Resistance

Adult plant resistance can be controlled by quantitative trait loci (QTL) (Pang and Halloran 1996). However, determination of quantitative resistance is difficult because of variables in field conditions. Despite the total 16 QTL have been identified, the position of QTLs varies depending on the population size and the samples used in studies (Jestin et al. 2012). Another issue with QTL analysis or QTL comparisons in *Brassica* for blackleg would be challenge due to the number of markers. In the recent study, stable QTL was observed in 10 linkage groups A1, A2, A4, A6, C1, C2, C4, C5 and C8 (Jestin et al. 2015). As means of durable resistance, the ideal method for blackleg management using genetic resistance would be the combination of qualitative resistance and high level of qualitative resistance (Brun et al. 2010).

2.5 Breeding for blackleg – Backcross Approach

A backcrossing approach has been applied effectively as breeders are interested in introducing a certain trait from a donor genotype into a recipient elite genotype (Hasan et al. 2015; Vogel 2009). This method is often initiated with intra- or interspecific crosses. According to theory of genome recovery in backcrosses, nearly complete genome of a recurrent parent will be recovered by sixth backcross generation BC_6, suggesting minimum five to six backcrosses are required for stable introgression of target trait. After that, selected lines showing the target trait will be self-pollinated to produce a homozygous for the trait.

In breeding programs, transfer favorable target genes could be derived from same species (intraspecific crosses) or from alien species (interspecific crosses), and has been successful in various crops (Gautam et al. 2014; Goodman et al. 1987). For example, hexaploid wheat

(*Triticum aestivum*) is originated from two wild diploid species and one of parent (*Aegilops tauschii speltoides*) is distant grass species (Shewry 2009). Another example is in corn (*Zea mays*) originated from crosses between cultivated corn and wild grass *Euchlaena* species (Whiting 1944). Similarly, *Brassica napus* L. is also originated from two wild species *B. rapa* and *B. oleracea*, and the species creation has been successful (Yu et al. 2012; Crouch et al. 1994).

Despite the fact that some interspecific crosses have been successful, in many other cases outcomes have included cross incompatibility such as low seed germination (Hakansson 1956), sterile pollen grains (Heslop-Harrison 1992), embryo abortion (Sharma et al. 1996), and low seed yield (Choudhary and Joshi 1999) have been discussed previously. Similar results have also been found in other important crops such as corn, rice, and cotton (Efisue et al. 2008; Shao and Jiang 1980). Moreover, difficulties in maintaining additional genome from interspecific crosses due to the meiotic disturbances have been reported by several researchers (Navabi et al. 2010; Schelfhout et al. 2006; Dixelius and Wahlberg 1999).

However, Heuer et al. (2003) suggest that interspecific incompatibility can be overcome by producing two or more backcross generations, which was proved in other crop breeding studies such as rice. As a conventional breeding method, introgression of a certain trait is achieved through hybridization followed by several backcrosses. With use of new technologies such as marker assisted backcrossing, the similar outcomes from conventional backcrossing can be achieved by reducing at least two generations although a large number of progeny is required (Hasan et al. 2015; Frisch 1999).

Molecular markers can be used to increase breeding efficiency and accuracy. However, the marker assisted selection (MAS) method has failed in many studies because of the blocking effect of QTLs and the marker numbers for qualitative traits (Wei et al. 2013). Particularly, in *Brassica*, use of new technologies is still challenging since closely linked markers to useful traits are lacking and genome resequencing is required for further analysis such as the discovery of single nucleotide polymorphism (SNP) and DNA insertion and deletion (Indel), generation of high density map, and comparison of species evolution (Wei et al. 2013).

The objectives of the research reported in the thesis are i) to develop new resistant *B. napus* lines against *L. maculans* by introducing high levels of blackleg resistance from *B. juncea* containing the B-genome, ii) to analyze blackleg resistance in synthetic hexaploid *Brassica* lines and iii) to evaluate interspecific cross-compatibility in the crosses between two tetraploid *Brassica* species and between hexaploid and tetraploid *Brassica* species.

CHAPTER 3. INTROGRESSION OF RESISTANCE TO *LEPTOSPHAERIA MACULANS* FROM *BRASSICA JUNCEA* INTO *B. NAPUS*

3.0 Abstract

Blackleg (Phoma stem canker), caused by the fungal pathogen *Leptosphaeria maculans*, is one of the major diseases in canola. As a primary control method for blackleg, resistant cultivars have been effective. However, breakdown of single gene resistance due to the selection pressure was observed in Australia and France previously, and more recently in western Canada. To develop a new resistant canola line, high levels of blackleg resistance were introduced from *Brassica juncea* (AABB) into a susceptible *B. napus* L. cv. Westar (AACC) through interspecific hybridization. In the current study, ten *B. juncea* UM lines that are resistant to two *L. maculans* isolates 03-15-03 (carrying *Avrlm2*, *Avrlm6*, *Avrlm11* and *AvrlmJ1*) and PG4-1M (carrying *Avrlm6*, *Avrlm11* and *AvrlmJ1*) were used as a source of resistance. Through selection, resistant individual plants within a family line were identified by cotyledon inoculation with isolate 03-15-03 in the F_1, BC_1, BC_2, and BC_3 and with an additional isolate PG4-1M in the BC_3. Overall, blackleg resistance in *B. juncea* was successfully incorporated into Westar using a backcross approach. Although a pre-fertilization barrier (pollen fertility) and post-fertilization barrier (embryo development) to interspecific hybridization existed between *B. juncea* and *B. napus*, these barriers were overcome by producing two or more backcross generations. The advanced backcross population BC_3 still possessed high levels of blackleg resistance and out of 905 individual plants, 117 resistant plants were obtained in the BC_3. The introgression lines obtained in this study are likely to have novel gene resistance. Further breeding work and molecular analyses will be required to identify the resistance genes.

3.1 Introduction

Canola (*Brassica napus* L. or Argentine rapeseed) is known as the healthiest vegetable oil as it contains favorable fatty acid compositions compared to other oilseeds (Canola Council of Canada 2016). In early cultivation periods, rapeseed oil was used for both cooking and lighting. During Industrial Revolution, however, rapeseed was mostly used for lubricants because of its distinctive adherence to water and steam-washed metal surfaces. This latter reason is believed to occur because of the long-chain fatty acids, particularly erucic acid, in rapeseed oil (Cook and McMaster 2002). Rapeseed was widely grown in Europe as early as the 13th century because of its adaptability to extreme conditions (Kimber and McGregor 1995). In Canada, Argentine rapeseed was first introduced when the needs of rapeseed oil increased during the World War II. After the war, as the demand for rapeseed oil declined efforts toward developing rapeseed as a crop were made in Canada.

Wild type rapeseed contains relatively high (50%) levels of erucic acid. The first Canadian rapeseed cultivar (cv.), Golden, which contains erucic acid content of 40-45% and a glucosinolate content of 100 micromoles per gram in air-dried oil free meal was introduced in 1954 (McVetty et al. 2009). Initially, there was a concern about rapeseed as edible oil because its fatty acid profile was found to be different from other vegetable oils. Rapeseed meal has high levels of protein, which might be a great protein source for animal feed. However, the bitter taste of glucosinolates in rapeseed meal resulted in reduced feed intake from animals. Later studies have found that the high contents of erucic acid and glucosinolates would be harmful to humans and animals. Thereafter, *B. napus* rapeseed had been intensively bred for low erucic acid and low glucosinolates. In 1968, the *B. napus* cv. Oro was released as the first low erucic acid rapeseed,

which containing only 5% erucic acid (Ahuja and Banga 1993). The first "double-low", low erucic acid and low glucosinolates, *B. napus* cv. Tower was developed by researchers at the University of Manitoba in 1974 (Stefansson and Kondra 1975). Afterward, canola was defined by Canadian law as oil that must have less than 2% erucic acid and the seed should contain less than 30 micromoles of glucosinolates per gram (Feed Regulations Amendment 1983).

Although the cultivar Tower was successful to be used for edible oil, its agronomic performance was inferior to other cultivars developed previously (Eskin 2013). A superior *B. napus* cv. Westar was commercially available in 1982 and grown over 3.5 million hectares in western Canada after its release. This cultivar recorded the top canola variety by area during the period of 1960 to 2008 (Brewin and Malla 2012). Westar had characteristics of high yield and oil content, early maturity, resistance to disease and lodging (Klassen et al. 1987). Later, Westar was found to be susceptible to blackleg disease, which causes significant economic losses (Klassen et al. 1987; Juska et al. 1997). Blackleg disease is one of the most diseases in canola caused by the fungal pathogen *Leptoshpaeria maculans*. It was a serious concern in the late 1980s and early 2000s as significant crop losses of up to 100% were reported worldwide (Fernando et al. 2007). A recent study about blackleg disease caused yield loss showed that each 20% increase in blackleg severity would result in a 17 to 24% yield loss (Hwang et al. 2016).

Blackleg disease causes leaf lesions and produces black-dotted fruiting bodies at the young stage; stem cankers develop during the later growing season (Guo et al. 2008; West and Fitt 2005). Stem canker leads to significantly poor seed yield or plant death due to a lack of water and nutrient uptake (Duczek 1997). Variations in the incidence and severity of the disease depend on

a number of conditions: field history, growing regions, and environmental conditions. Generally, however, the fungus favors warmer temperatures (15 °C to 23 °C) and requires moisture (Relative Humidity, RH > 50%) for germination and infection (Sosnowski et al. 2005; West et al. 2001; Juska et al. 1997). *Leptosphaeria maculans* produces sexual ascospores and asexual pycnidiospores from black fruiting body psudothesia and pycnida respectively. Ascospores can be dispersed by wind to a distance of kilometres (West and Fitt 2005) whereas pycnidiospores are spread by rain splash to a relatively short distance (Travadon et al. 2007). Both asexual and sexual spores can infect plants as an initial inoculum (West et al. 2001). However, in western Canada, pycnidiospores are the primary source of inoculum as they are found in field stubble (Guo and Fernando 2005).

Most *Brassica* species are host plants of the pathogen *L. maculans*. To control blackleg, genetic resistance in plants has been effective along with chemical and cultural practices. In western Canada especially, genetic resistance has been successful with resistant cultivars and four-year crop rotation for the last two decades (Peng et al. 2015). On the other hand, a breakdown of single gene resistance due to the selection pressure has been observed in Australia and France (Rouxel et al. 2003; West et al. 2001). Thus, a need for breeding work with more various sources of resistance is important for canola breeders. Also, Marcroft et al. (2012) proved that rotating resistant cultivars which comprise different resistance genes would have a 50% positive impact on plant mortality caused by blackleg disease. Surprisingly, a recent study revealed that 55.9% of 104 Canadian *B. napus* accessions and 49% of 102 different canola seeds obtained from Manitoba farmers have the same gene resistance, *Rlm3* (Zhang et al. 2015). Thus, diversification of genetic resistance is important for canola growers to prevent blackleg outbreaks, and rotation

of resistant cultivars becomes a more important management strategy for blackleg while maintaining genetic resistance.

In crop breeding, alien species are widely used to introduce economically important traits such as disease resistance, crop yield and seed quality, and genetic transfer has been successful in various crops including wheat, tomato as well as *Brassica* crops (Gautam et al. 2014; Goodman et al. 1987). Interspecific hybridization between the relative *Brassica* species, *B. rapa* (AA) and *B. napus* (AACC) has been successful (Yu et al. 2012; Crouch et al. 1994). Previous studies have reported that all B-genome *Brassica* species, *B. nigra* (BB), *B. juncea* (AABB) and *B. carinata* (BBCC), have high levels of blackleg resistance (Chen et al. 2011; Li et al. 2004). In addition, the 'complete resistance' which presents both seedling and adult resistance have been seen in the B-genome *Brassica* species and not in the A-genome (Roy 1978, 1984). For that reason, efforts at introducing resistance to *L. maculans* from the B-genome *Brassica* species into *B. napus* have continued (Fredua-Agyeman et al. 2014; Navabi et al. 2010; Schelfhout et al. 2006; Somda et al. 1998; Chèvre et al. 1996; Struss et al. 1991; Roy 1978). The latest studies have identified that six major resistance genes (*Rlm1*, *Rlm2*, *Rlm3*, *Rlm4*, *Rlm7* and *Rlm9*) are located in the A genome of *B. napus* (AACC) and two resistance genes (*Rlm8* and *Rlm11*) are in *B. rapa* (AA). Other four resistance genes (*LepR1*, *LepR2*, *LepR3* and *LepR4*) were sourced from *B. rapa* subsp. *sylvestris* and no resistance genes were found in the C genome. To date, a few resistance genes were mapped in B-genome *Brassica* species, *B. nigra* (*Rlm10*) and *B. juncea* (*Rlm5* and *Rlm6*) (Raman et al. 2013).

The resistance genes (R-genes) in *Brassica* can be determined by testing with known avirulence

genes (Avr-gene) or *vice versa* since gene-for-gene interaction has been discussed for blackleg disease (Ansan-Melayah et al. 1998). This concept was described in flax studies by Flor (1971), and explained as resistance genes in the host (e.g. *Rlm1*) and the corresponding avirulence genes in the pathogen (e.g. *AvrLm1*) confer disease resistance (Howlett 2004). Resistance gene and avirulence gene profile is critical information for canola growers to choose effective resistant cultivars although only three resistance genes *Rlm1*, *Rlm2* and *Rlm3* have been found in current canola cultivars (Zhang et al. 2015). In the previous study, 96 *L. maculans* isolates collected from 1999 to 2005 across Canada were used to determine the frequency of Avr-genes, and four known Avr-genes *AvrLm2*, *AvrLm6*, *AvrLm9* and *AvrLm10* were found in most field isolates (Kutcher et al. 2010a). The frequency of Avr-genes in fields has been changed in 2012. A similar study conducted with 300 *L. maculans* isolates collected across Manitoba showed that six known Avr-genes *AvrLm2*, *AvrLm4*, *AvrLm5/AvrLmJ1*, *AvrLm6*, *AvrLm7* and *AvrLm11* were found in most isolates. Previously, high proportion of *AvrLm9* – carrying isolates (60.4%) was decreased to 3.3%. Low proportion of *AvrLm7* – carrying isolates (25%) was increased to 89.2%. Significant increase of *L. maculans* isolates carrying new Avr-genes *AvrLm5/AvrLmJ1* and *AvrLm11*, and reduced frequency of isolates carrying each *AvrLm1*, *AvrLm2* and *AvrLm3* were observed (Zhang et al. 2015; Kutcher et al. 2010a). Variations in frequency might be due to the different number of isolate samples and regional range of sample collection.

Despite the fact that 'complete resistance' can be found in B-genome *Brassica* species, breeding work between the B-genome *Brassicas* and *B. napus* has confronted barriers of interspecific incompatibility (Schelfhout et al. 2006; Struss et al. 1991; Roy 1978). This genetic incompatibility has also been found in other important crops such as corn, rice, and cotton

(Efisue et al. 2008; Shao and Jiang 1980). Interspecific hybridization often results in low seed set and plant fertility in earlier cross generations (Efisue et al. 2008; Heslop-Harrison 1992). However, previous studies do say that plant sterility would be overcome after two or more backcrosses (Heuer et al. 2003).

Backcross breeding is commonly used when introducing a desirable trait or allele from a donor parent to an elite recurrent parent while eliminating undesired traits (linkage drag) (Hasan et al. 2015; Vogel 2009). The development of new technologies accelerates and facilitates breeding programs, since conventional crossing methods take at least eight to ten generations in order to be registered as a new variety (Branca and Cartea 2011). Using new technologies in breeding work with *Brassica* species, however, would still be a challenge. Particularly, marker assisted selection (MAS) and genome selection is rarely successful in *Brassica* due to the limited markers closely linked to the target genes (Wei et al. 2013). In all *Brassica* species, only two R genes, *LepR3* and *Rlm2*, in the A genome of *B. napus* have been cloned recently (Larkan et al. 2013, 2015).

The main objective of this study is to develop new canola breeding lines that are resistant to *L. maculans* by introducing resistance from *B. juncea* through interspecific hybridization followed by backcrosses to *B. napus* L. cv. Westar. Genetic compatibility of interspecific hybridization was evaluated by examining seed germination, pollen viability, pod set and seed set. Blackleg resistance in each plant generation was assessed through cotyledon inoculation, and selected resistant individuals were carried forward to produce the next generation.

3.2 Materials and Methods

3.2.1 Plant Materials

One susceptible *B. napus* L. cv. Westar and ten resistant *B. juncea* accessions were used as parents of interspecific crosses. Westar has been used as a susceptible control in most breeding studies for blackleg resistance. Initially, fifty-four accessions of *B. juncea* from the collection at the University of Manitoba were selected for this study; hereafter, they will be referred to as UM lines. Ten UM lines UM3056, UM3063, UM3073, UM3095, UM3108, UM3122, UM3477, UM3541 and UM3544 were selected to cross with Westar. The reproductive parts of both parents show a distinctive morphological difference. At the flowering stage, Westar has large and longer (0.5 – 0.7 cm) flower buds compared to UM lines, which have relatively small and short (< 0.5 cm) flower buds (Figure 3.1). Both Westar and UM lines were grown in summer 2014.

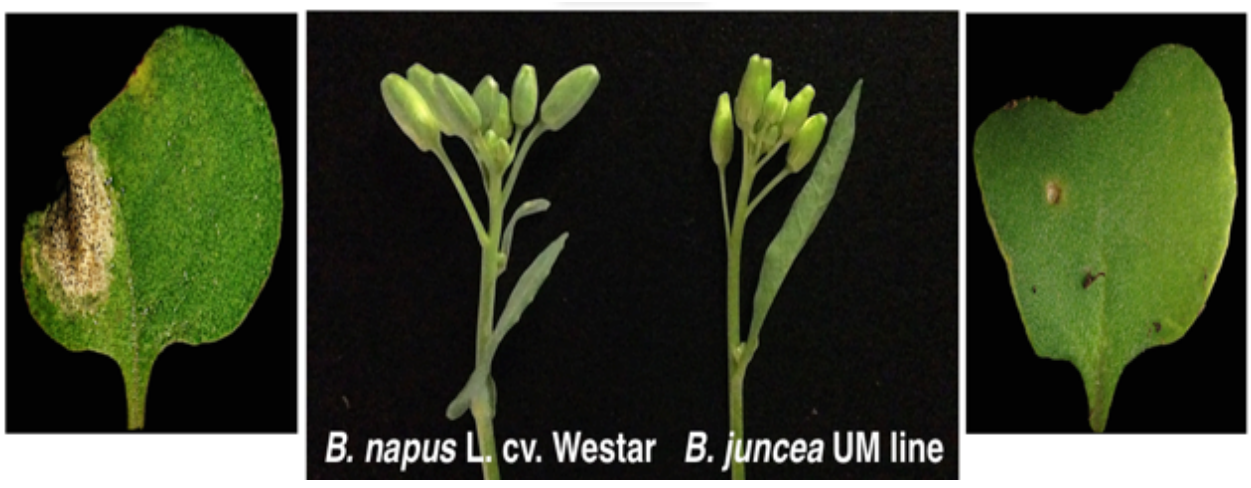

Figure 3.1. Morphological differences and cotyledon response to the *L. maculans* isolate 03-15-03 in *B. napus* L. cv. Westar and *B. juncea* UM line.

3.2.2 Plant Growth Condition

Seeds were sown 1cm deep in eight 3 x 4 cells filled with Sunshine Professional Growing Mix #4 soil (Sun Gro Horiculture, USA), pre-watered with ½ tablespoon of 20-20-20 (nitrogen-phosphorus-potassium) fertilizer per gallon of water. The soil mix contains Canadian sphagnum peat moss, coarse perlite, organic starter nutrient charge, dolomitic limestone and an organic wetting agent (Sun Gro Horiculture, USA). Seed trays were kept in a controlled growth chamber at 20 °C / 18 °C, 16 h light / 8 h dark with relative humidity (RH) greater than 50% facilitated by the Department of Plant Science, University of Manitoba. The young plants were lightly watered daily or as needed.

Seedlings at the 2 to 3 true leaf stage were transplanted individually into 6-inch paper towel lined standard plastic nursery pots containing a 2:2:1 mixture of soil, sand and peat moss incorporated with 20-16-14 (nitrogen-phosphorus-potassium) fertilizer. Eight pots in a row were placed into a greenhouse bench managed by the Department of Plant Science, University of Manitoba. The greenhouse setting remained the same for all growing season at 20 °C to 25 °C. Plants were watered daily and fertilized every second week with 1 tablespoon of 20-20-20 (nitrogen-phosphorus-potassium) fertilizer per gallon of water. The growth condition applied to all plant populations produced in this study.

3.2.3 Cross Populations

3.2.3.1 Production of F_1 Hybrid

A hand crossing method was used to produce F_1 hybrid lines derived from the crosses of Westar and UM lines. To achieve this successfully, the timing of pollination and choice of maternal

parent is critical (Brown et al. 1990; Efisue et al. 2008). After trying direct and reciprocal crosses, the resistant UM lines were used as female (seed) parents while Westar was used as male (pollen) parents. Emasculation of the female plants was performed when the flower bud tip was fully closed and colored green.

After crossing, flower buds were covered with a glassine bag to avoid cross-contamination. After 7 to 10 days, the bags were removed and the successful pollination was checked by looking for fertilized ovaries (Figure 3.2). Plant harvesting took place generally 40 to 50 days after bud pollination. When threshing, the following information was recorded to calculate pod set and seed set: the number of bud pollinations, seed pods and the seeds per pod.

Figure 3.2. Pistil of the fertilized ovary in the production of BC_3 generation. The picture was taken 7 to 10 days after bud pollination.

3.2.3.2 Production of Backcross Populations

F_1 seeds and the recurrent parent Westar were grown in fall 2014, and the seedlings were tested for blackleg resistance. After phenotyping, F_1 plants were grown under greenhouse conditions. Similar to the production of F_1 hybrids, a hand crossing method was used to produce backcross populations. To increase the success rate, crossing was made at appropriate time for pollination. The direct and reciprocal crosses were attempted, and the crossing parents were determined. Westar was used as pollen donor in the production of BC_1 and as maternal parents for BC_2 and BC_3 populations (Figure 3.3). Emasculation process, crossing procedure, and harvesting were performed as in the production of F_1 hybrids. While producing backcross generations, self-pollinated generations F_2, BC_1F_2 and BC_2F_2 were also produced to obtain homozygous resistant lines. Each backcross population was grown in the following seasons: BC_1 in winter through summer 2015, BC_2 in fall 2015 and BC_3 in winter 2016.

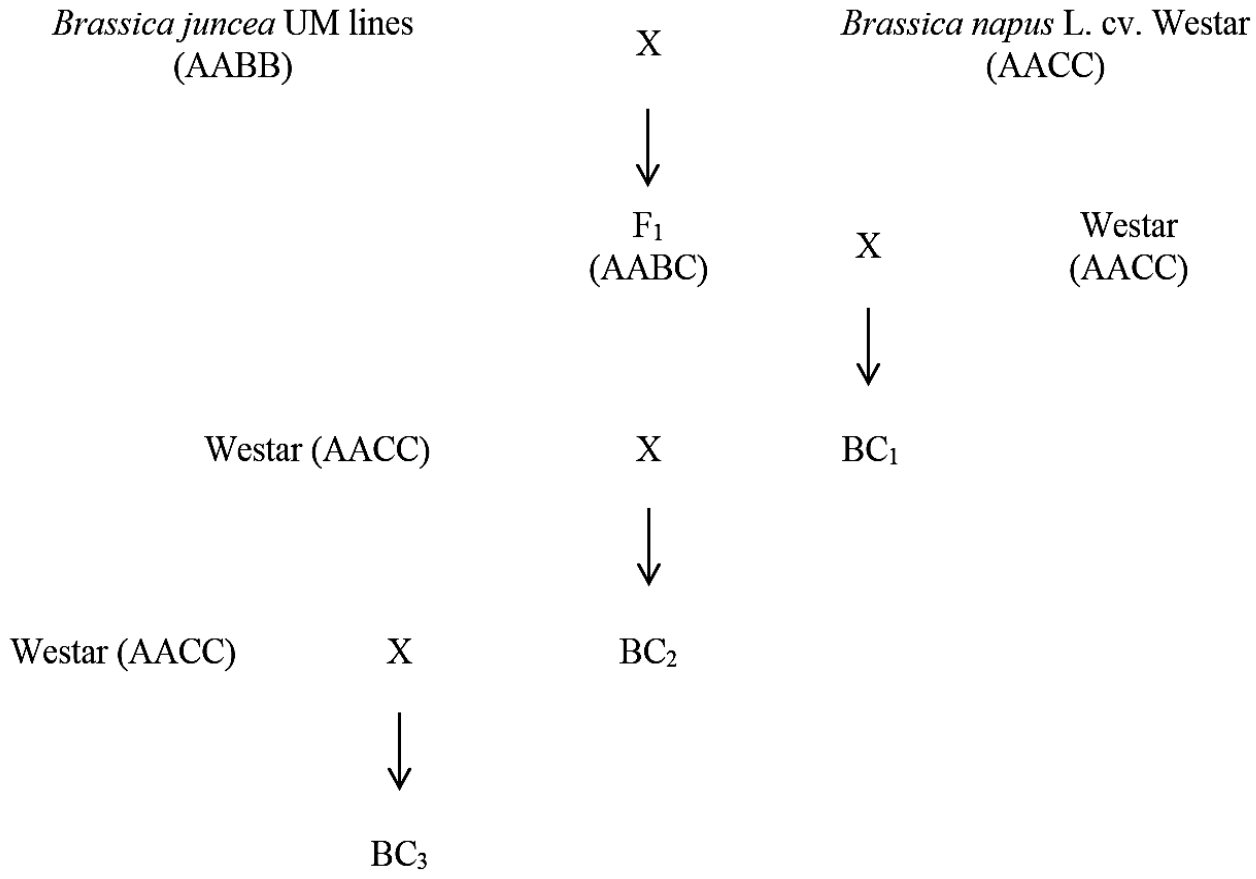

Figure 3.3. Crossing method for introducing blackleg resistance from *B. juncea* into *B. napus* L. cultivar Westar using the backcross breeding method. Direct crossing way is always expressed female x male plant.

3.2.4 Interspecific Cross-compatibility

3.2.4.1 Seed Germination

Seed germination (expressed as a percentage) was examined to test the degree of embryo development. Failure of seed germination is caused by disruption of endosperm development in the interspecific crosses (Håkansson 1956). The germination rate was calculated as the number of seeds emerging, divided by the number of seeds planted. Seeds that are non-germinated or germinated late were excluded in the screening for blackleg resistance. The results of germination rate were also used as a part of selection criteria of the UM lines for the cross

parents. Although the parent seeds of UM lines were stored in the cold seed storage room over decades, most of them germinated normally (Table 3.1). Seed germination was examined in F_1 and BC_1 generations for all ten UM lines and in BC_2 and BC_3 generations for the three selected UM lines (UM3073, UM3095 and UM3122).

Table 3.1. Germination rate of both parents' seeds *B. juncea* UM line and *B. napus* L. Westar.

Generation	UM line Number	No. of plants sown (A)	No. of plants germinated (B)	Germination rate (B/A x 100)
Parent	3056	16	15	94%
	3063	16	15	94%
	3073	22	22	100%
	3075	8	7	88%
	3095	20	20	100%
	3108	8	8	100%
	3122	20	18	90%
	3477	20	19	95%
	3541	20	19	95%
	3544	20	20	100%
	Average Germination Rate			95.6%
	Westar	40	40	100%

3.2.4.2 Pollen Viability

Pollen fertility is an important factor in the success of plant fertilization and seed set. Plant sterility due to infertile pollen often exists when interspecific crosses are made, and examining pollen quality is one way to estimate plant fertility (Heslop-Harrison 1992). Pollen quality test

supported the determination of male parent for the next cross. Pollen grains were investigated at early flowering stage using the acetocarmine staining technique (Shivanna and Tandon 2014). Fresh pollen grains of parent Westar and individual plants of F_1, BC_1 and BC_3 in the three UM lines (UM3073, UM3095 and UM3122) were used for the test. Three to five individual flowers from each plant generation were taken and touched on a separate microscope slide. Fresh pollen was stained with a drop of acetocarmine, followed by 5 minutes of drying (Shivanna and Tandon 2014). The stained image was observed through light microscope at a magnification of 40 X (ZEISS, Germany). Acetocarmine stains viable pollen grains with highly concentrated color in an oval shape; non-viable pollen grains were stained with a relatively diluted color in a shriveled shape (Shivanna and Tandon 2014; Chakraborty and Devakumar 2006).

3.2.4.3 Pod Set and Seed Set

Oilseed *Brassica* species form seed pods as a result of successful fertilization. In the current study, pod set (expressed as a percentage) was calculated as the number of seed pods formed, divided by the number of bud pollinations in each cross. The pod set rate was evaluated using five categories developed in this study, 0 – 20% (very poor, average 10%); 21 – 40% (poor, avg. 30.5%); 41 – 60% (moderate, avg. 50.5%); 61 – 80% (good, avg. 70.5%); 81 – 100% (very good, avg. 90.5%). Pod set data was presented for all ten UM lines in F_1 and BC_1 generations and the three selected UM lines (UM3073, UM3095 and UM3122) in BC_2 and BC_3 generations.

Seeds represent the successful reproduction of plants and the compatibility of female plants with the pollen. Seed set can be investigated to evaluate female fertility (Heslop-Harrison 1992). In this study, seed set was calculated as the number of seeds harvested per pod, and the seed set in

each cross generation was recorded to compare seed yields to the recurrent parent *B. napus* L. Westar. Seed set data was presented for the three selected UM lines (UM3073, UM3095 and UM3122) in all cross populations. In addition, seed set of self-pollinated generations F_2, BC_1F_2 and BC_2F_2 were evaluated.

3.2.5 Screening for Blackleg Resistance

3.2.5.1 Leptosphaeria maculans Isolates

Two *L. maculans* isolates 03-15-03 and PG4-1M were used in this study, and each belongs to PG2 and PG4 respectively. These isolates were originally obtained from Dr. S. R. Rimmer (former professor at the Department of Plant Science, University of Manitoba). Differential *B. napus* L. cultivars Westar, Glacier and Quinta can distinguish the two pathogenicity groups as they show different interaction of phenotypes. Westar is susceptible to both isolates, while both Glacier and Quinta show resistant responses against PG2 isolates and susceptible response to PG4 isolates (Koch et al. 1991; Mengistu et al. 1991).

All populations were screened for blackleg resistance along with the three controls, differential *B. napus* L. cultivars. The PG2 isolate 03-15-03 was used throughout the study while the PG4 isolate PG4-1M was used only for the advanced population BC_3 due to the small sample size. The two isolates showed different growth patterns (Figure 3.4). The key difference between the PG2 isolate 03-15-03 (Figure 3.4 a and c) and the PG4 isolate PG4-1M (Figure 3.4 b and d) was the growth patterns of a black fruiting body. The black fruiting body of isolate 03-15-03 grew in a circle around filter paper discs; the isolate PG4-1M did not form a circle line around the discs. In regards to Avr gene information, gene cloning and sequencing analysis work was done by Dr.

Tengsheng Zhou (a post-doctoral fellow, Department of Plant Science, University of Manitoba) and known Avr genes have been identified in both isolates 03-15-03 (*AvrLm2*, *AvrLm6*, *AvrLm11*, *and AvrLmJ1*) and PG4-1M (*AvrLm6*, *AvrLm11*, and *AvrLmJ1*).

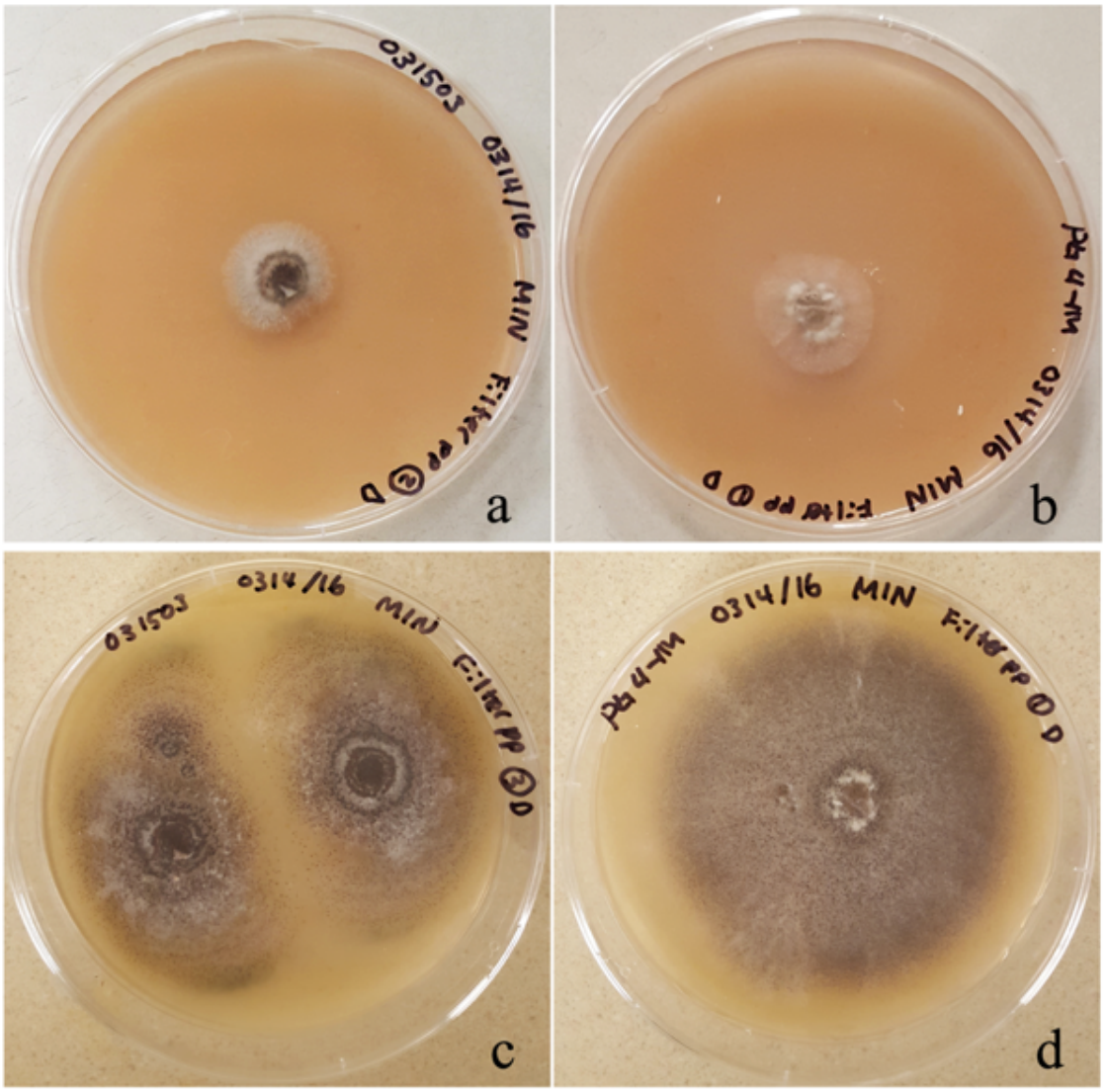

Figure 3.4. Culturing two *Leptosphaeria maculans* isolate 03-15-03 and PG 4-1M on V8 agar medium containing streptomycin antibiotics. a & b) 2-day-old isolate culture, c & d) 7-day-old isolate culture.

3.2.5.2 Inoculum Preparation

One way to preserve the virulence of *L. maculans* is to culture the fungus spores from the fresh cotyledon infected by the pathogen isolate (Nakasone et al. 2004). An initial inoculum prepared by culturing single spores was used to confirm parental phenotype. The infected cotyledon was surface sterilized with 70% ethanol for 5 seconds, then in 10% bleach for 2 minutes, and then it was rinsed with sterilized distilled water. The sterilized infected cotyledon was cut into less than 1 mm^2 size prior to placement on a V8 agar media. In 1L of V8 agar media, it contains 200 ml V8 juice (Campbell Soup Company Ltd. Toronto, ON), 800 ml distilled water, 15 g agar and 0.75 g calcium carbonate, 0.1 g streptomycin sulphate. The culture plates were then placed at room temperature for 4 to 7 days.

When masses of hyphae and pycnidia formed, a single spore collected using reusable inoculating loops were placed onto a fresh V8 agar media and grown as a single spore. After 7 to 14 days, when pink-purplish ooze (Figure 3.5) demonstrated the presence of pycnidiospores of *L. maculans*, the spores were harvested by flooding with sterilized distilled water, and scraping with a microscopic glass slide. The pycnidiospore suspension was filtered through cheesecloth into 50 ml screw cap centrifuge tubes (Fisher Scientific, Waltham, USA). The pycnidiospores were resuspended with a volume of 5 ml to 10 ml sterilized distilled water followed by centrifugation at 4000 rpm for 10 minutes. Blackleg inoculums were prepared for the final concentration of 2 x 10^7 pycnidiospores per mL using hemocytometer (Appendix III). A hemocytometer is designed to count cells and calculate cell density - critical information for proper inoculation (Hausser Scientific, USA). In order to minimize sampling errors, an average

of five 0.04 mm^2 per chamber was counted (Appendix IV). Concentrated inoculum was separated into 1.5 ml Eppendorf tube and stored at – 20 °C for subsequent use.

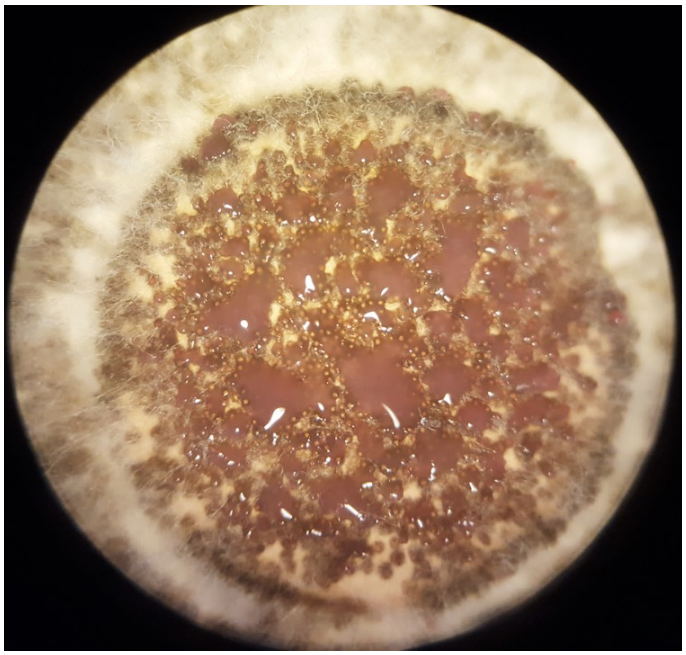

Figure 3.5. Formation of mycelium and black pycnidia of *Leptosphaeria maculans* on V8 agar medium. Pink ooze contains pycnidiospores.

Another way to maintain blackleg pathogenicity was by preparing isolate discs (Mengistu et al. 1993). Small discs (0.8 mm diameter in size) of autoclaved filter paper were immersed in concentrated inoculum and then dehydrated in a laminar hood. A separate disc was placed on a V8 media for culturing (Figure 3.4) or stored at – 20 °C for future use. Defrosting inoculum stored at – 20 °C should be allowed 15 to 30 minutes at room temperature before inoculation to catalyze fungal spore pathogenicity (Mengistu et al. 1993). Both isolates were cultured subsequently every 3 to 4 months to maintain the virulence of *L. maculans*.

3.2.5.3 Blackleg Inoculation

In six to seven days after planting, seedlings were prepared for inoculation (Figure 3.6). To standardize the symptoms of disease across each sample, a punctured infection site was created on each half of the cotyledons using pinpoint forceps. Cotyledon inoculation was performed by placing a 10 µl droplet of pycnidospore suspension on the wounded site of the leaf surface (Figure 3.6). The inoculated plants were placed back in the growth chamber following 12 to 24 hours of penetration period. Developing primary leaves were cut off to retain healthy green cotyledon leaves for appropriate evaluation at the cotyledon stage. Plants were monitored for disease symptoms regularly during the period of 3 to 21 days-post-inoculation (DPI).

Figure 3.6. Seven-day-old seedlings inoculated with 10µl-droplet ($2x10^7$ spores per mL) of *L. maculans* inoculum on the wounded site of cotyledons.

3.2.5.4 Initial Identification of Blackleg Resistance in B. juncea UM lines

A total of fifty-four UM lines were initially tested with cotyledon inoculation using *L. maculans* isolate 03-15-03 along with three differential *B. napus* L. cultivars Weser, Glacier and Quinta. Fifty-three showed complete resistance against 03-15-03 and one UM line (UM3086) was identified as partial susceptible to blackleg (Appendix II). Latter tests with the isolate PG4-1M

were also performed for the selected UM lines (UM3056, UM3063, UM3073, UM3095, UM3108, UM3122, UM3477, UM3541 and UM3544) and UM3086. The UM lines showed resistance to the isolate 03-15-03 also had resistance to PG4-1M. The line UM3086 was susceptible to the both isolates (Appendix II). Based on plant interaction with the fungal isolates and the symptoms of leaf lesion, the two isolates 03-15-03 and PG4-1M can be distinguished by the three *B. napus* L. cultivars, Westar, Glacier and Quinta (Figure 3.7). Cotyledon response against PG4-1M was slow and less severe compared to 03-15-03, which produced abundant black fruiting body pycnidia and more severe tissue collapse.

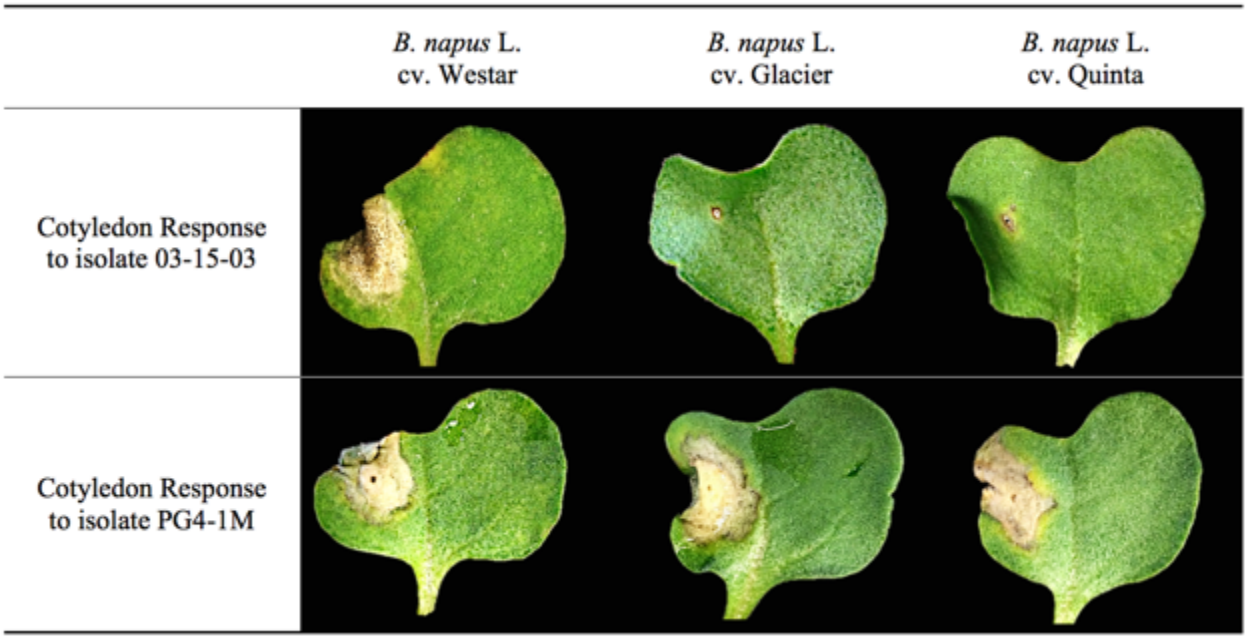

Figure 3.7. Differential interaction of *B. napus* L. cultivars Westar, Glacier and Quinta with *L. maculans* (isolate 03-15-03 and PG4-1M) at seedling stage.

3.2.5.5 Blackleg Evaluation and Selection Criteria

Inoculated plants were screened for cotyledon response to *L. maculans* during 7 to 14 DPI and rated on 10 ± 2 DPI using a 0 to 9 rating scale (Delwiche 1980). The scale is described below (Table 3.2); Figure 3.8 represents the blackleg symptoms on cotyledons according to the scale. Disease scoring was determined along with the symptoms on controls and both parents. In the current study, plants scoring 0 to 4 and 5 to 9 were defined as resistant and susceptible respectively (Table 3.3). Within resistant phenotypes, individual plants showing high levels of resistance (scored 0 or 1) and good seed set were randomly selected and carried forward for the next cross generation. After the selection, both resistant samples and susceptible or recurrent parents were transplanted individually into 6-inch plastic pots and grown under greenhouse conditions.

Table 3.2. Description of blackleg symptoms on cotyledon based on a 0 to 9 rating scale (Delwiche 1980).

Disease Score	Description
0	No darkening of tissue around wound, as in controls
1-2	Limited blackening around wound, lesion diameter 0.5 - 1.5 mm, faint chlorotic halo may be present, sporulation absent
3-4	Dark necrotic lesions, diameter 1.5 - 3.0 mm, chlorotic halo may be present, sporulation absent
5-6	Limited sporulation, lesions diameter 3.0 - 6.0 mm, may show grey-green tissue collapse or dark necrosis throughout
7-8	Grey-green tissue collapse 3.0 – 5.0 mm, sharply delimited, non-darkened margin
9	Rapid tissue collapse at about 10 days, accompanied by profuse sporulation in large, more than 5.0 mm, lesions with diffuse margin

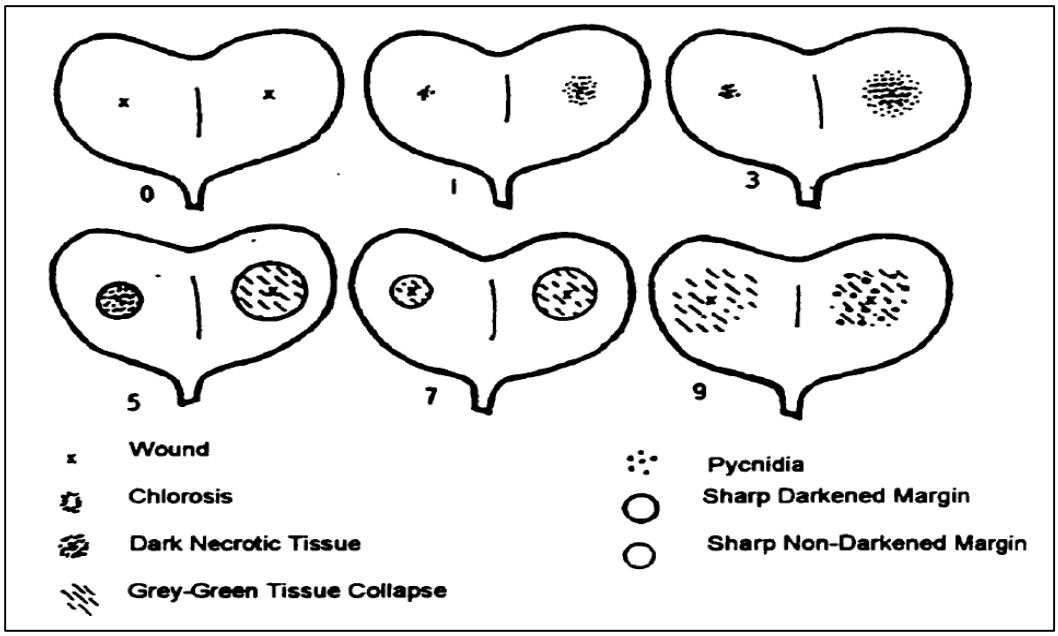

Figure 3.8. Schematic representation of cotyledon response against *L. maculans* on a 0 to 9 scale (Delwiche 1980)

Table 3.3. Phenotype categorized into two or four disease classes in the current study.

Rating Scale	Phenotype Classification	
0-2	Resistant	Resistant
3-4	Intermediate Resistant	
5-6	Intermediate Susceptible	Susceptible
7-9	Susceptible	

3.3 Results

3.3.1 Germination Rate

Out of fifty-four UM lines, fourteen UM lines which showed below 50% of germination rate and one susceptible UM line were excluded in the study (Appendix I). Within the remaining thirty-nine UM lines, ten lines showed about 70% of germination rate were chosen according to the germination rate tested with F_1 seeds and their parent UM lines (Table 3.4). The number of F_1 seeds planted in each UM line varied due to the different crossing frequency across all lines. However, the germination rate of F_1 seeds indicated no significant differences among the ten UM lines. When compared to the results of parents, the average germination rate was decreased from 95.6% to 86.1% in F_1 population. The three lines (UM3063, UM3108 and UM3541) showed 100% of germination rate and the UM3122 showed the lowest germination rate (69%).

Figure 3.9 represents the germination rate of seeds in each cross population F_1, BC_1, BC_2 and BC_3. Overall, most BC_1 seeds showed over 50% germination rate except UM 3075 derived seeds of which a significantly low germination rate (33%) was observed. The results of BC_2 and BC_3 population presented only for three UM lines (UM3073, UM3095 and UM3122). In the BC_2, the two UM lines UM3073 and UM3095 showed a similar or better germination rate compared to their BC_1 results. Surprisingly, however, a very poor germination (13%) in eight seeds was recorded for UM3122; improved seed germination nearly as the average was observed in the BC_3. The high levels of plant sterility observed in other seven UM lines resulted in zero to a few seeds, which limits further analysis. Thus, only UM3073, UM3095 and UM3122 were remained for further introgression of blackleg resistance.

Table 3.4. Germination rate of F_1 seeds of the cross between *B. juncea* UM lines and *B. napus* L. Westar.

Generation[a]	Parent UM line	No. of plants sown (A)	No. of plants germinated (B)	Germination rate (B/A x 100)
F_1	3056	16	14	88%
	3063	32	32	100%
	3073	35	29	83%
	3075	12	10	83%
	3095	8	6	75%
	3108	16	16	100%
	3122	16	11	69%
	3477	48	41	85%
	3541	12	12	100%
	3544	32	25	78%
	Average Germination Rate			86.1%

[a]: F_1 was produced by crossing between *B. juncea* UM lines (female parent) x *B. napus* L. cv. Westar (male parent).

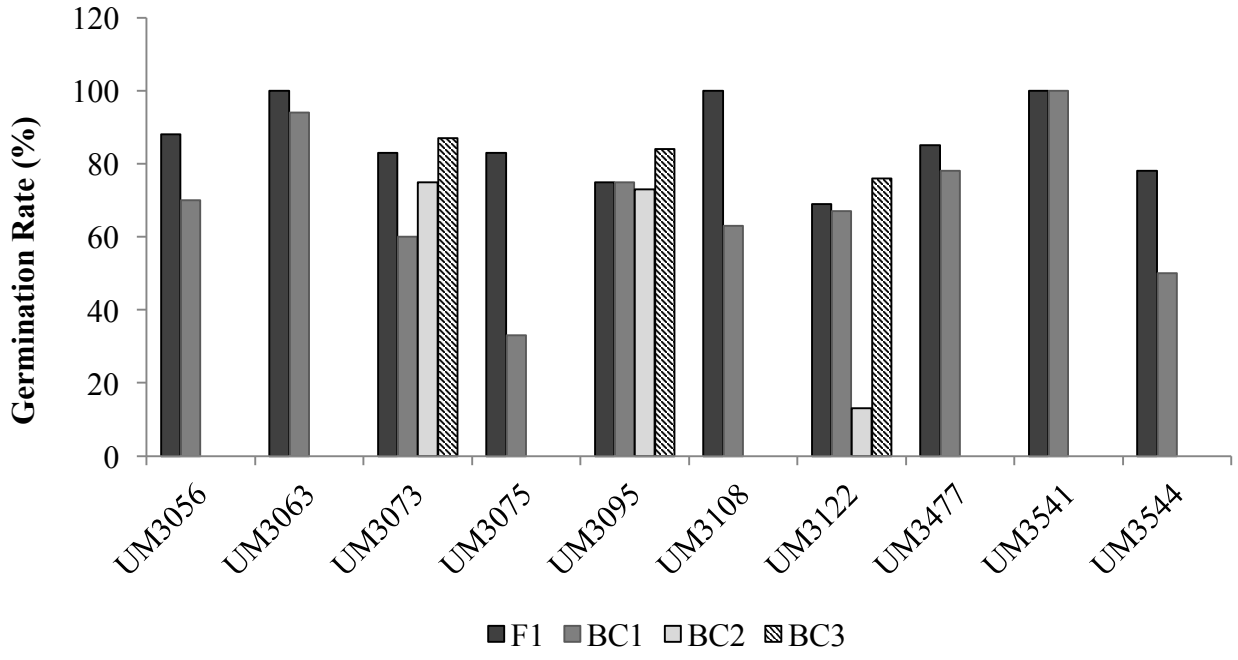

Figure 3.9. Germination rate of seeds in each cross population (F_1, BC_1, BC_2 and BC_3).

3.3.2 Pollen Viability

Acetocarmine staining images provided evidence of pollen sterility, which often occurs in interspecific hybridization. Pollen grains of the male parent Westar were stained in concentrated pinkish-red color, indicating viable pollen grains while viable pollen grains in F_1 hybrids derived from three UM lines (UM3073, UM3095 and UM3122) and the susceptible Westar were rarely observed (Figure 3.10 a and b), suggesting that F_1 plants were male sterile. Because of low pollen viability, F_1 plants were used as female parents in the production of the BC_1 generation. After backcrossing, pollen viability was noticeably improved in BC_1 (Figure 3.10 b and c). Although non-viable pollen grains were still observed in BC_1 plants, the plants were used as pollen donors for the BC_2 generation. In the advanced backcross generation BC_3, the frequency of non-viable pollen grains was significantly reduced: these results are consistent with the improved pod set as well as increased seed set in the BC_3 (Figure 3.10 d).

Based on the results of plant fertility, highly sterile plants were still found in BC_1 samples of the following UM lines: UM3063, UM3477 and UM3541. The pollen viability from UM3063, UM3477 and UM3541 might not be the same as those tested with acetocarmine staining.

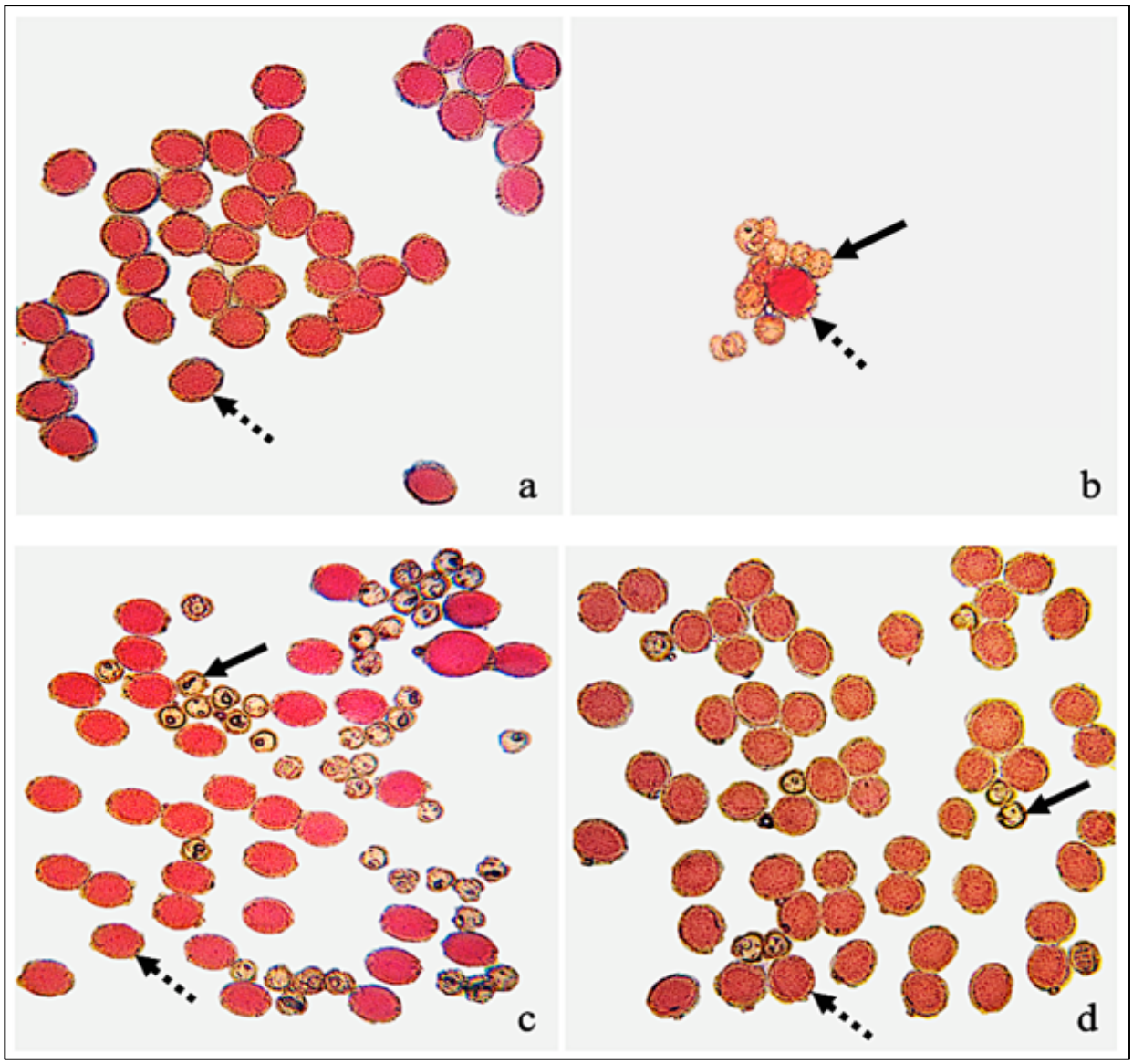

Figure 3.10. Acetocarmine staining image of pollen grain from the test populations under a light microscope at magnification 40X. a) *B. napus* parent Westar; b) F_1 hybrid of *B. juncea* x Westar; c) BC_1, F_1 backcrossed to Westar; d) BC_3, F_1 backcrossed three times to Westar. Viable pollen are stained in an intense color in an oval shape cell (dotted arrow), whereas non-viable pollen are shrunken and stained with a diluted color (full arrow).

3.3.3 Pod Set

The overall average pod set in F_1 crosses showed a moderate (50.5%) to good (70.5%) level (Figure 3.11). Four UM lines UM3056, UM3063, UM3073 and UM3075 were outstanding for their pod set rates. Almost half of the F_1 crosses in UM3544 showed a poor pod set while other five UM lines UM3095, UM3108, UM3122, UM3477 UM3541 and UM3544 produced average pod set rate. Interestingly, however, BC_1 crosses showed more variations in terms of pod set rate compared to F_1 results (Figure 3.12). Despite variations exhibited, pod set data of five UM lines UM3056, UM3073, UM3075, UM3095 and UM3122 in the BC_1 showed 5% to 25% good pod set. A very low pod set was also observed in all ten UM lines, which did not appear in F_1 data. All BC_1 crosses from two UM lines UM3541 and UM3544 showed extremely low pod set rates. Due to the low pod set rates, obtaining BC_2 seeds was unsuccessful in most UM lines.

Producing BC_1 generation by backcrossing to other parent *B. juncea* UM lines was failed in most UM lines (data not shown). The average pod set was very poor (34%) and most samples were recorded below 50%. The average seed set was 0.24 ± 0.38 and obtaining even a single seed was very difficult.

In BC_2 and BC_3 generations, pod set data obtained from three UM lines UM3073, UM3095 and UM3122 were assessed. Although over 50% of BC_2 samples from the three UM lines showed a good (70.5%) or better pod set rate, a low pod set rate was still observed in UM3122 (Figure 3.13). However, in the BC_3, the results from all three UM lines indicated nearly normal pod set rates (Figure 3.14).

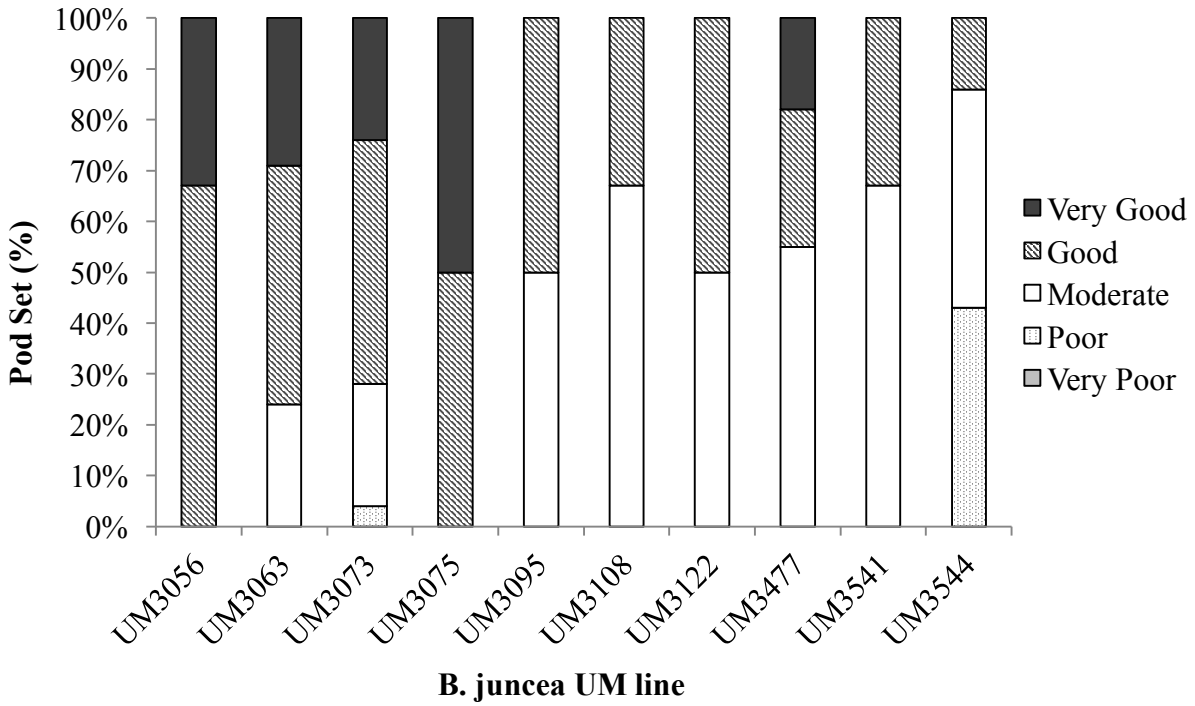

Figure 3.11. The average pod set in the F_1 crosses between *B. juncea* UM lines (female) and *B. napus* L. cv. Westar (male).

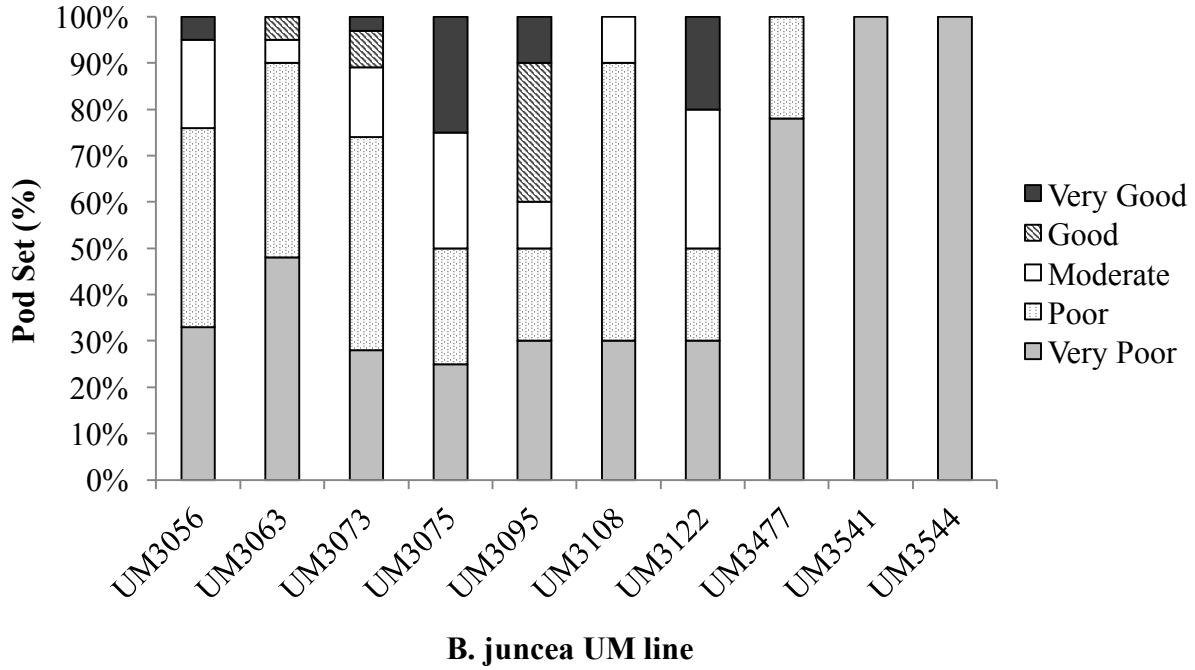

Figure 3.12. The average pod set in the BC_1 crosses, F_1 hybrids (*B. juncea* UM lines/*B. napus* L. cv. Westar) backcrossed to Westar.

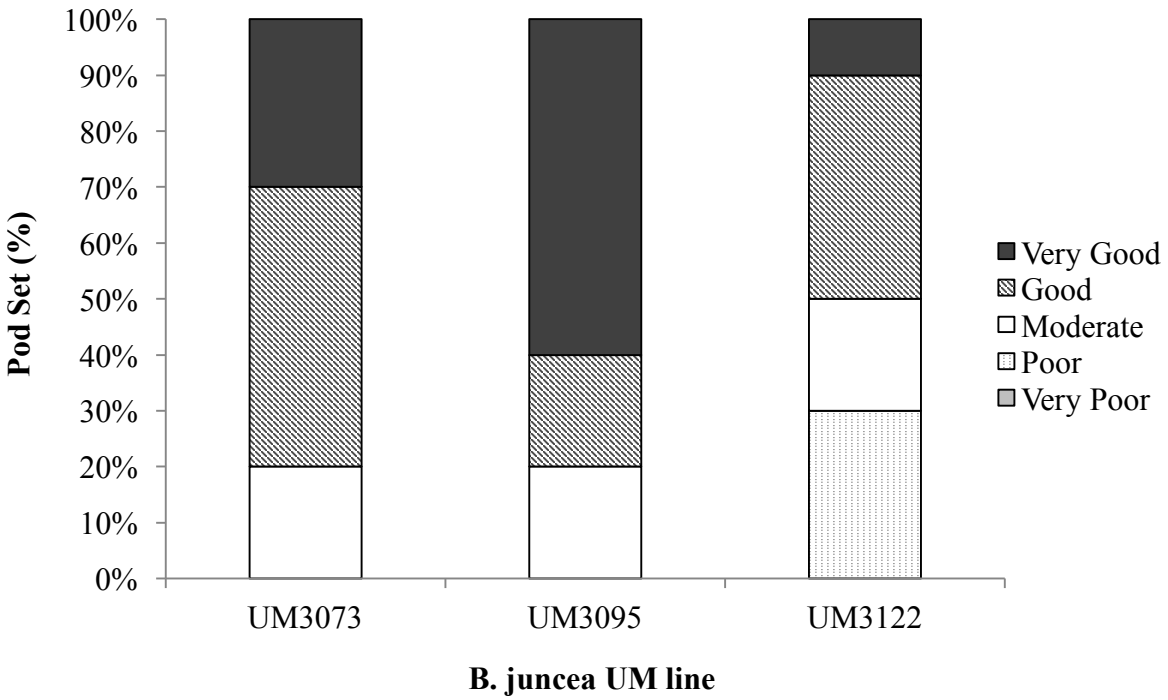

Figure 3.13. The average pod set in the BC_2 crosses, F_1 hybrids (*B. juncea* UM lines/ *B. napus* L. cv. Westar) backcrossed twice to Westar.

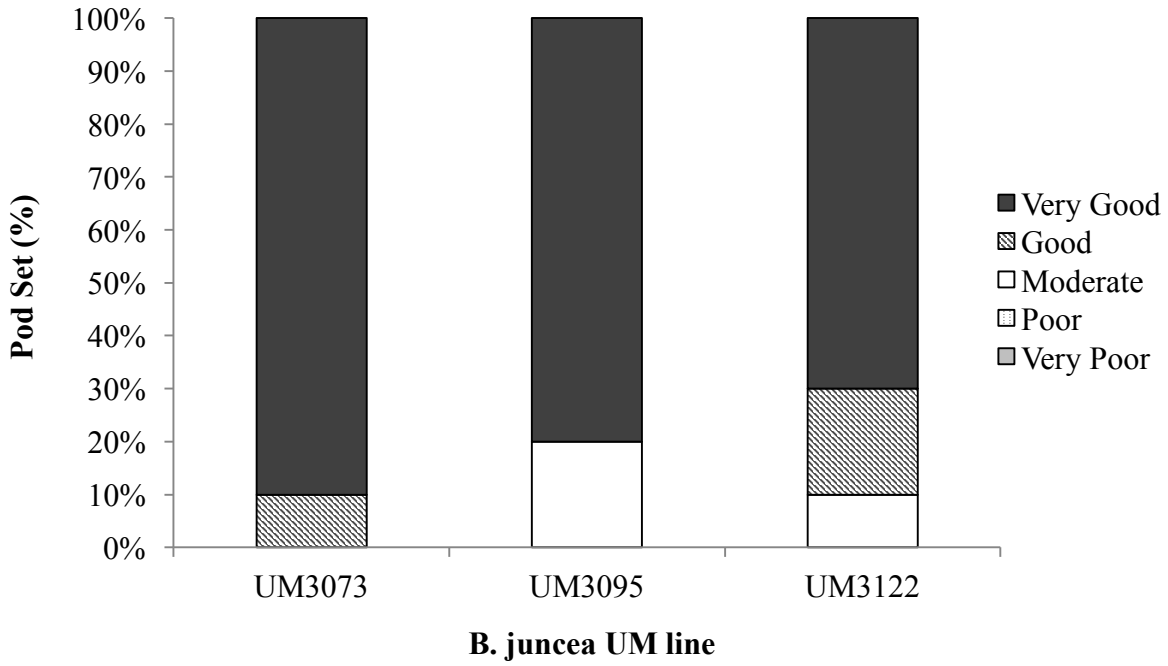

Figure 3.14. The average pod set in the BC_3 crosses, F_1 hybrids (*B. juncea* UM lines/ *B. napus* L. cv. Westar) backcrossed three times to Westar.

3.3.4 Seed set

Figure 3.15 presents the overall comparison of the average seed set in all crossed populations with *B. juncea* UM lines and *B. napus* L. cv. Westar. Each cross population data was pooled for the UM lines listed as followings: F_1 and BC_1 (UM3056, UM3063, UM3073, UM3075, UM3095, UM3108, UM3122, UM3477, UM3541 and UM3544); BC_2 and BC_3 (UM3073, UM3095 and UM3122); BC_4 (UM3073).

Obtaining progenies from interspecific crosses was more difficult in earlier generations. For example, an average 1.15 and 10.28 seeds were harvested in the BC_1 and BC_3 respectively. When compared to BC_1 data, a greater average seed set was discovered in F_1. However, the seed set results in the F_1 was only 15% of the recurrent parent Westar. The average number of seeds was increased in BC_2 and showed similar results as in the F_1. The low seed set in BC_1 was gradually increased in further backcross generations. A significant increase was observed in BC_3, and almost doubled number of seeds was counted in BC_4. Although the results of pod set obtained in the advanced backcross population BC_3 showed as high as 100%, a difference in seed yields was still found between the recurrent parent Westar and BC_3 progenies.

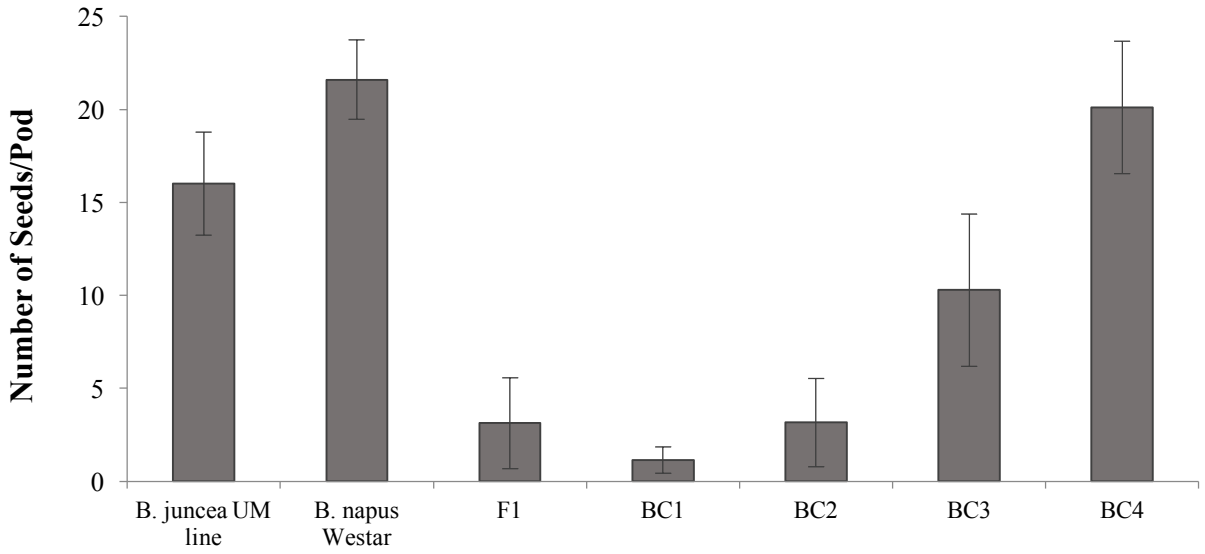

Figure 3.15. The average seed set in each cross generation (F_1, BC_1, BC_2, BC_3 and BC_4) and in both parent *B. juncea* and *B. napus* L. cv. Westar.

Table 3.5 indicates the average number of seeds per pod (seed set) in each cross generation of three UM lines UM3073, UM3095 and UM3122. There was a significant difference in the F_1 seed set between UM3095 and other two UM lines UM3073 and UM3122. This significant difference among the three UM lines did not appear in the BC_1 generation. The lower pod set observed in BC_1 crosses (Figure 3.12) was also confirmed with a lower number of seeds harvested compared to F_1 hybrids. Interestingly, zero to a few seeds were harvested in the BC_2 of UM3122 in spite of the fact that the pod set rate was moderate to good level. Increased seed set was clearly observed within backcross generations. Because of the small number of seed samples obtained in BC_2, more intensive crossing work focused on the three UM lines was carried out for the BC_3 population. As more backcrossing was attempted, variations in seed number were greater in the advanced generation BC_3 than the earlier generations. The maximum number of 15 seeds per pod was recorded in the BC_3 and approximately a hundred to two hundred seeds were harvested from the individual plants of each UM line.

Table 3.5. The average seed set in the cross generations F_1, BC_1, BC_2, and BC_3 of each UM line and in both parents *B. juncea* UM line and *B. napus* L. cv. Westar.

Generation[a]	Parent UM line	Avg. # of seeds/Pod
Brassica juncea UM line	-	16.00 ± 2.77
B. napus L. cv. Westar	-	21.60 ± 2.15
F_1	UM3073	5.44 ± 1.55
	UM3095	1.05 ± 0.55
	UM3122	5.76 ± 0.95
BC_1	UM3073	1.42 ± 0.36
	UM3095	1.61 ± 1.11
	UM3122	1.56 ± 0.51
BC_2	UM3073	2.98 ± 1.16
	UM3095	3.33 ± 3.24
	UM3122	0.62 ± 0.36
BC_3	UM3073	7.18 ± 4.37
	UM3095	12.72 ± 3.52
	UM3122	10.93 ± 2.23

[a]: *Brassica napus* L. cv. Westar was used as female recurrent parent for BC_2 and BC_3 generations while Westar was used as male parent for F_1 and BC_1 generations.

In self-pollinated generations, obtaining even one seed per plant was impossible in F_1 self-pollinated generation (F_2) (Table 3.6). This result also supports pollen viability observed in F_1 in the earlier results (Figure 3.10). After the first backcross, BC_1 plants showed improved pollen viability, and up to 24 seeds per plant were harvested in the first backcross self-pollinated generation (BC_1F_2). In the BC_2F_2 generation, an average of 42 seeds was harvested and the maximum 74 seeds per plant were harvested.

Table 3.6. The average number of seeds harvested per plant in each self-pollinated generation F_2, BC_1F_2 and BC_2F_2.

Self-Pollinated Generation	Avg. # of seeds/Plant (Range)
F_2	0.8 (0 – 10)
BC_1F_2	11.4 (1 – 24)
BC_2F_2	42.0 (10 – 74)

3.3.5 Blackleg Resistance in Cross Populations

A total of 187 resistant F_1 individual plants were obtained in ten UM lines UM3056, UM3063, UM3073, UM3095, UM3108, UM3122, UM3477, UM3541 and UM3544. In the current study, the rating scale from zero to four is defined as resistant phenotype. However, only F_1 plants showed high levels of resistance, scored zero to two, were selected and carried forward for BC_1 generation. Although the sample size varied among different UM lines, all F_1 progenies showed resistance to the *L. maculans* isolate 03-15-03 (Table 3.7).

Table 3.7. Phenotypic analysis of blackleg resistance against *L. maculans* isolate 03-15-03 in the F_1 derived from the crosses between *B. juncea* UM line and *B. napus* L. cv. Westar.

Generation[a]	*L. maculans* Isolate	Parent UM line	Total	Phenotype	
				R[b]	S[c]
F_1	03-15-03	UM3056	14	14	0
		UM3063	32	32	0
		UM3073	29	29	0
		UM3075	10	10	0
		UM3095	6	6	0
		UM3108	16	16	0
		UM3122	11	11	0
		UM3477	32	32	0
		UM3541	12	12	0
		UM3544	25	25	0
		Pooled	187	187	0

[a]F_1 was produced by crossing between *B. juncea* UM lines (female parent) and *B. napus* L. cv. Westar (male parent).
[b]R: Resistant phenotype (Disease score 0 to 4).
[c]S: Susceptible phenotype (Disease score 5 to 9).

High levels of blackleg resistance, however, were segregated in the BC_1 populations of ten UM lines (Table 3.8). A wide range of sample size was discovered in the BC_1 due to the number of seeds planted and seed germination rate. More frequent crosses were made in UM3073, which resulted in greater number of plant samples. The plant sample size in the BC_1 was mostly below ten except four lines UM3063, UM3073, UM3122 and UM3477.

Table 3.8. Phenotypic analysis of blackleg resistance against *L. maculans* isolate 03-15-03 in BC_1 population.

Generation[a]	*L. maculans* Isolate	Parent UM line	Total	Phenotype	
				R[b]	S[c]
BC_1	03-15-03	UM3056	7	3	4
		UM3063	15	5	10
		UM3073	45	7	38
		UM3075	2	1	1
		UM3095	9	7	2
		UM3108	5	3	2
		UM3122	10	5	5
		UM3477	14	4	10
		UM3541	4	4	0
		UM3544	3	1	2
		Pooled	114	40	74

[a]BC_1 was produced by crossing between resistant F_1 individual plants (female) and *B. napus* L. cv. Westar (male plant).
[b]R: Resistant phenotype (Disease score 0 to 4).
[c]S: Susceptible phenotype (Disease score 5 to 9).

In the earlier results, increased pollen viability was shown in backcrossed generations of three UM lines UM3073, UM3095 and UM3122. The UM lines showed a low survival rate of BC_1 plants and a poor germination of BC_2 progenies were excluded in the screening for blackleg resistance in the following generations. The phenotypic analyses of BC_2 populations of all three UM lines showed that eighteen out of a hundred seedlings have good resistance (Table 3.9). Due to the extremely low (13%) seed germination, only one BC_2 seedling in UM3122 was evaluated for blackleg resistance. This individual was shown to have good resistance.

Table 3.9. Phenotypic analysis of blackleg resistance against *L. maculans* isolate 03-15-03 in BC_2 population.

Generation[a]	*L. maculans* Isolate	Parent UM line	Total	Phenotype	
				R[b]	S[c]
BC_2	03-15-03	UM3073	52	9	43
		UM3095	47	8	39
		UM3122	1	1	0
		Pooled	100	18	82

[a]BC_2 was produced by crossing between *B. napus* L. cv. Westar (female recurrent parent) and resistant BC_1 individual plants (male parent).
[b]R: Resistant phenotype (Disease score 0 to 4).
[c]S: Susceptible phenotype (Disease score 5 to 9).

With approximately two hundred seeds obtained from BC_3 crosses of an individual plant family line, a separate inoculation test was performed with two *L. maculans* isolates 03-15-03 and PG4-1M. Two BC_3 family lines of 73-1 and 73-2, were tested with only one isolate 03-15-03 due to the small sample size. Different segregation patterns of blackleg resistance were observed within

the family lines of UM3073 (Table 3.10). Out of 553 individual plants, 75 showed resistance in BC_3 population. Two family lines 73-2 and 73-3 showed an approximate 25% resistant phenotypes whereas less than 10% of the hundred BC_3 progenies in the line 73-1 showed resistance.

A significantly different result was observed in the family line 95-1 indicating that a total loss of blackleg resistance to both isolates 03-15-03 and PG4-1M in all 176 progenies happened (Table 3.10 and Table 3.11). In the results of line 95-2, percentage of the resistant individual plants (17% in the total progenies tested) in BC_3 appeared to be the same as shown in BC_2; the doubled number of plant samples were evaluated in BC_3. The number of resistant plants was relatively low in family line 22-1 compared to other four family lines excluding the line 95-1.

Table 3.10. Phenotypic analysis of blackleg resistance against *L. maculans* isolate 03-15-03 in BC_3 population.

Generation[a]	*L. maculans* Isolate	Parent UM line	Family line	Total	Phenotype	
					R[b]	S[c]
BC_3	03-15-03	UM3073	73-1	100	9	91
			73-2	88	22	66
			73-3	95	23	72
		UM3095	95-1	90	0	90
			95-2	94	16	78
		UM3122	22-1	86	5	81
		UM Pooled		553	75	478

[a]BC_3 was produced by crossing between *B. napus* L. cv. Westar (female recurrent parent) and resistant BC_2 individual plants (male parent).
[b]R: Resistant phenotype (Disease score 0 to 4).
[c]S: Susceptible phenotype (Disease score 5 to 9).

In a separate screening for resistance against isolate PG4-1M, the proportion of resistant phenotype in each family line of BC_3 population was similar to the results with isolate 03-15-03 (Table 3.10 and Table 3.11). In two family lines 73-3 and 95-2, 17% and 18% of resistant plants were obtained respectively (Table 3.11). In family line 22-1, less number of resistant plants was obtained. The results suggested that plants showing blackleg resistance against the isolate 03-15-03 also were shown to have resistance to the isolate PG4-1M.

Table 3.11. Phenotypic analysis of blackleg resistance against *L. maculans* isolate PG4-1M in BC_3 population.

Generation[a]	*L. maculans* Isolate	Parent UM line	Family line	Total	Phenotype	
					R[a]	S[b]
BC_3	PG4-1M	UM3073	73-3	95	18	77
		UM3095	95-1	86	0	86
			95-2	87	15	72
		UM3122	22-1	84	9	75
		UM Pooled		352	42	310

[a]BC_3 was produced by crossing between *B. napus* L. cv. Westar (female recurrent parent) and resistant BC_2 individual plants (male parent).
[b]R: Resistant phenotype (Disease score 0 to 4).
[c]S: Susceptible phenotype (Disease score 5 to 9).

The results of phenotypic analysis using two *L. maculans* isolates in the BC_3 population were pooled (Table 3.12). In the BC_3, 13% (117 out of 905 individual plants) showed resistance response at the seedling stage. Variations on the number of resistant phenotypes existed among different UM lines as well as within a UM line. Within a UM3073 line, the percentage of

resistant phenotypes was 9% (9 individual plants in 100 samples) in the family line 73-1, 25% (22 individual plants in 88 samples) in the line 73-2 and 21.6% (41 individual plants in 190 samples) in the line 73-3. In the UM3095, the family line 95-2 obtained 17.1% of resistant plants in 181 samples. The family line 22-1 from the UM3122 showed only 8% (14 resistant phenotypes in 170 plants).

Table 3.12. Pooled data in BC_3 population.

Generation[a]	Parent UM line	Family line	Total	Resistant	Susceptible
BC_3	UM3073	73-1	100	9	91
		73-2	88	22	66
		73-3	190	41	149
	UM3095	95-1	176	0	176
		95-2	181	31	150
	UM3122	22-1	170	14	156
	UM Pooled		905	117	788

[a]BC_3 was produced by crossing between *B. napus* L. cv. Westar (female recurrent parent) and resistant BC_2 individual plants (male parent).

In this study, only individual plants showing a high level of blackleg resistance at the seedling stage (Figure 3.16) were carried forward to produce further backcrossed generations as well as self-pollinated generations. Most resistant plants grew well in the later growing season and appeared to be healthy plants (Figure 3.17). Typical symptoms of blackleg such as basal stem cankers, seedling root rot, black fruiting body, and grey lesions on leaves were observed in the

susceptible Westar (Figure 3.18); less severe symptoms were observed in a few resistant plants (data not shown). Resistant plants with symptoms at adult stages were excluded in the production of the next generation.

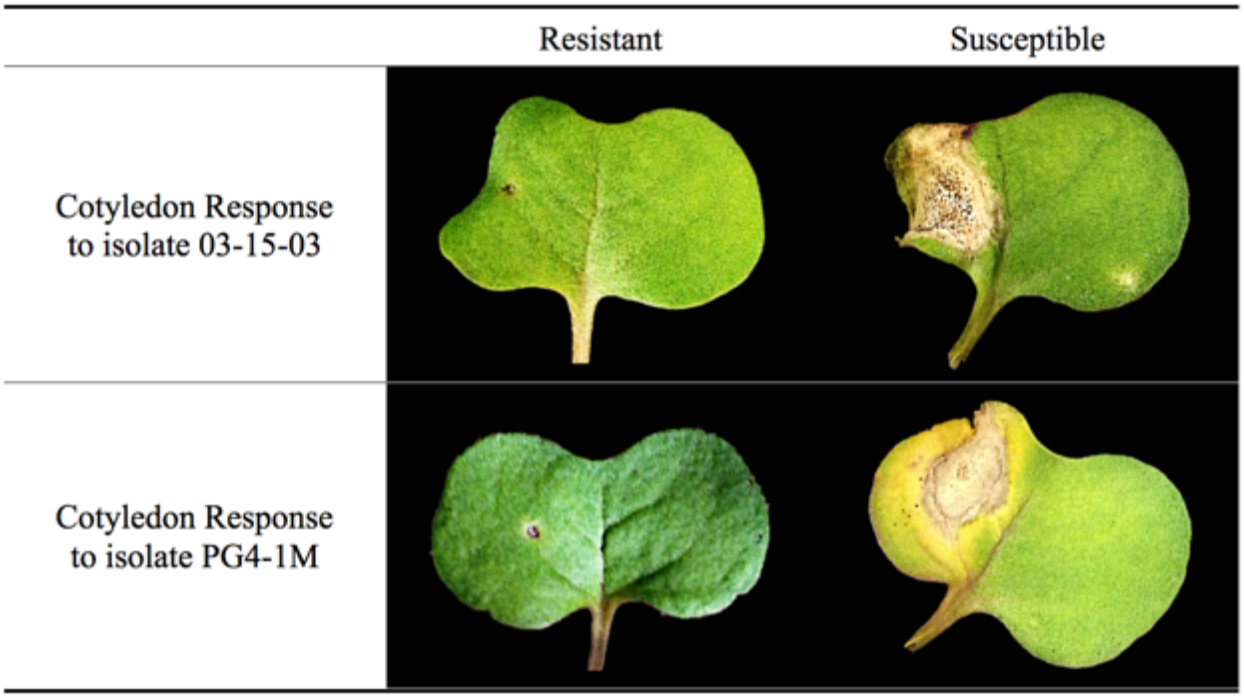

Figure 3.16. Screening results in the test population of BC_3 inoculated with two *L. maculans* isolates 03-15-03 and PG4-1M.

Figure 3.17. A healthy *Brassica* plant growing on a 6-inch plastic nursery pot.

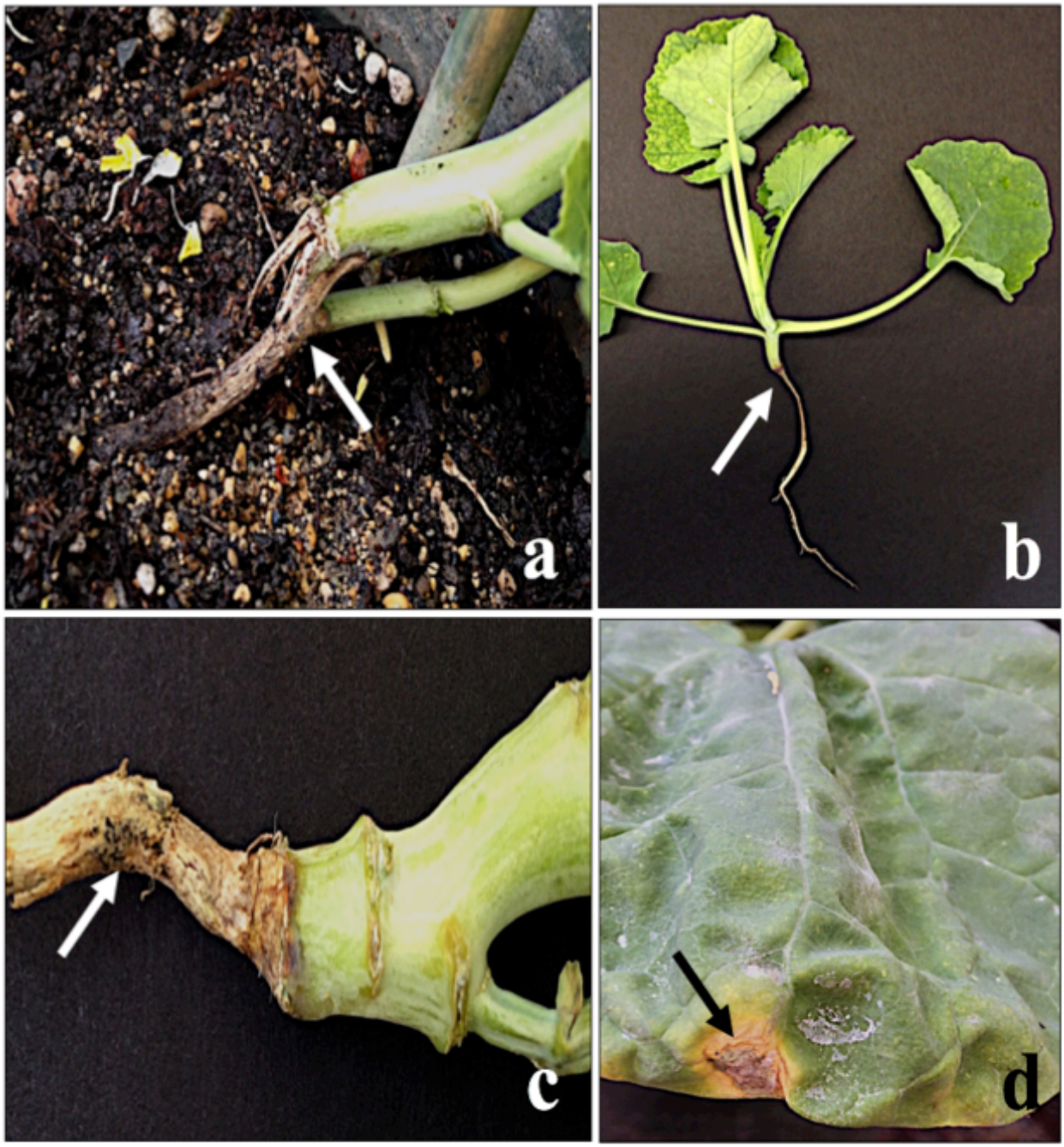

Figure 3.18. Symptoms of blackleg observed at adult plant stage of a susceptible *B. napus* L. cv. Westar a) basal stem canker; b) seedling root rot; c) presence of black fruiting body (pycnidia); d) grey leaf lesion.

3.4 Discussion

Morphological feature of *B. napus* and *B. juncea* provide basic information deciding the direction of a cross. As presented earlier in this study, *B. napus* has greater sized flower buds compared to *B. juncea* which makes emasculation easier with lower chances of damaging the female organs of a flower. However, Meng and Lu (1993) explain that interspecific failure between *B. napus* and *B. juncea* is caused by the heavy callose deposition of pollen tube in *B. juncea* which blocks the penetration of stigmatic papillae. This resulted in the development of poor embryos and endosperms in the production of F_1 seeds.

On the other hand, when *B. juncea* is used as a maternal parent, the success rate of plant fertilization and hybrid production was improved (Tsuda et al. 2014; Heenan et al. 2007; Meng and Lu 1993). In addition, previous studies show a higher percentage of true hybrids derived in *B. juncea* x *B. napus* (100%) compared to *B. napus* x *B. juncea* (58%), this finding strongly suggests the use of *B. juncea* as the maternal parent in interspecific hybridization (Mason et al. 2011).

In most F_1 hybrid plants, morphological appearance showed an intermediate phenotype between *B. juncea* and *B. napus* (data not shown). Flower morphology of crossed plants as an indication of genetic recombination can also provide an estimation of cross-productivity. Previously, Heenan et al. (2007) discuss that *B. napus* like F_1 hybrids show greater pollen fertility (78%) and significantly more seeds per pod (4.7) compared to the intermediate phenotype which result in low pollen fertility (25%) and very low seed yield (0.2). Another similar study also showed that *B. napus* like F_2 plants yield a mean of 36.6 g per plant whereas intermediate plant types yield 1

g per plant (Roy 1978). With similar variations, successful crosses between *B. juncea* and *B. napus* existed in the current study (Table 3.5). Previously, low pollen fertility (less than 30%) and fewer seeds per pod (range 0.0 – 4.4) have been discussed in interspecific hybridization studies of *Brassica* species (Mason et al. 2011; Tsuda et al. 2011; Heenan et al. 2007). Choudhary and Joshi (1999) also reported that obtaining even a single F_1 plant was nearly impossible in a specific cross combination.

Heuer et al. (2003) suggest that interspecific incompatibility can be overcome by producing two or more backcross generations. A similar study using *B. juncea* as recurrent parents discussed that nearly recovered seed productivity was shown in BC_3 generation (range 15.3 – 16.2) compared to their parent cross (15.8) (Song et al. 2010). In the present study, microscopic image of pollen grains showed a significant improvement of pollen fertility in BC_1 generation (Figure 3.10). In addition, the results from the average number of seeds harvested in self-pollinated generations and microscopic image of pollen grains supported the recovery of pollen fertility in backcross generations (Table 3.6).

Despite the fact that pollen viability and seed counts were significantly improved in the advanced backcross generation BC_3 (Figure 3.10 and Table 3.5), more severe incompatibility exists in the earlier backcross generations of BC_1 when compared to F_1. Interestingly, Song et al. (2010) discover that the significantly lower number of seeds (0.8) was harvested when an F_1 hybrid was used as a female parent in backcrossing to the recurrent parent *B. juncea* compared to its reciprocal cross (2.13). Another study on F_1 hybrid pollination with three different varieties of each *B. juncea* and *B. napus* explained the variations occurring with genotypes, which means one

cross-combination obtained increased seed number per silique in BC_1 (2.4 ± 2.0) than F_1 (1.6 ± 1.5); the other two cross-combinations produced very few seeds (0.1 ± 1.8) in BC_1 generation (Heenan et al. 2007). High variations in seed productivity in cross-generations were shown in different genotypes of *B. juncea* and *B. napus* in other previous studies (Mason et al. 2011; Tsuda et al. 2011; Choudhary and Joshi 1999).

According to Köhler et al. (2010), the ratio of genome composition from both parents is critical for normal growth and the development of an embryo. In the current study, the results of interspecific hybridization also suggested that the improved pollen quality in BC_1 is not necessarily expected to show increased seed productivity in BC_2 generations (Table 3.5). The theory also explains the abortion and abnormal development of embryo as a result of aborted seeds. Empty seed pods or aborted seeds observed mostly in the earlier cross generations such F_1 and BC_1 might be due to the additional B-chromosome from *B. juncea* in *B. napus*, and this showed no correlation between pod set and seed set (Figure 3.11, 3.12 and Table 3.5).

Amongst B-genome *Brassica* species, Scheffler and Dale (1994) concluded that *B. juncea* shows the highest cross-compatibility with *B. napus* when used as maternal parents in interspecific hybridization. However, introducing resistance gene in the *B. juncea* B-genome into *B. napus* is not easy due to the low frequency of homeologous pairing between B-genome and A- and C-genome (Mason et al. 2010; Meng et al. 1998) and high frequency of multivalent chromosome association (Cui et al. 2012; Mason et al. 2010). The genome composition of F_1 hybrids between *B. juncea* (AABB) and *B. napus* (AACC) could be AABC (Tsuda et al 2012; Mason et al. 2010) or AAB or AABBC (Sabharwal and Doležel 1993). This different composition resulted

depending on the choice of parental plant. Mason et al. (2010) also explained that the variations in the frequency of allosyndesis between the genome of A, B and C within three AABC interspecific hybrid lines, which derived between *B. juncea* and three different *B. napus* cultivars, may be affected by the different combinations of parental genes and genome.

The authors describe that there is a higher rate of chromosome pairing and bivalent events between A- and C-genome within the combinations of *Brassica* genome A, B and C (Cui et al. 2012). In the present study, the genome composition of F_1 hybrids would be AABC as the crossing way was considered, and the better seed set results observed in BC_1 generation which F_1 was backcrossed to the *B. napus* parent (AABC x AACC) than to the *B. juncea* parent (AABC x AABB) could also provide an evidence of the higher compatibility between *Brassica* A- and C-genome than A- and B-genome.

Many interspecific hybridization studies have used genome specific SSR markers to confirm true introgression of the B-genome in the hybrids (Fredua-Agyeman et al. 2014; Navabi et al. 2011; Christianson et al. 2006; Schelfhout et al. 2004). However, previous studies have reported difficulties in maintaining *Brassica* B-genome in the AC genome background because of their inheritance pattern as B-genome chromosomes are segregating as a whole or with terminal deletion (Navabi et al. 2010; Schelfhout et al. 2006). Another study also showed that only 10% of the B-genome loci were retained in BC_3 generation where *B. juncea* was used as a donor parent in *B. napus* background (Dixelius and Wahlberg 1999).

The theory about the recovery rate of recurrent genes in backcrosses explains the lower chances of having the B-genomes as an additional chromosome in which BC_3 progenies contain only 6.25% of genes from their donor parent (Hasan et al. 2015; Vogel 2009). Although molecular tests for additional B-genome in the hybrids are worthwhile to confirm true hybrids, the confirmation test was not valuable in the present study since the resistance observed in all F_1 hybrids of *B. juncea* and complete susceptible *B. napus* cultivar indicates indirectly the successful introgression of *B. juncea* B-genome.

In this study, blackleg resistance in *B. juncea* B-genome was successfully incorporated into the susceptible *B. napus* cv. Westar using the backcross approach. Although pre-fertilization barrier (pollen fertility) and post-fertilization barrier (embryo development) to interspecific hybridization existed between *B. juncea* and *B. napus*; these barriers could be overcome by producing more backcross generations. Further crossing work will be continued in order to stabilize the introgressed resistance gene(s) in backcross generations, and various molecular analyses relating to the resistance will be conducted for future studies.

CHAPTER 4. ANALYSIS OF BLACKLEG RESISTANCE IN SYNTHETIC HEXAPLOID *BRASSICA* SPECIES

4.0 Abstract

The genus *Brassica* is an important species used for cooking oil, condiment and vegetable crops. The six species have been cultivated, three diploid species (*B. rapa* – AA, *B. nigra* – BB, and *B. oleracea* – CC) and three allotetraploids derived from combinations of two diploid species (*B. napus* – AACC, *B. juncea* – AABB, and *B. carinata* – BBCC). In crop breeding, polyplodization has been beneficial to produce vigorous, superior and advantageous species in many crops such as sugar beets, cotton and bread wheat. With similar approaches, hexaploid *Brassica* (AABBCC) have been synthesized by crossing between *B. rapa* and *B. carinata* in order to introduce various beneficial traits into *B. napus*, a valuable oilseed crop worldwide, as well as to increase genetic diversity. No reports on blackleg resistance in synthetic hexaploid materials have been published yet. In the current study, blackleg resistance in synthetic hexaploid lines were evaluated at the seedling stage using *L. maculans* isolate 03-15-03 by crossing between three synthetic hexaploid lines and a susceptible *B. juncea* accession UM3086. The F_1 hybrids showed an extremely low level of embryo development due to the post-fertilization barrier. However, the F_2 plants produced as many as 95 seeds per plant. The results of phenotypic analysis in F_2, BC_1, BC_2 and BC_2F_2 populations suggested that two dominant genes controlling blackleg resistance segregated in backcross populations and each gene conferred the same level of resistance. Although three hexaploid lines C15, C21 and C28 were derived from different crosses, phenotypic analysis indicated the same pattern of genetic inheritance of blackleg resistance. Understanding the mechanism of synthetic hexaploid lines will lay a foundation to introduce the blackleg resistance in hexaploid lines into *B. napus*.

4.1 Introduction

Brassica species are economically important for cooking oil, condiment and vegetable crops. There are six *Brassica* species, three diploids *(B. rapa* – AA, *B. nigra* – BB and *B. oleracea* – CC) and three allotetraploids (*B. napus* – AACC, *B. juncea* – AABB and *B. carinata* – BBCC) (U, 1935). The allotetraploid *B. napus* has been cultivated for centuries (Downey 1966) and originated from the crosses between *B. rapa* and *B. oleracea* (U, 1935). Canola (*B. napus* L.) is one of the most valuable oilseed crops in the world to qualify as "Canola", the oil must contain less than 2% erucic acid and the seed should contain less than 30 micromoles of glucosinolates per gram (Feed Regulations Amendment 1983). To date, canola seeds are produced for the healthiest vegetable oil, a good protein meal, and the potential bioenergy source (Canola Council of Canada 2016).

However, due to the intensive cultivation, the genetic diversity in *B. napus* has been limited compared to other *Brassica* species (Rahman 2013). To broaden genetic diversity and improve seed quality, the efforts toward developing new *B. napus* lines by crossing various genotypes of *B. rapa* and *B. oleracea* have been made (Rahman et al. 2015; Rahman 2013; Zou et al. 2010; Qian et al. 2006; McVetty and Scarth 2002; Crouch et al. 1994).

In addition to the interspecific hybridization, polyploidization of a plant species has been successful for species evolution in various important crops such as sugar beets, cotton and bread wheat (Sleper and Poehlman 2006; Liu et al. 2001; Guzy et al. 1989) and polyploids are often more vigorous than their progenitors (Zou et al. 2010; Comai 2005; Guzy et al. 1989). Similarly, polyploidization has been also used in *Brassica* species to develop a new *B. napus* line by

crossing with doubled trigenomic haploids (ABC) in order to introduce agronomically important traits not found in *B. napus* (Zou et al. 2010; Chen et al. 2010; Li et al. 2005; Rahman 2001; Meng et al. 1998). Malek et al. (2012) and Tian et al. (2010) reported the heterosis in synthetic hexaploid *Brassica* lines, although some degree of chromosome instability existed. In *Brassica* polyploidization, more stable chromosome pairing and higher plant fertility were observed in the hexaploid *Brassica* lines synthesized from the crosses between *B. carinata* (BBCC) and *B. rapa* (AA) compared to *B. napus* (AACC) and *B. nigra* (BB).

As blackleg disease causes significant yield losses in most canola fields (Gugel and Petrie 1992), canola breeders have searched various genetic sources of blackleg resistance. Many studies report that high levels of resistance to *L. maculans* is in the B-genome of *Brassica* species and the introgression of blackleg resistance from the B-genome *Brassica* species into *B. napus* has been made (Fredua-Agyeman et al. 2014; Navabi et al. 2010; Schelfhout et al. 2006; Somda et al. 1998; Chèvre et al. 1996; Struss et al. 1991; Roy 1978). However, the introgression of blackleg resistance through interspecific hybridization between two allotetraploids, for example *B. napus* and *B. juncea* or *B. carinata*, has been achieved at a very low frequency (Fredua-Agyeman et al. 2014; Navabi et al. 2010, 2011; Schelfhout et al. 2006; Struss et al. 1991; Roy 1978) due to the chromosome incompatibility between the B-genome and the A- or C-genome (Mason et al. 2014; Cui et al. 2012; Mason et al. 2010). Also, previous researchers reported difficulties in maintaining *Brassica* B-genome in the AC genome background (Navabi et al. 2010; Schelfhout et al. 2006; Dixelius and Wahlberg 1999).

To increase chromosome stability and B-genome retainability in interspecific crosses, synthetic hexaploid *Brassica* lines are beneficial for many breeding programs. Yet, blackleg resistance in *B. carinata* is unclear and no resistance genes have been identified (Raman et al. 2013; Rimmer 2006). Previously, four identified resistance genes (*LepR1*, *LepR2*, *LepR3* and *LepR4*) were originated from *B. rapa* subsp. *sylvestris* (Yu et al. 2005, 2007, 2008) and two genes, *Rlm8* and *Rlm11*, were found in one *B. rapa* commercial cultivar (Balesdent et al. 2002) and one *B. rapa oleifera* accession (Balesdent et al. 2013) respectively. In general, however, no resistance to *L. maculans* is found in *B. oleracea* (Badawy et al. 1991; Roy 1978).

The main objective of this research is to study the genetic inheritance of blackleg resistance in three hexaploid *Brassica* lines, synthesized by crossing between *B. carinata* and *B. rapa*, through interspecific hybridization with a susceptible *B. juncea* followed by backcrosses to the susceptible parent. Blackleg resistance in each plant generation was assessed through cotyledon inoculation, and the resistant individual plants showing high levels of resistance were selected for the next generation. Genetic compatibility of interspecific hybridization between hexaploid and tetraploid *Brassica* was evaluated by examining seed germination, self-incompatibility, pod set and seed set. The results within the three synthetic lines were also compared to analyze differences and similarities.

4.2 Materials and Methods

4.2.1 Plant Materials

Three synthetic hexaploid *Brassica* accessions were obtained from Dr. Jinling Meng (Huazhong Agricultural University, Wuhan, China) and initially tested with cotyledon inoculation using the *L. maculans* isolate 03-15-03. All showed high levels of resistance at the seedling stage. Hereafter, synthetic accessions will be referred to as codes C15, C21 and C28. All hexaploid lines have been synthesized with different *B. carinata* and *B. rapa* (Table 4.1).

Table 4.1. Three hexaploid *Brassica* accessions and their parents.

Code	Seed Type	Parent of	
		B. carinata	*B. rapa*
C15	Hexaploid	CGN03953	Baiguotianyoucai
C21	Hexaploid	CGN03983	Wulitianyoucai
C28	Hexaploid	CGN03995	Baijian 13

One *B. juncea* accession UM3086 from the collection at the University of Manitoba was identified as susceptible to the isolate 03-15-03 in the initial screening for blackleg resistance (Figure 4.1). Although the rating results of UM3086 varied from disease score 5 (intermediate susceptible) to 7 (susceptible), it was considered as a susceptible *B. juncea*. By using UM3086 in the crosses with hexaploid lines, the C-genome will be likely eliminated in backcross generations due to its chromosome incompatibility in the A- and B-genome background. Yet, blackleg resistance has not been found in the C-genome of *Brassica* species (Long et al. 2011). Both hexaploid lines and *B. juncea* UM3086 were grown in summer 2014.

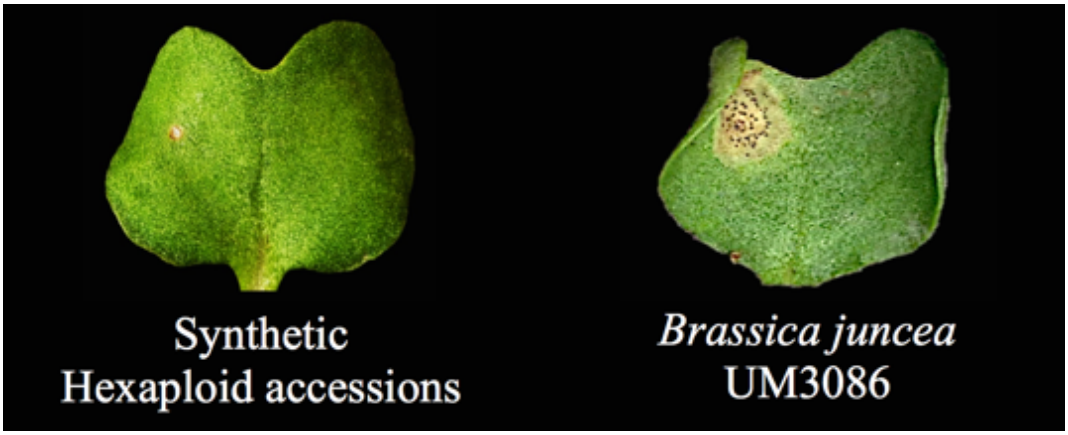

Figure 4.1. Analysis of blackleg resistance in hexaploid *Brassica* lines (C15, C21 and C28) and *B. juncea* (UM3086) against the *L. maculans* isolate 03-15-03.

4.2.2 Plant Growth Condition

Seeds were planted 1 cm deep in eight 3 x 4 cells filled with Sunshine Professional Growing Mix #4 soil, pre-watered with ½ tablespoon of 20-20-20 (nitrogen-phosphorus-potassium) fertilizer per gallon of water. Seed trays were placed in a controlled growth chamber at 20 °C / 18 °C, 16 h light / 8 h dark with relative humidity (RH) greater than 50% facilitated by the Department of Plant Science, University of Manitoba. The young plants were lightly watered daily.

Plants at the 2 to 3 true leaf stage were transplanted into 6-inch paper towel lined standard plastic nursery pots containing a 2:2:1 mixture of soil, sand and peat moss incorporated with 20-16-14 (nitrogen-phosphorus-potassium) fertilizer. Plants were then grown under greenhouse conditions (Department of Plant Science, University of Manitoba). The greenhouse setting remained the same for all growing season at 20 °C to 25 °C. Plants were watered daily and

fertilized every second week with 1 tablespoon of 20–20–20 (nitrogen-phosphorus-potassium) fertilizer per gallon of water.

4.2.3 Cross Populations

4.2.3.1 Production of F1 Hybrid

To produce F_1 hybrids derived from the crosses of three hexaploid lines and *B. juncea* UM3086, a hand crossing method was used. Timing of pollination is critical to accomplish fertilization (Brown et al. 1990). Emasculation of the female plants was performed when the flower bud tip was fully closed and colored green. Previously, a better embryo development was observed when maternal parents are higher ploidy than the parental parent (Håkansson 1956). After trying both direct and reciprocal crosses, hexaploid lines were used as maternal (seed) plants and the susceptible *B. juncea* UM3086 was used as parental (pollen) plants. Neither crossing mechanism showed a significant difference regarding pod set.

Bud-pollination was performed and the pollinated buds were covered with a glassine bag to avoid cross-contamination. After 7 to 10 days, fertilized ovaries produced seed pods indicating a sign of successful fertilization. However, all of seed pods were either empty with dark brown shrunken ovules or further produced an aborted embryo. Consequently, embryo rescue was attempted to generate hybrid plants.

As a conventional breeding method, cell culture techniques such as embryo rescue or ovule culture are widely used in interspecific crosses to obtain hybrid plants when F_1 embryo aborts at the early stage of development (Jing et al. 2008; Sharma et al. 1996). As 16-to-20 days after

pollination (DAP) is found to be the optimal time for successful embryo rescue in *Brassica* intercrosses (Jing et al. 2008; Rahman 2004), embryo rescue of the F_1 hybrid was performed by harvesting immature pods at 18 to 20 DAP.

Immature pods were surface sterilized with 70% ethanol for 3 minutes followed by 1% sodium hypochlorite (NaOCl) solution with a drop of Tween 20 (polysorbate 20) surfactant for 3 minutes. Treated pods were rinsed with sterilized distilled water three times. Using a stereo microscope, ovules were carefully dissected from the pod under aseptic conditions. Developing embryos at globular or heart-shaped stage were excised and cultured on a half-strength of Murashige and Skoog (MS) medium containing 1% sucrose (Murashige and Skoog 1962). Culture plates were placed on a bench under UV light at room temperature. Some embryos grew normally with developing shoots and roots; a few of them were shrunken and apparently dead (Figure 4.2). Germinated embryos with shoots and roots were then transferred to 3 x 4 cells filled with Sunshine Professional Growing Mix #4 soil (Sun Gro Horiculture, USA).

Figure 4.2. Embryo development of the F_1 hybrids on a half-strength of MS medium. An embryo develops shoot (right full arrow) and root (left full arrow); the shrunken seed indicates an aborted embryo (dotted arrow).

4.2.3.2 Production of Backcross Populations

An average of ten F_1 hybrid plants in each hexaploid line and the *B. juncea* parent UM3086 were grown in fall 2014. Because F_1 seedlings were produced through embryo culture, the F_1 was not tested for cotyledon response to *L. maculans*. With the assumption that all F_1 plants are resistant to blackleg based on Mendel's law of segregation, randomly selected F_1 plants were then backcrossed to the susceptible UM3086. The *B. juncea* UM3086 was used as a male plant in BC_1 crosses and as a female plant in BC_2 crosses (Figure 4.3). Similar to the production of F_1 hybrids, the same protocols for emasculation and hand-crossing method were used in all other cross generations. BC_1 crosses and onwards seemed to grow normal and were produced intact seeds. After screening for blackleg resistance in backcross populations, twelve resistant BC_1 individual

plants (four for each hexaploid line) were selected to produce BC_2 populations and eighteen resistant BC_2 plants (six for each hexaploid line) were selected and produced the self-pollinated generation BC_2F_2. Instead of producing another backcross generation BC_3, BC_2F_2 were produced in order to confirm that blackleg resistance is controlled by one dominant gene which 3:1 resistant-to-susceptible ratio is expected from BC_2 family lines showing the 1:1 ratio.

Harvesting took place generally 40 to 50 days after bud pollination. It was done when the plant seed pod turned yellow or brown; this was followed by 1 or 2 days of drying at 37 °C in the oven. When threshing, the following information was recorded to calculate pod set and seed set: the number of bud pollination, seed pods and the seeds per pod. Each backcross population was grown in the following seasons: BC_1 in winter 2015, BC_2 in summer through fall 2015 and BC_2F_2 in winter 2016.

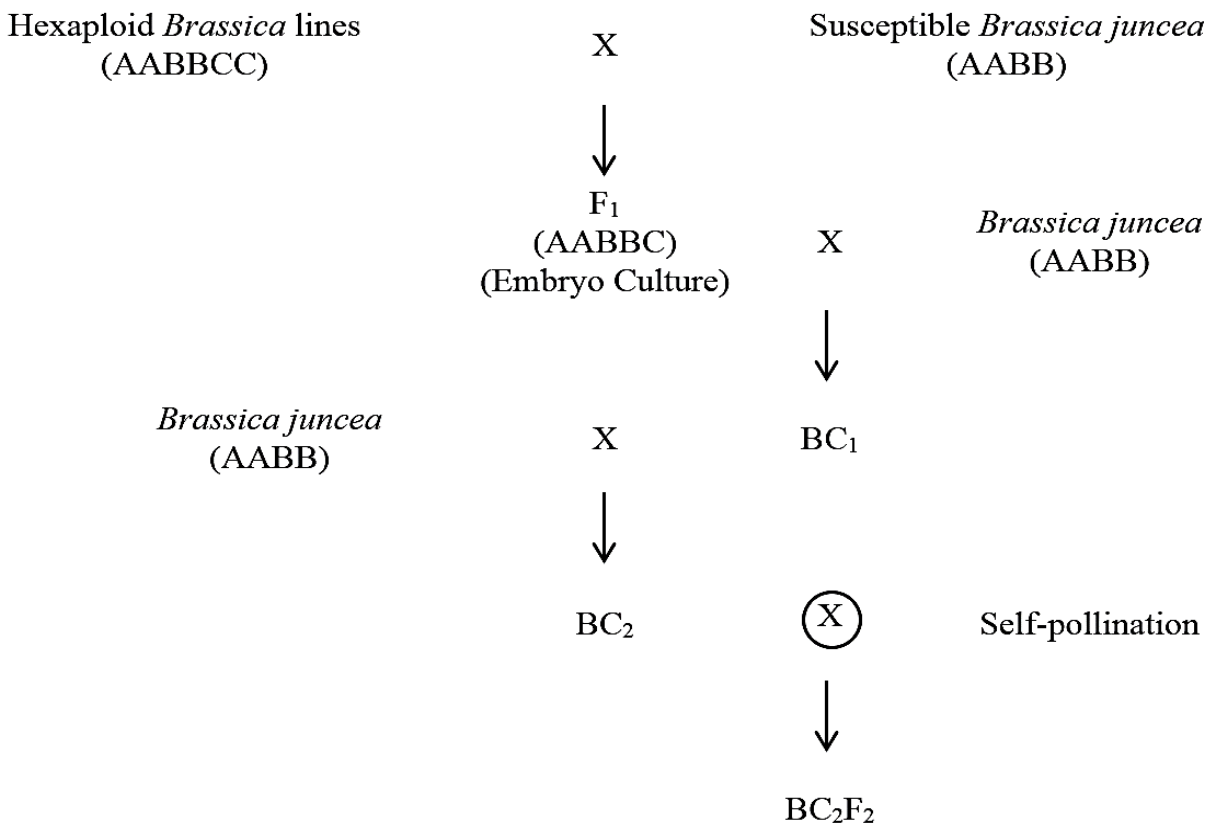

Figure 4.3. Crossing method for analysis of blackleg resistance in hexaploid *Brassica* lines using susceptible *B. juncea*. Direct cross is always expressed as female x male plant.

4.3.4 Interspecific Cross-compatibility

4.3.4.1 Seed Germination

To test the degree of embryo development, seed germination (expressed as a percentage) was investigated. In interspecific crosses, failure of seed germination is sometimes observed due to the disruption of endosperm development (Håkansson 1956). The germination rate was calculated as the number of seeds emerging, divided by the number of seeds planted. Seeds that are non-germinated or germinated late were excluded in the screening for blackleg resistance. Seed germination was examined in the following generations: F_2, BC_1, BC_2 and BC_2F_2.

4.3.4.2 Pollen Viability

Pollen quality is important for the successful plant reproduction. Testing pollen quality allows to predict the level of plant fertility Plant sterility due to infertile pollen often exists when interspecific crosses are made, and examining pollen quality is one way to estimate plant fertility (Heslop-Harrison 1992). F_1 hybrid plants obtained through embryo culture were self-pollinated to produce the F_2 generation and watched for pods and seed development. The number of seeds harvested in the F_2 was evaluated for pollen fertility indirectly.

4.3.4.3 Pod Set and Seed Set

Seed pods and seeds represent the successful reproduction of plants and the compatibility of female plants with the pollen. In this study, pod set (expressed as a percentage) was calculated as the number of seed pods formed, divided by the number of bud pollination in each cross, and seed set was calculated as the number of seeds harvested per pod. The pod set data was presented for F_1, BC_1 and BC_2 generations. The seed set in each cross generation was recorded to compare seed yields to both parent hexaploid lines and *B. juncea* UM3086. Seed set data was presented for F_1, BC_1 and BC_2 as well as the self-pollinated generations F_2 and BC_2F_2.

4.2.5 Screening for Blackleg Resistance

4.2.5.1 Leptosphaeria maculans Isolates

A *L. maculans* isolate belongs to PG2, 03-15-03, was used for all populations. This isolate was originally obtained from Dr. S. R. Rimmer (former professor at the Department of Plant Science, University of Manitoba). Three *B. napus* L. cultivars are well known for the differential interaction of phenotypes against *L. maculans* PG2 isolates. Westar is susceptible, while Glacier

and Quinta show resistant responses at the cotyledon stage (Koch et al. 1991; Mengistu et al. 1991). Differential *B. napus* L. cultivars Westar, Glacier and Quinta as well as both parent hexaploid lines and UM3086 were used as control phenotypes in this study. Figure 4.4 shows the cotyledon response of *B. napus* L. cultivars to the isolate 03-15-03. In regards to Avr gene information, gene cloning and sequencing analysis work was done by Dr. Tengsheng Zhou (a post-doctoral fellow, Department of Plant Science, University of Manitoba) and known Avr genes in the isolate 03-15-03 have been identified as follows: *AvrLm2*, *AvrLm6*, *AvrLm11*, *and AvrLmJ1*.

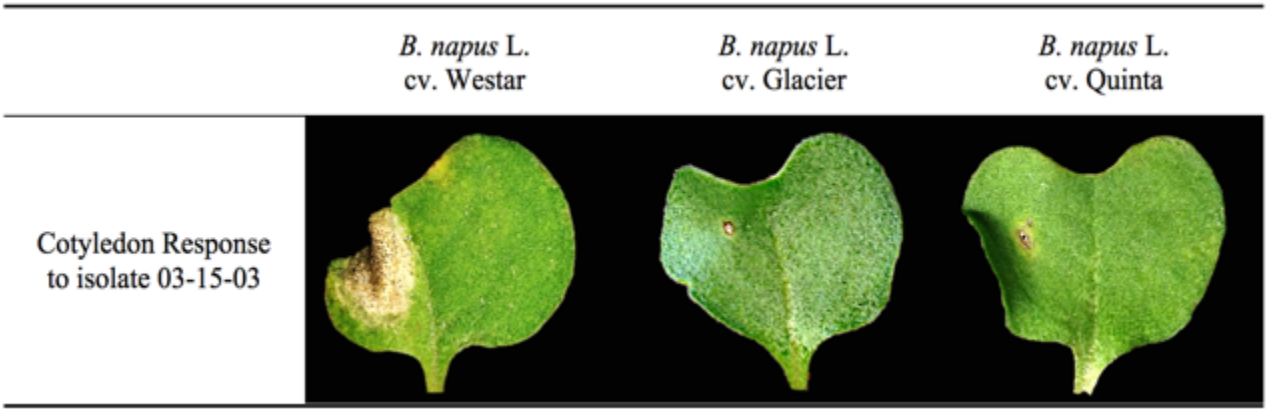

Figure 4.4. Differential interaction of *B. napus* L. cultivars Westar, Glacier and Quinta with the *L. maculans* isolate 03-15-03 at the seedling stage.

4.2.5.2 Inoculum Preparation

Pycnidiospores of *L. maculans* from the infected cotyledon can be cultured directly on a V8 agar media after the surface sterilization with 70% ethanol for 5 seconds, then in 10% bleach for 2 minutes followed by rinsing with sterilized distilled water. The sterilized cotyledon was cut into less than 1 mm^2 size prior to placement on a V8 agar media. Another way to culture fungus

spores is to prepare isolate discs (0.8 mm diameter in size). A separate disc was placed on a V8 media for culturing (Figure 4.6) or stored at – 20 °C for future use. 1L of V8 agar media contains 200 ml V8 juice (Campbell Soup Company Ltd. Toronto, ON), 800 ml distilled water, 15 g agar and 0.75 g calcium carbonate, 0.1 g streptomycin sulphate. The culture plates were placed at room temperature for 7 to 14 days. When black pycnidia of *L. maculans* produced pink-purplish ooze (Figure 4.5), the pycnidiospores were harvested by flooding with sterilized distilled water. Blackleg inoculums were adjusted to the final concentration of 2 x 10^7 pycnidiospores per mL using hemocytometer (Appendix III). Subsequent sub-culture of the isolate was performed every 3 to 4 months to maintain the virulence of *L. maculans*.

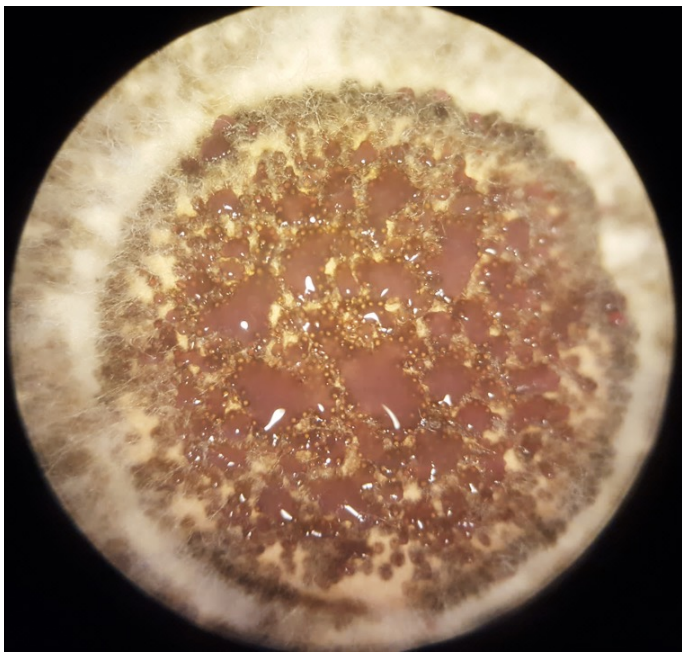

Figure 4.5. Formation of mycelium and black pycnidia of *Leptosphaeria maculans* on V8 agar medium. Pink ooze contains pycnidiospores.

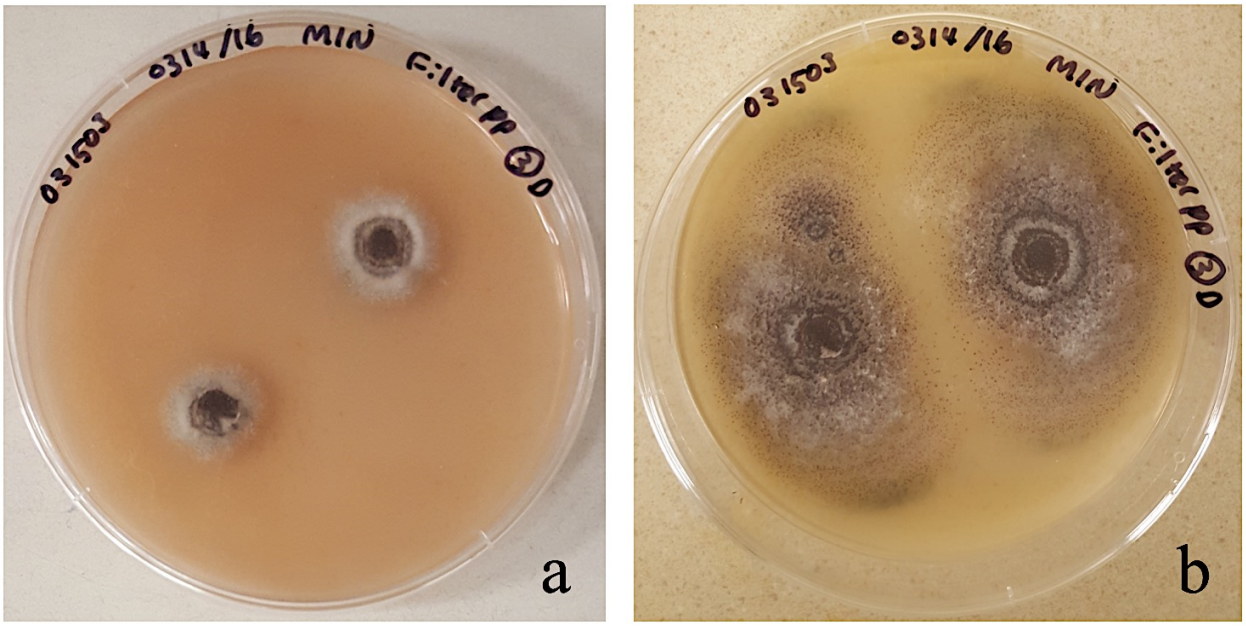

Figure 4.6. Culturing the *Leptosphaeria maculans* isolate 03-15-03 on V8 agar medium containing streptomycin antibiotics. a) 2-day-old isolate culture, b) 7-day-old isolate culture.

4.2.5.3 Blackleg Inoculation

Seedlings in ninety-six cell at six to seven days were prepared for inoculation (Figure 4.7). A punctured infection site was created on each half of the cotyledons using pinpoint forceps. Cotyledon inoculation was performed by placing a 10 µl droplet of pycnidiospore suspension on the wounded site of the leaf surface (Figure 4.7). After the inoculation, plants were monitored during the period of 3 to 21 days-post-inoculation (DPI), and developing primary leaves were removed during this period to retain healthy green cotyledon leaves for appropriate evaluation at the cotyledon stage.

Figure 4.7. Seven-day-old seedlings inoculated with 10µl-droplet ($2x10^7$ spores per mL) of *L. maculans* inoculum on the wounded site of cotyledons.

4.2.5.4 Blackleg Evaluation and Selection Criteria

Approximately one hundred plant samples from each hexaploid line were tested in the F_2 and BC_1 generations. In BC_2 and BC_2F_2 populations, close to a hundred plant samples were evaluated in each family line. Inoculated plants were rated on 10 ± 2 DPI using a 0 to 9 rating scale (Delwiche 1980). The scale is described below (Table 4.2); Figure 4.8 represents the blackleg symptoms on cotyledons according to the scale. Disease scoring was determined along with the symptoms on controls and both parents. In the current study, plants scoring 0 to 4 and 5 to 9 were defined as resistant and susceptible respectively (Table 4.3). Within resistant phenotypes, individual plants showing high levels of resistance (scored 0 or 1) were randomly selected and carried forward for the next cross generation. After the selection, both selected resistant plants and susceptible parents were transplanted individually into 6-inch plastic pots and grown under greenhouse conditions.

Table 4.2. Description of blackleg symptoms on cotyledon based on a 0 to 9 rating scale (Delwiche 1980).

Disease Score	Description
0	No darkening of tissue around wound, as in controls
1-2	Limited blackening around wound, lesion diameter 0.5 - 1.5 mm, faint chlorotic halo may be present, sporulation absent
3-4	Dark necrotic lesions, diameter 1.5 - 3.0 mm, chlorotic halo may be present, sporulation absent
5-6	Limited sporulation, lesions diameter 3.0 - 6.0 mm, may show grey-green tissue collapse or dark necrosis throughout
7-8	Grey-green tissue collapse 3.0 – 5.0 mm, sharply delimited, non-darkened margin
9	Rapid tissue collapse at about 10 days, accompanied by profuse sporulation in large, more than 5.0 mm, lesions with diffuse margin

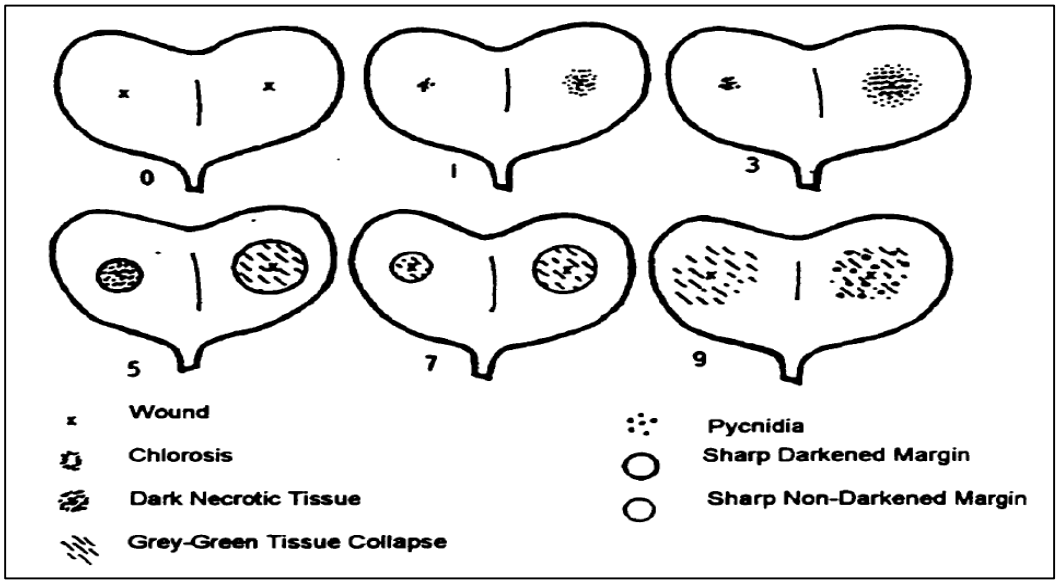

Figure 4.8. Schematic representation of cotyledon response against *L. maculans* on a 0 to 9 scale (Delwiche 1980)

Table 4.3. Phenotype categorized into two or four disease classes in the current study.

Rating Scale	Phenotype Classification	
0-2	Resistant	Resistant
3-4	Intermediate Resistant	
5-6	Intermediate Susceptible	Susceptible
7-9	Susceptible	

4.2.6 Statistical Analysis

A chi-square goodness-of-fit test was conducted for the F_2, BC_1, BC_2 and BC_2F_2 populations. Statistical discovery software *JMP*, a division of the statistical analysis system (SAS), version 12, was used for all chi-square tests (SAS Institute Inc.).

4.3 Results

4.3.1 Germination Rate

In general, all test population showed 50% or higher percentage of seed germination. Although F_1 plants were obtained through tissue culture, F_2 seeds particularly derived from the hexaploid line C28 were germinated as normal plants (Table 4.4). A similar rate of seed germination was observed between F_2 and BC_1 generations in each hexaploid line except the line C15. Within the three hexaploid lines, the line C28 showed the highest germination rate; the lowest rate was recorded in the line C15. However, variations in germination rate within hexaploid lines did not appear in the advanced backcross generation BC_2 and its self-pollinated generation BC_2F_2. Overall, within the three hexaploid lines, the line C15 showed a poor germination rate compared to other two lines.

Table 4.4. Germination rate of F_2 seeds, self-pollinated generation of F_1 derived between hexaploid *Brassica* lines and the susceptible *B. juncea* UM3086, and backcross progenies BC_1, BC_2 and the BC_2 self-pollinated generation BC_2F_2.

Generation	Parent hexaploid line	No. of plants sown (A)	No. of plants germinated (B)	Germination rate (B/A x 100)
F_2	C28	112	105	94%
	C21	128	100	78%
	C15	144	101	70%
BC_1	C28	176	149	85%
	C21	192	135	70%
	C15	264	148	56%
BC_2	C28	440	376	85%
	C21	418	396	95%
	C15	488	404	83%
BC_2F_2	C28	592	558	94%
	C21	576	564	98%
	C15	600	560	93%

4.3.2 Pollen Viability

Pollen quality was evaluated by examining seed set in the self-pollinated generation F_2. Most F_1 crosses showed normal seed pod development, indicating a successful fertilization. During embryo development, however, the significant abortion of embryo at early stage was observed in this study. Tissue culture technique allowed normal development of the F_1 embryo. These F_1 plants grew as their parents, and most flower branches were used for BC_1 crosses. Some of branches were self-pollinated and produced F_2. In F_2, the similar average was resulted regardless of hexaploid lines and a maximum of 95 seeds were harvested in an individual plant (Table 4.5).

Table 4.5. The average number of seeds harvested per plant in the F_2 of crosses of hexaploid *Brassica* line and *B. juncea* UM3086.

Self-Pollinated Generation	Hexaploid line used	Avg. # of seeds/Plant (Range)
F_2	C28	29.33 (10 – 95)
	C21	23.42 (6 – 80)
	C15	28.45 (2 – 95)

4.3.3 Pod Set

Overall, 70% or higher percentage of pod set was recorded in the BC_1 and BC_2 crosses, and the similar results were observed in all three hexaploid lines (Figure 4.9). However, the results of F_1 crosses showed a high variation in the different hexaploid lines. The two hexaploid lines C28 and C21 showed a good pod set 100% and 75% respectively. On the other hand, only about 50% of crosses were developed seed pods in the line C15. The pod set results showed that the lower pod set in F_1 generation was recovered in backcross generations.

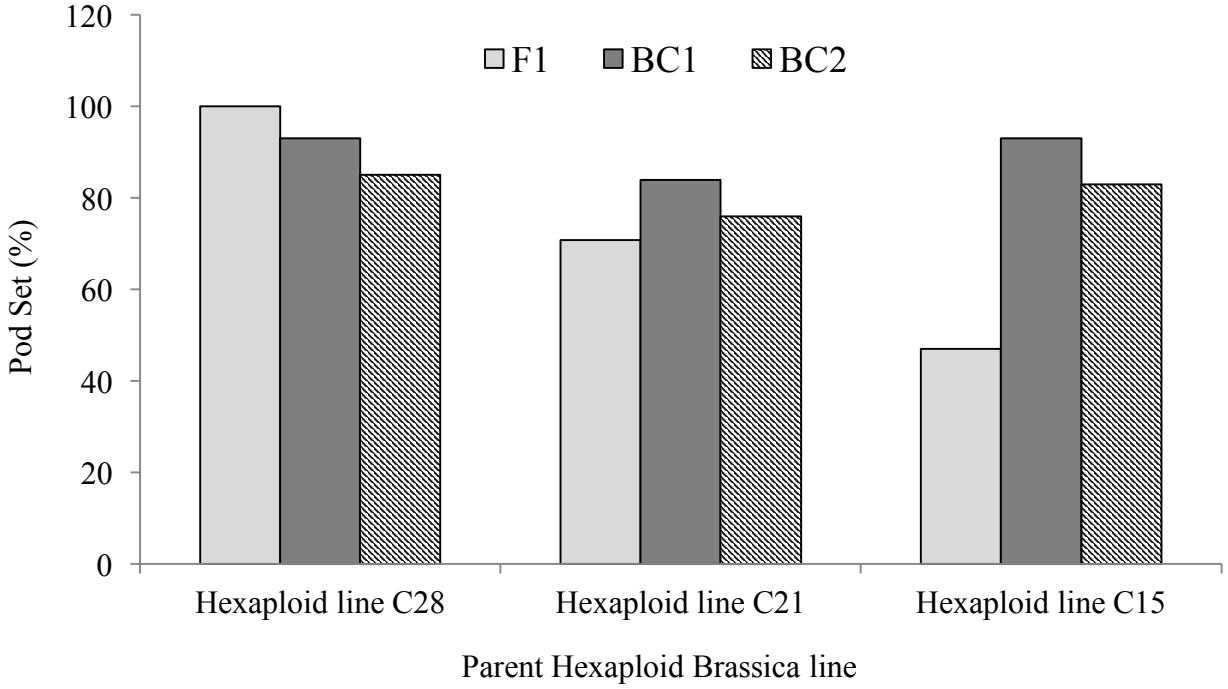

Figure 4.9. The average pod set in the crosses of F_1, BC_1 and BC_2 in each hexaploid *Brassica* line crossed with *B. juncea* UM3086.

Twelve resistant family lines (four resistant BC_1 individual plants from each hexaploid line) were produced. On average, all lines showed a good pod set (75% or higher percentage) in the BC_2 crosses, and no significant difference was observed within the same hexaploid line (Figure 4.10). Generally, however, a better pod set was observed in the hexaploid line C28. In addition, the R-line #1 in the hexaploid line C28 was dominantly high in pod set compared to all other lines.

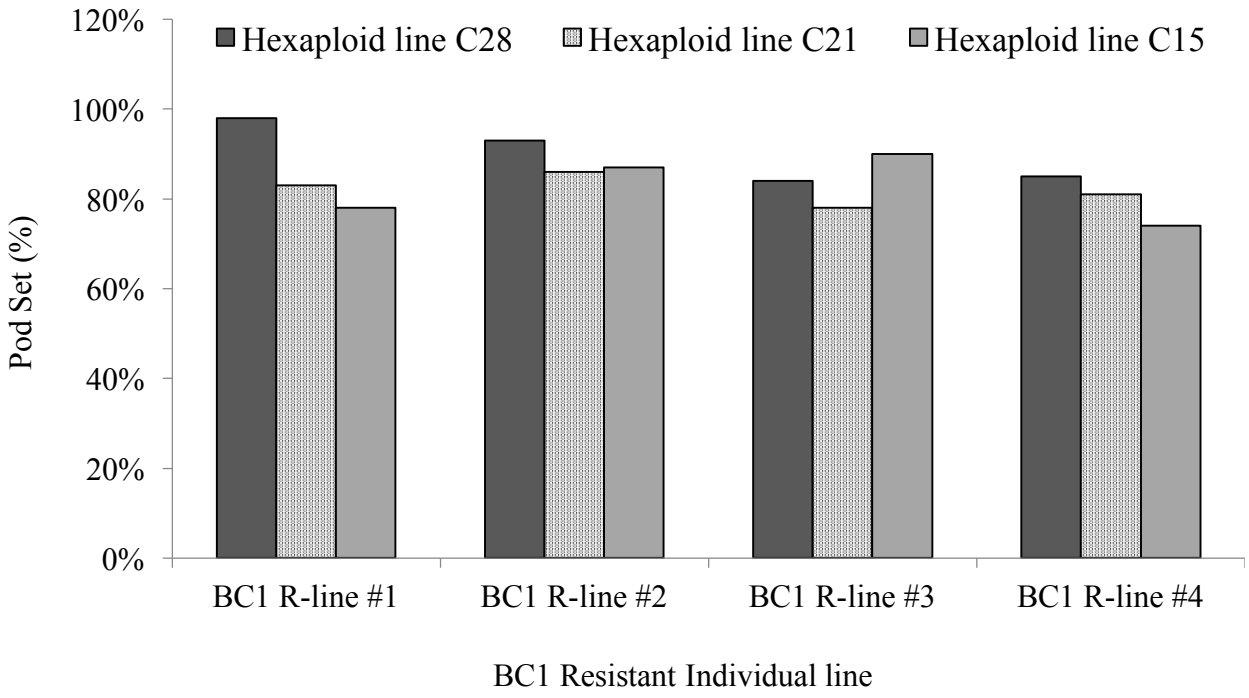

Figure 4.10. The average pod set in the BC_2 crosses between four individual resistant plants in each hexaploid *Brassica* line and *B. juncea* UM3086.

4.3.4 Seed Set

Figure 4.11 presents the overall comparison of the average seed set in all crossed populations with both parental hexaploid lines and *B. juncea* UM3086. Each cross population data was pooled for the three hexaploid lines (C15, C21 and C28). The seed set results in F_1 population were calculated as the number of embryos developing as intact seeds through embryo rescue per pod. In comparison of resistant and susceptible *B. juncea* UM lines, the seed set in the susceptible UM3086 (14.00 ± 2.42) was slightly lower than the resistant *B. juncea* UM lines (16.00 ± 2.77). Not surprisingly, since tissue culture was used to overcome embryo degeneration at the early stage from interspecific hybridization, relatively a low number of seeds were

obtained in the F_1 generation. On the other hand, a similar or better seed set was observed in the backcross generations BC_1 and BC_2 compared to the parent hexaploid lines.

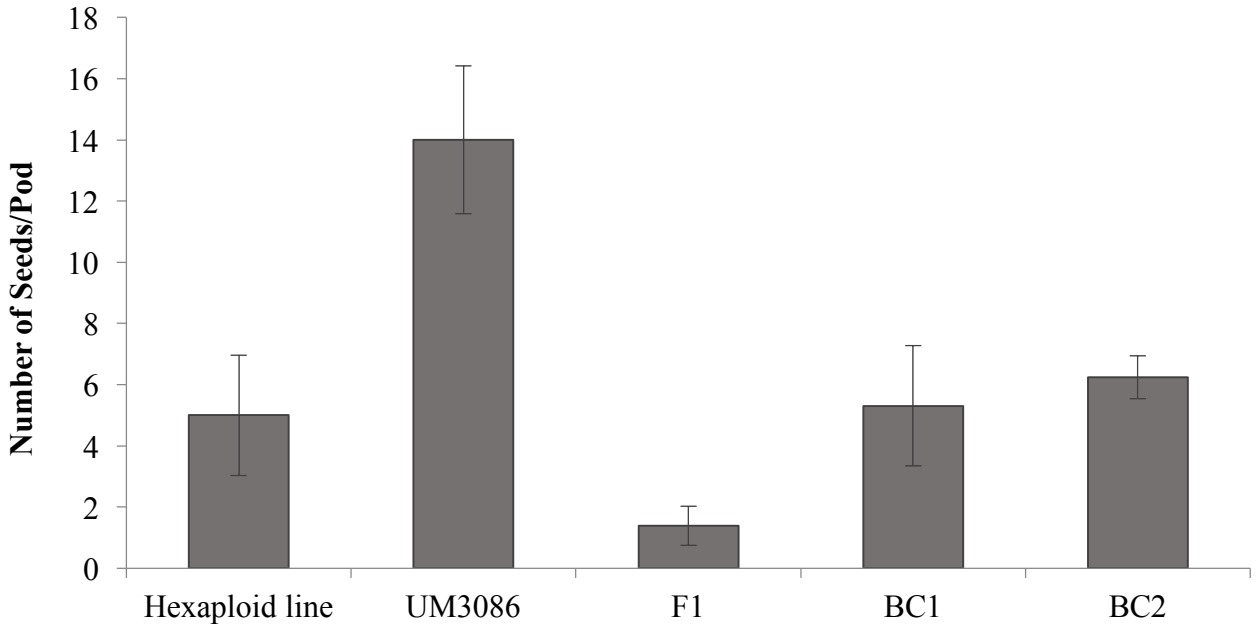

Figure 4.11. The average seed set in each cross generation (F_1, BC_1 and BC_2) and in both parent hexaploid *Brassica* line and *B. juncea* UM3086.

The seed set results in each hexaploid line showed variations in F_1 and BC_1 generations (Table 4.6). In the F_1, the hexaploid line C15 showed a significantly low seed set (0.83 ± 0.29) compared to the other two lines. Interestingly, the lowest average seed set in the BC_1 was observed in the hexaploid line C28, which showed an overall good pod set. For the BC_1 data, a total of fourteen crosses were analyzed in both hexaploid line C28 and C21. Twenty crosses were analyzed for the line C15. The results in BC_2 generation did not show a significant difference among the three hexaploid lines. The average seed set of each hexaploid line in the BC_2 was analyzed with the following numbers of samples: hexaploid line C28 – 91 crosses made with 13 resistant lines from BC_1; hexaploid line C21 – 101 crosses made with 16 BC_1 R-lines; hexaploid

line C15 – 59 crosses made with 9 BC_1 R-lines.

Table 4.6. The average seed set in the cross generations F_1, BC_1, and BC_2 and in both parents hexaploid *Brassica* line and *B. juncea* UM3086.

Generation[a]	Parent Hexaploid line	Avg. # of seeds/Pod
Hexaploid *Brassica* line	-	5.00 ± 1.97
B. juncea UM3086	-	14.00 ± 2.42
F_1	C28	2.08 ± 0.59
	C21	1.25 ± 1.06
	C15	0.83 ± 0.29
BC_1	C28	3.36 ± 2.02
	C21	7.27 ± 3.77
	C15	5.30 ± 1.80
BC_2	C28	6.28 ± 2.31
	C21	5.51 ± 2.00
	C15	6.92 ± 2.20

[a] F_1 was produced through embryo culture; susceptible *B. juncea* UM3086 was used as male parent for BC_1 generation and female parent for BC_2 generation.

Out of the various numbers of samples in the BC_2, seed set data from the four selected BC_1 resistant lines in each hexaploid line was compared within a hexaploid line and across the three hexaploid lines. The overall results of the selected lines showed that the greater number of seeds was harvested than the BC_2 average (Table 4.6) except the R-line #1 in the hexaploid line C15 (Table 4.7). Similar to the pod set results, the hexaploid line C28 was recorded as an outstanding line for seed set among other lines. Within a same hexaploid line, the similar results of seed set were observed in the line C28 and C21. However, the greater difference within the hexaploid

line C15 was discovered than in the other two lines. The lowest average number of seeds was 4.45 and the highest average was 10.11 seeds per pod in the line C15 (Table 4.7).

Table 4.7. The average seed set in BC_2 generation of the four selected resistant lines in each hexaploid *Brassica* line.

Generation[a]	BC_1 Resistant Plant line #	Avg. # of seeds/Pod		
		Parent Hexaploid line		
		C28	C21	C15
BC_2	1	10.14 ± 2.65	9.02 ± 1.97	4.45 ± 2.29
	2	8.33 ± 1.46	7.73 ± 2.24	7.82 ± 3.25
	3	9.72 ± 3.10	7.52 ± 2.76	10.11 ± 2.94
	4	8.79 ± 4.27	6.94 ± 4.03	7.67 ± 3.95

[a] BC_2 was produced by crossing between susceptible *B. juncea* UM3086 (female recurrent parent) and resistant BC_1 individual plants.

4.3.5 Blackleg Resistance in Cross Populations

Since F_1 embryos were rescued through tissue culture, no cotyledon inoculation was performed for the F_1 population. Instead, the F_2 population was evaluated for blackleg resistance. In the current study, two possible models were proposed: a model of one dominant gene and of two dominant genes (Table 4.8). Based on Mendel's law of segregation, if the resistance is controlled by a dominant single gene, a 3:1 resistant-to-susceptible ratio is expected in F_2 generations. If two dominant genes are present, a 15:1 resistant-to-susceptible ratio is expected in F_2 populations (Griffiths et al. 2000). The proposed genotypes of F_1 plants are 'Aa' for the one-gene model and 'AaBb' for the two-gene model respectively assuming that resistant parent was homozygous at one or both loci.

Table 4.8. Proposed gene model and genotypes for blackleg resistance in the present study.

Proposed gene model	Proposed genotype of resistant parent hexaploid line	Proposed genotype of susceptible parent *B. juncea*	Proposed genotype of F_1 plants	Expected F_2 R:S ratio[a]
One	AA	aa	Aa	3:1
Two	AABB	aabb	AaBb	15:1

[a]R:S ratio: The ratio of resistant phenotype (disease score 0 to 4) to susceptible phenotype (disease score 5 to 9).

In all test population, resistant individual plants scoring zero to two were only selected for the next generation even though rating score up to 4 was considered as resistant phenotype. Figure 4.12 indicated blackleg severity level at the seedling stages observed in the test population.

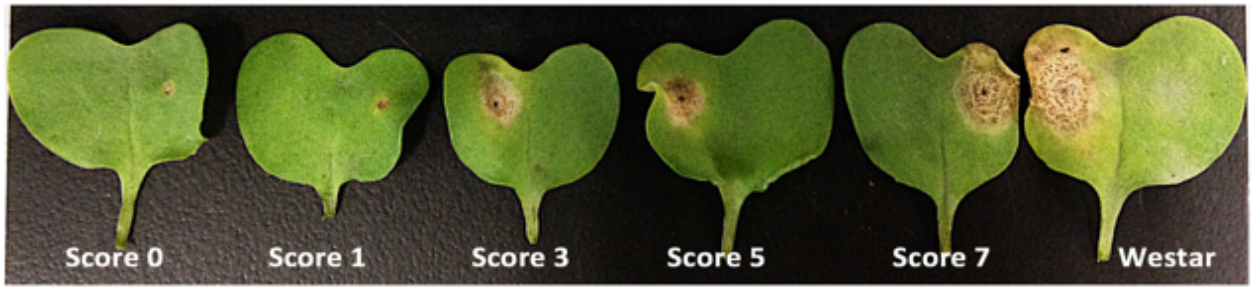

Figure 4.12. Screening results of cotyledon response to the *L. maculans* isolates 03-15-03 in the test populations F_2, BC_1, BC_2, BC_2F_2 along with the control phenotype *B. napus* L. cv. Westar.

In F_2 population, a similar number of resistant plants were observed in each hexaploid line. Out of an average 102 plant samples, over 90% of individual plants showed resistance to the *L. maculans* isolate 03-15-03 (Table 4.9). Within the resistant plants, most samples recorded a

disease score of zero to two; susceptible phenotypes showed more severe symptoms than the parent *B. juncea* UM3086 (Figure 4.1 and Figure 4.12).

The results of chi-square tests for a 15:1 ratio showed strong evidence that the tested population follows the two-gene model (Table 4.9). In addition, the pooled data also did not show significant difference to reject the hypothesis of the two-gene model. Thus, only the two-gene model was tested in the BC_1 and both one- and two-gene models were tested in the BC_2 and BC_2F_2 for the expected resistant-to-susceptible ratio based on the hypothesis that two genes might separate in the advanced generations.

Table 4.9. Phenotypic analyses of blackleg resistance against *L. maculans* isolate 03-15-03 and the results of chi-square goodness-of-fit-test for a 15:1 ratio in F_2 population.

Generation[a]	*L. maculans* Isolate	Parent Hexaploid line	Total	Phenotype		$X^2_{15:1}$[d]	P_{sig}[e]
				R[b]	S[c]		
F_2	03-15-03	C28	105	96	9	0.966	NS
		C21	100	95	5	0.267	NS
		C15	101	92	9	1.221	NS
		Pooled	306	283	23	0.838	NS

[a]F_2 was produced by selfing F_1 individual plants, originally crossed between hexaploid *Brassica* line (female parent) and susceptible *B. juncea* UM3086 (male parent).
[b]R: Resistant phenotype (Disease score 0 to 4).
[c]S: Susceptible phenotype (Disease score 5 to 9).
[d]$X^2_{15:1}$: Chi-square value of goodness-of-fit test for a 15:1 ratio (Critical value $X^2_{df=1}$: 3.841).
[e]P_{sig}: Probability of rejecting hypothesis at a significance level. * ($P \leq 0.05$), ** ($P \leq 0.01$), *** ($P \leq 0.001$), NS = not significant ($P > 0.05$).

According to Mendel's law of independent assortment, each dominant gene or allele is segregating independently in the next generation. In the case of the two-gene model, the

expected resistant-to-susceptible ratio is 3:1 for two genes in the BC_1 and BC_2 and for one gene in the BC_2F_2 families, 1:1 ratio for one gene in the BC_2 and 15:1 ratio for two genes in the BC_2F_2 families (Table 4.10). This also assumed that a single dominant gene is still effective for blackleg resistance at the cotyledon stage and the genotype would be heterozygous with the presence of at least one dominant gene.

Table 4.10. The expected resistant-to-susceptible ratio of the two-gene model in BC_2 and BC_2F_2 populations.

Proposed gene model	Proposed genotype of BC_1 plants	Expected BC_1 R:S ratio[a]	Proposed genotype of selected BC_1 resistant plants	Expected BC_2 R:S ratio[a]	Expected BC_2F_2 R:S ratio[a]
Two	AaBb Aabb aaBb aabb	3:1	AaBb	3:1	15:1
			Aabb aaBb	1:1	3:1

[a]R:S ratio: The ratio of resistant phenotype (disease score 0 to 4) to susceptible phenotype (disease score 5 to 9).

The results of phenotypic analysis in BC_1 population showed that an average of 144 plants was tested in each line and 70% to 80% of plants showed resistance (Table 4.11). The hexaploid line C28 obtained 74.5% of resistant plants in 149 samples. In the hexaploid line C21 and C15, resistant phenotypes were observed 80% and 71.6% respectively. The pooled data for all three hexaploid lines showed 75% resistant and 35% susceptible phenotypes. As expected, the chi-square test for a 3:1 ratio in the BC_1 population showed a good fit in each hexaploid line as well as in the pooled data (Table 4.11).

Table 4.11. Phenotypic analysis of blackleg resistance against *L. maculans* isolate 03-15-03 and the results of chi-square goodness-of-fit test for a 3:1 ratio in BC_1 population.

Generation[a]	*L. maculans* Isolate	Parent hexaploid line	Total	Phenotype		$X^2_{3:1}$[d]	P_{sig}[e]
				R[b]	S[c]		
BC_1	03-15-03	C28	149	111	38	0.020	NS
		C21	135	108	27	1.800	NS
		C15	148	106	42	0.901	NS
		Pooled	432	325	107	0.012	NS

[a]BC_1 was produced by crossing between F_1 individual plants (female parent) and susceptible *B. juncea* UM3086 (male parent).
[b]R: Resistant phenotype (Disease score 0 to 4).
[c]S: Susceptible phenotype (Disease score 5 to 9).
[d]$X^2_{3:1}$: Chi-square value of goodness-of-fit test for 3:1 ratio (Critical value $X^2_{df=1}$: 3.841).
[e]P_{sig}: Probability of rejecting hypothesis at a significance level. * ($P \leq 0.05$), ** ($P \leq 0.01$), *** ($P \leq 0.001$), NS = not significant ($P > 0.05$).

In the BC_1, four resistant individual plants were selected in each hexaploid line. Each plant was backcrossed with the susceptible UM3086 and at least a hundred seed from BC_2 crosses were produced. A total of four family lines in each hexaploid line were tested with the isolate 03-15-03 (Table 4.12). Approximately 200 resistant plants were obtained in the hexaploid line C28. Similarly, 216 and 230 resistant plants were observed in crosses derived from hexaploid lines C21 and C15 respectively. The proportion of resistant plants varied among the four family lines within a hexaploid line (Table 4.12).

Table 4.12. Phenotypic analysis of blackleg resistance against *L. maculans* isolates 03-15-03 in the BC_2 population,

Generation[a]	*L. maculans* Isolate	Parent hexaploid line	Plant Family line	Total	Phenotype	
					R[b]	S[c]
BC_2	03-15-03	C28	M5.BC_2-1	92	37	55
			M5.BC_2-2	94	43	51
			M5.BC_2-3	94	63	31
			M5.BC_2-4	96	54	42
		C21	M7.BC_2-1	103	57	46
			M7.BC_2-2	105	82	23
			M7.BC_2-3	94	51	43
			M7.BC_2-4	91	26	65
		C15	M8.BC_2-1	101	66	35
			M8.BC_2-2	94	65	29
			M8.BC_2-3	95	49	46
			M8.BC_2-4	90	50	40

[a]BC_2 was produced by crossing between susceptible *B. juncea* UM3086 (female parent) and resistant BC_1 individual plants (male parent).
[b]R: Resistant phenotype (Disease score 0 to 4).
[c]S: Susceptible phenotype (Disease score 5 to 9).

Two chi-square goodness-of-fit tests were conducted for the BC_2 population. In the results of chi-square tests, most family lines showed either 1:1 or 3:1 resistant-to-susceptible ratio as expected based on the theory (Table 4.13). The results of good fit of a 1:1 ratio represents that the blackleg resistance is controlled by a single gene; the results of good fit of a 3:1 ratio represents that the blackleg resistance is controlled by two dominant genes.

In the hexaploid line C28, the family lines M5.BC_2-1, M5.BC_2-2 and M5.BC_2-4 satisfied a 1:1 ratio; the family line M5.BC_2-3 showed a good fit of 3:1 ratio. Out of four family lines in hexaploid line C21, two family lines M7.BC_2-1 and M7.BC_2-3 satisfied a 1:1 ratio whereas line M7.BC_2-2 showed a good fit of 3:1 ratio while one family line M7.BC_2-4 did fit neither 1:1 nor 3:1 ratio. Similarly, in hexaploid line C15, two family lines M8.BC_2-3 and M8.BC_2-4 showed a good fit of 1:1 ratio, and the line M8.BC_2-2 satisfied a 3:1 ratio. The remaining family line M8.BC_2-1 in the hexaploid line C15 did not fit either 1:1 or 3:1 ratio.

Therefore, based on the chi-square results of twelve family lines in BC_2 population, two dominant genes are present in the following three family lines M5.BC_2-3, M7.BC_2-2 and M8.BC_2-2. These lines were self-pollinated to produce BC_2F_2, although blackleg evaluation did not perform. In seven family lines M5.BC_2-1, M5.BC_2-2, M5.BC_2-4, M7.BC_2-1, M7.BC_2-3, M8.BC_2-3 and M8.BC_2-4, the blackleg resistance is controlled by a single dominant gene as it satisfied 1:1 resistant-to-susceptible ratio in BC_2. In addition, the same level of resistance was observed as in BC_1 population, which was controlled by two dominant genes.

Table 4.13. The results of chi-square goodness-of-fit test for 1:1 and 3:1 ratio in BC_2 population.

Generation[a]	Parent hexaploid line	Plant Family line	$X^2_{1:1}$[b]	P_{sig}[d]	$X^2_{3:1}$[c]	P_{sig}[d]
BC_2	C28	M5.BC_2-1	3.522	NS	-	-
		M5.BC_2-2	0.681	NS	-	-
		M5.BC_2-3	-	-	3.192	NS
		M5.BC_2-4	1.500	NS	-	-
	C21	M7.BC_2-1	1.175	NS	-	-
		M7.BC_2-2	-	-	0.537	NS
		M7.BC_2-3	0.681	NS	-	-
		M7.BC_2-4	16.714	***	104.619	***
	C15	M8.BC_2-1	9.515	**	5.020	*
		M8.BC_2-2	-	-	1.716	NS
		M8.BC_2-3	0.095	NS	-	-
		M8.BC_2-4	1.111	NS	-	-

[a]BC_2 was produced by crossing between susceptible *B. juncea* UM3086 (female parent) and resistant BC_1 individual plants (male parent).
[b]$X^2_{1:1}$: Chi-square value of goodness-of-fit test for a 1:1 ratio (Critical value $X^2_{df=1}$: 3.841).
[c]$X^2_{3:1}$: Chi-square value of goodness-of-fit test for a 3:1 ratio (Critical value $X^2_{df=1}$: 3.841).
[d]P_{sig}: Probability of rejecting hypothesis at a significance level. * ($P \leq 0.05$), ** ($P \leq 0.01$), *** ($P \leq 0.001$), NS = not significant ($P > 0.05$).

For the self-pollinated generation BC_2F_2, three BC_2 resistant plants from each of two family lines were selected in each hexaploid line, and the selection was made with strong evidence of a good fit with the expected ratio of 1:1. Based on the proposed genotype (Table 4.10), the resistant BC_2 individual plants are controlled by single dominant gene. The selected family lines in BC_2

population were M5.BC_2-2 and M5.BC_2-4 in the hexaploid line C28, M7.BC_2-1 and M7.BC_2-3 in the line C21, and M8.BC_2-3 and M8.BC_2-4 in the line C15 (Table 4.13). Thus, a total of six family lines in each hexaploid line were evaluated for blackleg resistance and each family line was tested with close to a hundred seed (Table 4.14). Out of 558 plants in hexaploid line C28, approximately 83% showed resistance. The resistant phenotype was observed about 77% in 563 plants and 76% in 560 plants in hexaploid line C21 and C15 respectively.

The results of the conducted chi-square tests indicated that most family lines satisfied the 3:1 ratio, although a few lines did not show a good fit of 3:1 ratio (Table 4.14). In BC_2F_2 population, the family lines M5.BC_2F_2-2b and M5.BC_2F_2-4b in hexaploid line C28, M7.BC_2F_2-1a in the line C21 and M8.BC_2F_2-4b in the line C15 did not satisfied the expected ratio of 3:1. Overall, the hexaploid line C28 satisfied the 3:1 ratio at 5% significance level; one family line M5.BC_2F_2-2b satisfied the ratio at 1% significance level. Most family lines in the hexaploid line C21 and C15 showed a good fit of 3:1 ratio in BC_2F_2 except two family lines, one from each hexaploid line. The two lines M7.BC_2F_2-1a and M8.BC_2F_2-4b were not significant at 0.1% significance level. One exceptional family line M5.BC_2F_2-4b was significant to reject the hypothesis at 0.1% significance level.

Table 4.14. Phenotypic analysis of blackleg resistance against *L. maculans* isolates 03-15-03 and the results of chi-square goodness-of-fit test for a 3:1 ratio in BC_2F_2 population.

Generation[a]	Parent hexaploid line	Plant Family line	Total	Phenotype		$X^2_{3:1}$[d]	P_{sig}[e]
				R[b]	S[c]		
BC_2F_2	C28	M5.BC_2F_2-2a	93	70	23	0.004	NS
		M5.BC_2F_2-2b	90	77	13	5.348	*
		M5.BC_2F_2-2c	97	80	17	2.890	NS
		M5.BC_2F_2-4a	96	80	16	3.556	NS
		M5.BC_2F_2-4b	88	80	8	11.879	***
		M5.BC_2F_2-4c	94	75	19	1.149	NS
	C21	M7.BC_2F_2-1a	96	83	13	6.722	**
		M7.BC_2F_2-1b	92	74	18	1.450	NS
		M7.BC_2F_2-1c	92	62	30	2.841	NS
		M7.BC_2F_2-3a	94	75	19	1.149	NS
		M7.BC_2F_2-3b	95	68	27	0.593	NS
		M7.BC_2F_2-3c	94	74	20	0.695	NS
	C15	M8.BC_2F_2-3a	94	74	20	0.695	NS
		M8.BC_2F_2-3b	91	74	17	1.938	NS
		M8.BC_2F_2-3c	90	71	19	0.726	NS
		M8.BC_2F_2-4a	96	74	22	0.222	NS
		M8.BC_2F_2-4b	96	61	35	6.722	**
		M8.BC_2F_2-4c	93	69	24	0.032	NS

[a] BC_2F_2 was produced by selfing resistant BC_2 individual plants.
[b]R: Resistant phenotype (Disease score 0 to 4).
[c]S: Susceptible phenotype (Disease score 5 to 9).
[d]$X^2_{3:1}$: Chi-square value of goodness-of-fit test for a 3:1 ratio (Critical value $X^2_{df=1}$: 3.841).
[e]P_{sig}: Probability of rejecting hypothesis at a significance level. * ($P \leq 0.05$), ** ($P \leq 0.01$), *** ($P \leq 0.001$), NS = not significant ($P > 0.05$).

4.4 Discussion

Synthetic hexaploid *Brassica* lines (AABBCC) show poor seed set as a result of chromosome incompatibility among the three *Brassica* genomes of A, B and C in interspecific crosses as well as in self-pollinated generations of hexaploid *Brassica* lines (Tian et al. 2010; Li et al. 2005). Li et al. (2005) observed the extremely low seed set (0.62 to 0.86 seeds per bud pollination) in the first hexaploid generation (H_1) crossed between *B. carinata* (BBCC) and *B. rapa* (AA). Better seed set results (2.7 and 7.9 seeds per bud pollination) were obtained in Malek et al. (2012). Also, Tian et al. (2010) reported that the seed set in H_1 was highly varied and most lines showed very poor seed set with low fertility; some hexaploid lines produced 50 seeds per plant. Hexaploid *Brassica* lines (2n = 54) derived from various combinations of parental genotypes are not stable at the chromosome level and most lines show incomplete ploidy levels (2n < 54) (Chen et al. 2011). Different genotypes within the same *Brassica* species may primarily affect this result.

In the present study, a similar result of hexaploid instability was observed as the low seed yields (5 seeds per pod) from the self-pollinated generation of three hexaploid lines (hexaploid line C28, C21 and C15) (Table 4.6). The average number of seeds per pod in three hexaploid lines was lower than *B. carinata* (13 seeds), *B. rapa* (22 seeds) (Malek et al. 2012; Li et al. 2005) and the susceptible *B. juncea* UM3086 (14 seeds). The poor embryo development observed in F_1 hybrids of hexaploid lines and *B. juncea* might be a result of the cytological instability observed in hexaploid lines. Previously, Håkansson (1956) observed a higher frequency of embryo abortion at early stage in the crosses between higher ploidy plants, for instance crosses between two tetraploids compared to two diploids. It has been observed that embryo abortion is

accompanied by retarded development of endosperm, which in turn, may cause an arrest in seed development. Likewise, the pollen fertility of F_1 hybrids was relatively good as F_2 plants produced a good number of seeds, but shrunken ovules and aborted embryos were observed within a seed pod resulting empty seed pods in F_1 hybrids. These results supported the post-fertilization barrier occurred in F_1 crosses.

As embryo culture technique has been widely used to obtain the hybrid plants from interspecific crosses (Jing et al. 2008; Sharma et al. 1996), most F_1 hybrids showing early abortion were successfully produced through embryo rescue. The extremely low level of embryo development observed in F_1 hybrids could be overcome with embryo culture. In addition, this phenomenon as a result of interspecific hybridization could be overcome by producing backcross populations.

To analyze blackleg resistance in synthetic hexaploid lines originally crossed between *B. carinata* and *B. rapa*, susceptible *B. juncea* (AABB) was used in the present study. The genome composition of advanced backcross generations would likely be tetraploid *Brassica* (AABB) as the C-genome is being eliminated in backcrosses. It has been believed that the high levels of blackleg resistance are from the B-genome of *Brassica* species (i.e. *B. carinata*) because *B. rapa* has absence of the resistance (Badawy et al. 1991; Roy 1978) and the *B. juncea* UM3086 showed susceptibility to the *L. maculans* 03-15-03 (Appendix II). Additionally, the parent *B. rapa* used in the crosses of hexaploid lines (Jiang et al. 2007) and the *B. rapa* previously showing resistance to *L. maculans* (Yu et al. 2005, 2008; Crouch et al. 1994) are from different geographic origins.

In this study, blackleg resistance in synthetic hexaploid *Brassica* lines was analyzed at the seedling stage. The results of phenotypic analysis indicated that most tested populations satisfied the expected genetic ratios in each cross generation. Based on the expected ratio in backcross generations (Figure 4.14), each one family line from BC_2 in hexaploid line (M5.BC_2-3, M7.BC_2-2 and M8.BC_2-2) indicated that blackleg resistance is controlled by two dominant genes since 3:1 resistant-to-susceptible ratio was observed in BC_1 and BC_2 population. The most remaining BC_2 family lines suggested that a single dominant gene is segregating in these families as the lines fit a 1:1 resistant-to-susceptible ratio in BC_2 and showed a 3:1 ratio in BC_2F_2 populations.

In the BC_2F_2 family lines, high levels of blackleg resistance were still observed and the results suggested that each of two dominant genes confer the same level of blackleg resistance at the seedling stage. Overall, the genetic inheritance of blackleg resistance in hexaploid lines was identified with the proposed two gene model. The current study suggested that blackleg resistance in hexaploid lines C28, C21 and C15 is controlled by two dominant genes and each gene can confer the same level of resistance. Although three hexaploid *Brassica* lines C28, C21 and C15 were derived from different crosses, the screening results of blackleg resistance indicated no significant differences among three hexaploid lines in terms of degree of resistance. Further molecular analyses will be required to confirm that the resistance in hexaploid lines is originated from the B-genome of *Brassica.*

CHAPTER 5. GENERAL DISCUSSION

5.0 GENERAL DISCUSSION

Despite the existence of new technologies for crop breeding, conventional breeding methods are still important and useful for genetic analysis especially in *Brassica* species. *Brassica* crops have relatively short cultivation history compared to other important crops such as wheat, rice and corn, and because of that not many molecular markers closely linked to the target traits were available until recently. Using conventional breeding methods, transferring a desirable trait, e.g. blackleg resistance, from *B. juncea* into *B. napus* through interspecific hybridization followed by backcrossing was successfully achieved. Also, determination of blackleg resistance in synthetic hexaploid *Brassica* lines was studied using genetic ratios. Further molecular analyses will ensure the results of phenotypic analysis studied in this thesis in accordance with genetic information.

In the first project of this thesis, the successful introgression of blackleg resistance from the B-genome *Brassica* (*B. juncea*) into *B. napus* L. cv. Westar was achieved. Although frequency of crossovers between B-chromosome and A- or C-chromosomes was low, high levels of resistance against two *L. maculans* isolates 03-15-03 and PG4-1M were observed in the BC_3 populations. Resistant plants obtained in this study are likely to have novel gene resistance, but this claim needs to be confirmed in future research work. Two previously mapped resistance genes in *B. juncea* (*Rlm5* and *Rlm6*) may also appear. In order to determine whether the resistance identified in this study are previously-identified R genes or represent novel R genes, genetic mapping would be required after producing further backcross generations.

In the second project of this thesis, as the first report, genetic inheritance of blackleg resistance in hexaploid *Brassica* lines was determined using a susceptible *B. juncea*. In this project, firstly, the

crossing method allowed maintaining the B-genome in the hexaploid hybrids (AABBC). Secondly, as of yet, no known blackleg resistance is found in the C-genome of *B. carinata* and generally no resistance has been found in the A genome of *B. rapa*. Backcrossing demonstrated that the genetic resistance was present in the B-genome of hexaploid *Brassica* lines. As the first backcross generation BC_1 showed a 3:1 resistant-to-susceptible ratio, this study suggested that blackleg resistance is controlled by two dominant genes. By testing self-pollinated generation of the BC_2 populations showing a 1:1 ratio, it was confirmed that each dominant gene confers the same level of resistance.

To conclude this thesis with two projects, introgression of blackleg resistance seemed easier and beneficial when synthetic hexaploid *Brassica* was used as a source of resistance in the interspecific crosses with allotetraploid *Brassica* species. In this thesis, the crosses between hexaploid lines and *B. juncea* showed 3-fold greater hybrid efficiency than in the crosses between two allotetraploid *B. juncea* and *B. napus* (Table 5.1). Although a tissue culture technique had to be complied for production of F_1 plants, the better pollen fertility (from F_2 seed set results), seed set and germination rate were evident to say that interspecific barriers can be improved by using hexaploid materials. Since many researchers strive to improve quality of canola oilseeds, hexaploid *Brassica* materials would be a feasible tool to introduce not only blackleg resistance but also other desirable traits that are not found in current canola cultivars.

Table 5.1. Comparison of interspecific crossability and hybrid efficiency between tetraploid and hexaploid *Brassica* species.

Original cross	Generation[a]	Hybrid efficiency[b]	Average seed set	Germination rate (%)	F_2 seed set
B. juncea[1] x *B. napus*[2] (AABB) (AACC)	BC_2	18/100 (18%)	2.31 ± 1.47	54%	0.8
Hexaploid[4] x *B. juncea*[3] (AABBCC) (AABB)		643/1149 (56%)	8.19 ± 1.58	88%	27.07

[1]*B. juncea*: Resistant UM lines (collection at the University of Manitoba): refer to the chapter 3
[2]*B. napus*: *B. napus* L. cultivar Westar used in the chapter 3.
[3]*B. juncea*: Susceptible *B. juncea* UM3086 used in the chapter 4.
[4]Hexaploid line: Data pooled for hexaploid lines C28, C21 and C15: refer to the chapter 4.
[a]BC_2 was produced by crossing between susceptible *B. juncea* UM3086 (female parent) and resistant BC_1 individual plants (male parent).
[b]Hybrid efficiency: Successfully introgressed resistance observing/total plant samples tested, a bracket presents the percentage.

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APPENDICES

Appendix I. Germination rate of *B. juncea* parent UM lines and three differential *B. napus* L. cultivars Westar, Glacier and Quinta.

No.	UM line Number	No. of plants sown (A)	No. of plants germinated (B)	Germination rate (B/A x 100)
1	3045	8	8	100%
2	3046	8	8	100%
3	3047	8	8	100%
4	3048	8	8	100%
5	3050	8	8	100%
6	3051	8	6	75%
7	3055	8	4	50%
8	3056	16	15	94%
9	3063	16	15	94%
10	3064	8	3	38%
11	3066	8	8	100%
12	3067	8	7	88%
13	3068	8	2	25%
14	3070	8	8	100%
15	3072	8	6	75%
16	3073	30	30	100%
17	3074	8	8	100%
18	3075	8	7	88%
19	3076	12	8	67%
20	3082	8	8	100%
21	3083	12	9	75%
22	3084	8	8	100%
23	3085	8	8	100%
24	3086	38	38	100%
25	3087	8	8	100%
26	3088	8	8	100%
27	3089	8	8	100%

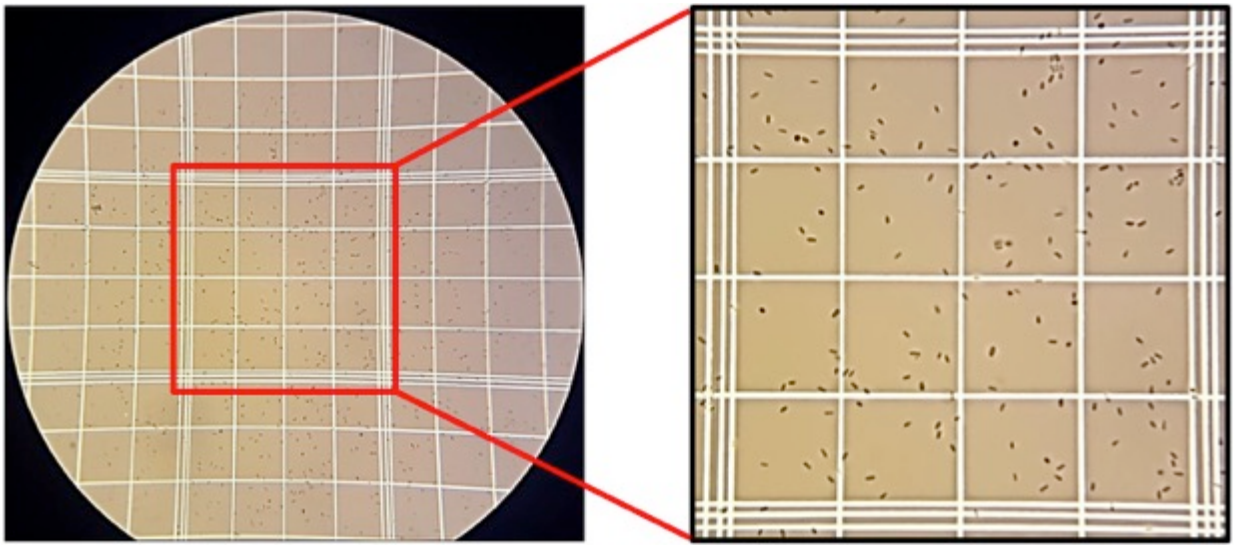
28	3090	8	1	13%
29	3091	8	4	50%
30	3094	8	2	25%
31	3095	28	28	100%
32	3096	8	1	13%
33	3097	8	6	75%
34	3098	8	4	50%
35	3100	5	3	60%
36	3102	8	4	50%
37	3104	8	8	100%
38	3105	8	8	100%
39	3106	8	8	100%
40	3107	8	5	63%
41	3108	8	8	100%
42	3121	8	8	100%
43	3122	28	26	93%
44	3123	8	8	100%
45	3132	16	16	100%
46	3154	8	8	100%
47	3477	20	19	95%
48	3482	10	6	60%
49	3510	8	1	13%
50	3511	8	8	100%
51	3516	8	8	100%
52	3533	8	8	100%
53	3541	20	19	95%
54	3544	20	20	100%
Control	Glacier	24	24	100%
Control	Quinta	24	24	100%
Control	Westar	40	40	100%

Appendix II. Phenotype identification of *B. juncea* parent UM lines and three *B. napus* L. cultivars Westar, Glacier and Quinta using *L. maculans* isolate 03-15-03 and PG4-1M.

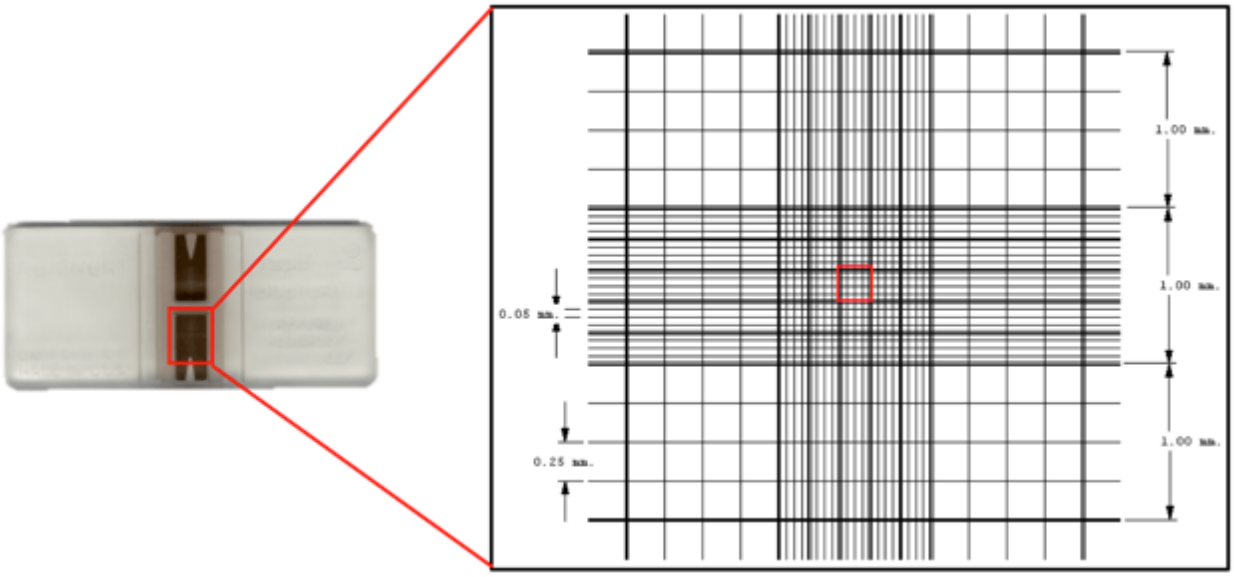
No.	UM line Number	Cotyledon Response to 03-15-03	Cotyledon Response to PG4-1M
1	3045	R	-
2	3046	R	-
3	3047	R	-
4	3048	R	-
5	3050	R	-
6	3051	R	-
7	3055	R	-
8	3056	R	R
9	3063	R	R
10	3064	R	-
11	3066	R	-
12	3067	R	-
13	3068	R	-
14	3070	R	-
15	3072	R	-
16	3073	R	R
17	3074	R	-
18	3075	R	-
19	3076	R	R
20	3082	R	-
21	3083	R	-
22	3084	R	-
23	3085	R	-
24	3086	S	S
25	3087	R	-
26	3088	R	-
27	3089	R	-

28	3090	R	-
29	3091	R	-
30	3094	R	-
31	3095	R	R
32	3096	R	-
33	3097	R	-
34	3098	R	-
35	3100	R	-
36	3102	R	-
37	3104	R	-
38	3105	R	-
39	3106	R	-
40	3107	R	-
41	3108	R	-
42	3121	R	-
43	3122	R	R
44	3123	R	-
45	3132	R	R
46	3154	R	-
47	3477	R	R
48	3482	R	-
49	3510	R	-
50	3511	R	-
51	3516	R	-
52	3533	R	-
53	3541	R	R
54	3544	R	R
Control	Glacier	R	S
Control	Quinta	R	S
Control	Westar	S	S

*R: Resistant (Disease score 0 to 4); S: Susceptible (Disease score 5 to 9); '-': not tested.



Appendix III. Counting pycnidiospores using hemocytometer is to calculate the number of spores per mL for inoculum preparation. Five of 16 small squares were counted to minimize sampling errors. Cells on the bottom and right line did not count to avoid duplicate counting.



Appendix IV. Top and bottom hemocytometer counting chamber (left) in which each is 9 mm^2, depth of 0.1 mm. Central 1 mm^2 square consists of 25 groups of small square highlighted in red (right). Each small square is divided into 16 of 0.0025 mm^2 squares. (Hausser Scientific, PA, USA)