University of Alberta

Introgression of Blackleg Resistance into *Brassica napus* from *Brassica carinata*

by

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Abstract

Blackleg, caused by *Leptosphaeria maculans*, is one of the most damaging diseases of oilseed rape, *Brassica napus*. Interspecific hybridization between *B. napus* and *B. carinata* was done to transfer resistance to PG4 type blackleg pathotype from *B. carinata* into *B. napus. In vitro* ovule culture and *in vivo* seed set techniques were applied for the production of interspecific hybrids, where ovule culture was more efficient than *in vivo* seed set; and ovule culture in NN liquid medium was more efficient than B5 solid medium. All the interspecific F_1 hybrids were resistant to blackleg. The F_1 hybrids were recurrently backcrossed to *B. napus* and selection for cotyledon and adult plant resistance performed in each generation. In the backcross generations, significant number of seedlings with cotyledon resistance was found to be susceptible at the adult plant stage suggesting that cotyledon and adult plant resistance is under different genetic control in *B. carinata*. The proportion of resistant plants decreased with the progression of backcrossing- apparently due to loss of *B. carinata* chromosome(s) carrying the resistance.

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Symbols and Abbreviations

⊗	Self pollination
♂	Male parent
♀	Female parent
±	Plus/minus
°C	Degrees Celsius
µL	Microliter
µmol/g	Micromoles per gram
χ^2	Chi-square test statistic
BAC	Bacterial artificial chromosome
BC_1	First backcross generation
BC_2	Second backcross generation
BC_3	Third backcross generation
BC_4	Fourth backcross generation
BC_2S_1	First self-pollinated generation after second backcross
BC_2S_2	Second self-pollinated generation after second backcross
BC_3S_1	First self-pollinated generation after third backcross
cm	Centimeter
Cot.	Cotyledon
cv.	Cultivar
DAP	Days after pollination
DH	Doubled haploid
DAI	Days after inoculation
DNA	Deoxyribose nucleic acid
dNTP	Deoxynucleotide triphosphate
F_1	First filial generation
Fig.	Figure
FISH	Fluorescent in situ hybridisation
g	Gram
GDP	Gross domestic product
GISH	Genomic in situ hybridisation
h	hour
H_2O	Water

L	Liter
m^2	Square meter
mg	Milligram
M ha	Million hectare
M ton	Million ton
min	Minute
mL	Milliliter
mm	Millimeter
mM	Millimole
n	Haploid number of chromosomes
ng	Nanogram
No.	Number
P	Probability
PCR	Polymerase chain reaction
PMC	Pollen mother cell
poll.	Pollinations
QTL	Quantitative trait loci
RFLP	Restriction fragment length polymorphism
rpm	Revolutions per minute
s	Second
S.E	Standard Error
SSR	Simple sequence repeat
t	Test statistic for t-test
Taq (polymerase)	Polymerase from the bacterial species *Thermus aquaticus*
Temp.	Temperature
UV	Ultraviolet (light)
V	Volt
Var.	Variety
w/v	Weight to volume ratio

Chapter1
Literature Review

1.1 Introduction

The Brassica oilseed crops are an important source of edible oil in many parts of the world. Oilseed crops have been cultivated in the Indian sub-continent since the beginning of the 20th century B.C for cooking and lighting purposes. Earlier writings of European and Asian civilizations contain references to the use of rapeseed and other closely related plants. Cultivation of oilseed crops in Europe and North America became extensive following the development of steam engines in the 18th century for use of these plant oils as lubricant. Rapeseed oil was found to adhere to water or steam-washed metal surfaces better than other lubricants under extreme heat and steam. Therefore, this oil was in high demanded for use as marine lubricant oil. The ability of Brassica seeds to grow at low temperatures has made Brassica oilseed crops as important crops in many temperate countries (Kimber and McGregor 1995). Furthermore, for survival, Brassica oilseed crops require far fewer heat units than other oilseed crops. Therefore, this crop proved well adapted with significant production in the Canadian Prairies (Canola Council of Canada 2010a).

In Canada, three Brassica oilseed crops viz. *B. napus* L., *B. rapa* L. and *B. juncea* (L.) Czern & Coss are grown, but *B. napus* (rapeseed) is by far the largest crop in terms of both acreage and production. *Brassica rapa* was first cultivated in Saskatchewan in 1936 by a farmer who had migrated from Poland, and therefore, this species is commonly called as Polish rape in Canada. During World War II,

there was an acute shortage of rapeseed oil in Canada and so to alleviate the shortage, 19 tones of *B. napus* rapeseed were imported from the United States of America. These seeds originated from Argentina, and therefore *B. napus* is commonly called as Argentine rape.

Traditional rapeseed oil is unfit for human consumption as it contains a high proportion of erucic fatty acid (40-50%). In addition, the seed meal fraction left over after crushing contains a group of compounds called glucosinolates which inhibits growth in livestock. Therefore, concerted efforts were made to improve its seed oil and meal quality. In the 1970's, using traditional breeding techniques, Canadian plant breeders succeeded in developing rapeseed cultivars with significantly reduced amount of erucic acid (<2%) in oil and glucocinolate (<30µmol/g) in seed meal (Canola Council of Canada 2010e). The first low erucic acid and low glucosinolate cultivar Tower was developed at the University of Manitoba in 1974 and this type of cultivars are generally called double low type. The first double low Polish cultivar Candle was developed by Agriculture and Agri-food Canada (AAFC), Saskatoon. In 1977, the Canola Council of Canada branded all double low cultivars as canola (Canola Council of Canada 2010b). However, the term double low is still used by the European breeders. Canola quality *B. juncea*, having the fatty acid profile and glucosinolate levels comparable to that of canola *B. napus* and *B. rapa*, was developed by AAFC, Saskatoon and Saskatchewan Wheat Pool in 2002 (Canola council of Canada 2010c).

According to the Canola Council, the term canola refers to the seeds of *B. napus*, *B. rapa*, or *B. juncea* whose oil contain less than 2% erucic acid in its fatty acid profile and the meal contain less than 30 micromoles of any one or any mixture of 3-butenyl glucosinolate (gluconapin), 4-pentenyl glucosinolate (glucobrassicanapin), 2-hydroxy-3 butenyl glucosinolate (progoitrin), and 2-hydroxy- 4-pentenyl glucosinolate (gluconapoleiferin) per gram of meal on air-dry oil-free solid basis. 1n 1985, Canadian canola achieved the GRAS (Generally Recognized As Safe) status by the United States Food and Drug Administration (FDA). Henceforth, canola quality cultivars have been extensively cultivated in Australia, China, Europe and North America. It is the second largest oilseed crop after soybean in the world, and its production has increased over the last decades much faster than any other edible oilseed crops (USDA 2010).

In Canada, canola is planted in May and harvested in September to October. Canola contributes about $14 billion to the Canadian economy and generates more than 216,000 jobs annually in Canada (Canola Council of Canada 2009a). Intensive plant breeding efforts and exploitation of hybrid vigour has increased canola seed yield significantly over the last few years, and this has made the crop highly profitable to canola growers. Consequently, in Canada, per hectare production of canola has increased more than 10 fold since its introduction and cultivation in the 1950's (Fig. 1.1). Recently, there is an increasing interest in the production of bio-fuels and biodegradable plastics from canola oil. Therefore, canola has become the second important cash crop in Canada (Fig. 1. 2). In recent years, canola has been cultivated on over 5 million

hectares per annum, as shown in Fig. 1. 3. In 2009, Canadian growers harvested a record of 11.83 million tonnes in 6.11 million hectares, with Saskatchewan, Alberta and Manitoba producing about 48%, 26 % and 24% of the total crop, respectively. The remaining 1-2% canola was produced in British Columbia, Ontario and Quebec (Canola council of Canada, Provincial acreage and yield 2009b). In 2008, Canada was the highest canola producing country (Table 1.1). According to the Food and Agricultural Organization (FAO 2011), Canada was the largest canola exporting country in 2008 and traded about 42% of the global canola export. The price of canola seed and oil is projected to increase in the future due to expected increase in the demand for healthy oil and the use of this oil in the production of environmentally friendly bio-fuel (G8 Summit, Hokkaido –Tokyo 2008). The Canola Council of Canada has set a target known as 'Growing Great 2015' to increase canola production to 15 million tonnes by 2015 (Canola Council of Canada 2009c). This is an ambitious target that can only be realized through integrated efforts from agronomists, breeders, pathologists, policy makers, and more importantly by getting growers to cultivate improved seeds and also adopt the best agricultural practices.

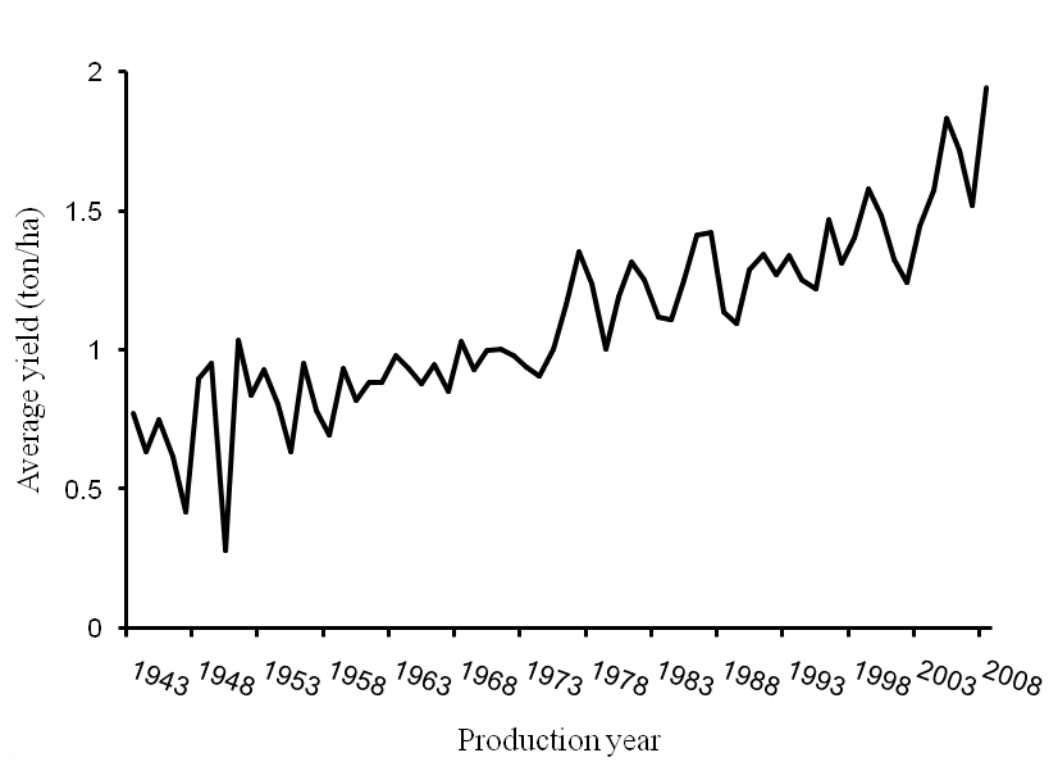

Fig. 1.1 Trends in rapeseed/canola yield in Canada since 1943

(Statistics Canada, January, 2010)

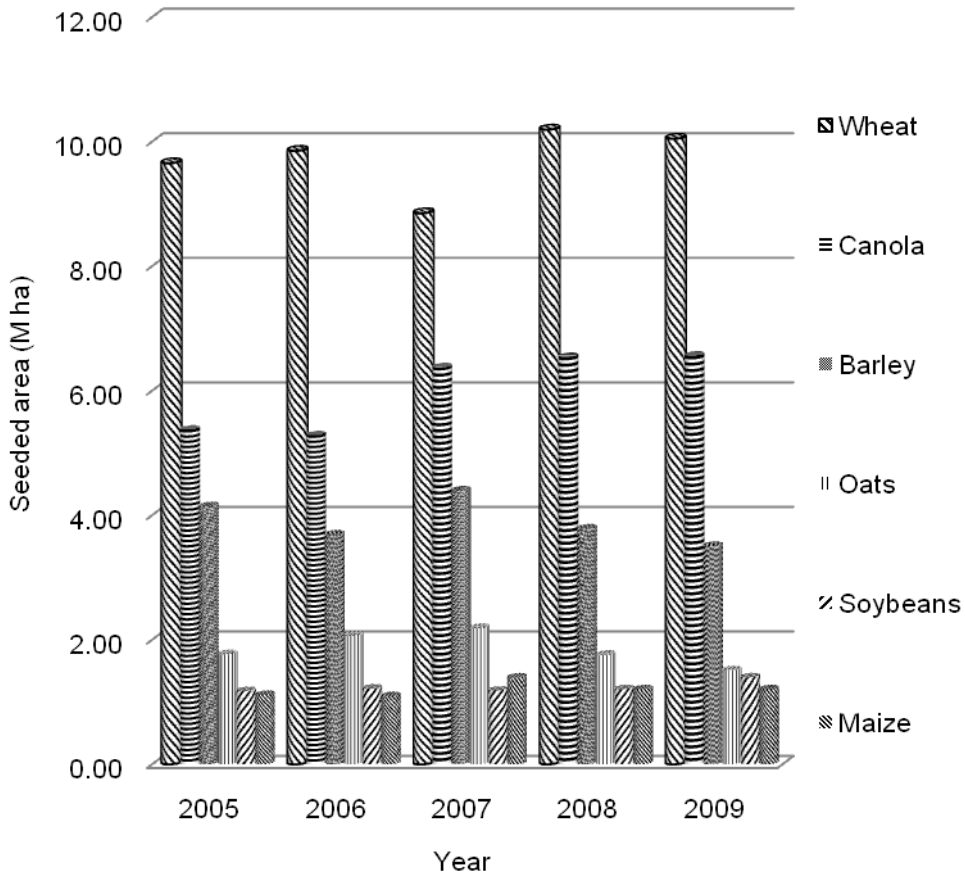

Fig.1.2 Comparison of seeded areas for the major field crops in Canada

(Statistics Canada, January, 2010)

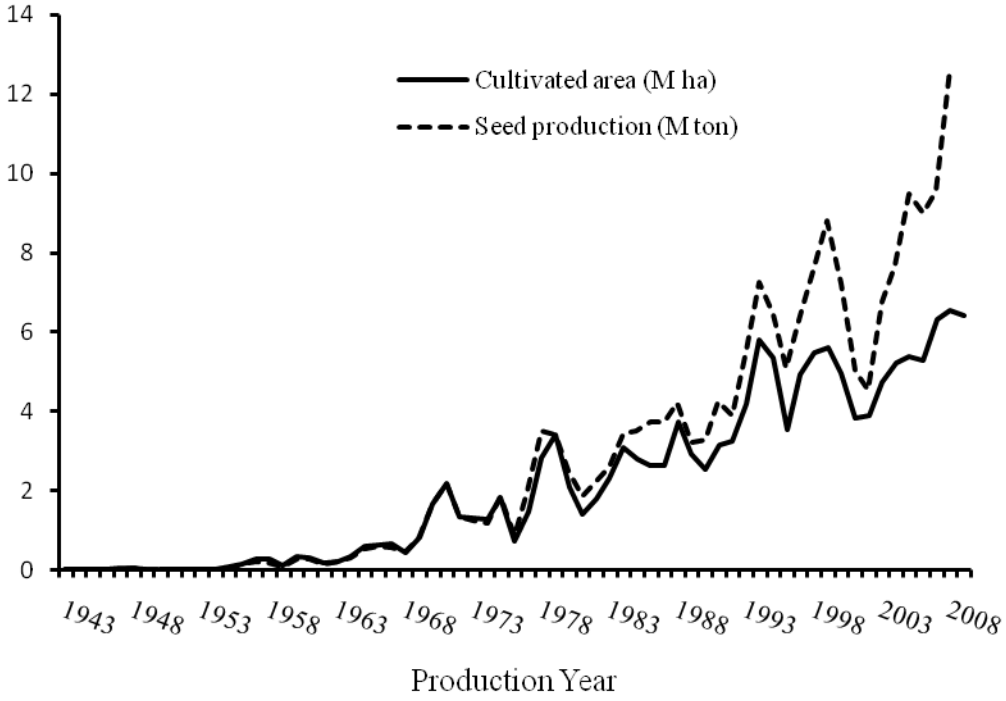

Fig. 1.3 Annual production of rapeseed/canola in Canada since 1943

(Statistics Canada, January, 2010)

Table 1.1 List of top ten *Brassica* oilseed producing countries in 2008

Country	Production (Million ton)
Canada	12.64
China	12.10
India	5.83
Germany	5.15
France	4.72
Ukraine	2.87
Poland	2.11
Australia	1.85
United Kingdom	1.97
Czech Republic	1.05

Source: FAO, January, 2011

In Canada, canola production is affected by the diseases such as blackleg, sclerotinia, alternaria, and clubroot (Agriculture and Agri-Food Canada 2005). The most devastating of these diseases are blackleg and clubroot. While clubroot disease is an emerging problem, the disease which farmers have struggled to contain over the years is blackleg. Therefore, if Canada is to achieve the 'Growing Great 2015' agenda then it needs to overcome blackleg disease as well as the other diseases of canola. Blackleg is caused by the fungus *Leptosphaeria maculans*, and the pathogen is present in the canola producing areas of Saskatchewan, Manitoba, Alberta and British Columbia (Gugel and Petrie 1992). According to the Canola Council of Canada, 90% of the fields with an average of 52% of the plants were infected by blackleg in Saskatchewan in 1989. The few fields which had 100% infection reported yield losses greater than 50%. Intensive breeding efforts over the last decade resulted in several blackleg resistant cultivars; however, this disease was still observed in 2009 in 38% of the crops surveyed in Saskatchewan (Dokken-Bouchard et al. 2010), and in 56% of the crops surveyed in Manitoba (McLaren et al. 2010)

In 2008-09, Canada's largest canola seed market was China, with an export of 2.8 million tonnes translating to a value of $1.3 billion. However, in November 2009, China imposed an emergency quarantine order to block the importation of Canadian and Australian canola carrying *L. maculans.* This restriction was imposed to stop the spread of blackleg disease in China. For the 2010 crop, China has indicated that it will accept canola from Canada if the seed is free of the blackleg pathogen (Canola Council of Canada 2010d). This may severely limit or

even close Canada's access to the Chinese market. Therefore, strong research effort is needed to develop blackleg resistant cultivars for the production of *L. maculans* free seeds.

Several sources of blackleg resistance have been reported. For example, the Australian canola (*B. napus*) cultivars Maluka and Shiralee, winter canola (*B. napus)* cultivar Major, *B. rapa* ssp. *sylvestris,* and all Brassica species carrying the B genome. Using Australian sources of resistance, several *B. napus* cultivars (Q2, Quantum, HiQ etc.) were developed at the University of Alberta (Stringam et al. 1995; Stringam et al. 1999; Stringam et al. 2000). These cultivars carry resistance to PG2 blackleg pathotype, which is conferred by the resistance gene *Rlm3*. However, the emergence of new blackleg pathotypes, e.g. PG3, PG4 and PGT, in the Canadian Prairies has become a major concern to the canola growers in Canada (Kutcher et al. 2007), and therefore, in recent years, high disease severity in previously resistant cultivars has been reported by growers (Kutcher et al. 2010). Breakdown of race specific resistance has also been reported in Australia, Europe and Canada. Therefore, the development of new canola cultivars resistant to different blackleg pathotypes is highly desired, but a great challenge to the breeders.

1.2 Genome relationship between Brassica species

Cytological analysis of chromosomes can be used for distinguishing different plant species. However, the identification of Brassica chromosomes is difficult and laborious due to their small size and similar morphology. Despite

these limitations, cytological analysis by Japanese scientists revealed the relationship between the diploid and amphidiploid Brassica species. Based on chromosome paring in Brassica interspecific hybrids, Morinaga (1934) (cited by Kubik 1999) hypothesized that *B. napus* (2n=38, AACC), *B. juncea* (2n=36, AABB) and *B. carinata* (2n=34, BBCC) are the amphidiploids of the diploid species *B. nigra* (2n=16, BB), *B. oleracea* (2n=18, CC) and *B. rapa* (2n=20, AA). U (1935) successfully synthesized these amphidiploids species from interspecific crosses between the diploid species, and proposed the genome relationship between the Brassica species that is now known as U's triangle (Fig 1.4).

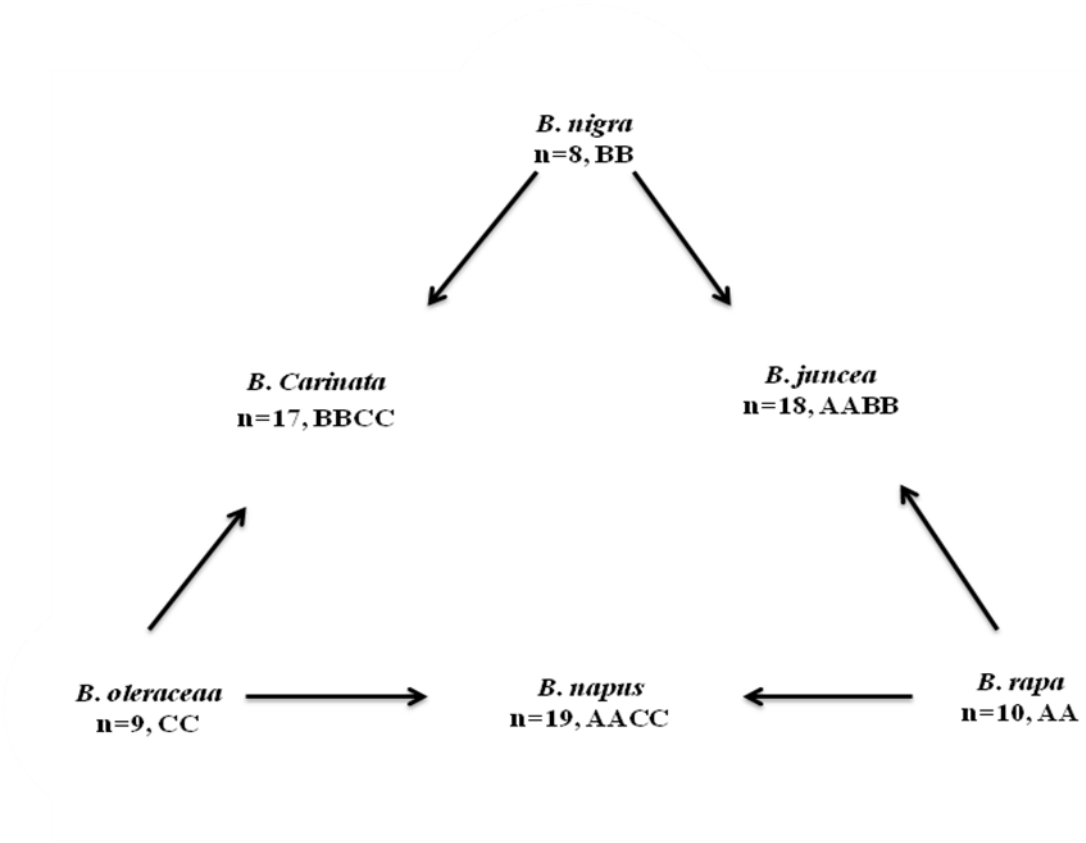

Fig. 1.4 The "Triangle of U" showing the genome relationships between the six *Brassica* species (U 1935)

Recently, molecular markers have been used to reveal the complex relationships between different *Brassica* species. In a phylogenetic study using Restricted Fragment Length Polymorphism (RFLP) markers, Song et al. (1988) confirmed that the three amphidiploid species of U triangle are the result of hybridizations between the three diploid species. Based on chloroplast genome analysis, Warwick and Black (1991) studied the cytodeme relationship between the diploid species and their evolutionary relationship and found that the diploid species evolved from a common prototype following two lineages: one led to the evolution of *B. rapa* and *B. oleracea*, while the other lineage led to the evolution of *B. nigra*. The Brassica B-genome has diverged significantly from the A and C genomes (Warwick and Black 1991) and is considered to have very limited homoeology with these two genomes (Warwick et al. 1992). Cytogenetic analysis has also revealed that the B-genome chromosomes have very little tendency to pair with chromosomes of the A or C genomes in Brassica interspecific hybrids, while the A and C genome chromosomes pair frequently (Attia and Röbbelen 1986; Busso et al. 1987). This genome relationship between the A/C and B genome chromosomes has also been confirmed by the molecular cytological FISH-BAC/ GISH technique (Mason et al. 2010). However, based on molecular markers, Lagercrantz and Lydiate (1996) and Panjabi et al. (2008) demonstrated that the B-genome share some homoeology with the A- and C genomes. The study by Struss et al. (1996) on the alien B-genome chromosomes of *B. nigra*, *B. carinata* and *B. juncea* in a *B. napus* background showed that translocations and recombination occurs between the A/C chromosomes with the B genome

chromosomes. These studies reveal that the B-genome chromosomes are more diverse and have little homoeology with the A and C genome chromosomes; therefore, transfer of trait(s) from this genome into the A or C genome of *B. napus* would be a challenging task.

1.3 Blackleg disease in *Brassica* crops

Blackleg, caused by the fungus *Leptosphaeria maculans*, is one of the most devastating diseases of canola worldwide (Howlett 2004; Fitt et al. 2006). This fungus is endemic to canola growing regions in Canada, Australia, United Kingdom, France, Germany and many other countries, where it causes significant yield loss (Gugel and Petrie 1992). *Leptosphaeria maculans* isolates have a world-wide distribution because of its transmission through air and on seeds of *B. napus*, *B. oleracea*, *B. rapa* and other Brassica crops. In Canada, it was first identified in the province of Saskatchewan in 1975 (McGee and Petrie 1978). Over the years, it spread to Alberta, Manitoba and British Columbia (Gugel and Petrie 1992).

Leptosphaeria maculans generally exists with at least two reproductively isolated populations which are similar in morphology but different in culture (Rimmer 2006). The strains of this pathogen have been classified into two pathotypes, A and B, based on their ability to cause stem cankers on *B. napus* and also to produce the phytotoxin serodesmin PL. The *L. maculans* strains that cause stem canker are called aggressive or virulent, and classified as group A; while the strains that do not cause stem canker on canola are called non-aggressive, weakly

virulent or avirulent and fall under group B (Howlett et al. 2001). Shoemaker and Brun (2001) reclassified the B group as *Leptosphaeria biglobosa* which is commonly found in canola fields in the Canadian prairies (Rimmer 2006) and causes superficial stem cankers (Johnson and Lewis 1994). The virulent or aggressive strains can infect canola from germination through to maturity.

The *Leptosphaeria maculans* isolates were originally classified into various pathogenicity groups (PGs) based on their differential virulence on cotyledons of the *B. napus* cultivars Westar (spring type), Glacier and Quinta (winter types) (Mengistu et al. 1991; Koch et al. 1991). Isolates which are non-aggressive or weakly virulent and do not cause disease on Westar were assigned to PG1 (*L. biglobosa*), while the isolates virulent on all of three differential cultivars were assigned to PG4. Isolates virulent on Westar but avirulent on Glacier and Quinta were classified as PG2. The isolates which are virulent on both Westar and Glacier but avirulent on Quinta were classified as PG3. A fourth pathogenicity group was found in western Canada in the late 1990's (Keri et al. 2001). This isolate was virulent on Quinta but avirulent on Glacier and named PGT (Kutcher et al. 2007). Other researchers have included many other varieties as differentials, for example Badawy et al. (1991) included 'Jet Neuf' as a fourth differential for classifying isolates. This PG system of classifying *L. maculans* isolates has been a valuable means of detecting changes in the pathogen population (Kutcher et al. 2007). However, the PG system does not account for variability caused by the sources of resistance not found in the differential varieties. To date, 14 resistance genes in various Brassica spp. have been reported,

and theoretically the number of possible pathotypes or races could be 2^{14} or 16,384 (Rimmer 2007).

Balesdent et al. (2005) proposed a new classification system to overcome the limitation of the PG system. The proposed classification was based on the existence of resistance (R) genes in the host and the corresponding avirulence (Avr) genes in the pathogen. In this new classification, a race is identified by its Avr allele composition as the distinct Avr genes govern the recognition between the *L. maculans* and its host (Balesdent et al. 2002). According to Rimmer (2007), 14 avirulence alleles can be detected against the 14 resistant genes that have been identified to date. For example, isolates IBCN85 and NzT belong to PG4 as both are virulent on Glacier and Quinta. However, there are differences in the allele composition between each isolate. The isolate IBCN85 carries avirulence alleles *AvrLm5*, *AvrLm6* and *AvrLm7*, while the isolate NzT carries avirulence alleles *AvrLm5, AvrLm6 and AvrLm8*. According to new classification system, the isolate IBCN85 is identified as the race Av5-6-7 and isolate NzT, as race Av5-6-8.

1.3.1 Life cycle and infection mechanism of *Leptosphaeria maculans*

The blackleg fungus usually survives the winter on infected canola stubble. In the field, it can take up to four years or longer for the larger stem pieces of canola stubble to decompose. Therefore, blackleg-infested stubble can continue to produce ascospores of *L. maculans* until the stubble is completely decomposed. Ascospores are released during the growing season. The spores can

be carried over long distances by air currents, but most are deposited close to their origin. Spores that land on susceptible canola plants or weeds may cause infection. Infection of seedlings occurs through stomata or wounds on the cotyledons and/or young leaves (Howlett et al. 2001). Once a plant become infected by the pathogen, additional spores are produced on the dead tissue of leaves, stems, and pods as thousands of black, pin-head sized asexual fungal fruiting bodies called pycnidia (Hammond and Lewis 1987, cited by Howlett et al. 2001). The pycnidiospores act as secondary inoculum; spread to short distance within a field mainly by splashing rain, and thus cause additional leaf and stem lesions on nearby plants. Hail injury may intensify the infection levels. Following initial infection of the leaf, the fungus colonizes the intercellular space between the mesophyll cells. The fungus then grows down the petiole in the xylem vessels or between the xylem parenchyma and cortex, and finally invades and kills the cells of the stem cortex resulting in a black canker. The canker can completely girdle the base of the stem, and is therefore, named as blackleg disease (Howlett et al. 2001).

Spread of the disease into a new area occurs through the movement of infected seed (Canola Council of Canada, 2010f). When blackleg-infested seed is sown, the seedlings may get infected on cotyledon, leaf or stem. The infected seedling produces spores which spread to the surrounding plants. A seed-lot with a low level of infection easily spread the disease throughout the entire field. Infection before the six-leaf stage usually results in significant yield loss (Ministry of Agriculture, SK 2010)

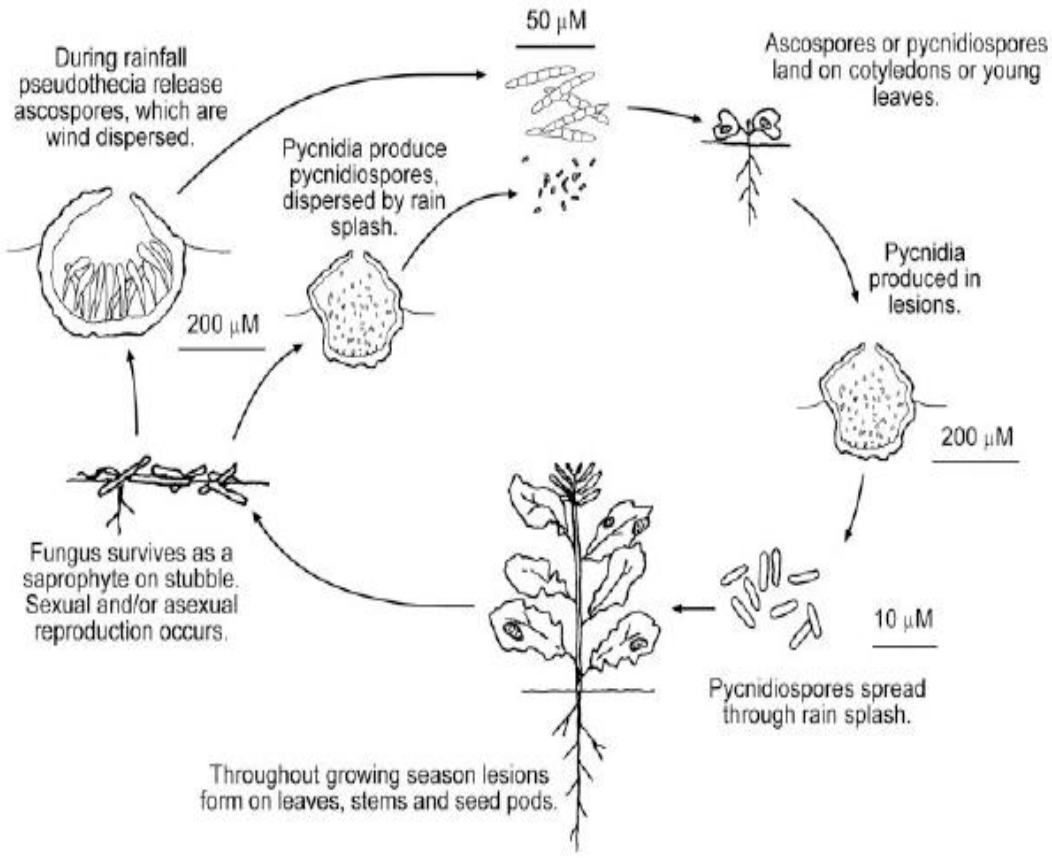

Fig. 1.5 Life cycle of *Leptosphaeria maculans* (Howlett et al. 2001).

1.3.2 Blackleg resistance genes

Breeding of blackleg resistant cultivars is an effective way of controlling this disease (Delourme et al. 2006). *Brassica napus* germplasm resistant to blackleg disease has been identified and characterized. Dixelius and Wahlberg (1999) mapped blackleg resistance in the three Brassica species carrying the B-genome, viz. *B. nigra, B. juncea* and *B. carinata.* Based on BC_1, BC_2 and BC_3 generation population, derived from interspecific crosses of these species with *B. napus*, and via the use of restriction fragment length polymorphism (RFLP)

markers, they detected six loci on three linkage groups of the B genome conferring resistance to this disease.

Ferria et al. (1995) mapped the blackleg resistance gene LEM1 (= *Rlm4,* Delourme et al. 2006) of *B. napus* cultivar Major on the linkage group 6 of their map (=N7, Delourme et al. 2006). Several RFLP markers linked to the resistant gene, *LmFr1,* from the spring *B. napus* cultivar Crésor have been identified by Dion et al. (1995); however, the position of this gene on the chromosome has not been confirmed. Mayerhofer et al. (2005) mapped the blackleg resistance gene *LmR1* of the Australian spring *B. napus* cultivar Shiralee on the linkage group N7 which is equivalent to linkage group 6 of Ferreira et al. (1995). To date, several blackleg resistance genes(e.g. *Rlm1* (N7), *Rlm2* (N10) *&* *Rlm3* (N7), *Rlm4* (N7), *Rlm7* (N7) and *Rlm9* (N7)) from different *B. napus* cultivars and breeding lines have been mapped by different researchers (Table 1.3, for review, see Delourme et al. 2006). With the exception of *Rlm2*, the other five genes are mapped to the linkage group N7 of the A genome. In addition to these genes from *B. napus*, resistance genes have also been identified in other chromosomes of the A genome, e.g. *LepR1* (N2) and *LepR2* (N10) from *B. rapa* ssp. *sylvestris* (AA, 2n=20) (Yu et al. 2005). Race-specific resistant genes in plants are matched by avirulence genes (*Avr*) in the pathogen postulating a hypersensitive response . Race specific resistance is effective in protecting the crop if the corresponding avirulence gene is predominant in the local pathogen population. Seven fungal avirulence (Avr) genes have been mapped to two gene clusters, *Avrlm*1-2-6 and *Avrlm*3-4-7-9, on

the *L .maculans* chromosomes (Balesdent et al. 2002, cited by Rouxel and Balesdent, 2005).

Table1.2 Summary of different blackleg (*Leptosphaeria maculans)* resistance genes identified in different *Brassica* species

Resistance Gene	Linkage Group	Brassica Species	Reference
Rlm1	N7	*B. napus*	Ansan-Melayah et al. 1998; Delourme et al. 2006
Rlm2	N10	*B. napus*	Ansan-Melayah et al. 1998; Delourme et al. 2006
Rlm3	N7	*B. napus*	Delourme et al. 2004, 2006; Balesdent et al. 2002
Rlm4 (*=LEM1*)	N7	*B. napus*	Delourme et al. 2006; Ferreira et al. 1995
Rlm4	N7	*B. napus*	Balesdent et al. 2001; Delourme et al. 2004, 2006
LMJR1	J13	*B. juncea*	Christianson et al. 2006
LMJR2	J18	*B. juncea*	Christianson et al. 2006
Rlm7	N7	*B. napus*	Balesdent et al. 2002; Delourme et al. 2004
Rlm9	N7	*B. napus*	Delourme et al. 2004, 2006
Rlm10	Chromosome 4	*B. nigra*	Chevere et al. 1996, cited by Leflon et al. 2007

1.3.3 Introgression of blackleg resistance

The most common and effective method of controlling blackleg disease is through the development of resistant cultivars (Delourme et al. 2006). Therefore, efforts have been made by different researchers to introgress blackleg resistance genes from different resistant sources including allied Brassica species into *B. napus* cultivars. A review of the efforts by different researchers for the introgression of blackleg resistance from different sources into *B. napus* and the key results are summarized in Table 1.4.

Brassica species carrying the B-genome generally possess high levels of resistance to blackleg disease (Christianson et al. 2006). Therefore, several researchers have attempted to transfer this B-genome resistance from *B. juncea, B. nigra* and *B. carinata* into *B. napus* through interspecific hybridization (Roy 1978, 1984, Jahier et al. 1989, Sacristan and Gerdemann 1986). It is apparent from the Table 1.3 that compared to *B. nigra* and *B. juncea*, very limited efforts have been made for introgression of resistance from *B. carinata* into *B. napus*. Chèvre et al. (1997) reported stable introgression of the B genome cotyledon resistance from *B. juncea* into *B. napus*; while Struss et al. (1996) reported introgression of blackleg resistance from the B genome of *B. nigra*, *B. juncea* and *B. carinata* into *B. napus*. Plieske et al. (1998) also reported blackleg resistant *B. napus* lines carrying resistance from *B. nigra*, *B. juncea* and *B. carinata*. In all three cases, resistance behaved as monogenic dominant and no difference in the level or mechanism of resistance was found due to the source of resistance. A gene conferring resistance to blackleg pathotypes PG2 has also been

transferred from *B. carinata* into *B. napus* cv. Westar through interspecific cross between these two species followed by backcrossing of the interspecific hybrids to Westar (Rahman et al. 2007).

Table 1.3 Summary of research on blackleg resistance in different Brassica species

Materials used	Institution	*L. maculans* pathotypes	Place and stage of BL test	Main results	References
Somatic hybrids of *B. napus*, cv. Hanna (S) and *A. thaliana* Columbia (Col-0) (R) backcrossed to *B. napus* and selfed (BC_1S_4)	Swedish University of Agricultural Sciences, Upsala, Sweden	PHW 1245	Growth chamber; leaf of 19 days old seedling	The BC_1S_4 generation was significantly more-resistant than *B. napus*	Bohman et al. 2002
Segregating *B. napus* population derived from. Maluka (R) x Niklas (S) cross.	University of Melbourne, Australia	MB2	Greenhouse; adult and cotyledon	Involvement of dominant/ epistatic gene effect in the control of resistance. Cotyledon test in screening for adult plant resistance would have limited value.	Pang and Halloran 1996
Segregating population from Maluka (R) x Niklas (S) cross.	University of Melbourne, Australia	MB2	Green house; adult stage	A single incompletely dominant major gene controls the resistance.	Pang and Halloran 1996
Segregating *B. napus* population from the crosses between Surpass 400 (R) and Westar (S) cross	The University of Western Australia, South Perth, Australia	Unspecified isolate	Field; cotyledon and adult	A major dominant gene controls resistance in both seedling and adult plants.	Li et al. 2003

R= Resistant, S= Susceptible, BL= Blackleg

Table 1.3 Summary of research on blackleg resistance in different Brassica species (contd.)

Materials used	Institution	*L. maculans* pathotypes	Place and stage of BL test	Main results	References
Brassica napus resynthesized from different accessions of *B. rapa* and *B. oleracea*	John Innes Centre for Plant Science, and University of East Anglia, UK	Aggressive isolate Lm11; less aggressive isolate Lm3, Lm 6, Lm Gl and Lm G2	Greenhouse and field; cotyledon	Two wild accession of *B. rapa* carry resistance	Crouch et al. 1994
Brassica napus-B. nigra chromosome addition lines	INRA 35653 Le Rheu Cedex, France	Isolates # 314, #290, #447 and #813	Greenhouse; cotyledon and adult	Monosomic addition line carrying *B. nigra* chromosome 4 of *B. nigra* showed significant resistance	Chèvre et al. 1996
Near-isogenic *B. napus* lines carrying resistance from *B. juncea* and *B. nigra*	Swedish University of Agricultural Sciences Upsala, Sweden	PG2 isolate #Lm 1245	Greenhouse; adult	One dominant gene control resistance in the *B. napojuncea* lines, while two independent dominant loci control resistance in *B. naponigra* line.	Dixelius 1999

R= Resistant, S= Susceptible, INRA= National Institute for Agricultural Research, BL= Blackleg

Table 1.3 Summary of research on blackleg resistance in different Brassica species (contd.)

Materials used	Institution	*L. maculans* pathotypes	Place and stage of BL test	Main results	References
Segregating population based on *B. juncea* resistance in *B. napus* (S) genome	INRA 35653 Le Rheu Cedex, France.	Isolate #314 (Highly virulent)	Greenhouse; cotyledon	Provided evidence that resistance from *B. juncea* introgressed into the *B. napus* genome by homologous recombination.	Barret et al. 1998
Recombinant lines of *B. napus* L. containing *B. juncea* resistance crossed with the *B. napus* cv. Samouraii (S).	INRA 35653 Le Rheu Cedex, France	d314 (A-group)	Greenhouse and field; cotyledon and adult	Monosomic control of the B-genome resistance of *B. juncea* introgressed into *B. napus.* Resistance is effective under field condition.	Chèvre et al. 1997
B. juncea mapping population from cross between R and S genotypes	Department of Biological sciences, University of Alberta, Canada.	PL86–12	Greenhouse; cotyledon	Resistance in *B. juncea* controlled by two independent genes - one dominant and one recessive, mapped on J13 and J18 respectively.	Christianson et al. 2006
Segregating population of *B. juncea* derived from cross between R and S genotypes	Department of Plant Science, University of Manitoba, MB, Canada	Pl85-9 and Pl86-14	Greenhouse; cotyledon	Two gene pairs with dominant recessive epistatic gene action involved in the control of resistance.	Keri et al. 1997
Segregating populations derived from crossing of *B. napus* line, carrying *B. juncea* like resistance with the susceptible *B. napus* cv. Tower.	University of Melbourne, Australia	MB2	Greenhouse adult stage	Three genes from *B. juncea* with complex interaction control the resistance.	Pang and Halloran 1996

R= Resistant, S= Susceptible, BL= Blackleg

Table 1.3 Summary of research on blackleg resistance in different Brassica species (contd.)

Materials used	Institution	*L. maculans* pathotypes	Place and stage of BL test	Main results	References
Two different 'B-genome/*B. napus'* recombinant lines carrying blackleg resistance genes either of *B. juncea* or of *B. carinata*	Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK), Gatersleben, Germany	-	-	Suggested location of the resistance genes on the A-genome of the 'B-genome / *B. napus'* lines. This provides evidence that introgression of the B-genome resistance into the A-genome is possible.	Plieske and Struss. 2001
B. napus lines carry B-genome chromosome of *B. juncea*	Institut für Pflanzengenetik und Kulturp flanzenforschung (IPK), Gatersleben, Germany	IBCN3, T12aD34, R17G9 and GSA Rethmar	Growth chamber; cotyledon	Resistance controlled by single recessive gene, designated as *rlm2*	Saal et al. 2004
B. napus x *B. juncea* and *B. napus* x *B. carinata* crosses and backcrossing the hybrids to *B. napus*	Institut fiir Angewandte Genetik, Freie Universitat Berlin, Germany.	Not specified	Greenhouse; cotyledon	Resistance transferred in the progeny of *B. napus* x *B. juncea* cross; however, resistance lost in the *B. napus* x *B. carinata* cross progeny	Sacristan and Gerdemann 1986

R= Resistant, S= Susceptible, BL= Blackleg

1.4 Interspecific hybridization in *Brassica* and production of hybrids

Interspecific hybridization in *Brassica* has been done by several researchers to introgress different traits from the allied species into our cultivated crop species. In some interspecific crosses, hybridization barriers do not exist and viable hybrids can be obtained without the application of special techniques (Downey et al. 1980). However, in many cases hybridization barrier exists, and these are grouped as pre- and post-fertilization barriers (Rahman, 2004). An incompatible pollen–pistil interaction is the major pre-fertilization barrier (Inomata, 1993). Post-fertilization barriers are common in many interspecific hybridization where embryo growth and development become arrested due to lack of embryo development and/ or an imbalance between the developing embryo and the endosperm. As a result, the abortion of the hybrid embryo occurs in Brassica interspecific crosses (Rahman 2004; Singh et al. 1990). Inomata (1993) reported that ovary, ovule, and embryo culture techniques can be applied for the rescue of hybrid embryos and thus, the efficiency of the production of Brassica interspecific hybrids can be increased. Ayotte et al. (1987) performed interspecific hybridizations between *B. napus* and *B. oleracea*, and found that pollen tube growth and fertilization occur frequently in this interspecific cross, however, without any seed set. They were able to produce viable hybrids through the application of an *in vitro* ovule culture technique. Diederichsen and Sacristan (1994) made reciprocal crosses between *B. rapa* and *B. oleracea* to produce synthetic *B. napus*. They applied both ovule and embryo culture techniques to

rescue the hybrid embryos and reported that ovule culture was significantly more efficient than the embryo culture technique. Similarly, Takeshita et al. (1980) (cited by Bajaj et al. 1984) investigated the efficiency of embryo, ovule, and ovary culture techniques for the production of hybrids between *Brassica* and *Raphanus* and found that ovule culture is the most efficient method among the three. Bennett et al. (2008) applied ovule culture technique to produce the F_1 and BC_1 hybrids from a *B. napus* x *B. oleracea* cross and found the ovule culture technique was highly efficient compared to *in vivo* seed set. In the case of *in vitro* embryo rescue, the culture medium is very important for the growth of hybrid embryos under culture conditions. Kameya and Hinta (1970) (cited by Bennett et al. 2008) reported that ovule culture in liquid medium was more efficient than ovule culture on solid medium for the production of Brassica interspecific hybrids.

1.5 Research objective

On the basis of the literature reviewed above, it is apparent that many efforts have been made to develop blackleg resistant oilseed *B. napus* through the use of resistance found in *B. napus* and its allied species. In Canada, several PG2 resistant cultivars have been developed through the use of Australian *B. napus* resistance source (Stringam et al. 1999, 1995 and 2000). PG2 resistance has also been introgressed from *B. carinata* into *B. napus* (Rahman et al. 2007). Race specific resistance is often under monogenic control; and breakdown of single gene resistance has been reported in Europe and Australia (Sprague et al. 2006,

Salisbury et al. 1995). In Canada, new *L. maculans* pathotypes, namely PG3 (Fernando and Chen 2003; Kutcher et al. 2007), PG4 (Chen and Fernando 2005) and PGT (Keri et al. 2001) have evolved over the last 15 years; this require the introgression of resistance genes against these pathotypes into canola *B. napus*. A *B. carinata* accession in the canola program of the University of Alberta was found to carry resistance to these recently evolved blackleg pathotypes.

The primary objectives of this research were:

1. Apply an *in vitro* ovule culture technique for the production of F_1 hybrids from reciprocal crosses between *B. carinata* and *B. napus*, and BC_1 hybrids from F_1 x *B. napus* crosses, with the objective of transferring PG4 resistance from *B. carinata* into *B. napus*.
2. Investigate the potential of introgression of the PG4 resistance from *B. carinata* into the *B. napus* genome through interspecific hybridization between these two species, followed by recurrent backcrossing of the hybrids to the *B. napus* parent.
3. Study the inheritance of the PG4 resistance and other agronomic traits in different backcross generations.

The underlying hypotheses tested in this study were

In Chapter 2,

1. *In vitro* ovule culture technique is more efficient than *in vivo* seed set in producing F_1 and BC_1 hybrid.
2. *In vitro* ovule culture in Nitsch and Nitsch (1967) (NN) medium (liquid) is more efficient than *in vitro* ovule culture on B5 medium (solid).

In Chapter 3 and 4

1. The blackleg resistance gene from the B genome of *Brassica carinata* can be introgressed into the A or C genome of *B. napus* through homeologous recombination

1.6 References

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Chapter 2

Efficiency of the *in vitro* Ovule Culture Technique for the Production of *Brassica carinata* x *Brassica napus* Interspecific F_1 and BC_1 Hybrids

2.1 Introduction

Wild species of the family Brassicaceae are reservoirs of genes for resistance to biotic and abiotic stresses, which have been utilized by several researchers through interspecific hybridization for the improvement of our cultivated crop species. The major challenges for the introgression of traits from allied species into our cultivated crop species are: (i) the production of interspecific hybrids, and (ii) the stable introgression of allied genes into the crop genome. The success of interspecific crosses and introgression of traits largely depends on the genetic relatedness of the species (Yao et al. 2010). To date, several interspecific or intergeneric hybridizations have been conducted by different researchers and desirable traits have been introgressed into *B. napus* (e.g. blackleg resistance from *B. juncea* (Chèvre et al. 1997), Ogu-INRA restorer gene from radish (Primard- Brisset et al. 2005), yellow seed colour gene from *B. rapa* (Rahman 2001), self incompatibility genes from *B. rapa* and *B. oleracea* (Rahman 2004), etc). Production of interspecific hybrids has been greatly facilitated through the application of different *in vitro* cell and tissue culture techniques (e.g. ovary culture (Bajaj et al. 1986), ovule culture (Bennett et al. 2008) and embryo culture (Rahman 2004)). All of these techniques primarily focus on the rescuing of interspecific hybrid embryos before the embryos are aborted due to the lack of

endosperm development and/or imbalance between the developing embryo and endosperm (Rahman 2004). Bajaj et al. (1986) reported that the best stage for ovary culture is 8 to 9 days after pollination (DAP) when rescuing hybrid embryos from the reciprocal crosses of *B. napus* x *B. juncea.* Bennett et al. (2008) obtained the greatest number of hybrids from *B. oleracea* (♀) x *B. napus* (♂) crosses through application of ovule culture at 16 DAP. In the case of interspecific embryo culture, Rahman (2004) reported that the greatest number of hybrids from *B. oleracea* (♀) x *B. rapa* (♂) crosses could be obtained through the rescue of embryos at 20 to 24 DAP. Among these three techniques, ovule culture was found to be 3 to 6 times more efficient than embryo culture when producing hybrids from reciprocal crosses of *B. japonica* x *B. oleracea* and from *B. oleracea* x *B. japonica.* Ovary culture was the least efficient (Takeshita et al. 1980). Diederichsen and Sacristan (1994) applied both ovule and embryo culture techniques to reciprocal crosses of *B. rapa* and *B. oleracea* and found significantly greater efficiency of the ovule culture over the embryo culture technique. Momtaz et al. (2000) reported the high efficiency of the ovule culture technique while producing *Brassica* x *Sinapis* intergeneric hybrids.

The objective of this study was to investigate the efficiency of the *in vitro* ovule culture technique for the production of interspecific F_1 and BC_1 hybrids of *B. napus* x *B. carinata* crosses with the goal of transferring blackleg resistance from *B. carinata* into *B. napus*. Furthermore, the effectiveness of using NN liquid and B5 solid culture media in *in vitro* ovule culture was also investigated.

2.2 Materials and methods

2.2.1 Parent materials, crossing and production of F_1 hybrids

One *Brassica carinata* line (98-14513), which showed excellent resistance to multiple pathotypes of *L. maculans*, and two blackleg susceptible *B. napus* cultivars, Westar and Polo were used as parent materials in this study. Seeds of *B. carinata* and Westar were obtained from the germplasm collection of the Canola Breeding Program of the University of Alberta, and Polo was obtained from NPZ, Lembke, Germany. Three plants of each of Westar, Polo and *B. carinata* were grown in 12.5cm plastic pots filled with Metro Mix® 290 potting mixture (Grace Horticultural Products, Ajax, Ontario, Canada). The plants were kept in a growth chamber at 20°C/15°C (day/night) with a 16 hour photoperiod. Photosynthetic photon flux density in the cabinet was 450 μmoles m^{-2} s^{-1} at plant level. The plants were fertilized every second week with 200 ppm 20-20-20 (N-P-K) complete fertilizer with micronutrients (Plant Products, Brampton, Ontario). The cultivars Westar and Polo flower about 3 weeks earlier than *B. carinata*. Therefore, the *B. carinata* line was seeded 3 weeks before seeding the *B. napus* parents to synchronize flowering, and reciprocal crosses were made between these two species. For this, unopened flower buds, one day prior to anthesis, of the female plants were emasculated and pollinated with fresh pollen from newly opened flowers of the male plants. After pollination, buds were bagged immediately with cellophane bags to prevent cross-fertilization with pollen from other plants. Cellophane bags were removed three days after pollination, and the developing siliques were used either for ovule culture or left to mature for *in vivo* seed set.

Application of *in vitro* ovule culture

The Ovule culture technique for the rescue of hybrid embryos was employed according to Bennett et al. (2008). For this, siliques at 8 to 18 days after pollination (DAP) were excised, surface sterilized with 7% (w/v) calcium hypochlorite [Ca $(OCl)_2$] solution for 10 min in sterile 50 mL conical tubes, and rinsed twice with distilled water. The siliques were longitudinally dissected using a sterile surgical blade. The developing (fertilized) ovules were excised and counted. The fertilized ovules that looked healthy and had a non-shrunken appearance were either cut into two pieces or a small incision made on the non-micropylar end, and were cultured either on liquid or solid medium. All procedures were conducted under aseptic conditions in a laminar flow hood.

In case of ovule culture on NN medium which is referred as liquid medium, the rescued ovules were floated on 5 mL liquid culture medium in a Petri dish (60 mm x 15 mm). The liquid culture medium consisted of Nitsch and Nitsch (1967) medium (NN medium) (cited by Bennet et al. 2008) supplemented with 300 mg L^{-1} casein hydrolysate, 200 mg L^{-1} glutamine, and 13% sucrose. The Petri dishes were sealed and placed on a shaker set at 60 rpm for 2 to 3 weeks. Embryos at the torpedo stage were gently picked with forceps from the liquid culture medium and placed lightly on solid B5 medium containing 0.1 mg L^{-1} GA_3, 20 g L^{-1} sucrose and 8 g L^{-1} agar (Coventry et al. 1988) in Petri dishes. Transfer of embryos was done under sterile conditions in a laminar flow hood. The sealed Petri dishes were initially placed in a cold room maintained at 4°C and 16 h photoperiod with 30 μmol) m^{-2} s^{-1} photosynthetic flux density for 2 to 4 days and then moved to a

growth room maintained at 22 to 25°C and 16 h photoperiod with 70 μmol m^{-2} s^{-1}. The embryos remained in the growth room for 3 to 4 weeks until they had fully germinated and had developed roots. The seedlings were then transplanted to 12.5-cm pots containing soil free medium composed of peat moss, vermiculate, coarse sand, dolomite, superphosphate, asbestos and trace elements, and placed in a growth chamber set at 20°C/15°C day/night temperature and 16 h photoperiod until maturity. To protect the seedlings from damage during watering as well as from desiccation, the transplanted seedlings were covered with transparent plastic cups for 1 to 2 weeks.

When carrying out ovule culture on B5 solid medium, the excised ovules were directly placed on B_5 solid medium containing 0.1 mg L^{-1} GA_3, 20 g L^{-1} sucrose and 8 g L^{-1} agar (Coventry et al. 1988). The sealed Petri dishes were placed in a growth room maintained at 22 to 25°C (16 h photoperiod) as mentioned above. The ovules were kept under this condition for 3 to 4 weeks until they were fully germinated and had developed roots. The seedlings were then transplanted to 12.5cm pots containing soil-free growth medium and placed in a growth chamber set at 20°/15°C day/night and 16 h photoperiod.

2.2.2 Confirmation of the F_1 hybrid with molecular markers

DNA extraction: Young leaves from the parents and F_1 plants were collected in 2mL Eppendorf tubes and stored at -80^{0}C until use. Genomic DNA of the samples was extracted using the GenElute™ Plant Genomic DNA Miniprep Kit (Sigma-Aldrich Co, St. Louis, MO, USA), following the instructions of the manufacturer.

Molecular marker analysis: Genomic DNA of the parental and F_1 plants was amplified by PCR using B genome specific simple sequence repeat (SSR, microsatellites) molecular markers. PCR was performed in a total volume of 25 mL containing 3 to 10 ng of genomic DNA, 1 x *Taq* buffer, 2.5 mmol/L $MgCl_2$, 200 mmol/L each dNTP, 0.2 mmol/L of each forward and reverse primer and 1.25 U of *Taq* polymerase (Promega Corp.). The reactions were carried out in a GeneAmp® PCR system 9700 DNA Engine Thermal Cycler (Applied Bio System, Life technologies Inc., Carlsbad, California, USA); and amplifications consisted of an initial denaturation for 5 min at 95 ^{0}C, 35 cycles of 1 min at 95 ^{0}C, 1 min at 56 ^{0}C, and 1 min 30 s at 72 ^{0}C, and a final extension of 30 min at 72 ^{0}C.

After completion of the PCR amplification, 2 µL of 10X DNA loading buffer was added, and the samples were run on 2% agarose gel at 90 V for 2 to 3 h. The gels were stained with ethidium bromide for 30 min followed by washing in water for 15 min. The amplified fragments were visualized and photographed under ultraviolet light using a Floro Chem ^{TM} SP system (Alpha InfoTech, Cell Biosciences Inc., Santa Clara, California, USA).

2.2.3 Production of first backcross hybrids

In vitro ovule culture as well as *in vivo* seed set technique, as described in section 2.2.1, was applied for the production of BC_1 hybrids. For this, F_1 plants of *B. carinata* x *B. napus* cv. Westar and *B. napus* cv. Polo x *B. carinata*, obtained from seeds produced in *in vivo*, were grown in 12.5cm plastic pots filled with potting mixture. The plants were kept in a growth chamber set at 20°C/15°C (day/night) and 16 h photoperiod. Photosynthetic flux density in the cabinet was

450 μmol m^{-2} s^{-1} at the plant level. The plants were fertilized every second week with 200 ppm 20-20-20 (N-P-K) complete fertilizer with micronutrients (Plant Products, Brampton, Ontario). All plants were backcrossed to *B. napus* cv. Westar using the F_1 plants as female; the fertilized siliques were subjected to *in vitro* ovule culture or left to maturity for the production of BC_1 hybrids/seeds.

2.3 Results

2.3.1 *In vivo* and *in vitro* F_1 hybrid production

Reciprocal crosses were made between the parental species *B. carinata* and *B. napus* for the production of F_1 hybrids. Using *B. carinata* as the female and *B. napus* cv. Westar as the male, 62% silique set was obtained from 278 crosses (Table 2.1). All developing siliques were used in the *in vitro* ovule culture, and a total of 72 embryos were obtained, which corresponded to 0.259 embryos/ pollination. About 83% of the embryos yielded hybrid plants. Thus, the number of F_1 hybrids obtained through the application of the ovule culture technique was 0.216 per pollination (Table 2.1), while it was only 0.003 per pollination from *in vivo* seed set (Table 2.2). Thus, the application of the *in vitro* ovule culture technique was highly effective compared to *in vivo* seed set for the production of F_1 plants from this interspecific cross. In the case of the reciprocal cross, where *B. napus* was used as female and *B. carinata* as male, 0.413 F_1 hybrid seeds per pollination were obtained without the application of the ovule culture technique.

Table 2.1 Production of F_1 plants of *Brassica carinata* (♀) x *Brassica napus* cv. Westar (♂) through the application of the *in vitro* ovule culture technique

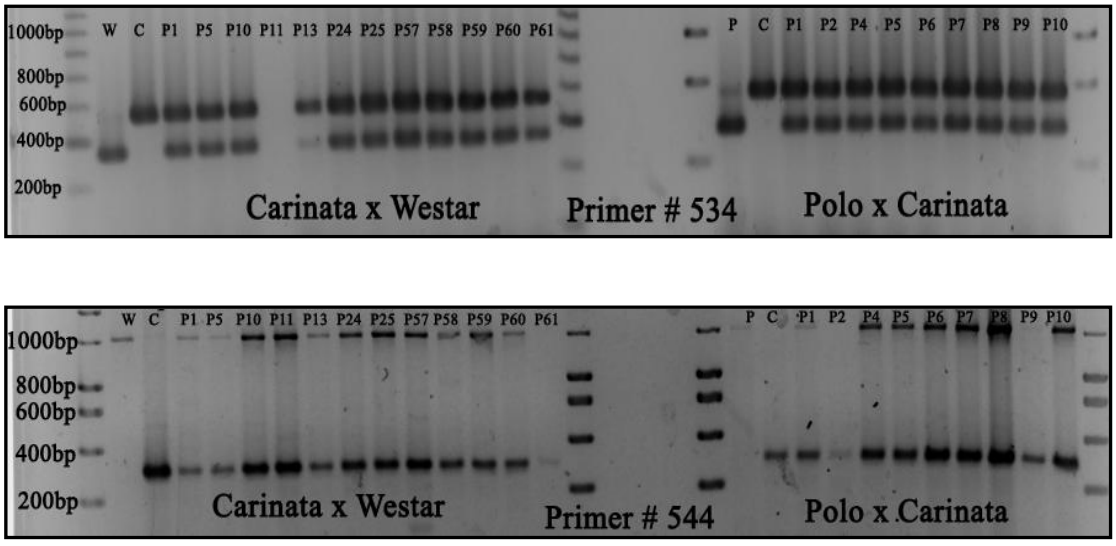
No. poll.	No. silique set	% silique set	No. ovule obtained	No. ovule/ poll.	No. ovule/ silique	No. embryo obtained	No. embryo rescued/poll.	No. F_1 plants obtained	No. F_1 hybrid/ poll.	% embryo yielded hybrid plants
278	171	62	818	2.942	4.784	72	0.259	60	0.216	83

Table 2.2 Production of *in vivo* F_1 seeds of *B. carinata* x *B. napus* cv. Westar and *B. napus* cv. Polo x *B. carinata* interspecific crosses

Cross (♀ x ♂)	No. pollination	No. F_1 seed obtained	No. F_1 seeds/ pollination
B. carinata x *B. napus* cv. Westar	307	1	0.003
B. napus cv. Polo x *B. carinata*	160	66	0.413

2.3.2 Confirmation of F_1 hybrid

A total of 23 F_1 plants, obtained from *in vivo* seed and *in vitro* ovule culture from reciprocal *B. napus* x *B. carinata* crosses were grown. Of these, 21 plants, comprising 12 of *B. carinata* x Westar cross and 9 of Polo x *B. carinata* cross, were evaluated for hybridity by the use of 14 B-genome specific SSR markers (Appendix A). Among these, the markers sJ0338-F and sB1728-F produced clearly different size fragment from the two parental species and were used for confirmation of the hybrid plants (Fig. 2.1). All interspecific cross derived plants produced the DNA fragments of the two parental species, as could be expected for a co-dominant marker, and thereby confirmed to be hybrid.



C= *B. carinata*; W= Westar; P= Polo

Fig. 2.1 Confirmation of the F_1 hybrids of *B. napus* x *B. carinata* by the use of SSR markers sJ0338-F (# 534) and sB1728-F (# 544).

2.3.3 Production of BC_1 hybrids through ovule culture

Twelve F_1 plants of *B. carinata* x Westar and 11 plants of Polo x *B. carinata* were backcrossed to the recurrent parent Westar, and *in vitro* ovule culture technique was applied for the production of BC_1 hybrids. Two culture media were used for this purpose: liquid and solid media. A total of 477 ovules from 2157 pollinations of the two backcrosses were cultured in NN liquid medium, which yielded 13 embryos, i.e. 0.006 embryos/ pollination. In the case of ovule culture on B5 solid medium, 0.001 embryos per pollination were obtained (Table 2.3). The effect of the type of medium on *in vitro* ovule culture is also evident in individual crosses. In the case of *B. carinata* x Westar and Polo x *B. carinata* crosses, 0.006 and 0.002 BC_1 hybrids per pollination were obtained from the use of NN liquid medium, respectively. However, ovule culture on B5 solid medium yielded 0.001 and 0.000 BC_1 hybrids per pollination from *B. carinata* x Westar and Polo x *B. carinata* crosses, respectively. Thus, the *in vitro* ovule culture on NN liquid medium was found to be more efficient than B5 solid medium in the present study.

Table 2.3 Efficiency of *in vitro* ovule culture technique in the production of first backcross (BC_1) hybrids of *Brassica* interspecific crosses

Parentage (♀ x ♂)	Culture media	No. poll.	% silique set	No. ovule plated	No. embryo obtained	No. BC_1 plants obtained	No. embryo / poll.	No. BC_1 plants/poll.
(B.c x W) x W	Liquid	1245	16.71	315	10	8	0.008	0.006
(B.c x W) x W	Solid	1157	16.68	312	3	1	0.003	0.001
(P x B.c) x W	Liquid	912	14.58	162	3	2	0.003	0.002
(P x B.c) x W	Solid	919	13.71	156	0	0	0.000	0.000
Total	Liquid	2157	15.81	477	13	10	0.006	0.005
	Solid	2076	15.37	468	3	1	0.001	0.000

B.c = *B. carinata* ; P= Polo; W= Westar

2.4 Discussion

According to Downey et al. (1980), hybrid production in a *B. napus* (♀) x *B. carinata* (♂) cross is more effective than in a *B. carinata* (♀) x *B. napus* (♂) cross. They reported that when using *B. napus* as female and *B. carinata* as male, 0.002 hybrids per pollination were obtained; while the reciprocal cross, i.e. *B. carinata* as female and *B. napus* as male usually fail to produce hybrid plants. Although a good number of hybrids from the *B. napus* (♀) x *B. carinata* (♂) cross can be obtained without the application of cell and tissue culture techniques; production of hybrids from the *B. carinata* (♀) x *B. napus* (♂) cross, however, might be desired for the development of an alloplasmic *B. napus* line carrying the cytoplasm of *B. carinata*. The effect of alien cytoplasm on male sterility (Banga et al. 2003; Deol et al. 2003; Prakash et al. 2001), seed dormancy, seed weight and oil content (Chang et al. 2009), and triazine herbicide tolerance (Beversdorf et al. 1980) has been reported earlier. Therefore, the *in vitro* ovule culture technique was applied in the present study to produce F_1 hybrids from the *B. carinata* (♀) x *B. napus* (♂) cross. This technique was found to be 72 times more efficient compared to F_1 hybrid production from *in vivo* seed set in this study. This agrees with earlier researchers that the application of the embryo rescue technique facilitates the production of *Brassica* interspecific hybrids. Bennett et al. (2008) reported that application of the ovule culture technique was 10 times more effective compared to *in vivo* seed set for the production of F_1 hybrids from the *B. napus* x *B. oleracea* interspecific crosses. Chen et al. (1988) also found the *in*

vitro embryo rescue technique more efficient than *in vivo* seed set while producing hybrids from the cross between *B. alboglabra* and *B. rapa.*

When applying embryo rescue techniques, especially in the case of *in vitro* ovule culture, the efficiency of the technique largely depends on the type of culture medium being used. In the present study, liquid and solid media were evaluated for the production of BC_1 hybrid**.** The use of liquid medium yielded significantly greater number of BC_1 hybrids compared to the use of solid medium. This is in accordance with Kameya and Hinta (1970) who reported ovule culture in liquid medium is more efficient than on solid medium for the production of hybrid embryos from *B. chinensis* x *B. pekinensis* cross.

Thus, the present study demonstrates that application of the *in vitro* ovule culture technique facilitates the production of interspecific hybrids of *B. carinata* x *B. napus* crosses; and the use of NN liquid culture medium further increases effectiveness.

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Chapter 3

Evaluation of F_1 and Backcross Populations of the *B. napus* x *B. carinata* Interspecific Cross for Resistance to *Leptosphaeria maculans*

3.1 Introduction

Blackleg disease, caused by the fungus *Leptosphaeria maculans*, is one of the most important diseases affecting the Brassica oilseed crops worldwide. This pathogen has been reported to be endemic to some canola growing regions of Canada, Australia, United Kingdom, France, Germany and many other countries where it causes significant yield loss (Gugel and Petrie 1992). This fungus was first reported in Canada in 1975 (McGee and Petrie 1978).

To date, different sources of blackleg resistance have been identified to be effective against Canadian blackleg pathotypes. Among these sources the PG2 resistance of the Australian *B. napus* cvs. Maluka and Shiralee have been used extensively in the Canadian canola/rapeseed breeding programs for the development of resistant cultivars (Stringam et al. 1995; Stringam et al. 1999; Stringam et al. 2000). The breakdown of PG2 resistance and the evolution of new blackleg pathotypes in the Canadian prairies, e.g. PG3 (Fernando and Chen 2003; Kutcher et al. 2007), PG4 (Chen and Fernando 2005) and PGT (Keri et al. 2001) is of increasing concern to growers and breeders. Therefore, introgression of resistance to these newly evolving pathotypes into Canadian canola/ rapeseed germplam is urgently needed. As reviewed in Chapter 1, the Brassica species carrying the B genome, viz. *B. carinata*, *B. nigra* and *B. juncea*, often show a high

level resistance against different blackleg pathotypes. Several efforts have been made to transfer resistance from these species, especially from *B. juncea*, and *B. napus* lines with stable introgression of resistance against the European blackleg pathotypes has been achieved (Chèvre et al.1997). The amphidiploid species *B. carinata*, carrying the B genome, often shows resistance against multiple diseases, e.g. blackleg (Christianson et al. 2006) and sclerotinia stem rot (Yang et al. 2010;Navabi et al. 2010). Despite this, as reviewed in Chapter 1, very limited efforts have been made to introgress resistance from this species into *B. napus*. In the Canola Breeding Program at the University of Alberta, a *B. carinata* accession showing resistance to multiple diseases, including resistance to different blackleg pathotypes, has been identified.

The objective of this research was to introgress PG4 type blackleg resistance from *B. carinata* into the *B. napus* background, and to investigate the prospect of developing a euploid *B. napus* line carrying cotyledon and adult plant resistance. As a part of this project, different PG4 type *L. maculans* isolates were evaluated to identify the most virulent isolate for challenging the interspecific cross derived populations.

3.2 Materials and methods

3.2.1 Identification of *L. maculans* isolate for screening the *B. napus* x *B. carinata* cross derived populations

Isolates and genotypes: Five *Leptosphaeria maculans* isolates were evaluated for virulence. The isolates PG4- 166 and PGT- 165 were in the collection of the Canola Breeding Program of the University of Alberta, and the isolates 290CDN,

BL03-02RK and BL05-08RK were kindly supplied by Dr. Randy Kutcher, Agriculture and Agri-Food Canada, Melfort, SK. Five *Brassica* genotypes, *Brassica napus* cvs. Westar, Polo, Glacier and Quinta, and *B. carinata* accession #98-14513, were challenged with these five isolates. The most pathogenic isolate amongst the five was selected and used for screening the interspecific populations of *B. napus* x *B. carinata* crosses.

Preparation of *L. maculans* isolates: The five *L. maculans* isolates were cultured on V8-agar medium in separate 9 cm diameter Petri plates under light at 20°C. The medium was composed of V8 juice (200 ml/ L), rose bengal (0.05 g/L), agar (20 g/L), calcium carbonate (3.0 g/L) and distilled water (400 ml/L). After 15-20 days, sporulating cultures were flooded with 5 mL sterile distilled water and the surface of the plates was scraped gently with a flamed glass rod to dislodge the pycnidiospores. The pycnidiospore suspension was filtered through sterile 'cheese cloth (Fisher Scientific Canada, Edmonton, Canada) into sterile 5 mL tubes. The concentration of the spore suspension was determined with a haemocytometer, and adjusted to $1x10^7$ pycnidiospores per ml with sterile H_2O.

Seeding and experimental design: The five *Brassica* genotypes were seeded in plastic trays filled with Metro Mix® 290 potting mixture (Grace Horticultural Products, Ajax, Ontario, Canada). A total of five 48-cell trays were seeded. The size of each cell was 6.35cm x 3.81cm x 5.72cm (length x width x height). Each tray contained all five genotypes of eight seedlings of each genotype. Each cell contained one seedling. The seedlings in a tray were inoculated with one isolate, and thus the five trays were inoculated with five isolates. The trays of seedlings

were kept in a growth chamber at 20°C/15°C (day/night) with 16 h photoperiod. Photosynthetic flux density in the cabinet was 450µmol m^{-2} s^{-2} at plant level. The experiment was repeated. For statistical analysis, the two seeding were considered as temporal blocks and data analysis was done following randomized complete block design (RCBD).

Inoculation of seedlings: Inoculation was done following Bansal et al. (1994) with slight modifications. Seedling cotyledons at the age of seven days after seeding were inoculated with five blackleg isolates. For this, lobes of the two cotyledons were wounded in the centre with a No. 1 entomological needle. Inoculations were made by dispensing 10 µL blackleg spore suspension on each wound. The trays of inoculated seedlings were kept in the growth chamber and the seedlings were covered with dark plastic tops for 24 h to maintain high humidity.

a **b** **c** **d**

Fig 3.1 Inoculation of *Brassica* seedlings with *Leptosphaeria maculans* isolates**.** (a) Seedlings ready for inoculation, (b) wound made on cotyledon, (C) dispensing spore suspension in the wound, (d) and suspension of spores on each lobe

Disease score and statistical analysis: Disease symptoms, based on lesion size, tissue collapse, and necrosis or chlorosis was scored 10 days after inoculation on a 0 to 9 scale (Delwiche 1980). The description of the rating scale is presented in the Table 3.1. Score 0 was assigned when there were no visible disease symptoms, similar to the resistant parent *B. carinata*; and score 9 was assigned to

the seedlings where lesion size was >5 mm with profuse sporolation (Table 3.1). Data were analyzed by using SAS software version 9.2 (SAS Institute Inc., 2008) and using the command Proc GLM. The statistical model used to estimate the disease severity was as follows:

$Y_{ijk} = \mu + T_i + I_j + G_k + IG_{jk} + e_{ijk}$

Y_{ijk} = Disease severity

μ = Over all mean

T_i = Trial effect, where i = 1, 2

I_j = Isolate, where j = 1,…,5

G_k = Genotype, where k = 1,. ..,5

IG_{jk} = Isolate*Genotype

e_{ijk} = error

Means of disease score were ranked by applying the t-test ($LSD_{0.5}$).

1 **3** **5** **7** **9**

Figure 3.2 Disease symptom evaluation scale (0 to 9) of Brassica cotyledons inoculated with *Leptosphaeria maculans*. For detailed description, see Table 3.1

3.2.2 Recurrent backcrossing of *B. napus* x *B. carinata* hybrids to the *B. napus* cv. Westar.

The F_1 and BC_1 hybrids obtained through *in vitro* ovule culture and *in vivo* seed, as described in Chapter 2, were used in this recurrent backcross program. Recurrent backcrossing of the interspecific *B. napus* x *B. carinata* hybrid to the *B.*

napus cv. Westar was done with selection of blackleg resistant plants in each backcross generation for introgression of resistance from *B. carinata* into the blackleg susceptible cv. Westar (Fig. 3.3). For this, the seedlings/plants of different genotypes obtained from *in vivo* seed were evaluated for blackleg resistance, and the progeny of the resistant plants were taken into the subsequent generations.

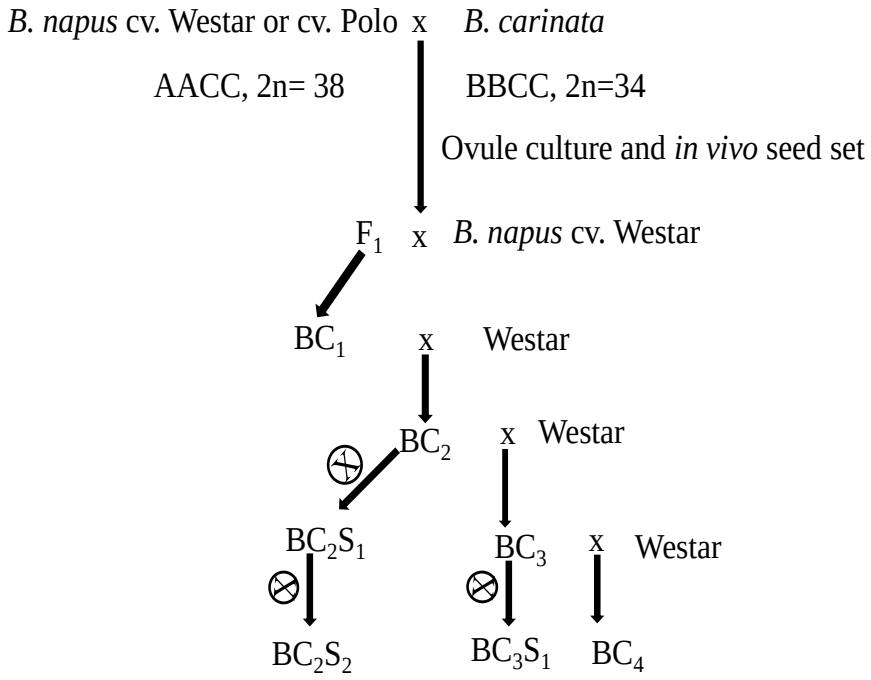


Fig 3.3 The outline of interspecific recurrent backcrossing scheme of (*B. napus* x *B. carinata*) x *B. napus* for introgression of blackleg resistance from *B. carinata* into *B. napus*

3.2.3 Evaluation of F_1 and backcross generation populations

Isolate preparation: The *L. maculans* isolate 290CDN (PG4 type) was used in this research for evaluation of the progenies derived from *B. napus* x *B. carinata* interspecific cross. The detail of the preparation of the isolate for inoculation is given in Section 3.2.1.

Growing of seedlings: Seedlings were grown in trays as described in the Section 3.2.1. Westar was also seeded in each tray for comparison with the interspecific populations.

Inoculation: Cotyledons of the F_1 and backcross generations (BC_1, BC_2, BC_2S_1, BC_3), grown from *in vivo* seed, were inoculated with the *L. maculans* isolate 290CDN at 7 days after seeding, as described in Section 3.2.1.

Disease scoring:

Cotyledon resistance

Disease severity of the F_1 and BC_1 seedlings was scored at 10 days after inoculation (DAI) on a 0-9 scale by Delwiche (1980) (Table 3.1). Disease severity in BC_2, BC_2S_1 and BC_3 generations was recorded at 10 DAI and 13 DAI. Seedlings which did not show any disease symptoms by 13 DAI were scored as resistant.

Adult plant resistance

The plants resistant to blackleg in the cotyledon test, as well as the seedlings derived from *in vitro* ovule culture, were grown to maturity and evaluated for blackleg severity. For this, the plants were uprooted and cut at the base of the stem where the maximum disease symptoms were visible. Disease severity was

scored on a 0-5 scale where 0 indicates no disease and 5 indicate 100% infection of tissue (Table 3.2).

Table 3.1 Blackleg disease severity rating scale for evaluation of resistance at cotyledon stage as described by Delwiche (1980)

Disease Scale	Disease Description
0	No darkening of tissue around the wound. Typical response of non-inoculated cotyledon
1	Limited blackening around wound; lesion diameter 0.5-1.5 mm. Faint chlorotic halo may be present. Sporulation absent.
3	Dark necrotic lesion, 1.5-3.0 mm diameter. Chlorotic halo may be present. Sporulation absent.
5	Non-sporulating, 3.0-6.0 mm lesion, sharply delimited by darkened necrotic tissue. May show grayish-green tissue collapse characteristic of susceptible reactions (7 and 9), or dark necrosis throughout the leaves.
7	Greyish-green tissue collapse. Lesion 3.0-5.0 mm, with sharply delimited, non-darkened margins.
9	Rapid tissue collapse at about 10 days accompanied by profuse sporulation in large lesions (>5 mm) with diffuse, non-darkened margins.

Table 3.2 Blackleg disease severity rating scale for evaluation of resistance at adult plant stage as described byWestern Canada Canola/Rapeseed Recommending Committee (WCCRR)

Diseases score	Disease description
0	No diseased tissue visible in the cross section
1	Diseased tissue occupies 25% or less of the cross section
2	Diseased tissue occupies 26-50% of the cross section
3	Diseased tissue occupies 51-75% of the cross section
4	Diseased tissue occupies >75% of the cross section with little or no constriction of affected tissue
5	Disease tissue occupies 100% of the cross section with significant constriction of affected tissue: tissue dry and brittle, plant dead

Statistical analysis: Data analysis was done using SAS software version 9.2 (SAS Institute Inc., 2008). Comparisons between the interspecific populations and blackleg susceptible cv. Westar was made by t-test at 5% level of significance. In the case of BC_1 generation, Chi-square test for segregation for blackleg resistance was done following Strickberger (1976).

3.3 Results

3.3.1 Evaluation of blackleg isolates for virulence

The disease severity of the five genotypes (Westar, Polo, Glacier, Quinta, and *B. carinata*) tested against the five isolates (PG4-166, 290CDN, PGT-165, BL03-02RK and BL05-08RK) is presented in Table 3.3. The cultivar Westar was

found to be most susceptible, while *B. carinata* was resistant to all isolates and did not show any visible disease symptoms. Among the five isolates, the isolate 290CDN caused the highest infection (disease score 5.932) on the five Brassica genotypes. The aggressiveness of this isolate was significantly higher than the isolates BL03-02RK, BL05-08RK and PGT-165, but not different from the isolate PG4-166. The isolate BL5-08RK was found to be the least virulent (disease score 5.374) among the five tested isolates. Thus, based on mean disease score, the isolate 290CDN was selected for challenging the *B. napus* x *B. carinata* interspecific cross derived populations in this research.

Table 3.3 Blackleg disease severity (DS) caused by *Leptosphaeria maculans* isolates in *B. carinata* and four different *B. napus* cultivars Westar, Quinta, Glacier and Polo

Isolates	Brassica genotypes					$LSD_{(0.05)}$	Mean (DS)
	Westar	Polo	Glacier	Quinta	*B. carinata*	(Isolate)	
290 CDN	7.52	7.41	7.39	7.35	0	0.51	5.93a
PG4-166	7.91	7.50	7.11	6.85	0	0.35	5.87a
BL03- 02RK	7.16	7.01	7.06	7.13	0	0.22	5.67b
PGT-165	7.61	7.35	4.72	7.44	0	0.43	5.42c
BL05-08RK	7.69	7.41	4.77	7.01	0	0.36	5.37c
$LSD_{(0.05)}$ (Genotype)	0.24	0.45	0.36	0.60	0		
Mean (DS)	7.58a	7.33b	6.21d	7.15c	0		

In the last row and columns, the mean values followed by the same letter in row and column are not significantly different ($P \leq 0.05$) based on t-tests ($LSD_{0.05}$)

3.3.2 Production of different generation backcross seeds

The number of seeds set per pollination in different backcross generations is presented in Table 3.4. In the first backcross (BC_1), the F_1 plants of *B. carinata* (♀) x *B. napus* (♂) yielded 0.014 BC_1 plants/pollination as compared to 0.006 BC_1 plants/pollination from the *B. napus* (♀) x *B. carinata* (♂) cross (Table 3.4). A total of 25 BC_1 plants of the *B. carinata* (♀) x *B. napus* (♂) cross and 9 BC_1 plants of *B. napus* (♀) x *B. carinata* (♂) cross were used as female for producing BC_2 seeds. The BC_1 hybrids of the *B. carinata* (♀) x *B. napus* (♂) cross produced 0.963 BC_2 seeds/ pollination, while the hybrids of the *B. napus* (♀) x *B. carinata* (♂) interspecific cross produced 0.338 seeds/ pollination. Thus, a greater number of backcross hybrids were obtained from the interspecific plants which had *B. carinata* cytoplasm compared to the plants carrying the *B. napus* cytoplasm.

The difference in seed set/pollination due to cytoplasm of the female plants was less pronounced in BC_3 and BC_4 generations. For the BC_3 and BC_4 generations, the number of seeds/pollination was about five to six hundred-fold greater than the number of seeds/pollination in the BC_1 generation. It was clearly evident that the number of backcross seeds per pollination increased with the progression of recurrent backcrossing. However, the number seeds/pollination in the BC_4 generation (5.71 to 6.57) was still far less than the number seeds that could be expected for the intraspecific cross in *B. napus* (about 20 seeds/ pollination).

During the course of recurrent backcrossing, the backcross seeds were harvested only from the plants showing resistance to blackleg disease at the adult stage, and seeds of these plants constituted the next generation population.

Table 3.4 Number of seeds set per pollination in different backcross generations of *B. napus* x *B. carinata* interspecific crosses

Parentage of the F_1 (♀ x ♂)	Female plant generation	Harvested seed generation[1]	No. plants	No. poll.	No. fert. Silique	No. seed obtained	No. seeds/ poll.
B.c x W	F_1	BC_1	12	4841	648	67	0.014
P x B.c			11	1636	236	10	0.006
B.c x W	BC_1	BC_2	25	1003	545	966	0.963
P x B.c			9	432	125	146	0.338
B.c x W	BC_2	BC_3	15	208	152	1029	4.947
P x B.c			20	385	254	1699	4.413
B.c x W	BC_3	BC_4	10	135	109	887	6.570
P x B.c			6	78	67	445	5.705

[1]In each backcross generation, the *B. napus* cv. Westar was used as recurrent parent and as male in the cross.
B.c = *B. carinata*; W = *B. napus* cv. Westar;
P = *B. napus* cv. Polo

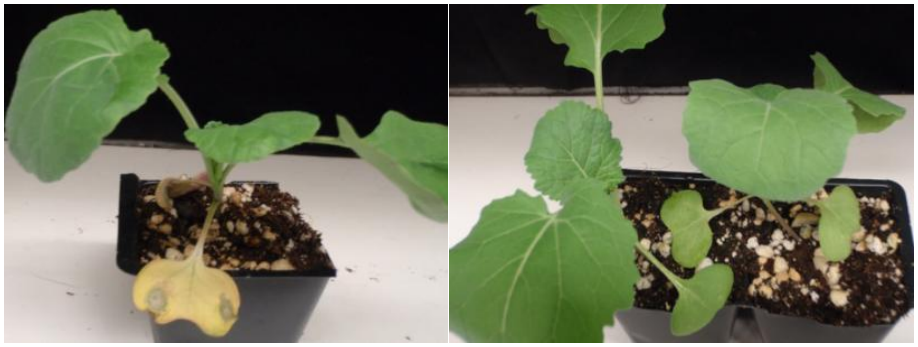
3.3.3 Evaluation of the F_1 of *B. napus* x *B. carinata* interspecific hybrids for blackleg resistance

A total of 29 seedlings, obtained from *in vivo* seed of the *B. napus* x *B. carinata* reciprocal crosses, were evaluated for cotyledon resistance against the *L. maculans* isolate 290CDN (Table 3.5). The parental genotypes: *B. carinata*, *B. napus* cvs. Westar and Polo were also inoculated for comparison. All F_1 seedlings showed resistance to this isolate, while the cultivars Westar and Polo were susceptible. All *B. carinata* seedlings were resistant, as expected.

3.3.4 Evaluation of BC_1 population for blackleg resistance

A total of 40 seedlings from seven BC_1 families of (*B. carinata* x *B. napus*) x *B. napus* were evaluated for cotyledon resistance, where 27 seedlings were found to be resistant and 13 susceptible (Table 3.6 and Appendix B). Chi-square test for goodness of fit was done, and all families followed a 1:1 segregation for resistant (R) and susceptible (S) phenotypes (Table 3.6). Chi-square test for heterogeneity indicated that the BC_1 families were homogeneous for this segregation (χ^2 = 2.36, P= 0.70-0.90), and thus, pooling data of these families could be justified. However, segregation in pooled data for resistant and susceptible phenotype deviated significantly from 1:1 ratio (27R:13S), where about two thirds of the total number of plants were deemed to be resistant (disease score zero).

Based on the disease resistance score, 27 BC_1 plants, showing no visible blackleg symptom (disease score zero) at the cotyledon stage were selected for further backcrossing to Westar. At adult stage, out of 27 plants, 20 plants did not show any disease symptom, and the BC_2 seeds harvested from these plants were used.



(a) (b)

Fig 3.4 (a) Blackleg susceptible seedling showing lesions and (b) the resistant seedling showing no lesion on the cotyledonary leaves

3.3.5 Evaluation of BC_2 population for blackleg resistance

Two hundred and forty nine seedlings derived from 20 BC_2 families of *B. carinata* (♀) x *B. napus* (♂) (Table 3.7) and 87 seedlings from 7 BC2 families of *B. napus* (♀) x *B. carinata* (♂) crosses (Table 3.8) were inoculated, and disease scoring was done at 10 and 13 DAI. In general, disease severity increased at 13 DAI compared to 10 DAI, and this difference was more pronounced in the susceptible check Westar compared to the BC_2 families.

For the 20 BC_2 families of the *B. carinata* (♀) x *B. napus* (♂) cross, 14 families showed significantly higher resistance to blackleg than Westar at 10 DAI; while at 13 DAI 17 families showed significantly higher resistance than Westar. This difference is primarily due to significantly greater disease severity in Westar at 13 DAI against which the BC_2 families were compared. When the disease score at 10 and 13 DAI were compared, only 3 of the 20 BC_2 families showed significantly greater severity at 13 than at 10 DAI; but in case of Westar, grown

with the BC_2 families, this difference was statistically significant in 18 of the 20 cases (Table 3.7). Of the 249 seedlings from 20 BC_2 families evaluated for cotyledon resistance, 68 seedlings from 17 families were found to be resistant. These seedlings did not show any visible disease symptom. The 68 resistant seedlings were grown to maturity, where 15 plants from eight families were found to be resistant at adult stage (Table 3.7 and Appendix C). Backcross (BC_3) and self pollinated (BC_2S_1) seeds harvested from these plants were used for further study.

For the 7 BC_2 families derived from the *B. napus* (♀) x *B. carinata* (♂) cross, 87 seedlings were inoculated, of which 24 seedlings from 6 families showed resistance at the cotyledon stage; and all seedlings from family 4.1 were found to be susceptible (Table 3.8). The 24 cotyledon resistant seedlings were grown to maturity, of which 20 plants showed resistance at adult plant stage (Table 3.8 and Appendix C). The BC_3 and BC_2S_1 seeds harvested from these plants constituted the next generation population.

Table 3.5 Cotyledon resistance of the parental and F_1 seedlings of *Brassica napus* x *B. carinata* interspecific crosses against the *Leptosphaeria maculans* isolate 290CDN

Materials	No. seedling	Disease score		No. seedlings	
		Range	Mean ± SE	Susceptible	Resistant
F_1 (P x B.c)	28	0.00-0.25	0.03 ± 0.01	0	28
F_1 (B.c x W)	1	0.00-0.00	0.00 ± 0.00	0	1
Polo	10	5.00-8.00	6.10 ± 0.29	10	0
Westar	10	5.00-7.50	5.95 ± 0.24	10	0
B. carinata	10	0.00-0.00	0.00 ± 0.00	0	10

B.c = *B. carinata*; W = *B. napus* cv. Westar; P = *B. napus* cv. Polo

Table 3.6 Cotyledon resistance against the *Leptosphaeria maculans* isolate 290CDN in the BC_1 generation of (*B. carinata* x *B. napus* cv. Westar) x Westar, generated from *in vivo* seed

BC_1 family	No. Seedling	Disease score at 10 DAI		No. seedlings		χ^2 test for 1:1	
		Range	Mean± SE	R	S	χ^2	P
Fam. 1	5	0.0- 6.0	1.20 ±1.09	4	1	1.80	0.10-0.20
Westar	8	2.3-4.0	2.88 ± 0.21*				
Fam. 2	6	0.0-7.0	2.17 ± 1.37	4	2	0.66	0.30 - .50
Westar	8	2.3-5.5	3.78 ± 0.42*				
Fam. 3	10	0.0-6.5	1.15 ± 0.77	8	2	3.60	0.05-0.10
Westar	8	2.5-5.5	4.03 ± 0.39*				
Fam.4	8	0.0-7.5	3.31 ± 1.26	4	4	0 .00	1.00
Westar	8	3.8-6.0	5.28 ± 0.25*				
Fam.5	4	0.0-4.5	1.13 ±1.13	3	1	1.00	0.30-0.50
Westar	8	3.8-6.0	4.91 ± 0.24*				
Fam.6	2	0.0-5.0	2.50 ± 2.50	1	1	0.00	1.00
Westar	8	3.5-5.5	4.63 ± 0.26*				
Fam.7	5	0.0-5.0	1.80 ± 1.11	3	2	0.20	0.30-0.50
Westar	8	5.0- 6.0	5.25 ± 0.13*				
Total BC_1 =	40			27	13	4.90	0.01-0.05
Heterogeneity[a]						2.36	0.70-0.90

[a]Pooled heterogeneity df= 6

Asterisk after Mean± SE indicates that disease symptom of Westar was significantly different from the BC_1 family with which Westar was grown (t-test, $P \leq 0.05$).

R= Resistant; S= Susceptible.

Table 3.7 Cotyledon resistance against the *Leptosphaeria maculans* isolate 290CDN at 10 and 13 days after inoculation (DAI) in BC_2 seedlings of *Brassica carinata* (♀) x *B. napus* (♂) interspecific cross

BC_2 Fam.	No. seedling	Disease score at 10 DAI		Disease score at 13 DAI		t-test	No. resistant plants	
		Range	Mean± SE	Range	Mean± SE	10 DAI vs.13 DAI	Cot.	Adult
Fam.1.1	8	0.0-5.0	1.84±0.67	0.0-5.0	2.19± 0.72	NS	3	0
Westar	8	2.3-4.0	2.88± 0.21*	3.0-6.0	4.41± 0.32*	*		
Fam.1.2	16	0.0-5.0	2.55±0.40	0.0-7.0	3.58± 0.59	NS	3	0
Westar	8	5.0-9.0	6.06±0.55*	6.5-9.0	7.50±0.31*	*		
Fam.1.3	6	0.0-5.0	2.79± 0.80	0.0-5.0	3.88± 0.78	NS	1	0
Westar	8	1.3-4.0	2.56±0.33	3.0-6.0	4.41± 0.32*	*		
Fam. 2.1	8	0.0-4.5	2.78± 0.63	0.0-5.5	3.16±0.74	NS	2	1
Westar	8	2.8-4.5	3.41± 0.23	5.0-6.0	5.34±0.14*	*		
Fam. 2.2	7	0.0-4.0	2.86± 0.37	2.5-4.5	3.54±0.31	NS	0	0
Westar	8	2.8-4.5	3.31± 0.24	5.5-8.5	7.00± 0.38*	*		
Fam. 2.3	7	0.0-5.0	2.86± 0.52	0.0-7.0	5.29±0.90	*	1	0
Westar	5	2.5-5.0	4.05± 0.16*	5.5-7.0	6.10± 0.29	*		
Fam. 3.1	16	0.0-7.5	4.23± 0.53	0.0-9.0	6.31± 0.54	*	1	0
Westar	8	3.5-7.5	5.69±0.57*	5.5-8.5	7.31± 0.39*	*		
Fam. 3.2	7	0.0-3.5	1.54± 0.59	0.0-5.5	2.71± 0.98	NS	3	0
Westar	5	2.5-7.0	4.25± 0.76*	5.5-7.0	6.10±0.30*	*		
Fam. 3.3	14	0.0-5.0	2.86±0.37	0.0-5.0	3.43± 0.40	NS	1	0
Westar	7	2.0-7.0	4.43± 0.61*	5.0-7.0	6.00± 0.39*	*		
Fam. 3.4	8	0.0-7.0	3.50± 1.32	0.0-9.0	4.50± 1.70	NS	4	1
Westar	6	3.0-6.0	4.42± 0.45	4.5-7.0	6.00± 0.38	*		
Fam. 4.1	20	0.0-4.5	2.38± 0.38	0.0-4.5	2.53± 0.40	NS	6	3
Westar	8	3.5-7.0	4.81± 0.35*	5.5-8.5	6.56± 0.36*	*		
Fam. 4.2	6	3.0-5.5	3.92± 0.31	4.0-6.5	5.17± 0.31	NS	0	0
Westar	6	3.0-6.0	4.42± 0.45	4.5-7.0	6.00± 0.48*	*		
Fam. 5.1	6	0.0-7.5	3.58±1.46	0.0-8.0	3.83± 1.64	NS	3	1
Westar	7	2.3-3.9	3.07±0.20	3.5-5.5	4.75± 0.34	*		
Fam. 5.2	20	0.0-7.0	2.13±0. 46	0.0-7.5	3.05± 0.56	NS	7	1
Westar	7	4.0-9.0	5.54± 0.60*	6.0-9.0	7.29± 0.36*	*		
Fam. 5.3	18	0.0-6.5	2.29± 0.58	0.0-8.0	3.26 ± 0.61	NS	5	0
Westar	7	3.8-8.5	6.46±0.27*	5.5-8.5	7.29± 0.63*	NS		
Fam. 6.1	16	1.0-7.5	4.84± 0.46	2.5-9.0	5.59± 0.45	NS	0	0
Westar	8	3.0-5.0	3.81±0.35*	5.5-7.5	6.56± 0.26*	*		
Fam. 6.2	20	0.0-5.5	1.09±0.43	0.0-7.0	2.10±0.63	NS	14	0
Westar	6	2.8-5.0	3.46±0.36*	3.0-7.5	6.25±0.51*	*		
Fam. 7.1	7	0.0-3.5	1.71±0.62	0.0-5.5	2.93±1.04	NS	3	2
Westar	7	1.5- 3.0	2.50± 0.21*	3.5-5.5	4.75± 0.19*	*		
Fam. 7.2	20	0.0- 4.5	1.64 ± 0.35	0.0-6.5	3.05± 0.48	*	5	2
Westar	8	3.0- 4.0	3.44± 0.11*	3.5-5.5	4.38± 0.20*	NS		
Fam. 7.3	19	0.0-5.0	1.83± 0.44	0.0-7.0	2.84±0.50	NS	6	4
Westar	7	3.3-7.5	5.04±0.66*	4.8-8.0	6.32± 0.53*	*		
Total BC_2	249	0.0-7.5	2.66±0.21	0.0-9.0	3.64±0.25	NS	68	15
Westar	142	1.3-9.0	4.18±0.26*	3.0-9.0	6.04±0.22*	*		

Asterisk after Mean± SE indicates that disease symptom of Westar was significantly different from the BC_2 family with which Westar was grown (t- test, $P \leq 0.05$); NS = Not significant

Table 3.8 Cotyledon resistance against the *Leptosphaeria maculans* isolate 290CDN at 10 and 13 days after inoculation (DAI) in BC_2 seedlings of *Brassica napus* (♀) x *B. carinata* (♂) interspecific cross

BC_2 family	No. Seedling	Disease score at 10 DAI		Disease score at 13 DAI		t-test 10 DAI vs. 13 DAI	No. resistant plants	
		Range	Mean± SE	Range	Mean ± SE		Cot.	Adult
Fam. 1.1	3	0.0-3.0	1.00 ± 1.00	0.0-5.5	1.83± 1.83	NS	2	2
Westar	6	4.0-5.8	4.54± 0.29*	5.5-7.0	6.46± 0.21*	*		
Fam. 2.1	9	0.0-2.0	0.50± 0.24	0.0-4.0	1.83± 0.60	*	3	3
Westar	6	4.0-5.5	4.75± 0.21*	6.0-7.0	6.58± 0.20*	*		
Fam. 4.1	3	0.0-2.0	0.83± 0.60	0.8-3.5	2.25± 0.80	NS	0	0
Westar	7	4.0-5.5	4.86± 0.21*	6.5-7.5	7.19± 0.13*	*		
Fam.6.1	13	0.0-3.0	1.21±0..29	0.0-5.0	3.08±0.43	*	2	2
Westar	8	5.5-6.5	6.00± 0.13*	6.5-8.0	7.63± 0.21*	*		
Fam. 7.1	21	0.0-1.8	0.31± 0.11	0.0-5.5	2.22± 0.51	*	7	6
Westar	7	5.0-6.5	5.57± 0.22*	6.0-8.0	7.21± 0.28*	*		
Fam. 8.1	25	0.0-4.5	0.48± 0.21	0.0-5.5	2.53± 0.50	*	9	6
Westar	6	4.5-6.5	5.50± 0.28*	7.0-8.0	7.42± 0.15*	*		
Fam. 9.1	13	0.0-3.0	1.33± 0.27	0.0-5.5	3.65± 0.44	*	1	1
Westar	7	3.8-5.5	5.11±0.27*	6.0-7.5	7.00± 0.28*	*		
Total BC_2	87	0.0-4.5	0.80± 0.15	0.0-5.5	1.77± 0.30	*	24	20
Westar	47	3.8-6.5	5.19± 0.20*	5.5- 8.0	7.07± 0.16*	*		

Asterisk after Mean±SE indicates that disease symptom of Westar was significantly different from the BC_2 family with which Westar was grown (t-test, $P \leq 0.05$); NS = Not significant

3.3.6 Evaluation of BC_2S_1 population for blackleg resistance

One hundred and thirty one BC_2S_1 seedlings from 10 BC_2S_1 families, derived from the *B. carinata* (♀) x *B. napus* cv. Westar (♂) interspecific cross, were evaluated for cotyledon resistance at 10 and 13 DAI (Table 3.9). Of the 131 seedlings evaluated, 16 seedlings from 6 families did not show any disease symptom and were like *B. carinata.* These cotyledon resistant seedlings were

grown to maturity. Ten of the 16 cotyledon resistant plants were also resistant at the adult plant stage. Disease severity at cotyledon and adult stages is shown in Table 3.9 and in Appendix D. Self pollinated seed of the resistant plants was harvested as BC_2S_2 seed families.

One hundred and fifty four seedlings from 15 BC_2S_1 families derived from the *B. napus* cv. Polo (♀) x *B. carinata* (♂) interspecific cross were evaluated for cotyledon resistance. Of these 154 seedlings, 24 seedlings from 9 families showed cotyledon resistance at 13 DAI (Table 3.10). These seedlings did not show any visible symptoms, and were self- pollinated to get BC_2S_2 seeds. However, at the adult stage, 11 of the 24 plants from 6 families were found to be resistant (Table 3.10 and Appendix D)

Table 3.9 Cotyledon resistance against the *Leptosphaeria maculans* isolate 290CDN at 10 and 13 days after inoculation (DAI) in BC_2S_1 seedlings of *Brassica carinata* (♀) x *B. napus* (♂) interspecific crosses

BC_2S_1 Family	No. seedling	Disease score at 10 DAI		Disease score at 13 DAI		t-test 10 DAI vs. 13 DAI	Total Resistant plants	
		Range	Mean± SE	Range	Mean ± SE		Cot.	Adult
Fam. 2.1.1S	13	0.0-5.5	2.25±0.56	0.0-5.5	2.94±0.68	NS	5	3
Westar	4	4.5-5.5	5.00±0.20*	5.5-7.0	6.50±0.35*	*		
Fam. 3.4.1S	14	0.0-6.0	2.59±0.63	0.0-6.5	3.96±0.72	NS	4	2
Westar	4	5.0-5.5	5.38±0.13*	6.0-6.5	6.10±0.10*	*		
Fam. 4.1.1S	14	0.0-5.5	4.18±0.24	4.8-6.5	5.82±0.14	*	0	0
Westar	4	4.5-5.5	4.88±0.24	4.5-6.0	5.38±0.31	NS		
Fam. 5.1.1S	12	0.0-7.0	4.00±0.65	0.0-8.0	5.63±0.58	*	2	1
Westar	4	5.0-5.5	5.13±0.13	5.5-7.0	6.38±0.21	*		
Fam. 5.2.1S	13	0.0-7.0	5.00±0.32	5.5-7.5	6.27±0.18	*	0	0
Westar	4	5.0-7.0	5.75±0.43	6.5-7.5	7.13±0.31	*		
Fam. 7.1.1S	14	0.0-6.0	4.16±0.24	0.0-8.0	5.61±0.49	*	1	1
Westar	4	4.5-5.5	5.00±0.20	7.0-7.5	7.25±1.37	*		
Fam. 7.2.1S	14	0.0-6.0	3.48±0.44	0.0-8.5	4.84±0.71	NS	2	1
Westar	4	5.0-6.0	5.38±0.24*	5.5-6.5	6.25±0.25*	*		
Fam. 7.3.1S	13	0.0-5.5	4.31±0.38	4.5-8.0	5.83±0.36	*	0	0
Westar	4	4.5-5.5	5.00±0.20	6.0-7.5	6.88±0.30	*		
Fam. 7.3.2S	11	0.0-5.5	3.20±0.62	0.0-7.5	4.34±0.81	NS	2	2
Westar	4	4.5- 5.5	5.25±0.25*	6.5-8.0	7.00±0.36*	*		
Fam. 7.3.3S	13	2.8-6.5	4.98±0.29	3.3-9.0	6.60±0.38	*	0	0
Westar	4	5.0-5.5	5.13±0.13	6.0-8.0	7.13± 0.50	*		
Total BC_2S_1	131	0.0-7.0	3.81±0.29	0.0-9.0	5.17±0.36	*	16	10
Total Westar	40	4.5-7.0	5.19±0.08*	4.5-9.0	6.41±0.28*	*		

Asterisk after Mean±SE indicates that disease symptom of Westar was significantly different from the BC_2 S_1 family with which Westar was grown (t-test, $P \leq 0.05$); NS = Not significant

Table 3.10 Cotyledon resistance against the *Leptosphaeria maculans* isolate 290CDN at 10 and 13 days after inoculation (DAI) in BC_2S_1 seedlings of *Brassica napus* (♀) x *B. carinata* (♂) interspecific cross

BC_2S_1 family	No. seedling	Disease score at 10 DAI		Disease score at 13 DAI		t-test 10 DAI vs. 13 DAI	No. resistant plants	
		Range	Mean± SE	Range	Mean ± SE		Cot.	Adult
Fam. 1.1.1S	11	0.0-6.0	3.95± 0.40	0.0-6.5	5.14±0.70	*	1	1
Westar	5	5.0-6.0	5.40± 0.25*	6.0-7.0	6.60±0.17*	*		
Fam. 1.1.2S	11	0.0-5.5	2.43 ±0.41	0.0-6.0	3.82±0.79	NS	2	0
Westar	5	5.0-6.0	5.60±0.17*	6.0-8.0	6.80±0.25*	*		
Fam. 2.1.1S	10	0.0-6.0	3.90± 0.33	3.5-7.5	5.60±0.45	*	0	0
Westar	4	4.5-6.0	5.25± 0.30*	6.0-8.0	6.63±0.37*	*		
Fam. 2.1.2S	10	0.0-6.0	3.75 ±0.41	0.0-7.0	4.55±0.48	NS	1	0
Westar	4	5.0-6.0	5.63±0.18*	6.0-8.0	7.00±0.15*	*		
Fam. 6.2.1S	11	0.0-5.0	2.91±0.44	0.0-7.0	4.86±0.51	*	2	2
Westar	4	5.5-6.0	5.75±0.27*	6.5-8.5	7.13±0.47*	*		
Fam. 7.1.1S	13	0.0-6.0	4.46±0.12	4.0-6.5	5.54±0.72	*	0	0
Westar	5	5.0-6.0	5.60±0.19*	6.0-7.8	6.85±0.41*	*		
Fam. 7.2.1S	11	0.0-5.0	2.68±0.30	0.0-7.0	4.30±0.21	*	2	1
Westar	5	4.5-6.0	5.20±0.31	6.5-7.0	6.60±0.16*	*		
Fam. 7.3.1S	3	2.3-3.0	2.50±0.25	4.5-5.0	4.67±0.17	*	0	0
Westar	5	4.0-6.0	5.10±0.33*	6.5-7.5	7.00±0.27*	*		
Fam. 7.5.1S	5	0.0-5.0	1.60±1.03	0.0-7.5	2.30±1.55	NS	3	1
Westar	4	4.5-5.5	5.00±0.20*	5.5-9.0	7.13±0.43*	*		
Fam. 7.6.1S	14	0.0-5.0	3.07±0.34	0.0-6.5	4.57±0.60	*	2	2
Westar	7	4.5-6.0	5.50±0.17*	7.0-8.0	7.50±0.19*	*		
Fam. 8.1.1S	13	0.0-5.0	3.35±0.43	2.5-6.0	5.08±0.44	*	0	0
Westar	6	5.0-5.5	5.17±0.12*	6.0-8.0	6.75±0.22*	*		
Fam. 8.3.1	14	0.0-5.0	2.80±0.67	4.0-6.5	4.93±0.80	*	0	0
Westar	5	5.0-6.0	5.40±0.29*	6.5-7.0	6.90±0.43*	*		
Fam. 8.4.1S	14	0.0-5.0	0.70±0.23	0.0-6.0	1.43±0.16	*	10	3
Westar	5	5.0-6.0	5.50±0.22*	7.5-8.0	7.70±0.35*	*		
Fam. 8.6.1S	7	0.0-5.0	3.18±0.38	0.0-6.5	4.39±0.53	NS	1	1
Westar	5	5.0-6.0	5.50±0.12*	7.0-8.5	7.70±0.31*	*		
Fam.9.1.1S	7	4.0-5.0	4.79±0.39	4.5-7.0	5.86±0.34	NS	0	0
Westar	5	5.0-6.0	5.70±0.16*	6.0-8.0	7.00±0.22*	*		
Total BC2S1	154	0.0-7.0	3. 15±0.25	0.0-9.0	4.87±0.29*	*	24	11
Total Westar	74	4.0-7.0	5.42±0.06*	5.5-9.0	7.01±0.09*	*		

Asterisk after Mean±SE indicates that disease symptom of Westar was significantly different from the BC_2 S_1 family with which Westar was grown (t-test, $P \leq 0.05$); NS = Not significant

3.3.7 Evaluation of BC_3 population for blackleg resistance

One hundred and seventy four BC_3 plants from 15 families of the *B. carinata* (♀) x *B. napus* cv. Westar (♂) interspecific cross were evaluated for cotyledon resistance at 10 and 13 DAI. At 13 DAI, 13 BC_2 families showed significantly greater resistance compared to Westar, and two families (4.1.2 and 5.2.1) were susceptible like Westar (Table 3.11). Disease severity in Westar increased at 13 DAI compared to 10 DAI, as expected. Of these 174 BC_3 seedlings evaluated, 23 seedlings from 11 families showed cotyledon resistance like *B. carinata*, and were grown to maturity, where10 plants showed adult plant resistance (Table 3.11 and Appendix E). The BC_4 and BC_3S_1 (self of BC_3) seeds were harvested from these 10 plants showing both cotyledon and adult plant resistance.

Two hundred and thirty two plants from 20 BC_3 families, of *B. napus* cv. Polo (♀) x *B. carinata* (♂) were evaluated for cotyledon resistance at 10 DAI and 13 DAI. Disease severity increased significantly in 6 BC_3 families at 13 DAI compared to 10 DAI. Of the 232 BC_3 seedlings, 37 from 13 families showed strong resistance with no visible disease symptom (Table 3.12). These seedlings were grown to maturity; however, 6 of the 37 cotyledon resistant plants showed resistance at adult plant stage (Table 3.12 and Appendix E). The BC_4 and BC_3S_1 seeds were harvested from these plants.

Table 3.11 Cotyledon resistance against the *Leptosphaeria maculans* isolate 290CDN at 10 and 13 days after inoculation (DAI) in BC_3 seedlings of *B. carinata* (♀) x *B. napus* (♂) interspecific cross

BC_3 family	No. seedling	Disease score at 10 DAI		Disease score at 13 DAI		t-test 10 DAI vs. 13 DAI	No. resistant plants	
		Range	Mean± SE	Range	Mean ± SE		Cot.	Adult
Fam. 2.1.1	14	0.0-5.5	3.96± 0.60	0.0-8.0	5.18±0.77	NS	3	1
Westar	5	5.0-6.0	5.40± 0.24*	6.0-7.0	6.60±0.18*	*		
Fam. 3.4.1	13	0.0-3.0	1.48 ±0.41	0.0-6.0	2.96±0.79	NS	6	4
Westar	5	5.0-6.0	5.43±0.17*	6.0-7.0	6.79±0.15*	*		
Fam. 4.1.1	14	0.0-5.5	3.36± 0.38	0.0-7.0	5.32±0.45	*	1	1
Westar	6	4.5-7.0	5.67± 0.36*	6.0-8.0	6.58±0.32*	NS		
Fam. 4.1.2	14	0.0-5.5	3.82 ±0.41	0.0-7.0	5.79±0.48	*	1	0
Westar	7	4.5-6.0	5.21±0.18*	6.5-7.5	6.79±0.15	*		
Fam. 4.1.3	14	0.0-7.0	4.68±0.44	0.0-7.5	5.96±0.51	NS	1	1
Westar	6	5.0-6.0	5.33±0.17	6.5-7.5	7.08±0.15*	*		
Fam. 5.1.1	13	0.0-7.0	3.87±0.61	0.0-7.5	5.42±0.69	NS	2	1
Westar	5	3.5-6.0	5.00±0.42	6.0-7.5	6.80±0.33*	*		
Fam. 5.2.1	13	0.0-7.0	4.58±0.36	5.0-8.0	6.65±0.21	*	0	0
Westar	5	4.0-6.0	5.17±0.31	6.5-7.5	6.92±0.16	*		
Fam. 7.1.1	13	0.0-6.5	3.23±0.62	0.0-8.0	4.88±0.80	NS	3	1
Westar	5	4.0-6.0	5.10±0.33*	6.0-7.5	7.00±0.27*	*		
Fam. 7.1.2	1	0.0-0.0	0.00±0.00	0.0-0.0	0.00±0.00	NS	1	0
Westar	3	4.0-5.0	4.67±0.33*	5.5-7.0	6.50±0.50*	*		
Fam. 7.2.1	14	0.0-5.5	4.21±0.34	5.0-7.0	6.07±0.18	*	0	0
Westar	5	5.0-6.0	5.50±0.17*	7.0-8.0	7.33±0.17*	*		
Fam. 7.2.2	14	0.0-5.5	2.89±0.43	0.0-6.5	4.82±0.44	*	1	0
Westar	5	5.0-5.5	5.20±0.12*	6.0-7.0	6.50±0.22*	*		
Fam. 7.3.1	9	0.0-6.5	3.03±0.67	0.0-7.5	4.11±0.84	NS	2	0
Westar	5	5.0-6.0	5.50±0.29*	5.0-7.5	6.60±0.43*	*		
Fam. 7.3.2	8	0.0-5.5	3.31±0.51	4.5-6.0	5.38±0.16	*	0	0
Westar	5	5.0-6.0	5.40±0.19*	5.5-7.0	6.20±0.34*	NS		
Fam. 7.3.3	10	0.0-3.5	1.95±0.38	0.0-4.5	3.15±0.53	NS	2	1
Westar	5	5.5-6.0	5.70±0.12*	6.0-6.5	6.20±0.12*	NS		
Fam. 7.3.4	10	0.0-5.00	3.75±0.37	3.5-6.0	4.75±0.35	NS	0	0
Westar	5	5.0-6.0	5.50±0.16*	6.0-7.5	6.70±0.30*	*		
Total BC_3	174	0.0-7.0	3.45±0.24	0.0-8.0	5.03±0.33	*	23	10
Total Westar	77	3.5-6.5	5.37±0.57*	5.0-8.0	6.70±0.08*	*		

Asterisk after Mean ± SE indicates that disease symptom of Westar was significantly different from the BC_3 family with which Westar was grown (t-test, $P \leq 0.05$); NS =Not significant

Table 3.12 Cotyledon resistance against the *Leptosphaeria maculans* isolate 290CDN at 10 and 13 days after inoculation (DAI) in BC_3 seedlings of *B. napus* (♀) x *B. carinata* (♂) interspecific cross

BC_3 family	No. seedling	Disease score at 10 DAI		Disease score at 13 DAI		t-test 10 DAI vs. 13 DAI	No. resistant plants	
		Range	Mean± SE	Range	Mean ± SE		Cot.	Adult
Fam. 1.1.1	14	0.0-6.5	3.52±0.53	0.0-6.5	3.96±0.54	NS	1	1
Westar	6	5.0-6.0	5.17± 0.28*	6.0-7.0	6.58± 0.20*	*		
Fam. 1.1.2	1	3.0	3.00±0.00	6.0	6.00±0.00	*	0	0
Westar	3	3.0-7.0	5.00±1.15*	6.5-9.0	7.83±0.88*	*		
Fam. 2.1.1	14	0.0-7.0	5.70 ± 0.36	4.3-7.5	6.38± 0.25	NS	0	0
Westar	4	5.0-7.0	5.50± 0.50	6.5-9.0	7.63± 0.62	*		
Fam. 2.2.1	14	0.0-5.5	4.34± 0.26	4.0-6.5	5.30± 0.24	*	0	0
Westar	5	5.0-5.5	5.38± 0.13*	5.5-9.0	7.17± 0.51*	*		
Fam. 2.3.1	13	0.0-7.0	4.15± 0.56	0.0-8.0	4.44± 0.58	NS	1	1
Westar	5	5.0-6.0	5.40± 0.19*	5.5-9.0	7.10± 0.63*	*		
Fam. 6.1.1	13	0.0-6.0	2.88± 0.55	0.0-7.0	4.08± 0.69	NS	3	0
Westar	6	4.0-6.0	5.2± 0.34*	5.0-9.0	7.33± 0.57*	*		
Fam. 6.2.1	7	0.0-6.0	2.71± 0.92	0.0-6.0	2.86± 0.99	NS	2	0
Westar	3	3.0-7.0	5.00± 1.15*	5.5-9.0	7.00± 1.04*	*		
Fam. 7.1.1	11	0.0-6.0	4.06± 0.52	3.0-7.5	5.29± 0.50	NS	0	0
Westar	5	5.0-7.0	5.50± 0.38*	6.0-8.5	7.20± 0.46*	*		
Fam. 7.2.1	12	0.0-5.5	3.31± 0.29	4.5-7.5	5.13± 0.31	*	0	0
Westar	6	5.0-6.0	5.40± 0.15*	6.0-9.0	7.35± 0.51*	*		
Fam. 7.3.1.	13	0.0-4.5	2.42± 0.36	0.0-5.5	3.77± 0.50	*	2	0
Westar	5	4.5-5.5	5.10± 0.24*	6.0-9.0	7.80± 0.51*	*		
Fam. 7.4.1	7	0.0-4.5	1.21± 0.79	0.0-5.5	1.43± 1.13	NS	5	0
Westar	5	4.5-6.0	5.20± 0.30*	7.0-8.5	7.30± 0.40*	*		
Fam. 7.5.1	14	0.0-5.0	2.36± 0.56	0.0-6.0	3.14± 0.69	NS	5	1
Westar	5	4.0-5.5	4.90± 0.29*	6.0-9.0	7.40± 0.51*	*		
Fam. 7.6.1	14	0.0-5.0	1.98± 0.41	0.0-7.0	3.34± 0.65	NS	2	2
Westar	5	3.5-6.0	5.00± 0.42*	6.0-8.0	7.10± 0.31*	*		
Fam. 8.1.1	14	1.5-3.5	3.05± 0.32	2.0-6.0	4.36± 0.33	*	0	0
Westar	5	5.5-6.0	5.63± 0.16*	6.0-9.0	7.00± 0.55*	*		
Fam.8.2.1	12	0.0-5.5	2.19± 0.53	0.0-6.5	3.04± 0.69	NS	4	0
Westar	5	3.0-5.5	4.60± 0.57*	6.0-9.0	7.10±0.49 *	*		
Fam. 8.3.1	14	0.0-5.5	3.00± 0.54	0.0-6.0	4.25± 0.53	NS	2	1
Westar	5	3.0-6.0	5.00± 0.88*	7.0-9.0	7.80± 0.33*	*		
Fam. 8.4.1	13	0.0-5.0	1.54± 0.57	0.0-5.5	1.77± 0.62	NS	7	0
Westar	5	5.0-5.5	5.30± 0.12*	7.0-8.0	7.20± 0.20*	*		
Fam. 8.5.1	13	0.0-6.0	3.77± 0.57	0.0-6.5	4.19± 0.61	NS	2	0
Westar	6	4.0-6.0	5.33± 0.31*	6.5-7.5	7.08± 0.33*	*		
Fam. 8.6.1	6	0.0-4.0	2.92± 0.61	0.0-5.5	3.92± 0.84	NS	1	0
Westar	5	3.5-6.0	4.50± 0.52*	5.0-7.0	6.30± 0.49*	*		
Fam. 9.1.1	13	3.0-7.0	4.25± 0.31	4.0-7.5	5.36± 0.31	*	0	0
Westar	5	4.0-5.5	5.00± 0.27	6.0-8.5	7.20± 0.63*	*		
Total BC_3	232	0.0-7.0	3.17± 0.24	0.0-9.0	5.40± 0.27	*	37	6
Westar	99	3.0-7.0	5.16± 0.06*	5.0-9.0	7.10± 0.09*	*		

Asterisk after Mean±SE indicates that disease symptom of Westar was significantly different from the BC_3 family with which Westar was grown (t-test, $P \leq 0.05$); NS = Not significant

3.3.8 Summary of cotyledon resistance of different backcross generations of the *B. napus* x *B. carinata* cross at 10 DAI and 13 DAI.

All Westar seedlings, grown with the backcross population showed disease susceptibility at 10 DAI while a significant number of backcross seedlings did not show any visible disease symptoms at this stage (disease score zero). Disease score at 13 DAI revealed that a small portion (1-4%) of these cotyledon resistant seedlings became susceptible at 13 DAI (Table 3.13) except the BC_2 population of *B. napus* x *B. carinata* where a much greater difference (14%) was found for the proportion of resistant seedlings between these two scoring dates.

Table 3.13 Comparison between 10 and 13 days after inoculation (DAI) for the proportion of cotyledon resistant seedlings in different backcross generation populations of *B. napus* x *B. carinata* crosses

Parentage of F_1	Generation	No. seedlings	% Resistant seedlings		Difference between 10 DAI and 13 DAI
			10 DAI	13 DAI	
B.c x W	BC_2	249	28	27	1
P x B.c	BC_2	87	44	28	14
B.c x W	BC_2S_1	131	16	12	4
P x B.c	BC_2S_1	154	19	16	3
B.c x W	BC_3	174	17	13	4
P x B.c	BC_3	232	18	16	2

B.c = *B. carinata*; W = *B. napus* cv. Westar; P = *B. napus* cv. Polo
DAI= Days after inoculation

3.3.9 Summary of adult plant resistance of the plants resistant at the cotyledon stage in different generation of *Brassica napus* x *B. carinata* interspecific crosses

Adult plant resistance of the cotyledon resistant plants in different backcross generations, as reported in Sections 3.4 to 3.7, are summarized in Table 3.14. As mentioned in these earlier sections, during the recurrent backcrossing program, only the cotyledon resistant plants were backcrossed to Westar, and the backcross and self- pollinated seeds of the plants showing good resistance at the adult plant stage (disease score zero) were retained for growing the subsequent generation populations. Data presented in Table 3.14 and Fig 3.5 show that the proportion of cotyledon and adult resistant plants decreased as backcrossing progressed. All F_1 plants from the reciprocal interspecific crosses showed excellent resistance at the cotyledon and adult plant stages. However, in BC_1 68 % of the seedlings showed cotyledon resistance; and 50 % plants of the total population showed resistance at the adult stage. This trend continued in BC_2, BC_2S_1 and BC_3 generation populations. In BC_3, 13-16% seedlings of the total population showed cotyledon resistance while only 3-6% plants showed resistance at the adult stage.

Table 3.14 Summary of the cotyledon and adult plant resistance of different backcross generations of *B. napus* and *B. carinata* interspecific crosses

Parentage of the F_1 (♀ x ♂)	Evaluated generation	No. families tested	% families showing cotyledon resistance	Cotyledon Resistance				Adult plant resistance[c]		
				No. seedlings	No. resistant	No. sus.	% Resistant seedlings	No. Resistant plant	No. susceptible plants	% resistant plant
B.c x W	BC_1	7	100	40	27	13	68	20	7	50
B.c x W	BC_1	NI	-	8[a]	-	-	-	-	-	-
P x B.c	BC_1	NI	-	9[b]	-	-	-	-	-	-
P x B.c	BC_1	NI	-	1[a]	-	-	-	-	-	-
B.c x W	BC_2	20	85	249	68	181	27	15	53	6
P x B.c	BC_2	7	86	87	24	63	28	20	4	23
B.c x W	BC_2S_1	10	60	131	16	115	12	10	6	8
P x B.c	BC_2S_1	15	60	154	24	130	16	11	13	7
B. c x W	BC_3	15	73	174	23	151	13	10	13	6
P x B.c	BC_3	20	35	232	37	195	16	6	31	3

B.c = *B. carinata*, W= *B.napus* cv. Westar, P= *B. napus* cv. Polo

NI= Not inoculated for blackleg resistance.

[a]Seedlings obtained from *in vitro* ovule culture and not inoculated for blackleg resistance.

[b]Seedlings obtained from *in vivo* but not inoculated for blackleg resistance.

[c]Only the cotyledon resistant plant were evaluated at adult plant stage.

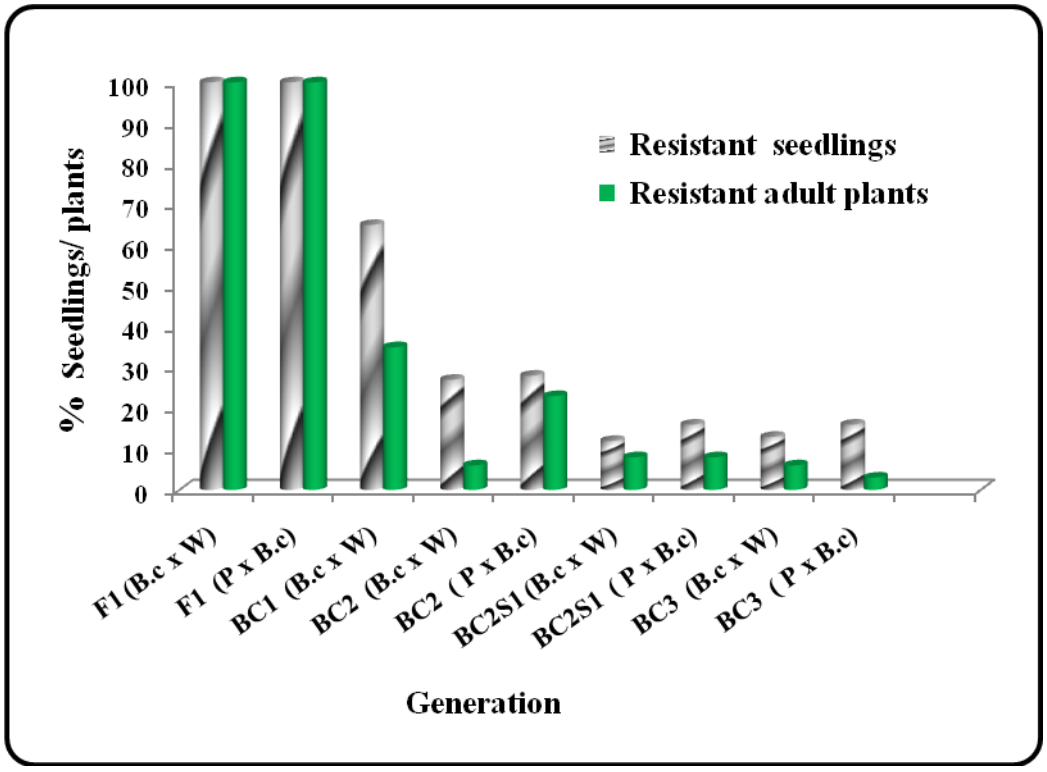


Fig. 3.5 Proportion of the seedlings that showed resistance at cotyledon stage and the cotyledon resistant seedlings that showed resistance at adult plant stage in F_1 and different backcross generations of *B. napus* x *B. carinata* interspecific crosses.
B.c = *B. carinata*, W= *B. napus* cv. Westar, P= *B. napus* cv. Polo
Parentage of the F_1's are given in brackets.

Fig. 3.6 (a) Stems of the resistant and (b) susceptible plants; and (c) three susceptible and (d) one resistant and two susceptible adult plants

3.4 Discussion

In the present study, reciprocal interspecific crosses between blackleg susceptible *B. napus* genotypes and a resistant *B. carinata* genotype were done, and the interspecific hybrids were recurrently backcrossed to the highly susceptible *B. napus* cv. Westar for introgression of cotyledon and adult plant resistance from *B. carinata* into the 'Westar' background. For this, in each generation, cotyledon resistant seedlings were retained and backcrossed to 'Westar' and/or self-pollinated and the plants at maturity were evaluated for adult plant resistance. Seeds harvested from the resistant adult plants constituted the next generation population. In the F_1 generation, all plants were resistance to the PG4 type *L. maculans* isolate 290CDN at the cotyledon as well as the adult plant stage. In the BC_1 generation, segregation for cotyledon resistance was observed. However, while Chi-square tests on the seven families individually fit to 1:1 segregation ratio for resistant and susceptible phenotypes, the pooled data of these seven families deviated significantly from a 1:1 segregation. Therefore, based on present data, it is difficult to conclude whether a single locus or more than one locus is involved in the control of blackleg resistance in *B. carinata*. Delwiche (1980) (cited by Pang and Halloran 1996) reported that resistance to cotyledon lesion development in *B. napus* is controlled by two dominant genes; while. Chèvre et al. (1997) reported monogenic control of cotyledon resistance in *B. juncea.* In the case of adult plant resistance, monogenic inheritance has been reported by Li and Cowling (2003), Dion et al. (1995), and Stringam et al. (1992). However, polygenic control of this trait has also been reported by several

researchers (Cargeeg and Thurling 1979; Sippell et al. 1991; Ferreira et al. 1995; Pang and Halloran 1996; Pilet et al. 1998; Sawatsky 1989).

During this recurrent backcross program, a significant number of the cotyledon resistant seedlings turned out to be susceptible at the adult plant stage. This suggests that cotyledon resistance and adult plant resistance in *B. carinata* might be under different genetic control. According to Rimmer and Van den Berg (1992), there are two types of blackleg resistance, cotyledon/seedling and adult plant resistance. Pang and Halloran (1996) reported that crown canker in mature plants is not related to cotyledon resistance. In the present research, the proportion of cotyledon resistant plant showing resistance at the adult plant stage decreased with the increasing number of backcrosses. This is apparently due to the lack of introgression of resistance from the *B. carinata* genome (s) into the *B. napus* genome, and elimination of the *B. carinata* chromosome(s) carrying adult plant resistance during backcrossing. Sacristan and Gerdmann (1986) also reported loss of adult plant resistance in BC_2 generations while transferring the blackleg resistance from *B. carinata* into *B. napus*, and explained this in the context of limited recombination between the A and B genome chromosomes.

In a breeding program, selection for adult plant resistance is important as blackleg disease severity at this stage causes yield loss. Although several studies suggest that seedling and adult plant resistance are under different genetic control, several authors have also found a significant correlation between the two traits (Newman and Bailey 1987; Mcnabb et al. 1993; Bansal et al. 1994; Li and Cowling 2003). Our study suggests that cotyledon and adult plant resistance to

PG4 type *L. maculans* isolate 290 CDN in *B. carinata* is under different genetic control, and introgression of adult plant resistance from *B. carinata* into *B. napus* by selecting cotyledon resistant seedlings might not be an effective strategy to achieve the goal.

An efficient and reliable screening method for the identification of truly resistant plants is a prerequisite for establishing a successful resistance breeding program. The time of disease scoring is therefore important. Bansal et al. (1994) found that disease scoring at 10 DAI is effective for screening at the cotyledon stage. Pang and Halloran (1996) assessed cotyledon lesion at 14 DAI and Fernando and Chen (2003) and Dusabenyagasani and Fernando (2008) assessed this at 12 DAI. Dixelious and Wahlberg (1999) scored the inoculated plants at 8-16 DAI. In this present study, screening for cotyledon resistance was done at 10 and 13 DAI for selecting the highly resistant seedlings. Selection based on disease scoring at 13 DAI was found to be slightly more effective compared to disease score at 10 DAI. At this stage, an additional 1-4% susceptible seedling which was resistant at 10 DAI, could be identified and discarded.

The production of backcross seed in different generations indicated that interspecific hybrid plants carrying the cytoplasm of *B. carinata* yielded a greater number of seeds per pollination compared to the plants carrying *B. napus* cytoplasm. Molecular analysis of chloroplast DNA of different *Brassica* species revealed that *B. carinata* carries the cytoplasm of *B. nigra* and *B. juncea* carries the cytoplasm of *B. rapa* (Palmer et al. 1983; Erickson et al. 1983; Hallden et al. 1993; Pradhan et al. 1992; Warwick and Black 1991). However, no strict

assignment could be made in the case of *B. napus* as to which diploid species contributed cytoplasm in this amphidiploid species. Chloroplast DNA of *B. napus* often shows close similarity with *B. oleracea* (Erickson et al. 1983; Hallden et al. 1993). Song and Osborn (1992) identified four major types of cytoplasm in the diploid species which were also observed in the *B. napus* accessions. However, most of the cultivated *B. napus* carried cytoplasm which is different from its cytoplasm; and these *B. napus* accessions contain the chloroplast genome as that of *B. montana* and the mitochondrial genome intermediate between *B. montana and B. rapa.* This suggests that *B. montana* or a close relative might be the common progenitor species from which the cytoplasm of *B. rapa* and *B. oleracea* were derived through changes in the cytoplasmic (cp) and mitochondrial (mt) genome, and consequently contributed the cytoplasm in *B. napus*. Narasimhulu et al. (1989) investigated the effect of the cytoplasm on shoot morphogenesis in *B. carinata* (BBCC) resynthesized from reciprocal crosses between *B. nigra* (BB) and *B. oleracea* (CC). They found that the shoot regeneration response of the cotyledon of the resynthesized *B. carinata* carrying *B. oleracea* cytoplasm was as much as twice of the cotyledon carrying *B. nigra* cytoplasm. On the other hand, Uprety et al. (1990) reported that the rate of photosynthesis in *B. carinata* carrying the *B. oleracea* cytoplasm was lower compared to *B. carinata* carrying *B. nigra* cytoplasm. In the case of *B. napus*, the effect of mitochondrial DNA (mtDNA) on linolenic acid content in oil, flowering time and protein content has been reported by Rajcan et al. (2002). Chang et al. (2009) reported significant effect of *B. napus* and *B. juncea* cytoplasm in the alloplasmic *B. carinata* lines,

where the effect of *B. napus* cytoplasm was found to be more deleterious than the effect *B. juncea* cytoplasm. Chang et al. (2011) reported that an alloplasmic *B. oleracea* line carrying *B. carinata* cytoplasm result smaller size petals. Thus, it is apparent that cytoplasm may interact differentially in different genetic background, and this might be one of the reasons for the occurrence of greater cytoplasmic effect on seed set per pollination in the earlier generation of backcross, viz. BC_1, and BC_2, compared to the later backcross generations; viz. BC_3 and BC_4. A positive effect of *B. carinata* cytoplasm on interspecific crossability is also evident from the data presented by Rahman (2001).

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Chapter 4

Morphological and Cytological Characteristics of the *Brassica napus* x *Brassica carinata* Interspecific Populations

4.1 Introduction

Among the Brassica species of U's triangle, *Brassica carinata* (BBCC, 2n=34) possesses many desirable traits (Jiang et al. 2007), which are lacking in *B. napus*. This species is generally resistant to blackleg disease caused by *Leptosphaeria maculans* (Christianson et al. 2006), tolerant to heat and drought stresses (Getinet et al. 1996) and possesses the yellow seed color trait (Gugel et al. 1990, Getinet and Rakow 1997, Rahman and Tahir 2010). These facts make the species an important genetic resource for the improvement of canola *B. napus*.

In this research project, reciprocal interspecific crosses were made between *B. napus* and *B. carinata* primarily to introgress blackleg resistance into *B. napus*. However, interspecific crossing can generate progeny with novel trait, as has been demonstrated by Rahman et al. (2011) through the development of an early flowering *B. napus* line by crossing *B. napus* with the late flowering species *B. oleracea*. Similarly, Xiao et al. (2010) reported the occurrence of novel morphological traits, e.g. apetalous flowers, male sterile flowers, large seeds, high oil content, high linolenic acid, etc., while developing new types of *B. napus* with the A or the C genome of *B. rapa* or *B. carinata*. Thus, it is also probable that the progeny of a *B. napus* x *B. carinata* interspecific cross, besides carrying blackleg resistance, may also carry novel traits that arose from genetic recombination between the genome of these two species.

The objective of this part of the study was to investigate different agronomic characteristics and fertility of the plants, including silique length and number of seeds per silique, derived from reciprocal crosses between *B. napus* and *B. carinata*. The assessment of silique and seed set in the progeny of an interspecific cross also provide a preliminary indication on the ploidy level and chromosomal stability of the plants (Rahman et al. 2011).

4.2 Materials and methods

4.2.1 Morphological traits

The F_1 and different backcross generation populations derived from reciprocal interspecific crosses between *B. napus* and *B. carinata* were evaluated for leaf morphology, days to germination, days to flowering, flower color, silique length and number of seeds per silique. The pedigree of these populations is described in Chapters 2 and 3. Leaf morphology was assessed based on the canola crop description by the Canadian Food Inspection Agency (CFIA) (2010). Data on days to germination were recorded for the seedlings derived from *in vivo* produced seeds. Days to flower was recorded for the cotyledon resistant plants as well as for the plants generated through ovule culture. In the case of the cotyledon resistant plants, this trait was recorded as number of days from seeding to opening of the first flower; while for the plants generated from ovule culture, this was recorded as the number of days required from transfer of the seedlings to the greenhouse to opening of the first flower. Petal colour was recorded as yellow (*B. napus* type) or creamy white (*B. carinata* type). Silique length and number of seeds

per silique were recorded for the plants resistant both at the cotyledon and adult stages. Silique length was measured following the description of CFIA (2010); and was recorded as the average of randomly chosen 5-10 siliques from each plant in all generations. Similarly, 5-10 siliques from each plant of F_1, BC_1 and BC_2 generations were chosen randomly and number seeds/silique was recorded, and the average data was used in statistical analysis. However, in case of BC_2S_1 and BC_3 generations, all siliques from a plant were used for estimation of the average number of seeds/silique.

4.2.2 Cytological analysis

Pollen viability:

The pollen viability of the blackleg resistant and susceptible BC_3 and BC_2S_1 plants were estimated following the standard acetocarmine staining technique. For this, flower buds at the age of one day prior to anthesis were used. The anthers were squashed in 1% acetocarmine solution on a microscope slide and the pollen grains studied under a Zeiss microscope (Zeiss Canada Ltd. Toronto, ON. Canada) at 10x magnification. The microscope was equipped with a camera 'Cool snap' (Zeiss Canada Ltd. Toronto, ON., Canada) and the images were captured by using Metamorph (version 5.6) software (www.Universal imaging.com). Pollen grains that stained red were counted as viable, while the shrivelled or malformed and unstained pollen grains were counted as unviable (Nelson et al. 2009). The pollen viability was determined by using the following formula:

$$\text{Pollen viability (\%)} = \frac{\text{Number of viable pollen}}{\text{Total number of pollen counted}} \times 100$$

Pollen mother cells and meiosis:

Two blackleg resistant BC_3 generation plants were studied for the behaviour of chromosomes in meiosis. For this, infloresences were collected from 6-8 week old plants in the morning (8 am to 10 am), and fixed in Carnoy's solution (3:1ethanol/glacial acetic acid) at 4^0C for 24 h. After 24 hrs, the buds were transferred to a modified solution (Carnoy's solution containing 0.011g mL^{-1} ferric chloride) and stored at 4^0C for 48 hours. The fixed buds of about 1mm in length were removed and stained in 1% acetocarmine solution in a water bath at 60°C for 4 h. The anthers were dissected and squashed with a needle in a drop of 1% acetocarmine on a slide. The debris was removed and a cover slip was placed on the slide and pressed gently. The underside of the slide was heated for few seconds over a burner flame and the cover slip was pressed gently to flatten the cells and to bring the cells in one plane. The slides were sealed with nail polish. The pollen mother cells (PMC) were studied under the above mentioned microscope.

4.2.3. Statistical analysis

Duncan's Multiple Range Test (DMRT) was done to compare agronomic characteristics of the interspecific progenies and their parents. For this, data were analyzed by using PROC GLM of SAS software version 9.2 (SAS Institute Inc.,

2008). Correlation between pollen viability and seed set were estimated with PROC CORR of SAS. The statistical model used to estimate the coefficient of correlation was as follows:

$$r_{XY} = \frac{Cov\ (X, Y)}{S_X S_Y}$$

Where,

r = Coefficient of correlation

X = Pollen viability

Y = No. of seeds/silique

Cov = Covariance between pollen viability and no. of seeds/silique

S_X = Standard deviation of pollen viability

S_Y = Standard deviation of no. of seeds/silique

4.3 Results

4.3.1 Morphological characteristics

The morphological description of the leaves, based on lamina shape, development of lobes, shape of margin and apex shape of the parents *B. carinata*, Westar and Polo and their F_1 hybrids is presented in Table 4.1. The leaves of *B. carinata* were dark green with violet veins and petiolate. The leaves of Westar were bluish green with strongly developed lobes; and the ratio of the width and length (W/L) of leaf lamina was greater than 0.80, i.e. the shape of lamina was orbicular. The leaves of Polo had naked petiole with almost no lobes, which make this cultivar quite distinct from Westar. The leaves of the F_1 plants of *B. carinata* x Westar had wide elliptic type lamina (W/L = 0.67-0.79, i.e. less than 0.80) with strongly

developed lobes (Fig. 4.1). The margin of the leaves was undulating with a round shaped apex. The leaves of the F_1 plants of Polo x *B. carinata* had few lobes (Fig. 4.2) and the shape of the lamina was orbicular. In general, the leaves of the F_1 plants were intermediate of their parents.

Brassica carinata had creamy white petals while *B. napus* cvs. Westar and Polo had yellow petals. However, all F_1 and backcross generations populations derived from these two interspecific crosses had yellow petals (Fig. 4.3).

Days to germination and days to flowering data, for the F_1 and backcross generation populations, are presented in Table 4.2. In general, *B. carinata* required a slightly longer time to germinate compared to the *B. napus* genotypes. The average number of days required for germination of the different backcross generation populations was similar to the recurrent parent Westar. The *B. napus* cvs. Westar and Polo flowered within 43- 50 days, while *B. carinata* required about 70 days to flower. The F_1 and backcross generation populations flowered within a range of 44-50 days, similar to the *B. napus* parents.

Silique length and number of seeds/silique for different generation populations are presented in Table 4.3. The average silique length of *B. carinata* was about 40 mm with about 17 seeds/silique. The recurrent parent Westar had significantly larger size silique (about 59 mm) with a greater number of seeds/silique (about 32 seeds/ silique). The average silique length of the F_1 plants was about 15 mm, producing only 0.02-0.03 seeds/ silique. However, the length of silique and number seeds per silique increased progressively with the increasing number of backcrosses to Westar. The average length of silique in the BC_3 generation increased more than 3-fold compared to the F_1 generation.

Similarly, the number of seeds per silique in the BC_3 generation increased about 30-fold compared to F_1. However, the length of a silique in the BC_3 generation was still significantly lower than the recurrent parent Westar, and none of the plants produced number seeds/silique similar to this *B. napus* genotype.

Table 4.1 Leaf morphology of *Brassica carinata* and *B. napus* cvs. Westar and Polo and their F_1 hybrids.

Genotype	Lamina shape	Development of lobes	Shape of margin	Apex shape
B. carinata	Orbicular	Medium	Rounded teeth	Intermediate
Westar	Orbicular	Strong	Undulating	Round
Polo	Orbicular	Very weak	Undulating	Round
F_1 (B.c x W)	Wide elliptic	Strong	Undulating	Round
F_1 (P x B.c)	Orbicular	Medium	Undulating	Round

B.c = *B. carinata* , W= *B. napus* cv. Westar, P = *B. napus* cv. Polo

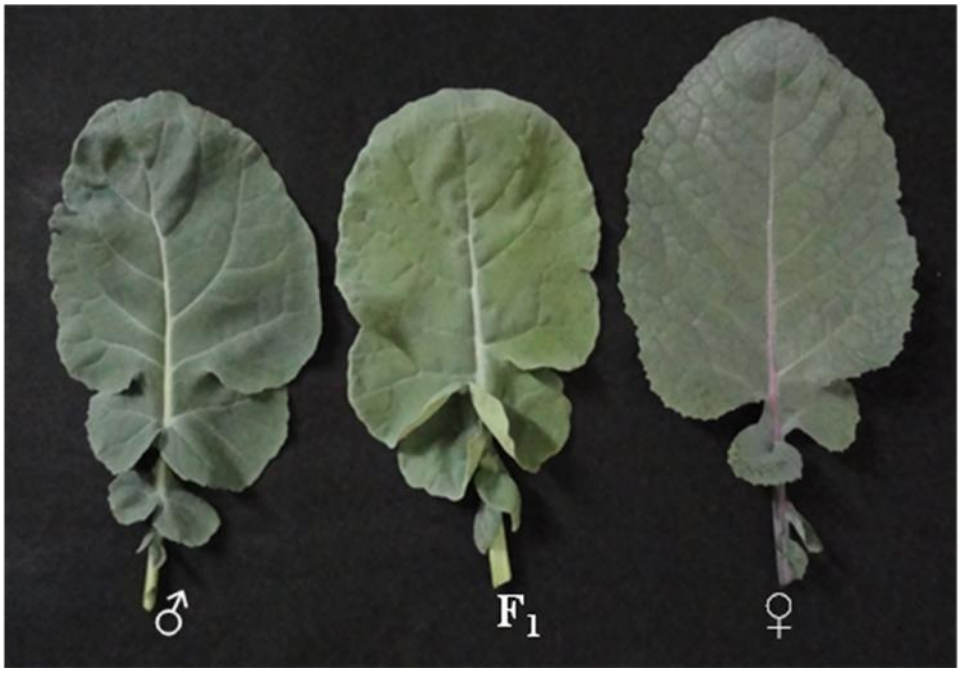

Fig 4.1 Leaf of *Brassica napus* cv. Westar (♂ parent, left), *B. carinata* (♀ parent, right) and the F_1 hybrid (centre)

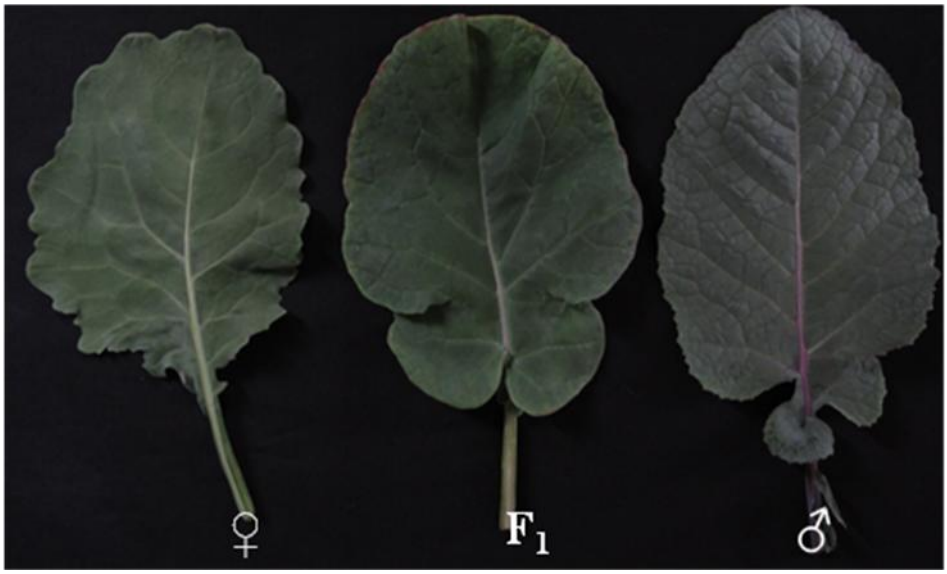

Fig. 4.2 Leaf of *Brassica napus* cv. Polo (♀ parent, left), *B. carinata* (♂ parent, right) and the F_1 hybrid (centre)

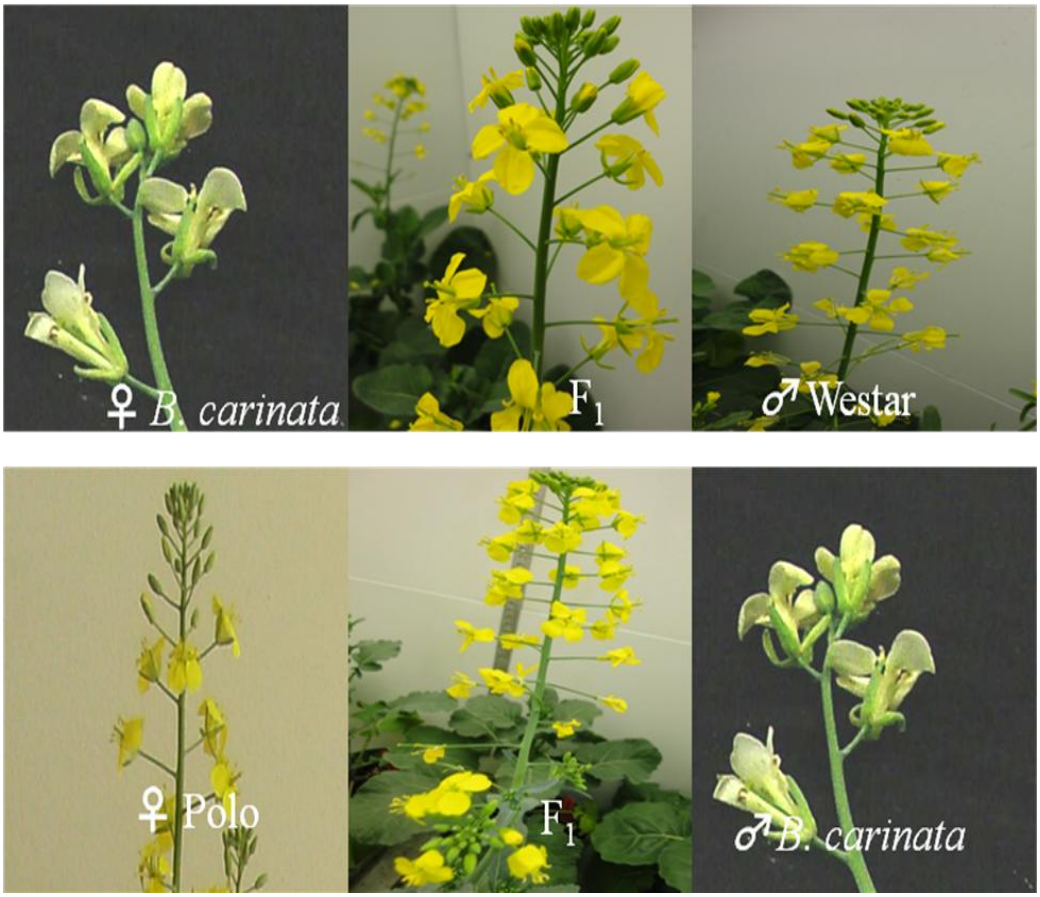

Fig. 4.3 Flower of the F_1 plant of *B. carinata* x *B. napus* cv. Westar and *B. napus* cv. Polo x *B. carinata* interspecific crosses

Table 4.2 Agronomic traits of the parents, F_1 and backcross generation populations of *B. carinata* x *B. napus***.**

Generation[1]	No. of plants	Days to germination		No. of plants	Days to flower	
		Range	Mean		Range	Mean
F_1 (B.c x W)	1	3	3.0a	5	48-50	48.6b
Westar	5	3-4	3.2a	5	44-49	47.0b
B. carinata	5	3-5	3.4a	5	69-73	71.60a
F_1 (P x B.c)	20	3-4	3.2b	10	45-48	46.6b
Polo	5	2-4	3.0b	5	45-49	46.6b
B. carinata	5	3-5	4.2a	5	70-75	71.6a
BC_1 (B.c x W)	20	3-4	3.2b	10	44-48	47.1b
Westar	5	2-4	3.0b	5	46-49	47.8b
B. carinata	5	3-5	3.6a	5	69-74	71.8a
BC_1 (P x B.c)	9	3-5	4.0b	9	44-49	45.0b
Westar	5	2-5	3.4b	5	43-48	46.6b
B. carinata	5	3-5	4.6a	5	70-75	71.4a
BC_2 (B.c x W)	50	2-3	2.8b	20	44-47	45.4b
Westar	5	2-5	3.4b	5	44-47	45.6b
B. carinata	5	3-5	4.4a	5	70-74	72.0a
BC_2 (P x B.c)	50	3-5	4.0b	20	40-48	45.0b
Westar	5	2-4	3.4b	5	43-48	46.2b
B. carinata	5	3-5	4.6a	5	70-77	72.4a
BC_2S_1 (B.c x W)	50	2-4	2.8b	20	45-47	46.0b
Westar	5	2-4	3.4b	5	44-47	45.4b
B. carinata	5	3-5	3.8a	5	69-74	71.8a
BC_2S_1 (P x B.c)	50	3-5	4.0b	20	44-49	45.3b
Westar	5	2-4	3.4b	5	45-49	46.6b
B. carinata	5	3-5	4.6a	5	70-75	72.0a
BC_3 (B.c x W)	50	2-3	2.8b	20	44-49	46.3b
Westar	5	2-4	3.0b	5	44-50	45.8b
B. carinata	5	3-5	4.4a	5	70-74	71.4a
BC_3 (P x B.c)	50	3-5	4.0b	20	44-49	45.0b
Westar	5	2-4	3.4b	5	45-49	46.6b
B. carinata	5	3-5	4.6a	5	70-75	71.6a

[1]Parentage of the F_1 is given in brackets.

In recurrent backcrossing, the F_1 plants were used as female and *B. napus* cv. Westar was used as male.

B.c = *B. carinata* , W= *B. napus* cv. Westar, P = *B. napus* cv. Polo

Within the group, mean values followed by same letter are not significantly different (Duncan Multiple Range Test, $P \leq 0.05$)

Table 4.3 Silique length of the parents, F_1 and backcrossing generation populations of *B. napus* x *B. carinata* interspecific crosses.

Generation[1]	No. plants	Silique length (mm)		No. seeds / silique	
		Range	Mean	Range	Mean
F_1 (B.c x W)	12	14.3-16.5	15.08c	0.0-0.3	0.02c
Westar	5	53.6-58.0	55.52a	31.3-33.7	32.60a
B. carinata	5	39.7-41.3	40.44b	15.5-20.5	17.80b
F_1 (P x B.c)	11	14.5-16.0	15.24 c	0.0-0.8	0.30c
Westar	5	53.6-56.5	54.56 a	30.5-33.8	31.76a
B. carinata	5	40.8-41.5	40.52b	16.3-20.2	17.68b
BC_1 (B.c x W)	20	20.5-32.5	30.98c	1.5-4.7	3.32c
Westar	5	53.7-57.3	55.28a	29.4-30.7	30.40a
B. carinata	5	39.4-41.1	40.20b	17.3-19.2	18.20b
BC_1 (P x B.c)	9	20.5-32.5	27.28c	0.7-1.7	1.28c
Westar	5	53.7-58.5	58.20a	31.2-33.0	32.20a
B. carinata	5	39.0-41.2	39.84b	14.6-19.0	16.76b
BC_2 (B.c x W)	15	51.0-57.8	52.54b	5.011.4	7.74c
Westar	5	53.7-58.5	56.44a	31.1-33.0	32.34a
B. carinata	5	38.2-40.3	38.92c	15.8-19.9	17.70b
BC_2 (P x B.c)	19	34.0-53.8	47.52b	3.7-12.0	6.18c
Westar	5	59.3-64.9	62.32a	30.6-34.5	32.06a
B. carinata	5	39.9-40.9	40.24c	16.7-18.8	17.68b
BC_2S_1 (B.c x W)	10	45.2-50.8	48.20b	0.0-7.7	2.98c
Westar	5	58.8-67.7	64.50a	29.8-33.1	31.84a
B. carinata	5	36.6-39.4	38.50c	16.9-18.5	17.70b
BC_2S_1 (P x B.c)	11	38.8-51.3	47.42b	0.0-10.4	3.36c
Westar	5	60.1-63.3	62.30a	31.4-34.2	32.80a
B. carinata	5	40.0-41.2	40.70c	15.8-19.6	17.86b
BC_3 (B.c x W)	10	49.4-64.0	59.24a	2.5-15.0	8.14c
Westar	5	57.0-62.2	60.30a	31.8-33.2	32.56a
B. carinata	5	42.2-41.9	41.24b	16.0-19.1	17.70b
BC_3 (P x B.c)	6	48.8-52.3	51.20b	2.6-10.6	6.64c
Westar	5	56.8-61.2	59.50a	31.4-33.1	32.30a
B. carinata	5	41.0-42.0	41.60c	18.2-19.1	18.60b

[1]Parentage of the F_1 is given in brackets.

In recurrent backcrossing, the F_1 plants were used as female and *B. napus* cv. Westar was used as male.

B.c = *B. carinata* , W= *B. napus* cv. Westar, P = *B. napus* cv. Polo

Within the group, mean values followed by same letter are not significantly different (Duncan Multiple Range Test, $P \leq 0.05$).

4.3.2 Estimation of pollen fertility in BC_3 and BC_3S_1 plants derived from *B. napus* x *B. carinata* interspecific crosses

Pollen viability and seed set were estimated in BC_3 and BC_2S_1 generation plants and in their parents. The three parents, *B. carinata*, Westar and Polo produced close to 100% viable pollen. However, pollen viability in the BC_3 plants varied from 59% to 79% (Table 4.5) and in the BC_2S_1 population, it varied from 50% to 73% (Table 4.4). In the case of BC_3 plants, there was no significant difference in the pollen viability between the resistant and the susceptible plants (Table 4.5).

A positive correlation between the number seeds/silique and pollen viability was observed in BC_2S_1 plants and this linear trend explained 72% of the total phenotypic variation (Fig. 4.4). A Similar correlation was found in the BC_3 plants. However, the linear model explained only 25% of the total variation (Fig. 4.5). On the other hand, no significant correlation between the number of seeds/silique and the pollen viability was found while backcrossing the BC_3 plants to Westar (Table 4.5, Fig. 4.6)

Table 4.4 Pollen viability and seed set in blackleg resistant BC_2S_1 plants derived from *B. carinata* x *B. napus* interspecific cross

Parentage of F_1	No. BC_2S_1 Family	No. plant	Pollen fertility (%)		BC_2S_2 seeds/silique		
			Range	Mean±SE	No. silique	Range	Mean±SE[a]
B.c x W	6	10	50-73	60.89±2.81	593	0.00-7.67	2.98±0.82
P x B.c	6	11	52-73	63.65±2.32	677	0.00-10.40	3.36± 0.94
B.c x W	Coefficient of correlation between pollen viability and seed set, r = 0.96* (df =9)						
P x B.c	Coefficient of correlation between pollen viability and seed set, r = 0.74* (df =10)						

B.c = *B. carinata*; W= *B. napus* cv. Westar; P = *B. napus* cv. Polo

[a]Calculation was done based on total number of siliques of each plant

*= Significant ($P \leq 0.05$)

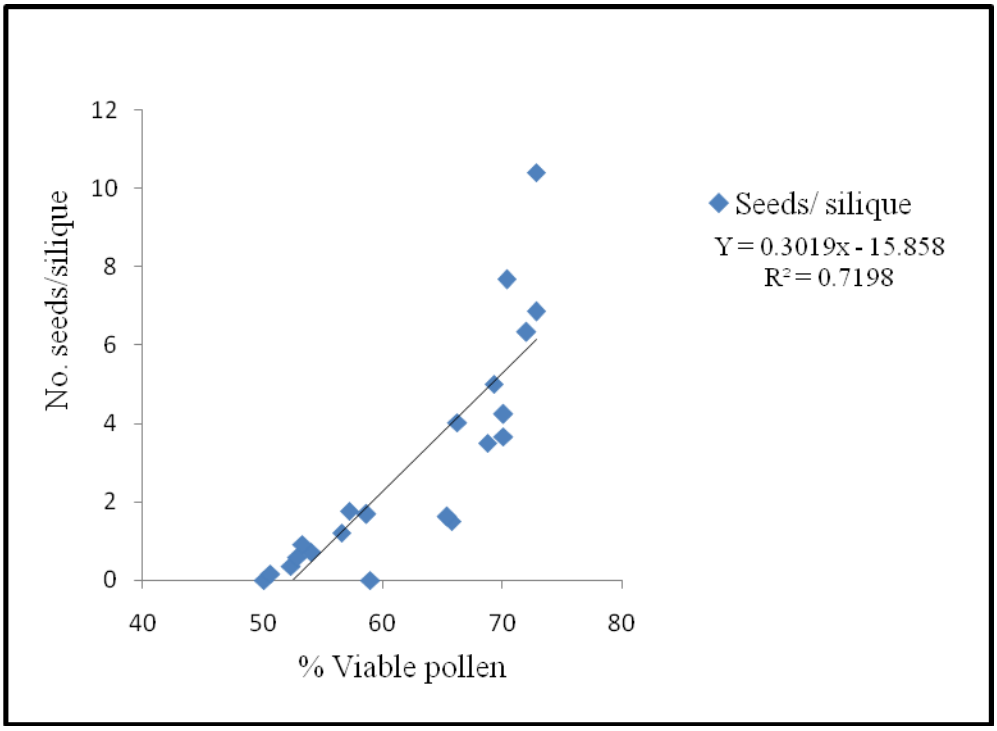

Fig. 4.4 Relationship between pollen viability and seed set on self- pollination under bag isolation in BC_2S_1 plants of a *B. napus* x *B. carinata* interspecific cross

Table 4.5 Pollen viability and seed set in BC_3 plants derived from a *B. napus* x *B. carinata* interspecific cross

Parentage of F_1	Blackleg resistance (R/S)	No. BC_3 family	No. plant	Pollen fertility (%)		Crossed (BC_4) seed/silique			Selfed (BC_3S_1)Seed/silique		
				Range	Mean±SE	No. silique	Range	Mean±SE[a]	No. silique	Range	Mean±SE[a]
B.c x W	R	7	10	66-79	73.11±1.16	109	2.50-15.00	8.14± 1.17	299	0.00-7.57	2.68±0.89
B.c x W	S[1]	9	12	65-74	68.77±0.74		-	-		-	-
P x B.c	R	6	6	71-75	72.83±0.70	67	2.62-10.64	6.64±1.27	163	0.00-1.88	0.93±0.36
P x B.c	S[1]	7	18	59-72	67.40±0.82		-	-		-	-
B.c	R	-	3	97-99	98.08±0.25		-				
W	R	-	3	95-98	96.57±0.39		-	-			
P	R	-	3	93-97	95.79±0.79		-	-			
Coefficient of correlation between pollen viability and BC_4 seed production , r= 0.13ns, df=15											
Coefficient of correlation between pollen viability and BC_3S_1 seed production , r= 0.66*, df=15											

B.c = *B. carinata*; W= *B. napus* cv. Westar; P = *B. napus* cv. Polo
R=Resistant; S= Susceptible at adult plant stage
r= Coefficient of correlation
[a]Calculation was done based on total number of siliques of individual plant
[1] No seed was harvested from susceptible plants
ns= not significant; *= Significant ($P \leq 0.05$)

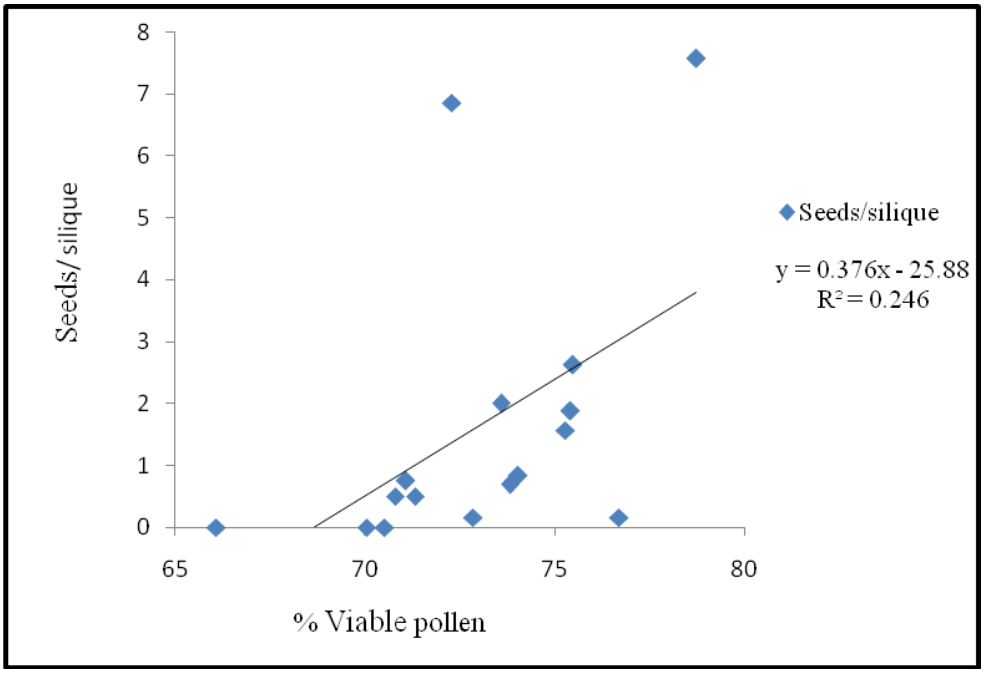

Fig. 4.5 Relationship between pollen viability and seed set on self pollination under bag isolation in BC_3 plants of *B. napus* x *B. carinata* interspecific crosses

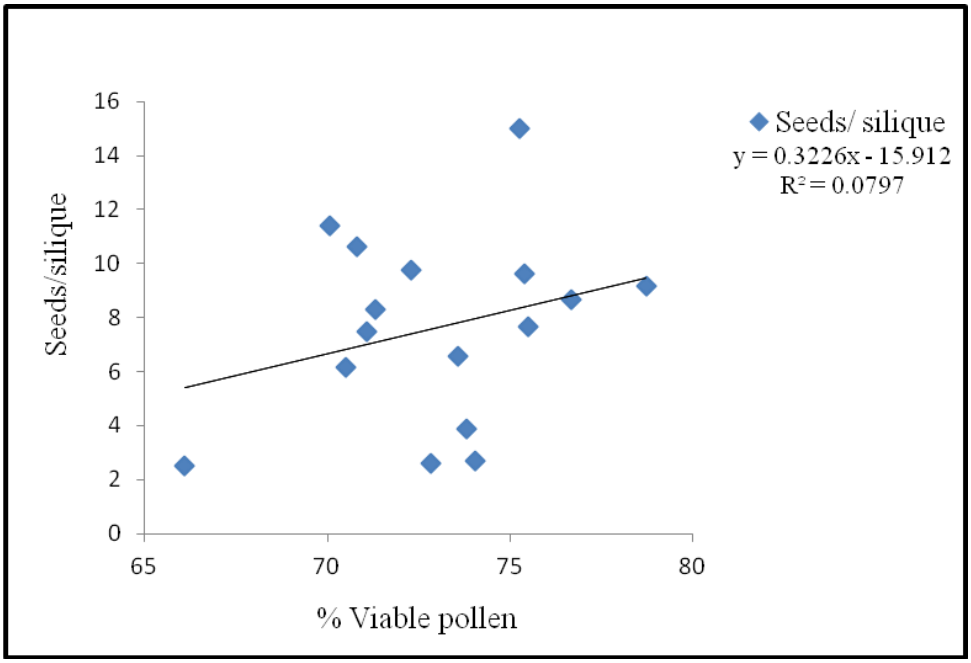

Fig. 4.6 Relationship between pollen viability and seed set on backcrossing of the BC_3 plants of *B. napus* x *B. carinata* interspecific crosses

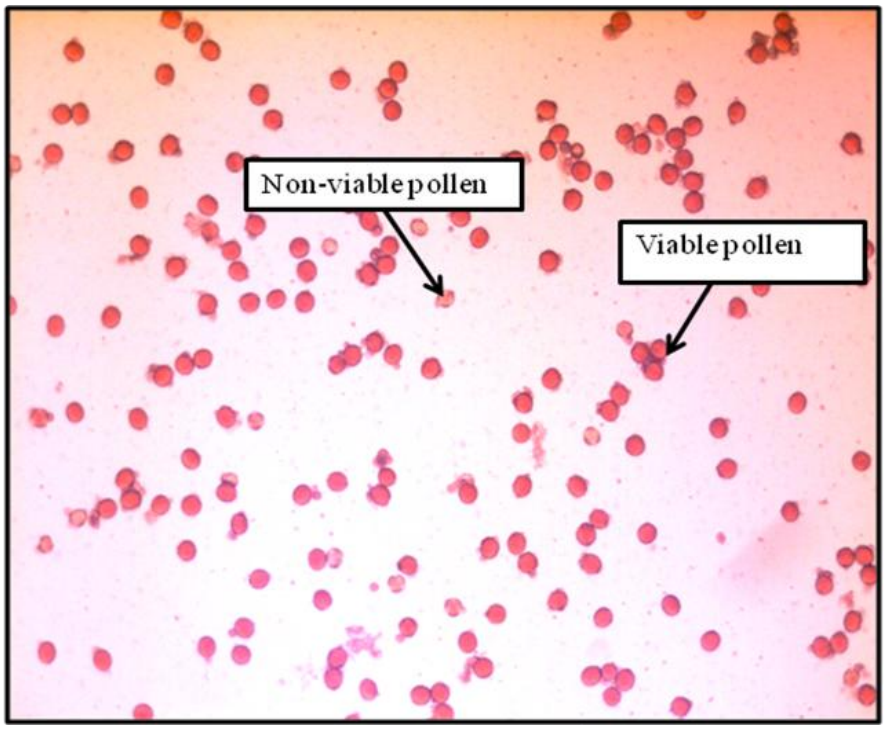

Fig. 4.7 Viable and non-viable pollen in a BC_3 plant of *B. napus* x *B. carinata* interspecific cross

4.3.3 Meiotic behaviour in BC_3 plants

Pollen mother cells (PMC) from two blackleg resistant BC_3 plants of *B. napus* x *B. carinata* were studied for behaviour of the chromosomes in meiosis. These two plants had 72% and 78% viable pollen and produced 0.75 and 7.57 seeds/ silique (BC_3S_1) on self pollination. The behaviour of the chromosomes in meiosis appeared to be normal in both plants in first (Fig. 4.8) and second (Fig. 4.9) meiotic divisions. In both meiotic stages the chromosomes moved normally to the two poles without leaving any laggard in the equitorial plate. However, the exact number of chromosomes in these plants was difficult to establish due to their small size (Fig. 4.8 and Fig. 4.9).

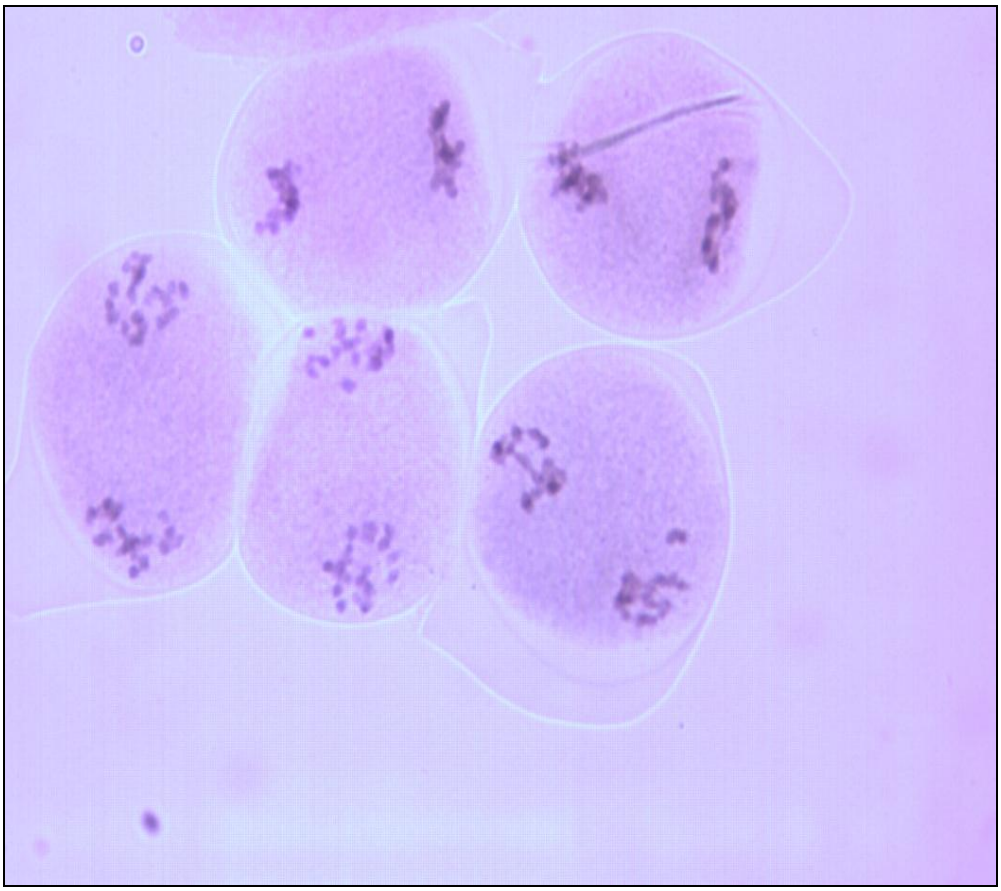

Fig. 4.8 First meiotic division in a BC_3 plant of *B. carinata* x *B. napus* showing normal meiotic orientation of the chromosomes in two poles

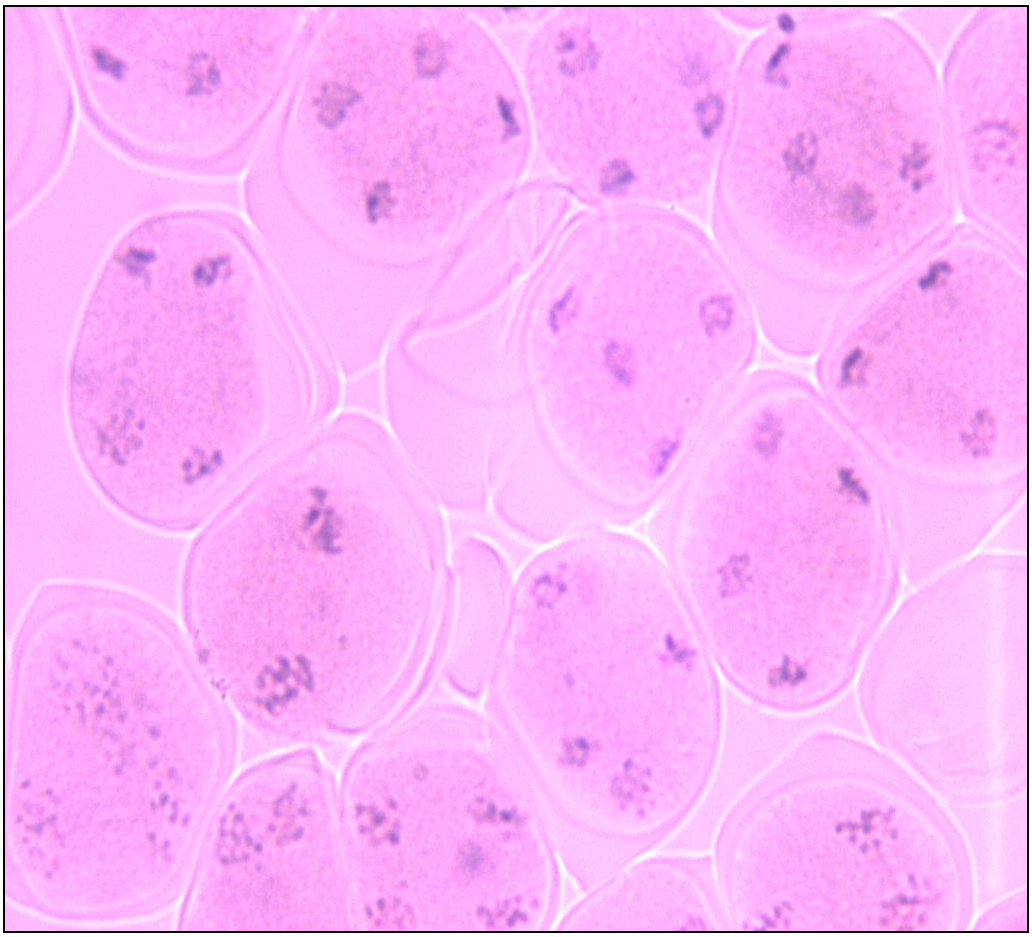

Fig. 4.9 First and second meiotic division in a BC_3 plants of *B. carinata* x *B. napus*

4.4 Discussion

The morphology of the F_1 plants of *B. napus* x *B. carinata* interspecific crosses was different from the parents in all stages of development. The hybrid plants could easily be identified based on petal colour, leaf morphology, plant height, stem thickness etc. Among the morphological characteristics, the petal colour was the most reliable and the easiest character for identifying hybrid plants, especially in the *B. carinata* x *B. napus* cross. The parent *B. carinata* had creamy white petals, while both *B. napus* cvs. Westar and Polo had yellow petals.

All plants derived from *B. carinata* (♀) x *B. napus* (♂) had yellow petals - a definite confirmation of their hybrid nature. All backcross generation plants derived from a recurrent crossing of the interspecific hybrids to Westar had only yellow flowers. This indicates that the yellow petal colour of *B. napus* is dominant over creamy- white colour of *B. carinata*. This is in agreement with Getinet et al. (1993) who reported that petal color in *B. carinata* is under simple Mendelian genetic control where creamy- white petal color is recessive to yellow petal color. Similarly, Rahman (2001) also reported that petal color in *B. rapa* is controlled by a single gene locus, and the homozygous recessive condition result creamy-white petals.

In the present recurrent backcrossing program, the length of silique and number seeds/silique was increasing with the progression of backcrossing. This indicates that recurrent backcross was leading the interspecific population towards the *B. napus* type plant, as expected. However, all BC_1, BC_2, BC_2S_1 and BC_3 plants produced significantly lower numbers of seeds/silique compared to the *B. napus* parent. This indicates that the plants, even in the BC_3 generation, were still aneuploids. Navabi et al. (2010) found that doubled haploid (DH) lines derived from a BC_2S_3 generation of (*B. napus* x *B. carinata*) x *B. napus* can still carry either intact or partly deleted B genome chromosomes. The DH lines carrying extra B genome chromosomes often show poor seed set compared to euploid DH lines (Navabi et al. 2010, Fredua-Agyeman, Canola Breeding Program, University of Alberta, personal communication). Ripley and Beversdorf (2003) reported a lower seed set in segregating backcross generation population of

(*B. napus* x *B. oleracea*) x *B. napus*, and explained that aneuploidy may have caused reduced male and/or female fertility and consequently poor seed set in the plants.

Pollen viability in the BC_3 population was slightly higher than in BC_2S_1. This might be due to the result of an additional generation of backcrossing of the interspecific hybrids to the recurrent parent Westar. McGrath and Quiros (1990) also reported higher pollen fertility in backcross population than in the F_1 of a *B. napus* x *B. rapa* interspecific cross. However, in both the BC_3 and BC_2S_1 populations, pollen viability was significantly lower than in the parents, indicating that the plants are still aneuploid. Lower pollen viability due to aberrant meiosis has been reported by Elling et al. (2010) in the aneuploid plants of *B. napus* x *B. rapa* interspecific crosses. Similarly, Ayotte et al. (1988) also reported low pollen fertility (mean 67.24%) in BC_1 hybrids of a (*B. napus* x *B. oleracea*) x *B. oleracea* cross. According to Plama-Silva et al. (2004), high pollen viability reflects regular meiosis in a genotype.

Although a significant correlation between pollen viability and self-pollinated seed set was found in the BC_3 and BC_2S_1 generations, the number seeds/silique in these generations were significantly less than in *B. napus.* This is apparently due to the aneuploid nature of the plants from these generations. In the interspecific populations, a strong correlation between pollen viability and seed set does not necessarily reflect the euploid nature of the plant. The poor correlation between the number of backcrossed seeds/silique and pollen fertility in BC_3 plants might be due to higher viability of the aneuploid gamets in the female

side than in the male side. In this case, pollen viability estimation was based on the male gametes of the BC_3 plants; while the backcrossed seeds are the result of the union of the female gametes of the BC_3 plants and male gametes of *B. napus* cv. Westar.

In this present study, silique and seed set data indicate that the blackleg resistant backcross derived plants are still aneuploids carrying the B genome chromosome(s) of *B. carinata*. Further backcrossing of the resistant plants would be needed for stable introgression of the resistance gene(s) from the B genome of *B. carinata* into *B. napus*. This further strengthens the results of earlier researchers (as reviewed in Chapter 1) that the *Brassica* B genome is very distinct from the A and the C genome; and introgression of a trait from this genome into *B. napus* is a challenging task.

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Chapter 5

General Discussion and Conclusions

5.1 General discussion

Yield loss in canola due to blackleg disease, caused by *Leptosphaeria maculans*, is one of the major challenges to the growers. This disease can reduce seed yield by more than 50% (Gugel and Petrie 1992); however, any economically viable cultural practices for the control of this disease have not been reported. Breeding of resistant cultivars is considered an effective strategy for the control of this disease (Delourme et al. 2006), and this has been proved to be safe for the environment. A number of *B. napus* genotypes resistant to blackleg disease have been identified and extensively utilized in the breeding programs for the development of blackleg resistant cultivars. However, breakdown of resistance has been reported in Canada, Europe and Australia due to the evolution of new pathotypes. Therefore, the development of canola cultivars resistant to these newly evolving races is needed. As reported in Chapter 1, among the Brassica species, the species carrying the B genome, viz. *B. nigra, B. carinata* and *B. juncea*, generally show high resistance to blackleg disease and carry other valuable agronomic traits. Efforts have been made by several researchers to introgress B genome blackleg resistance, especially from *B. juncea* into *B. napus*; however, very limited efforts have been made so far to utilize the blackleg resistance of *B. carinata*. Therefore, the focus of this research was to introgress the blackleg resistance of *B. carinata* into *B. napus* for the development of germplasm resistant to the newly evolving blackleg pathotypes.

In these *B. napus* x *B. carinata* interspecific crosses, the application of the *in vitro* ovule culture technique greatly facilitated the production of interspecific F_1 and BC_1 hybrid plants. This supports the results of Bennett et al. (2008), Diederichsen and Sacristan (1996), and Takeshita et al. (1980) that this technique increases the efficiency of interspecific hybrid production in Brassicas. Furthermore, in this research project NN liquid medium and B5 solid medium were evaluated for producing BC_1 hybrids, where the NN liquid medium was more efficient than the B5 solid medium.

The F_1 and the backcross generation populations obtained through *in vivo* produced seed were evaluated against the PG4 type isolate 290CDN for cotyledon and adult plant resistance. Based on segregation for cotyledon resistance in the BC_1 generation, it was difficult to conclude whether a single dominant gene or more than one single gene controlled this trait in *B. carinata*. This is primarily because of the small size of population used in this study, which is associated with the difficulty of producing a large number of BC_1 hybrids in this interspecific cross. Chèvre et al. (1997) reported monogenic control of cotyledon resistance in *B. juncea*, while Delwiche (1980) reported that this trait is controlled by two gene loci in *B. napus*. Furthermore, I found that, a significant number of cotyledon resistant BC_1 seedlings became susceptible at adult plant stage, and loss of this adult plant resistance also continued in advanced backcross generation populations. These results suggest that cotyledon and adult plant resistance in *B. carinata* is under different genetic control. Elimination of *B. carinata* chromosome(s) carrying the adult plant resistance during recurrent backcrossing

accompanied with selection for cotyledon resistance might be the reason for this loss of adult plant resistance. Sacristan and Gerdmann (1986) also reported the loss of adult plant resistance in BC_2 generations while backcrossing the *B. carinata* x *B. napus* hybrids to the *B. napus* parent. Similarly, Pang and Halloran (1996) reported that cotyledon resistance and adult plant resistance in *B. napus* to be under different genetic control.

Pollen viability and meiosis was investigated in some of the BC_2S_1 and BC_3 plants. Pollen viability in BC_2S_1 and BC_3 was significantly lower compared to the parents. In addition, seed set in these backcross generation plants was also significantly lower than the recurrent parent *B. napus* cv. Westar. These results suggested that the blackleg resistant plants obtained in the present study are still aneuploid and carrying extra chromosome(s) of *B. carinata*. Reduced pollen fertility sometimes associates with laggard chromosomes (Sheidai et al. 2006); however, no laggard chromosome was found in meiosis of the BC_3 plants. Significant correlation was found between pollen fertility and seed set in self-pollinated backcross generation plants. Therefore, a selection of self-pollinated backcross generated plants for pollen fertility similar to the *B. napus* recurrent parent may be used for the identification euploid (AACC, 2n=38) plants.

5.2 Conclusions

In this research, the materials that I have developed through interspecific crosses followed by recurrent backcrossing lay the foundation for introgression of blackleg resistance from *B. carinata* into *B. napus* for the development of

blackleg resistant canola cultivars. The novel findings from my research are listed below:

- Ovule culture in NN liquid medium is more efficient than in B5 solid medium.
- Seed set in interspecific hybrids, especially in early generations, e.g. F_1 and BC_1, is affected by the type of cytoplasm in the maternal plant.
- The cotyledon and adult plant resistance in *B. carinata* to the *L. maculans* isolate 290CDN is under different genetic control.

5.3 Future research

Seed set and fertility of the blackleg resistant plants indicate that these plants are still to be aneuploid and carrying extra or large segments of B genome chromosome(s). Therefore, for future research with this material, I would recommend the cytological characterization of the resistant plants for chromosome number and the behaviour of the chromosomes in meiosis. Backcrossing and self-pollination of the resistant plants would be needed for stable introgression of the B genome resistance in *B. napus* background. Finally, the development of molecular marker(s) for this resistance gene(s) and use in marker assisted selection would help canola breeders and researchers to develop canola cultivars with resistance to multiple races of the blackleg pathogen.

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Appendix A.

Table A-1. Microsatellite markers used for the detection of polymorphism in the parental lines *B. carinata*, *B. napus* cvs. Westar, Polo and in the *B. napus* x *B. carinata* interspecific hybrids

Primer number	Primer name	*B. juncea* linkage group	Expected product size	Genome
505	sJ0644	J11	438-451	B genome
508	sJ6846	J12	319-372	B genome
513	sB4817R	J12	250-364	B genome
515	sB1822	J13	250-267	B genome
517	sJ7046	J13	277-287	B genome
518	sB1990F	J13	491-498	B genome
520	sB2131	J14	311-321	B genome
526	sB0202I	J15	108-187	B genome
532	sB31138	J16	188-199	B genome
533	sJ7104	J16	322-337	B genome
534	sJ0338	J16	307-341	B genome
536	sJ3640I	J16	327-346	B genome
542	sB1937	J17	262-279	B genome
544	sB1728	J18	445-490	B genome

Source: Agriculture and Agri-Food Canada (AAFC)

Appendix B.

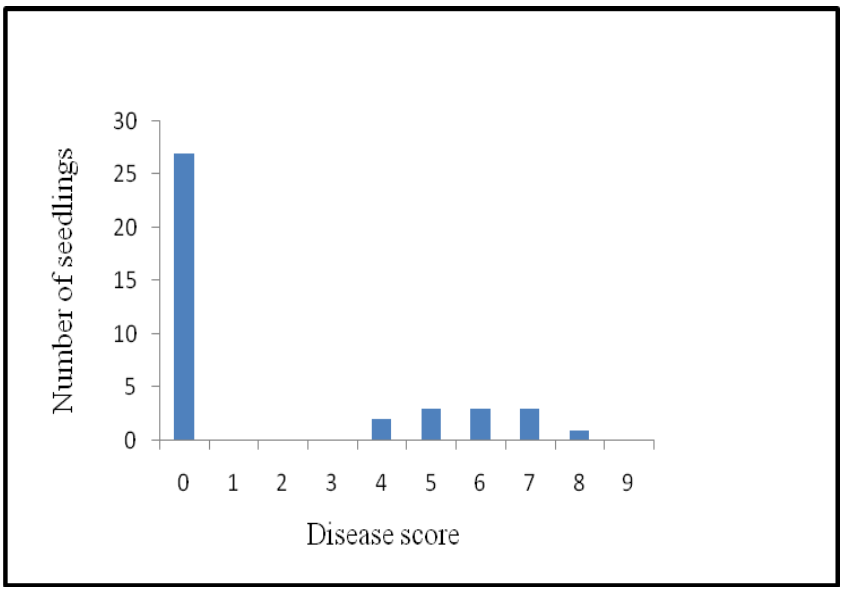

Fig. B1 Disease severity at cotyledon stage in BC_1 plants of *B. carinata* (♀) x *B. napus* (♂) interspecific cross

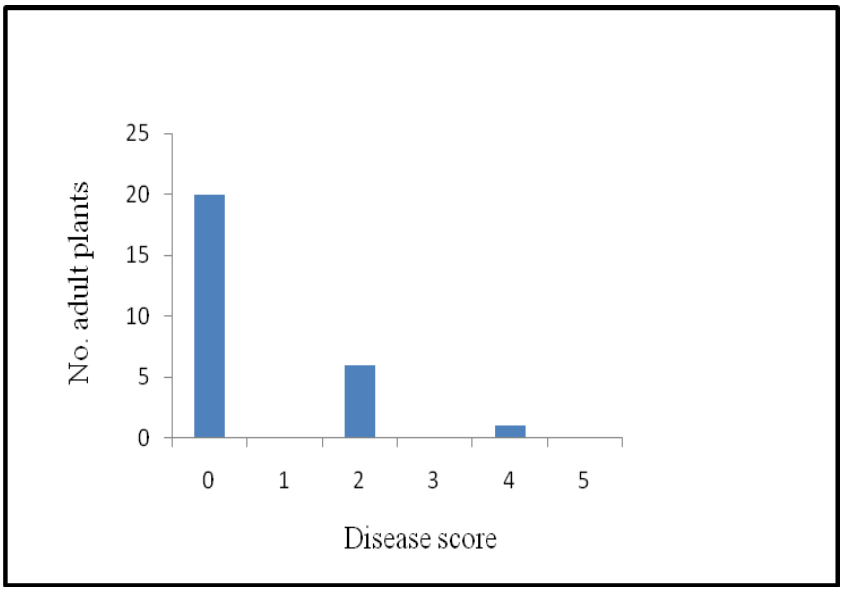

Fig. B2 Disease severity at adult stage in BC_1 plants of *B. carinata* (♀) x *B. napus* (♂) interspecific cross

Appendix C.

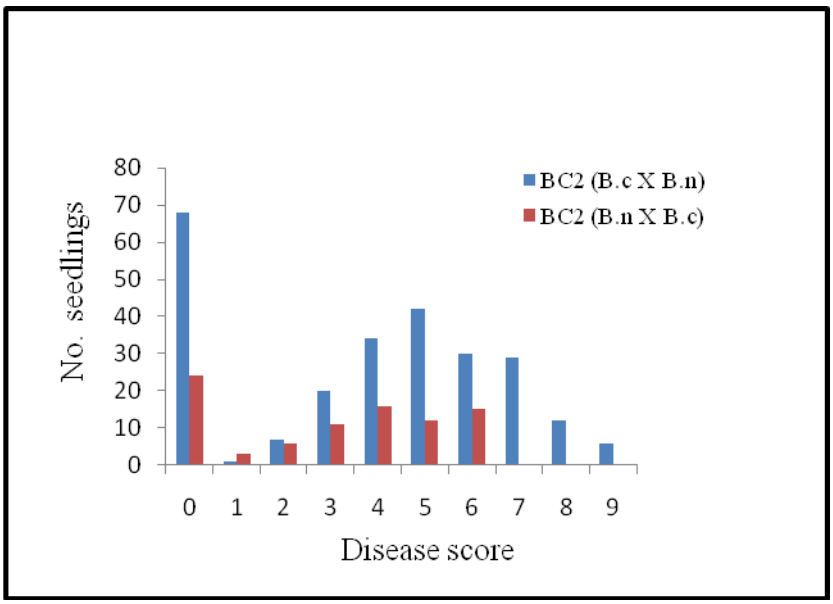

Fig. C1 Disease severity at cotyledon stage in BC_2 plants of *B. napus* x *B. carinata* interspecific cross; Parentage of the F_1 is given in brackets. B.c = *B. carinata*; B.n= *B. napus*

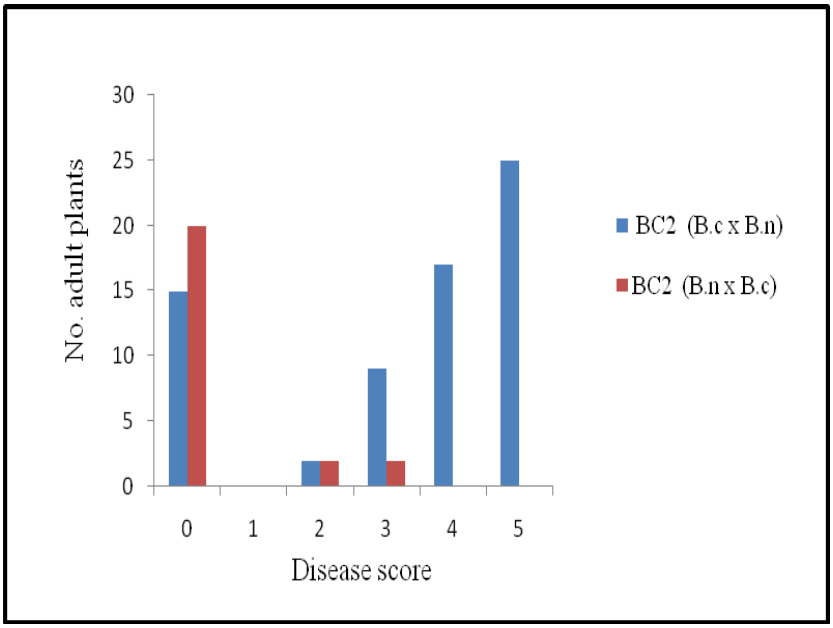

Fig. C2 Disease severity at adult stage in BC_2 plants of *B. napus* x *B. carinata* interspecific cross; Parentage of the F_1 is given in brackets. B.c = *B. carinata*; B.n= *B. napus*

Appendix D.

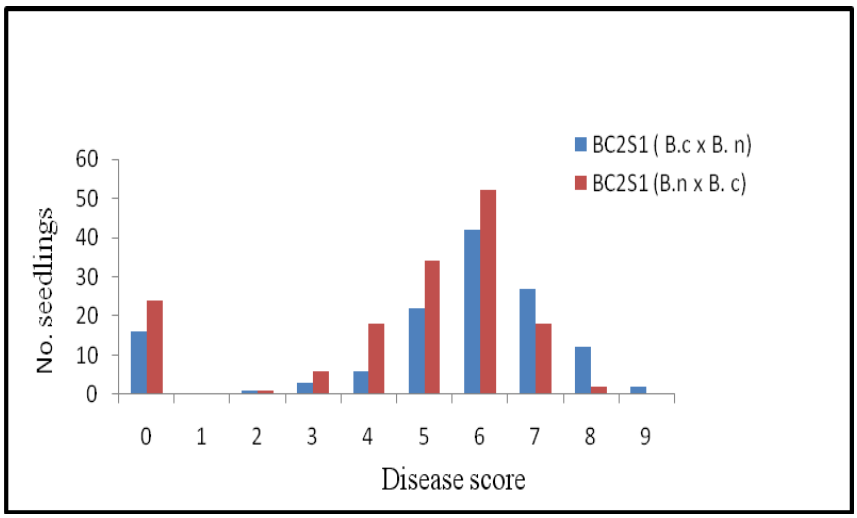

Fig. D1 Disease severity at cotyledon stage in BC_2S_1 plants of *B. napus* x *B. carinata* interspecific cross; Parentage of the F_1 is given in brackets.
B.c = *B. carinata*; B.n= *B. napus*

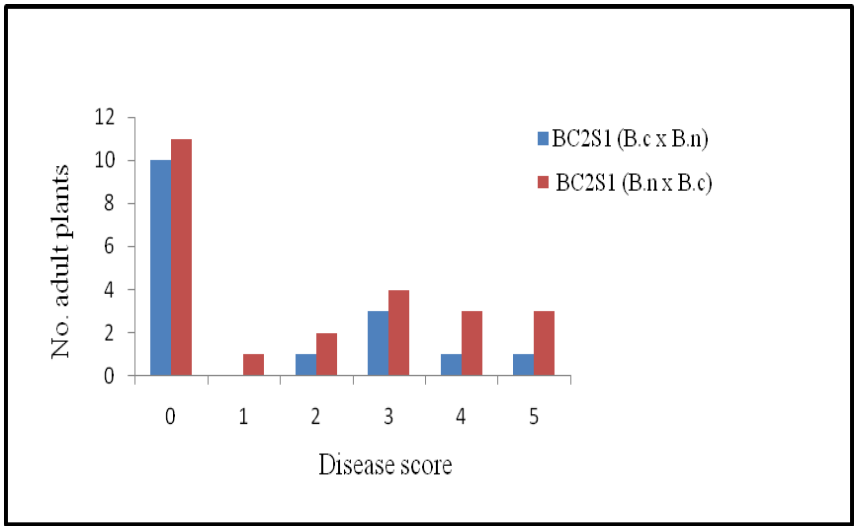

Fig. D2 Disease severity at adult stage in BC_2S_1 plants of *B. napus* x *B. carinata* interspecific cross; Parentage of the F_1 is given in brackets.
B.c = *B. carinata*; B.n= *B. napus*

Appendix E.

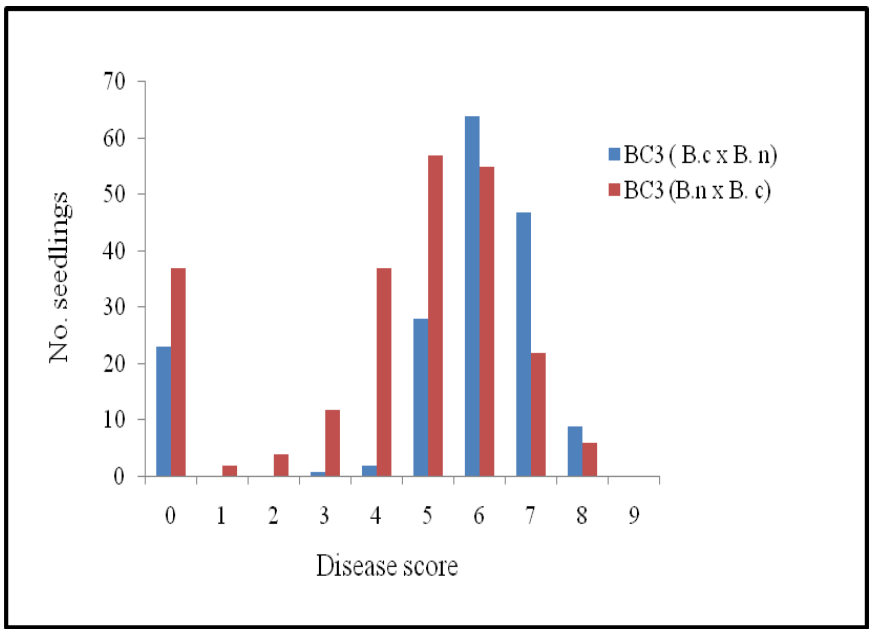

Fig. E1 Disease severity at cotyledon stage in BC_3 plants of *B. napus* x *B. carinata* interspecific cross; Parentage of the F_1 is given in brackets.
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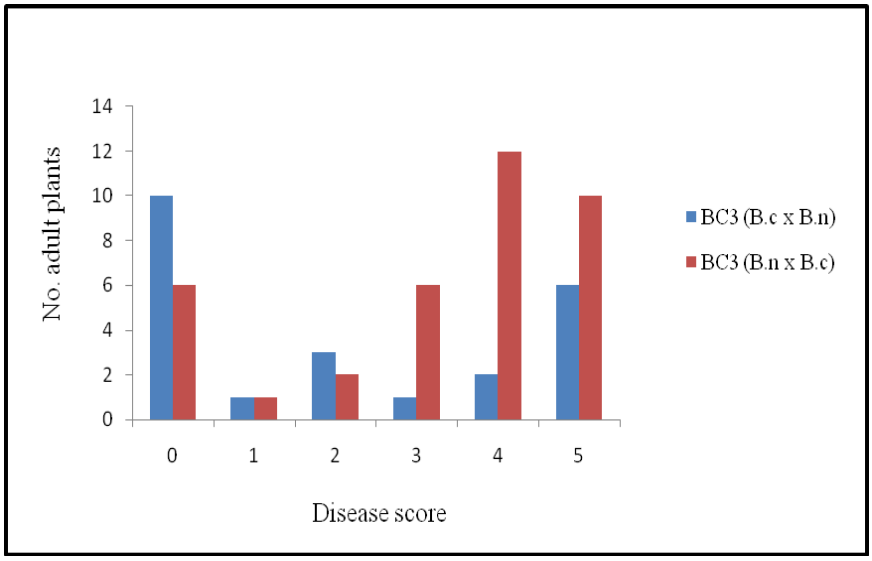

Fig. E2 Disease severity at adult stage in BC_3 plants of *B. napus* x *B. carinata* interspecific cross; Parentage of the F_1 is given in brackets.
B.c = *B. carinata*; B.n= *B. napus*