Introgression of clubroot resistance from *B. oleracea* into *B. napus* and study the inheritance of the resistance

by

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Abstract

Clubroot disease, caused by *Plasmodiophora brassicae*, is one of the most devastating threats to *Brassica napus* canola production worldwide. Growing clubroot-resistant cultivars is considered the most efficient way of managing this disease. All available clubroot-resistant canola cultivars carry resistance in their A-genome. Some of the qualitative resistances located in the A genome of canola became ineffective after growing only for a few years due to the evolution of new *P. brassicae* pathotypes. This highlighted the need of introducing the quantitative resistance from *Brassica oleracea* into the C genome of *B. napus* canola. The objective of this thesis research was to investigate the prospect of developing clubroot-resistant canola quality spring growth habit *B. napus* plants carrying resistance in the C genome through *B. napus* × *B. oleracea* interspecific cross. In addition to this, genetic analysis of clubroot resistance was carried out using canola lines from the canola breeding program to investigate the prospect of developing non-GM (non genetically modified) clubroot resistant canola.

This thesis research demonstrated that clubroot resistant euploid *B. napus* ($2n = 38$) lines carrying resistance in the C genome can be achieved in advanced generation population of the *B. napus* × *B. oleracea* interspecific cross. Mapping of this resistance identified four QTL located on the C genome chromosomes C04 (two loci), C05 and C08. Agronomic and seed quality analysis of the population showed that clubroot resistant *B. napus* lines that flower a day earlier than the *B. napus* parent and contain low erucic acid in seed oil and low glucosinolate in seed meal can be achieved from this population. The average oil and protein contents of the advanced generation population was low; however, these traits can be improved through breeding. Segregation for clubroot resistance in the F_2 and backcross ($B_1 = F_1 \times$ Resistant parent; $B_2 = F_1 \times$ Susceptible parent) populations derived from clubroot resistant × susceptible canola crosses

showed that a major locus involved in the control of resistance in these populations. This simpler genetic control of clubroot resistance suggested that clubroot resistant non-GM canola lines can be developed from these populations without facing much difficulty. Thus, the results from this thesis research demonstrated the feasibility of the development of clubroot-resistant spring growth habit *B. napus* lines carrying clubroot resistance in the C genome, and disclosed the inheritance of clubroot resistance in three canola populations. The knowledge and materials developed from this thesis research can be used in breeding clubroot resistant canola germplasm/cultivars.

Preface

This thesis is an original work by Minchien Tsai for the degree of Master of Science. Minchien Tsai carried out all the experiments, collected and analyzed data, wrote the first draft of all chapters, and incorporated the comments, and suggestions from her supervisor Dr. Habibur Rahman and mentor Dr. Aleya Ferdausi. Additional suggestions from the committee member Dr. Linda Gorim and arm-length examiner Dr. Jocelyn Hall were also incorporated in the final version of the thesis.

For chapter 2, Minchien conducted evaluation for clubroot resistance in greenhouse and fields, collected phenotyping data, and carried out seed quality and ploidy analysis. Dr. Muhammad Jakir Hasan and Dr. Junye Jiang provided training on clubroot resistance tests. An Vo provided training on ploidy analysis and helped in analysis of seed quality traits. Salvador Lopez and Hysent Nikang assisted in field trials. The interspecific cross population used in this experiment were developed by Zhongyang Zhang in the Canola program of the University of Alberta.

For chapter 3, Minchien carried out DNA extraction, genotyping, data collection, molecular analysis and figure visualization. Dr. Muhammad Jakir Hasan and Zhongyang Zhang provided the SSR markers. Dr. Berisso Kebede and Dr. Aleya Ferdausi provided training on molecular work and SSR marker analysis. Dr. Berisso Kebede also helped in QTL mapping analysis. For chapter 4, Minchien developed the segregating populations in greenhouse and growth chamber, performed clubroot resistance test and conducted segregating analysis. Dr. Berisso Kebede assisted with clubroot resistance test.

In addition, Dr. Habibur Rahman provided guidance and valuable suggestions on all research plans.

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This thesis can be completed, I would like to express the deepest gratitude to my supervisor Dr. Habibur Rahman for his guidance and funding support through the whole research work. I would also like to thank Dr. Aleya Ferdausi for her guidance and valuable suggestions to my thesis.

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In addition, I would like to acknowledge the continuous support from my parents, Chinchih Tsai and Yuyi Liu, my sister, Yulin Tsai, and my partner, Yash Toshniwal. If without them, I wouldn't be able to complete my Master's program.

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List of Abbreviations

2n	Diploid number of chromosomes
µl	Microlitre
µmol	Micromole
µmol/g	Micromole per gram
ABI	Applied Biosystems
AFLP	Amplified fragment length polymorphism
BC1	First backcross generation
BC1Fx	xth generation of BC1-derived population
bp	Base pair
CCD	Canadian Clubroot Differentials
cm	Centimeter
cM	Centi-Morgan
CR	Clubroot resistance
cv.	Cultivar
DAI	Days after inoculation
DNA	Deoxyribose nucleic acid
dNTP	Deoxynucleotide triphosphate
DSI	Disease severity index
ECD	European Clubroot Differentials
F1	First generation
Fx	xth generation of F2-derived population
g	Gram
GM	Genetically modified
Hi-Di	Highly Deionized Formamide
ICIM	Inclusive Composite Interval Mapping
LOD	Logarithm of odds
Mb	Mega base
mM	Millimole
ml	Millilitre
min	Minute
ng	Nanogram
ng/µl	Nanogram per microliter
p	Probability value
PCR	Polymerase chain reaction
QTL	Quantitative trait loci

R	R project for statistical computing
RAPD	Random amplified polymorphism DNA
RFLP	Restriction fragment length polymorphism
S.E.	Standard Error
SSR	Simple sequence repeat
SMA	Single Marker Analysis
USDA	United States Department of Agriculture
var.	Variety

Chapter 1 Literature Review

1.1 The biology of Canola and its economic value

Canola or rapeseed (*Brassica napus* L.) (AACC, 2*n* = 38) is a member of the Brassicaceae family. This is an amphidiploid species that evolved through interspecific hybridization between its diploid progenitor species *Brassica rapa* L. (AA, 2*n* = 20) and *Brassica oleracea* L. (CC, 2*n* = 18) (Morinaga, 1934). *Brassica napus* is cultivated worldwide for its oil-rich seed, comprising nearly 45% oil (Canola Council of Canada, 2019). The seed oil of wildtype *B. napus* contain a high content of erucic acid (>40% of the total fatty acids), which is undesirable for human nutrition (Knutsen et al., 2016). The seed meal of this crop contains a high level of glucosinolates (>100 μmol glucosinolates/g seed meal), which restricts the use of this protein-rich meal in animal feed (Velayudhan et al., 2017; Alexander et al., 2008). Therefore, Canadian breeders developed *B. napus* cultivars with low contents of erucic acid and glucosinolates during 1960s and 1970s, and labelled them with "Can. O., L. A." (Canadian oilseed, low acid) (Jahreis and Schäfer, 2011; Eskin et al., 2020). The name "Canola" is, therefore, established for branding cultivars carrying these improved traits.

Canola oil contains 6-7% saturated fatty acids (SFAs), 59-64% monounsaturated fatty acids (MUFAs) and 28-32% polyunsaturated fatty acids (PUFAs) (Zambiazi et al., 2007; Jahreis and Schäfer, 2011; for review, see Eskin et al., 2020). Canola oil with a low level of SFAs is considered as a healthy oil as compared to other commodity oils such as soybean (14.9%) and sunflower oils (11.3%) (Eskin et al., 2020). The PUFA content of canola oil is composed of linoleic acid (21-22%) and α-linolenic acid (10-11%). The human body is not able to generate these two fatty acids; therefore, they are considered essential fatty acids in human nutrition, and they must be obtained from food (Di Pasquale, 2009). Canola oil, therefore, can be an important

source of essential fatty acids. Not only does canola oil provide the essential fatty acids, but also possesses tocopherols and phytosterols. Tocopherols, the natural antioxidants, present mainly as α-tocopherol and γ-tocopherol in the canola oil; phytosterols, also known as plant sterols, can help prevent cardiovascular risks (Marwede et al., 2004; Eskin et al., 2020). These components also make this oil valuable for human nutrition.

After extraction of oil, canola seed meal remains as a by-product. This seed meal contains a high level of proteins and fibre, a low content of glucosinolates (<30 μmol/g), and trace amounts of minerals and vitamins (Canola Council of Canada, 2022). The breakdown products of glucosinolates affects the thyroid gland and interfere with iodine uptake that eventually reduce the performance of animals and their health (Velayudhan et al., 2017; Mawso et al., 1994, cited by Alexander et al., 2008).

According to USDA (2021), canola is the third largest source of vegetable oil after palm and soybean oil (Figure 1.1), and it is the second largest source of protein meal right after soybean meal (Figure 1.2). All parts of the canola plant can be used for various purposes. Canola seeds provides oil for human consumption or for production of biodiesel (Degfie et al., 2019) and the seed meal after oil extraction generally used as protein-rich animal feed (Canola Council of Canada, 2022). The waste biomass of the canola plants, such as the leaves and dried stem, can be used for textile fiber production (Shuvo et al., 2019). Furthermore, canola can be used as cover crop for the control of weeds (Agricultural Marketing Resource Center, 2018).

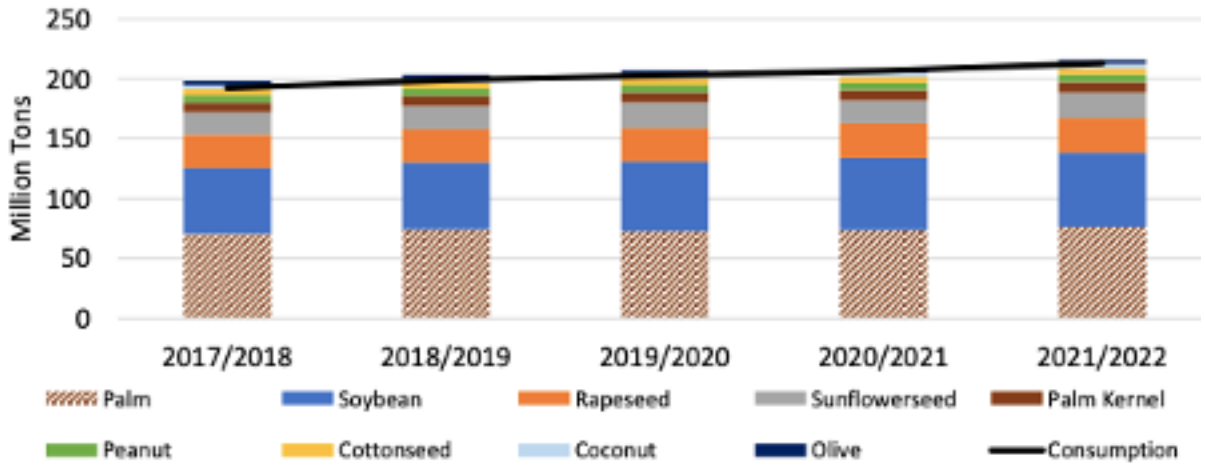

Figure 1.1 Global vegetable oil production and consumption during the period of 2017-18 to 2021-22 (USDA 2021; data retrieved on May 17, 2021). The bar graphs showing production, and the black line showing the consumption.

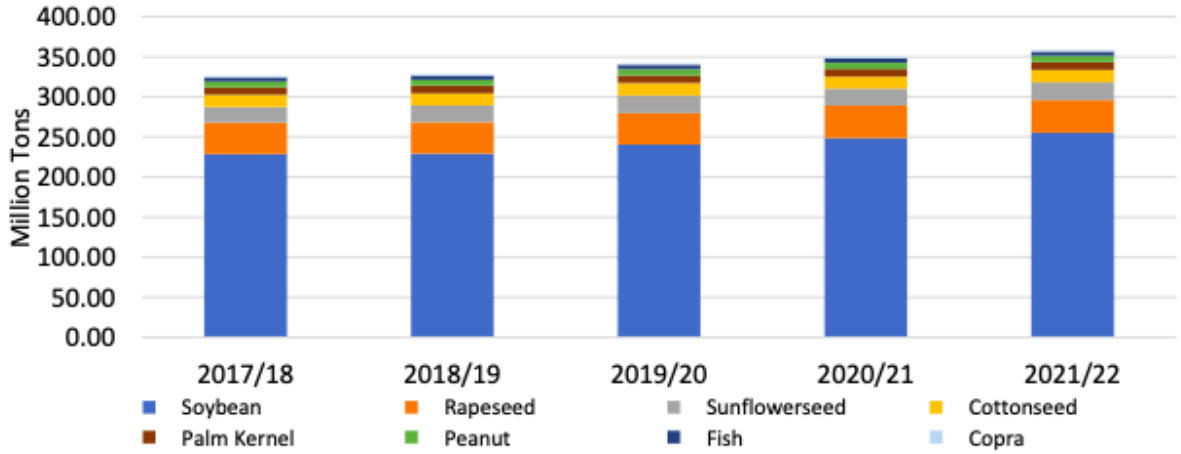

Figure 1.2 Global use of protein meal during the period of 2017-18 to 2021-22 (USDA 2021; data retrieved on May 17, 2021)

1.2 *Brassica* species and their genomic relationship

Brassica is the most economically important genus of the family Brassicaceae. It includes many economically important vegetable and oilseed crops, such as Chinese cabbage, turnip, canola, rutabaga, cauliflower, broccoli, and kale. In 1935, Nagaharu (1935, cited by Xue et al., 2020) explained the genome relationships among six *Brassica* species using a triangle which is commonly called 'U's triangle' (Figure 1.3). In this, the three amphidiploid species, *B.*

juncea (AABB, 2*n* = 36), *B. napus* (AACC, 2*n* = 38) and *B. carinata* (BBCC, 2*n* = 34) derieved from crossing of three diploid species *B. rapa* (AA, 2*n* = 20), *B. nigra* (BB, 2*n* = 16) and *B. oleracea* (CC, 2*n* = 18).

Song *et al*. (1988) proposed that the three diploid *Brassica* species originated following two evolutionary pathways. In this, *B. nigra* evolved from a common ancestor that led to *Hirschfeldia incana* or a relative to *Sinapis arvensis*, while *B. oleracea* and *B. rapa* originated from another common ancestor that gave rise to *Diplotaxis erucoides* or a closely related species (for review, see Prakash et al., 2011; Figure 1.4). Comparative analysis of DNA sequences of *D. erucoides*, *B. oleracea* and *B. rapa* further supported that *D. erucoides* might have been involved in the evolution of these two diploid *Brassica* species (Harbinder and Laksmikumaran, 1990). Cheung et al. (2009) carried out a comparative analysis of the genomic regions of the A genome of *B. rapa* and the C genome of *B. oleracea* and found that the genome of *B. oleracea* exhibits high collinearity with that of *B. rapa.* These findings also support Song *et al.*'s evolutionary model that *B. oleracea* and *B. rapa* might have evolved from a common ancestor.

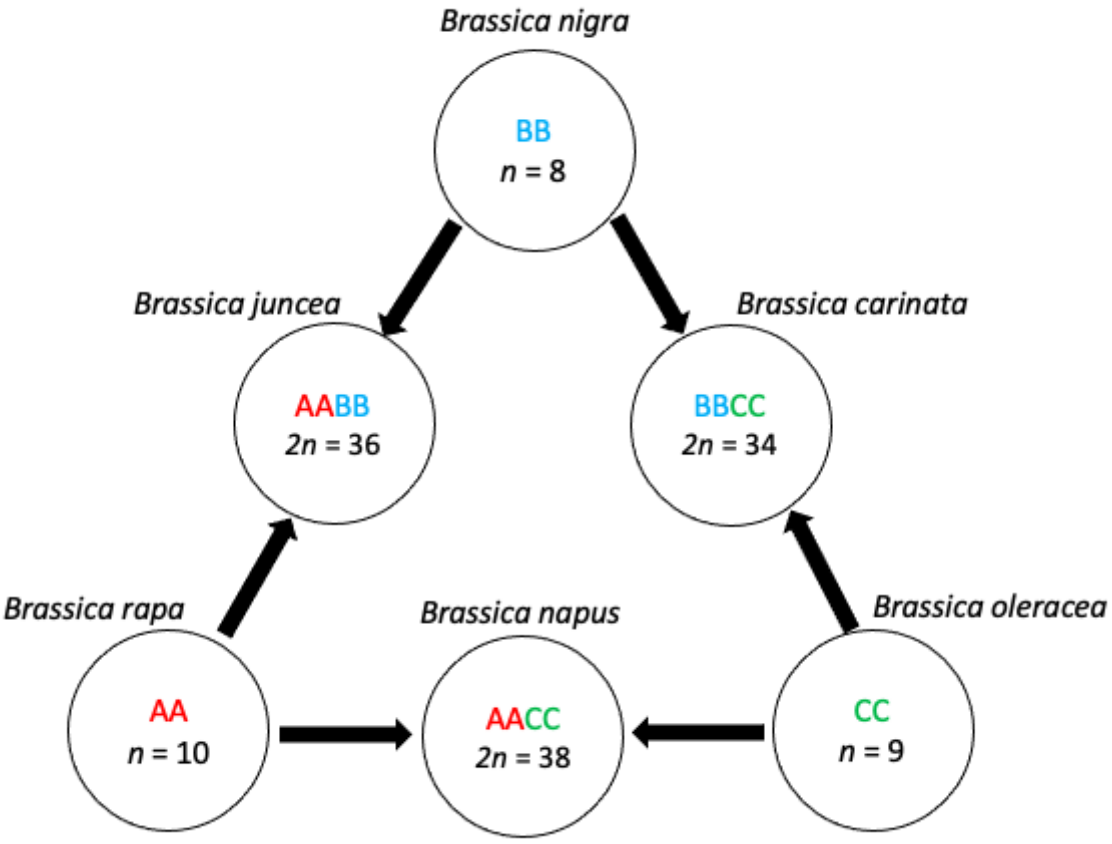

Figure 1.3 The genome relationships of six *Brassica* species. n = number chromosome sets (adapted from Nagaharu, 1935, cited by Xue et al., 2020).

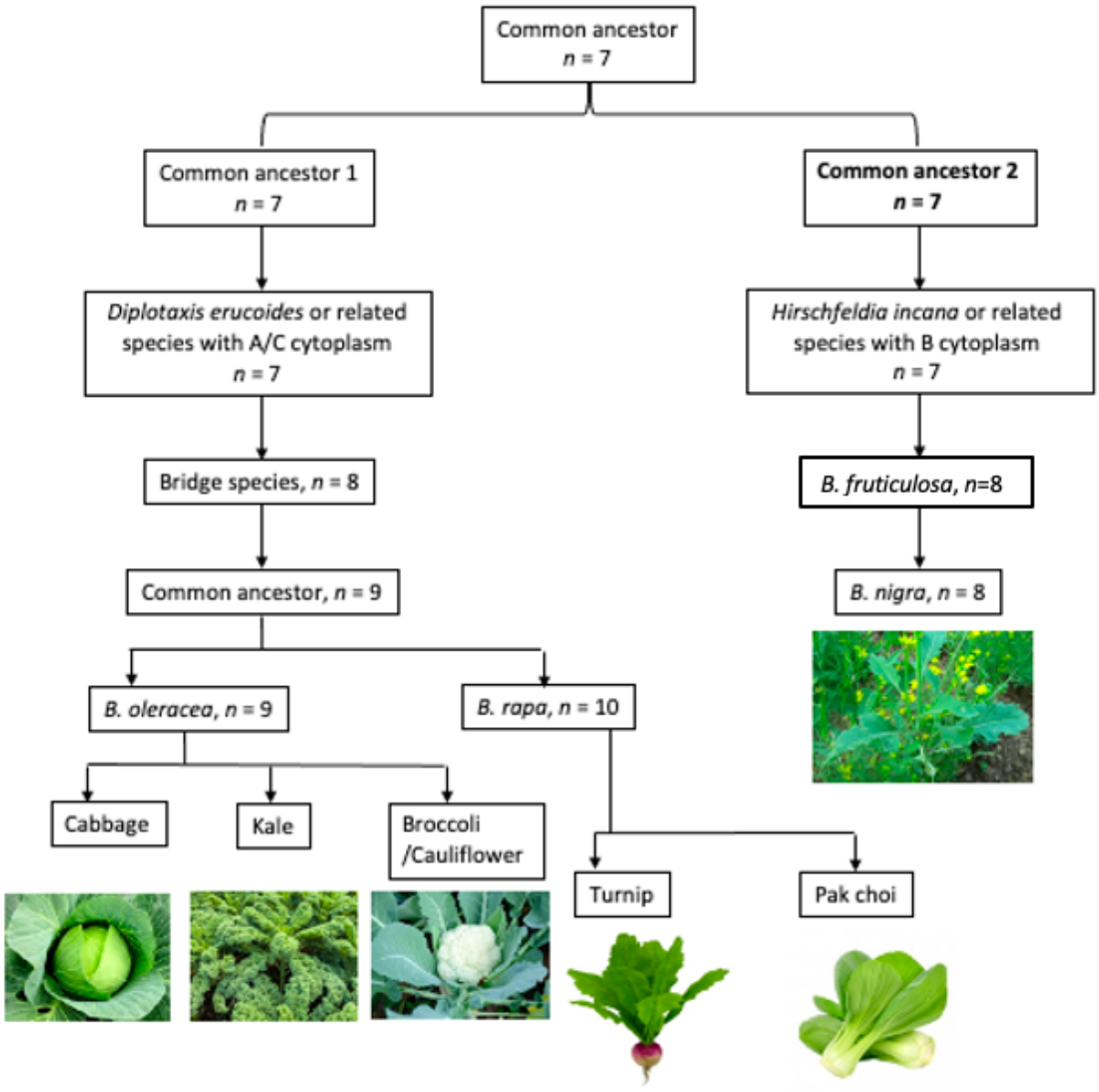

Figure 1.4 Hypothetical evolutionary pathways of the origin of three *Brassica* diploid species (developed from Prakash et al., 2011 and Song et al., 1988).

1.3 The relationship between *Brassica* and *Arabidopsis thaliana* genomes

The Brassicaceae family includes 349 genera belonging to 53 tribes (Hendriks et al., 2022), and the tribe Brassiceae includes the genus *Brassica* and *Arabidopsis* (Koch et al. 2018; Hendriks et al., 2022). These two genus evolved from a common ancestor following two separate lineages (Beilstein et al., 2006), and this split occurred approximately 20-24 million

years ago (Figure 1.5) (Beilstein et al., 2006; Ziolkowski et al., 2006). Thus, *A. thaliana* shares common ancestry with *Brassica* and the plant of this species is characterized by a short life cycle, carrying five chromosomes with a genome size of ~135 mega bases (Mb) (*Arabidopsis* Genome Initiative, 2000; https://plants.ensembl.org/Arabidopsis_thaliana/Info/Annotation/). It was the first plant whose genome has been sequenced (*Arabidopsis* Genome Initiative, 2000) and used as a model plant for plant biology and genetics research. Based on phylogenetic analysis of protein coding genes, Town et al. (2006) and Zhang et al. (2019) corroborated that *A. thaliana* share a common ancestry with *Brassica* species.

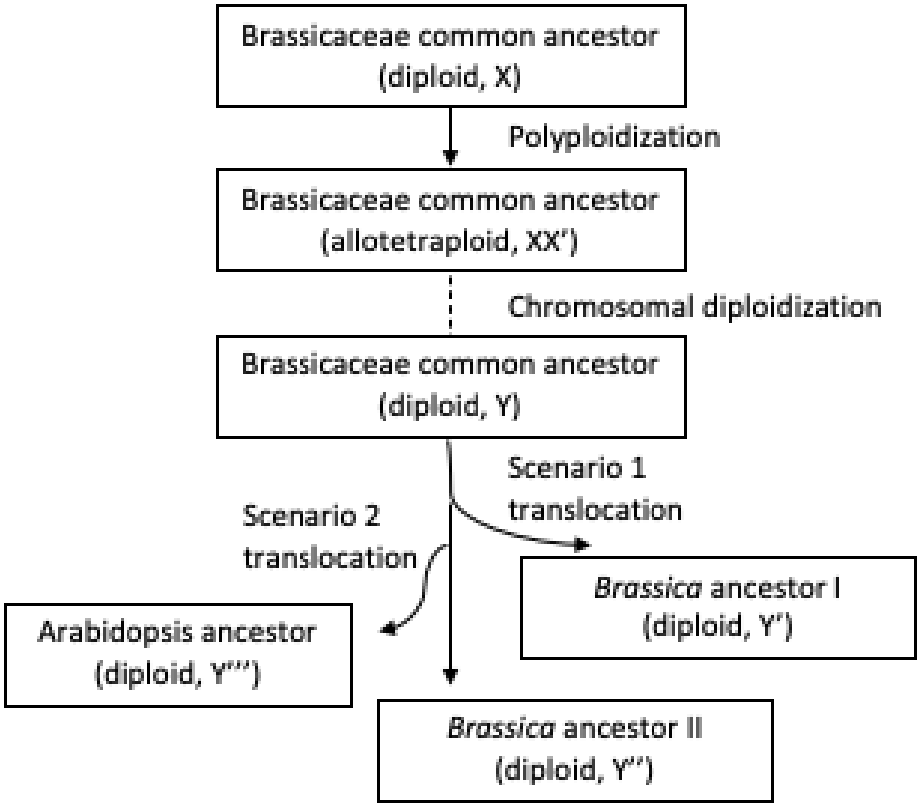

Figure 1.5 The evolution and divergence of *Arabidopsis* and *Brassica* (adapted from Ziolkowski et al., 2006).

1.4 Clubroot disease and its impact on canola

Canola is seeded on more than 9 million hectares of land in Canada in 2021 (Statistics Canada, 2022). The production of this crop is threatened by several biotic and abiotic stresses, of which clubroot disease, caused by *Plasmodiophora brassicae,* is one of the most devastating one. Dixon (2009) reviewed that the host range of *P. brassicae* is extremely broad covering all genera and over 3,700 species of the Brassicaceae family. Nevertheless, most studies on clubroot disease were carried out within the genera *Brassica*, *Raphanus* and *Arabidopsis* (Murakami et al., 2000; Malinowski et al., 2012; for review, see Javed et al., 2022). Clubroot is a soil-borne disease that results in the formation of large galls or swollen roots of the susceptible hosts, and this interferes with water and nutrient uptake causing stunting, yellowing, and wilting of the leaves and stems, or even death of the host plant (for review, see Javed et al., 2022).

Clubroot disease of *Brassica* crops has spread in the last decades in different countries and has been reported to cause about 10-15% yield loss worldwide (for review, see Dixon, 2009). Recently, it has emerged as a serious and devastating disease of canola in Canada. This disease was first identified in canola in Alberta in 2003, when only 12 infested fields were identified (Tewari et al., 2005). However, by 2019, clubroot disease had been identified in 3353 canola fields in Alberta (Strelkov et al., 2020) indicating its ability to spread very fast. Pageau et al. (2006) found that 80-91% yield loss can occur in canola when the crop is grown in severely infested fields in Quebec, Canada. Furthermore, this disease also reduces thousand-seed weight in canola (Botero-Ramírez et al., 2021).

1.5 Life cycle of *Plasmodiophora brassicae*

According to Wallenhammar (1996), *P. brassicae* is able to survive in the soil for up to 17 years without host plants. It overwinters in the soil as a resting spore, which is approximately

2.4 - 3.9 µm in diameter and is spherical in shape (Buczacki and Cadd, 1976). Under favourable condition, the resting spore germinates and produces primary zoospore, which is about 2.8 - 5.9 µm in diameter (Ayers, 1944). The primary zoospores are biflagellate with two unequal flagella (Ledingham 1934, cited by Macfarlane, 1970) and can move a short distance in humid soil to seek for potential host plants. Once zoospore reaches the surface of the host's root hairs or wounds, it penetrates the cell wall and injects its cellular content into the root hair; this is called primary infection or root hair infection (Kageyama and Asano, 2009).

In the root hairs, the primary zoospore develops into a primary plasmodium, a mass of naked protoplasm containing many nuclei. The plasmodia then transform into zoosporangia after a number of nuclear cleavages and divisions (Ingram and Tommerup, 1972). Each zoosporangium produces and releases 4 - 8 secondary zoospores (Ayers, 1944). The empty zoosporangia usually remain in the root hair while the secondary zoospores penetrate the root cortex tissues; this is called secondary infection (Kageyama and Asano, 2009). During secondary infection phase, the secondary zoospore forms a secondary plasmodium in the infected root cortex. As the secondary plasmodia proliferate through mitotic cell division, hyperplasia and hypertrophy occur in the infected cells that eventually give rise to the formation of distinct galls or clubbed roots (Kageyama and Asano, 2009). These galls interfere with the transportation of water and nutrients and leads to plant wilting and death of the roots and plants.

Later, the secondary plasmodia become mature and two haploid nuclei in plasmodia may fuse to form diploid plasmodia, which can give rise to haploid plasmodia again through meiotic cleavage (Buczacki 1983, cited by Kageyama and Asano, 2009). A vast number of resting spores are eventually produced in the plasmodia and are released into soil for a new cycle (Figure 1.6) when the infected roots are decomposed by soil microbes (Kageyama and Asano, 2009; Liu et al.,

2020).

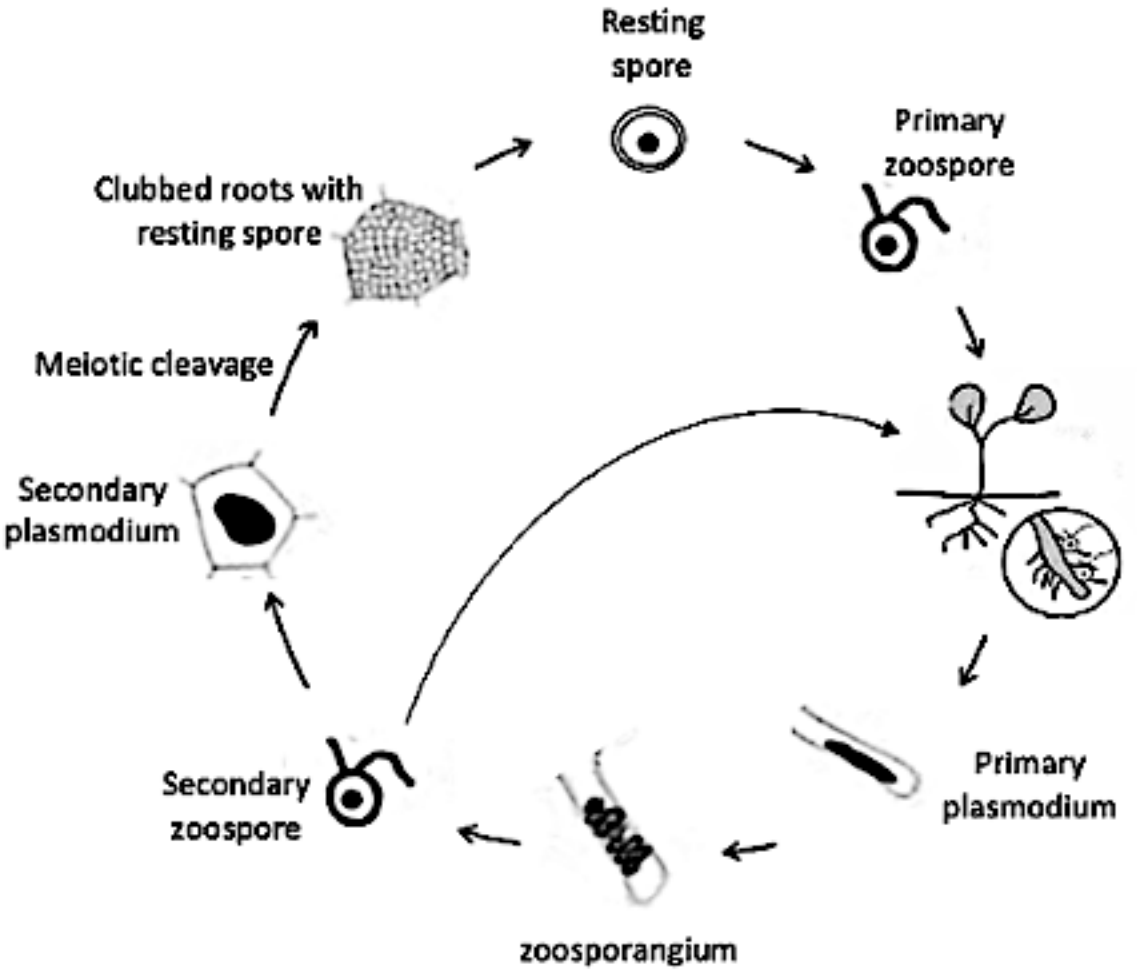

Figure 1.6 Life cycle of *Plasmodiophora brassicae* (adapted from Kageyama and Asano, 2009).

1.6 Virulence and classification of *P. brassicae*

A knowledge of virulence of the *P. brassicae* pathogen is critical for the development of clubroot resistant canola cultivars. Strains of *P. brassicae* can be identified by their virulence on a set of host differentials. Several host differential sets have been proposed to classify various *P. brassicae* pathotypes. Among them, Williams' differentials set (Williams 1966, cited by Hollman et al., 2021), European clubroot differential (ECD) set (Buczacki et al., 1975), and the differential set by Somé et al. (1996) have been used extensively worldwide. The Williams' differentials set includes two rutabaga and two cabbage cultivars to differentiate the pathotypes.

Thus, pathotype classification using this differential set is based on pathogen reaction with rutabaga and cabbage, not using canola. ECD set was developed by Buczacki et al. (1975) which includes 15 hosts belonging to *B. rapa*, *B. napus*, and *B. oleracea*. The differential set by Somé et al. (1996) is largely used in France and includes three *B. napus* cultivars, and based on this, they grouped 20 field collections of *P. brassicae* into five pathotypes (P_1 to P_5).

Of the above-mentioned differential sets, the Williams' set has been initially used in Canada for the identification of *P. brassicae* pathotypes. However, several strains of *P. brassicae* have evolved in canola fields in Alberta in the past few years, and it was not possible to classify them precisely by using Williams' differential sets. Therefore, the Canadian Clubroot Differential (CCD) set, which is based on 13 hosts, was developed by Strelkov et al. (2018). This set is composed of the differential hosts of Williams' (1966, cited by Hollman et al., 2021) and Somé et al. (1996), and selected eight hosts from the ECD set (Buczacki et al., 1975), along with the *B. napus* cultivars 'Brutor', 'Mendel', 'Westar' and '45H29'. According to Strelkov et al. (2018), the CCD classification system also enables *P. brassicae* populations to obtain the pathotype designations as per Williams' and Somé et al.'s set. By using CCD set, the pathogen isolates were classified into 17 pathotypes (A to P plus X); however, Hollman et al. (2021) and Askarian et al. (2021) have identified additional pathotypes by using this set.

On the basis of CCD set, Askarian et al. (2021) identified six novel pathotypes that can be related to three pathotypes (P_1, P_2 and P_3) of the Somé et al.'s (1996) differential system: pathotype 4A = pathotype P_1; pathotype 2A and 7A = pathotype P_2; pathotype 6A, 6B and 6C = pathotype P_3. The pathotypes 4A and 7A were reported from Canada for the first time. Subsequently, Hollman et al. (2021) also found nine novel pathotypes that can be related to five pathotypes (P_1, P_2, P_3, P_4 and P_5) as per differentials of Somé et al. (1996). Pathotypes P_4 and P_5

were the first time found in Canada. The results from these two studies denoted the emergence of novel *P. brassicae* pathotypes which can overcome the resistance of the clubroot resistant canola cultivars and can exert significant pressure on the canola industry in Canada. Apparently, this imposes a challenge to the canola breeders to maintain the durability of resistance in canola and sustainably manage the clubroot disease in the long term. However, breeding efforts for resistance to pathotype 3H demonstrated that the canola lines carrying resistance to this pathotype also exhibit resistance to several other recently pathotypes including 3D, 3O, 5G, 5I, 8E and 8P (Shaikh et al., 2020).

1.7 The management of clubroot disease

A susceptible host, a counterpart pathogen and favorable conditions are the basic requirements of clubroot disease development. Therefore, clubroot disease can be managed by the elimination of pathogen from soil or by growing resistant host plants or by the amendment of growing environment. In the early studies, researchers mostly focused on the removal of *P. brassica* pathogen and changing the environment to hinder the development of *P. brassica* (Peng et al., 2015; Hwang et al., 2015; Hwang et al., 2017; Wang et al., 2017; Liu et al., 2018; Ernst et al., 2019; Zahr et al., 2021; Liao et al., 2022). However, none of studies showed that pathogen can be completely removed from the soil and each method is not consistently effective in different situations (Ahmed et al., 2011; Fox et al., 2022). Hence, researchers are increasingly focusing on the development of resistant canola cultivars to control the clubroot epidemics (Rahman et al., 2011; Hirani et al., 2016; Hasan et al., 2021; Kaur et al., 2022).

Three different strategies were examined for the control of clubroot disease, and this includes cultural, chemical, and biological control methods. The half-life of *P. brassica* spore inoculum is about 3.6 years (Wallenhammar, 1996). Continuous growing of clubroot host plants

without break increases the level of *P. brassica* spores in the field. However, a >2-year crop rotation without susceptible host has been proven to reduce the concentration of spore inoculum significantly (Ernst et al., 2019) and increases the yield by 32% (Peng et al., 2015). A recent study showed that ultraviolet light exerts a negative effect on clubroot disease by making the *P. brassicae* spores less robust (Zahr et al., 2021). Ploughing the resting spores in the deep soil could effectively manage clubroot epidemics. However, this cultural control method is not feasible where canola is grown under zero-tillage condition, such as in Canada.

Application of chemicals, such as soil fumigants and fungicides, may be an effective way in managing clubroot, but it is not an environmentally friendly approach and not allowed in many countries. Soil fumigant 'dazomet' has been proven to be an effective chemical to control clubroot in canola (Hwang et al., 2018); however, the efficacy of 'dazomet' depends on the concentration of spore inoculum, more effective in the field with a low level of spore inoculum (Hwang et al., 2018). A variety of fungicides, such as cyazofamid, methiadinil and fluazinam, had been found to reduce the severity of clubroot disease (Wang et al., 2017; Liao et al., 2022; for review, see Chai et al., 2014; Donald and Porter, 2009); however, fluazinam and cyazofamid are the only two fungicides registered in Canada due to the strict process to apply in the fields (Peng et al., 2014).

Compared to chemical practices, biological control is an environment-friendly approach of controlling diseases. Biological substances released as products of the activity of living organisms and microorganisms themselves are natural sources to suppress the development of clubroot disease which are environment friendly. Liu et al. (2018) demonstrated that *Bacillus subtilis* XF-1 reduces the clubroot severity due to the release of biological substance, fengycin-type cyclopeptides, that collapses the resting spores of *P. brassica* (Li et al., 2013). Planting bait

crops is another example of biological controls of clubroot disease. A bait crop refers to a crop that attracts pests or stimulate the resting spore of pathogens to germinate. Bait crops, infected by *P. brassica*, followed by the removal from fields, hampered the life cycle of *P. brassica* and showed reduction of spore concentration (Murakam et al., 2000; Hwang et al., 2015; for review, see Hwang et al., 2014). Bait crops together with crop rotation and lime treatments maintain the effectiveness (Hwang et al., 2015; for review, see Donald and Porter, 2009); however, growing bait crops alone is not a practical solution in the case of severe infection (Ahmed et al., 2011).

The spore germination rate of *P. brassica* was significantly declined when soil pH was increased to neutral (Niwa et al., 2008). Therefore, the change in soil pH may reduce the severity of clubroot epidemics. Similarly, increasing soil pH to 6.0, 6.5, 7.0 and 7.5 with different lime treatments has successfully reduced the root infections (Fox et al., 2022). Moreover, Porter et al. (2004, cited by Donald and Porter, 2009) indicated that lime with higher level of calcium carbonate can effectively manage clubroot epidemics in Australia, and a finely ground lime was found to be more effective as compared with a coarse ground lime (Tremblay et al., 2005).

The above-mentioned strategies have been examined to support the clubroot disease management (Peng et al., 2014, Peng et al., 2015; Hwang et al., 2015; Hwang et al., 2017; Wang et al., 2017; Liu et al., 2018; Ernst et al., 2019; Zahr et al., 2021; Liao et al., 2022; Fox et al., 2022), but they were not efficient or economically viable. Therefore, the development of clubroot-resistant canola cultivars and cultivation under appropriate management practices has been considered the best strategy of combating clubroot. Currently, the commercially available clubroot-resistant canola cultivars are mostly developed based on qualitative resistance genes; however, these cultivars imposed a significant selection pressure on *P. brassicae* population, giving rise to the emergence of the new virulent pathotypes (Strelkov et al., 2016) which found

to render these resistance genes ineffective. Therefore, introgression of quantitative resistance into canola is needed for sustainable production of this crop.

1.8 Clubroot resistance (CR) in *Brassica* germplasm

Several studies, as reviewed above, have showed that clubroot disease is hard to manage by only traditional, chemical, and biological approaches. Therefore, it is imperative to develop clubroot-resistant cultivars to meet the current industrial needs. For this, the identifications of resistant genes are essential for breeding resistant cultivars. *B. rapa* and *B. oleracea* are the progenitor diploid species of *B. napus.* The available clubroot resistance in these two species (Hasan et al., 2012; Fredua-Agyeman et al., 2020; Farid et al., 2020) make them valuable for breeding clubroot-resistant *B. napus* cultivars. To date, the A genome of *B. rapa* and the C genome of *B. oleracea* had been revealed to carry several CR genes. In this section, I reviewed the genetic basis of clubroot disease resistance in these two *Brassica* species.

1.8.1 Clubroot resistance (CR) in the A genome of *B. rapa*

Researchers had identified several CR genes from the A-genome of several cultivars and lines of *B. rapa*. For example, the locus *CR6a* was identified on chromosome A1 of *B. rapa* which contributes resistance to *P. brassicae* pathotype 6 (Lee et al., 2002); *Crr2* was also identified on chromosome A1 of *B. rapa* but only confer resistance to *P. brassicae* pathotypes 4 when this locus co-exists with *Crr1* located on chromosome A8 (Suwabe et al., 2003). *CRc* and *Rcr8* loci located on the chromosome A2 of *B. rapa* as reported by Sakamoto et al. (2008) and Yu et al. (2017), and these two loci confer resistance to *P. brassicae* pathotype 2 and 5X, respectively.

Several CR loci were mapped on chromosome A3: four loci *CRk*, *CRa, Crr3* and *CRq* were identified which associated with resistance to *P. brassicae* pathotype 2 (Sakamoto et al.,

2008; Ueno et al., 2012; Hirai et al., 2004; Saito et al., 2006; Yuan et al., 2015, cited by Hasan et al., 2021); five loci *Rcr1*, *Rcr2*, *Rcr5*, *CRb* and *Bra012688* were mapped and found to confer resistance to *P. brassicae* pathotype 3 (Chu et al., 2014; Huang et al., 2019; Huang et al., 2017; Kato et al., 2013; Hasan et al., 2021); two loci *CRd* and *CR6b* were reported to be associated with resistance to *P. brassicae* pathotype 4 and 6, respectively (Pang et al., 2018; Lee et al., 2002); the locus *Rcr4* was reported to be conferring resistance to *P. brassicae* pathotype 2, 3, 5, 6 and 8 (Yu et al., 2017). Subsequently, Fredua-Agyeman et al. (2020) identified two loci clustered in the genomic regions where the *CRa*/*CRb*^{Kato} are mapped and confer resistance to *P. brassicae* pathotype 5X and 5G.

Two CR loci *CrrA5* and *Crr4* were mapped on chromosome A5 and A6, respectively (Nguyen et al., 2018; Suwabe et al., 2006). Later, Zhu et al. (2019) identified two QTL *qBrCR38-1* and *qBrCR38-2* associated with resistance to *P. brassicae* pathotype 7 on chromosome A7 and A8 of *B. rapa*, respectively. Four loci, *Crr1, Rcr3, CRs, Rcr9*, were identified on chromosome A8 and found to confer resistance to pathotypes 2, 3, 4 and 5X, respectively (Suwabe et al., 2003; Karim et al., 2020; Laila et al., 2019; Yu et al., 2017). The genomic region where the *Crr1* is located further revealed that, in fact, it includes two loci, *Crr1a* and *Crr1b* (Hatakeyama et al., 2013).

Thus, to date, researchers had reported a total of 26 gene loci and mapped them on seven A-chromosome, except for A4, A9, A10. Of 26 CR loci, 13 loci have been identified on chromosome A3, six on chromosome A8, two loci on chromosomes A1 and A2 respectively, and each one locus on chromosomes A5, A6 and A7. However, some of the loci reported from the same chromosome may be located in the same genomic region; however, designated as different loci due to the use of different pathotypes in the experiments. For example, the precise locations

of *CRa* and *CRb* were controversial until 2017, when researchers revealed that these two loci are in fact a single locus located on A3 of *B. rapa* (Hatakeyama et al., 2017).

1.8.2 Clubroot resistance in the C genome of *B. oleracea*

Besides the clubroot resistance from *B. rapa*, researchers also found several loci on all C-genome chromosomes from several *B. oleracea* cultivars. For example, Voorrips et al. (1997) reported two QTL *pb-3* and *pb-4* associated with resistance to *P. brassicae* pathotypes ECD 16/3/30 as per the differentials set of Buczacki *et al.* (1975) and mapped them on chromosomes C3 and C1 of *B. oleracea*. Moriguchi et al. (1999) also located one QTL on chromosome C3 that was associated with *P. brassicae* pathotypes 1 and 3 as per Williams' differentials set (1966, cited by Hollman et al., 2021). Rocherieux et al. (2004) identified nine QTL (*Pb-Bo1, Pb-Bo2, Pb-Bo3, Pb-Bo4, Pb-Bo5a, Pb-Bo5b, Pb-Bo8, Pb-Bo9a* and *Pb-Bo9b*) and mapped them on chromosomes C1, C2, C3, C4, C5, C8 and C9. Of the nine QTL, the QTL *Pb-Bo1* conferred resistance to five *P. brassicae* isolates that corresponds to pathotypes P_1, P_2, P_4 and P_7 as per differentials of Somé et al. (1996); while the other eight QTL were associated with isolate-specific resistance to one, two or three isolates only.

The loci on C6 and C7 were first reported after the year 2010. Nagaoka et al. (2010), Peng et al. (2018) and Dakouri et al. (2018) identified several CR QTL on chromosomes C2, C3, C5, C7 and C8 that confer resistance to *P. brassicae* pathotype 3, 4 and 5X L-G2. Later, Farid et al. (2020) identified ten QTL on chromosomes C3, C4, C6, C7, C8 and C9 of which six QTL (designated as *PbC4.1*, *PbC6*, *PbC7.1*, *PbC7.2*, *PbC8* and *PbC9.1*) found to confer resistance to *P. brassicae* pathotype 3A, while the remaining four QTL (*PbC3*, *PbC4.2*, *PbC7.3* and *PbC9.2*) found associated with resistance to *P. brassicae* pathotypes 5X L-G2.

Clubroot resistance in the C-genome of *B. oleracea* mostly controlled by multiple gene

loci; however, the locus *Rcr7* mapped on chromosome C7 reported to confer qualitative resistance (Dakouri et al., 2018). Compared to the A-genome clubroot resistance from *B. rapa*, the clubroot resistance from *B. oleracea* covers all nine chromosomes of the C-genome.

1.9 Research objectives

As reviewed above, most of the studies, to date, were focused on the introduction of clubroot resistance from the A genome of *B. rapa* into *B. napus*. The rapid evolution of new virulent *P. brassicae* pathotypes rendering the qualitative resistance of the A genome relatively ineffective highlighing the need for the use of the quantitative resistance of the C genome of *B. oleracea* in the breeding of clubroot resistant *B. napus* canola. However, studies on the introgression of clubroot resistance from the C genome of *B. oleracea* into *B. napus* is limited. The objectives of this study were to develop a stable clubroot resistant euploid (2n = 38) *B. napus* line, carrying resistance in the C genome, from a segregating population of *B. napus* × *B. oleracea* interspecific cross. In addition to this, genetic analysis of a clubroot resistant line identified in the canola program has also been carried out.

The hypothesis of this M.Sc. thesis research were the followings:

1. The *B. napus* genome includes the C genome of *B. oleracea*; therefore, clubroot resistance from *B. oleracea* can be introgressed into the C-genome of spring *B. napus*.
2. The *B. napus* parent used in the *B. napus* × *B. oleracea* interspecific is a canola quality type; therefore, a stable canola quality euploid (2*n* = 38) *B. napus* line carrying resistance in the C genome can be developed from the progeny of this interspecific cross through selection for the canola quality traits.
3. Analysis of a segregating population derived from clubroot resistant × susceptible *B. napus* lines will disclose the genetic control of the resistance.

Chapter 2 Development of clubroot resistant *Brassica napus* lines from the progeny of a *B. napus* × *B. oleracea* interspecific cross

2.1 Introduction

Brassica napus L. (AACC, 2*n* = 38), evolved through interspecific hybridization between the diploid species *Brassica rapa* L. (AA, 2*n* = 20) and *Brassica oleracea* L. (CC, 2*n* = 18) (Morinaga, 1934), is an important crop for use its oil as human food and its seed meal as animal feed. Phylogenetic analysis between *B. rapa* and *B. oleracea* has been reported by several researchers (Li et al., 2017; Zhang et al., 2019) supporting that these two species might have evolved from a common ancestor. The model plant *Arabidopsis thaliana* also shares ancestry with *B. rapa* and *B. oleracea* (Song et al., 1988; Koch et al., 2000; Beilstein et al., 2006; Ziolkowski et al., 2006; Town et al., 2006; for review, see Franzke et al., 2011; Zhang et al., 2019).

Among the different threats to the production of *B. napus* canola, the clubroot disease caused by *Plasmodiophora brassicae* Woronin is one of the most important one (Dixon 2009; Pageau et al., 2006; Strelkov et al., 2007). The resting spore of this pathogen can survive in soil for up to 17 years (Wallenhammar 1996). Different crop management practices including cultural, biological and chemical controls have been found not to be consistently effective to control this disease (Ahmed et al., 2011; Hwang et al., 2017). Therefore, the development of clubroot-resistant canola cultivars is essentially needed, although the maintenance of the resistance in the cultivars is challenging due to evolution of new virulent pathotypes only in a few years (Strelkov et al., 2016, 2018; *Askarian et al., 2021;* Hollman et al., 2021). Clubroot resistances of the winter canola cv. Mendel and rutabaga as well as *B. rapa* have been used by different researchers in the breeding of spring *B. napus* canola (Rahman et al., 2011a; Chu et al.,

2013; Hasan and Rahman, 2016; Hirani et al., 2016; Hasan et al., 2021b; Yu et al., 2021; Kaur et al., 2022; Zhan et al., 2022). Resistance to this disease can also be found in *B. oleracea* (Hasan et al., 2012; Farid et al., 2020); however, the commercially available canola cultivars in Canada carry resistance located only in the A genome (for review, see Rahman et al., 2014b; Hasan et al., 2021a). Farid et al. (2020) showed that *B. oleracea* carry resistance to the recently evolved *P. brassicae* pathotypes such as pathotypes 3A and 5X; therefore, this diploid species can be used to broaden the genetic base of clubroot-resistance in *B. napus* canola. Thus, the objective of this study was to develop a genetically stable clubroot-resistant euploid ($2n = 38$) *B. napus* line from a segregating population of *B. napus* × *B. oleracea* interspecific cross carrying the resistance in its C genome.

2.2 Materials and Methods

2.2.1 Plant materials

For my MSc thesis research, I received seeds of a F_5 population of *B. napus* × *B. oleracea* interspecific cross from the Canola Program of the University of Alberta (Zhang, 2022). This population was developed by crossing a canola quality (zero erucic acid in oil and <15 µmol glucosinolates (GSL) per g/seed) spring type *B. napus* line A04-73NA with a clubroot resistant non-canola quality (>40 % erucic acid in seed oil and >80 µmol GSL/g seed) *B. oleracea* var. *acephala* cv. Winterbor line AM114 (Farid et al., 2020) using *B. napus* as female. The F_1 plants were obtained through application of in vitro ovule culture technique, and the F_2 population was subjected to pedigree breeding with selection for resistance to *P. brassicae* pathotype 3H and spring growth habit plants. The details of the development of this population can be found in Zhang (2022). I obtained F_5 seeds from the Canola Program of the University of Alberta. During this MSc thesis research, I evaluated the F_5 and their progeny generation populations in

greenhouse and field for resistance to clubroot disease, plant fertility and ploidy level of the plants, as well as for agronomic and seed quality traits aiming at the development of a canola quality clubroot resistant euploid ($2n = 38$) *B. napus* line (Fig. 2.1).

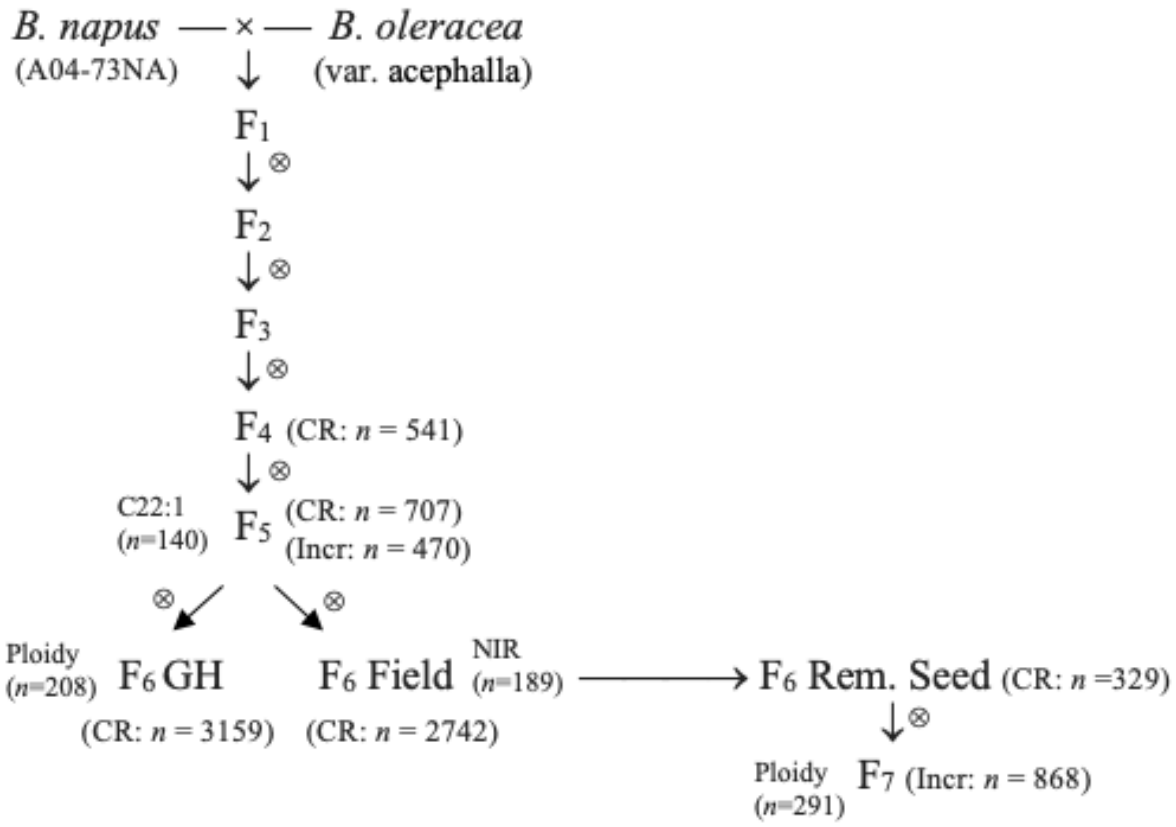

Figure 2.1 Flow diagram of *Brassica napus* canola line development from *B. napus* × *B. oleracea* interspecific cross. The number in the brackets shows the number of plants evaluated for clubroot resistance in greenhouse or in field, or grown for seed increase. In case of erucic acid, ploidy and NIR analysis, the number indicates the number of families evaluated. C22:1 = erucic acid content estimated by gas chromatographic technique; Ploidy = flow cytometric analysis for approximate ploidy level of the plants; NIR = near-infrared spectroscopy for estimation of oil, protein and glucosinolate contents; CR = clubroot resistance test in greenhouse; Incr = seed increase in greenhouse; Rem. Seed = remnant seed; S canola = susceptible canola)

2.2.2 Inoculum preparation for greenhouse test

Single spore isolates of *P. brassicae* pathotype 3 as per Williams' (1966, cited by Strelkov et al., 2018) differential system or pathotype 3H as per Canadian Clubroot Differential

system (Strelkov et al., 2018) was received from Plant Pathology Laboratory of the University of Alberta and used in the present study. Resting spores of this pathogen were preserved in the form of galls of the susceptible *B. napus* cultivar Hi-Q and stored at -20°C. Inoculum was prepared one day before inoculation from the preserved galls following modified version of William's technique as described by Strelkov et al. (2007). Briefly, about 36 g of galls was ground with 1000 ml distilled water in a blender (Ninja® Professional Blender 1100 W) at medium speed for 5 min or until all galls were ground properly. The homogenate was filtered through a multi-layer cheesecloth (American Fiber and Finishing Inc., Albemarle, NC, USA) and resting spore concentration in the suspension was quantified by a hemocytometer (VWR, Mississauga, ON, Canada) and adjusted to 1×10^7 spores/ml, which is known as the optimal concentration for inoculation according to Voorrips and Visser (1993). The spore suspension was stored at 4°C overnight for inoculation on the next day.

2.2.3 Inoculation in greenhouse

The F_5, F_6 and selected F_6, based on field trial, generation populations were evaluated for clubroot resistance in a greenhouse of the Faculty of Agricultural, Life and Environmental Sciences of the University of Alberta in fall 2020, spring 2021 and winter 2021-22, respectively. For this, 8-16 plants, depending on the availability of seeds, of each family were grown along with one to two plants of the susceptible cultivar Hi-Q in 32-cell trays (tray size: 52 cm × 26 cm × 8 cm, L × W × D; cell size: 6.5 cm × 6.5 cm × 8 cm, L × W × D) filled with Sunshine Professional Growing Mix (Sunshine Horticulture, Bellevue, USA). The greenhouse conditions were follows: 21/15 ± 2 °C (day/night), 16 h photoperiod, and light intensity of 450 µmol/m^2s at plant level. The *B. napus* cultivar Hi-Q was used as susceptible check. The seedlings were inoculated at 7-10 days after germination by pipetting (Lamers and Toxopeus, 1977, cited by

Voorrips and Visser, 1993) one ml inoculum at the base of each seedling. To ensure successful infection, inoculation was repeated on the following day. In the first week after inoculation, the cells were kept saturated with water to ensure favorable environment for germination of the resting spore and root infection by zoospores; afterwards, watering was done once a day until maturity. The plants were fertilized once a week with 15-30-15 (N-P-K) fertilizer (Plant Products, Brampton, Ontario).

2.2.4 Evaluation for clubroot resistance in field

The F_6 population was also grown in a clubroot disease infested field in Spruce Groove, Alberta in summer 2021 for resistance to *P. brassicae* field populations. This field is known to carry different pathotypes including pathotype 5X (personnel communication, Crop Diversification Centre North, Edmonton, Alberta). Seeding for 257 F_6 family was done in single row 3-meter-long plots with row spacing of 50 cm and with one replication; the check cultivar Hi-Q was seeded at every 15^{th} plot. Standard crop management practices including fertilizer application was followed for growing a good crop.

2.2.5 Disease evaluation

Evaluation of the plants, grown in greenhouse, for clubroot resistance was performed at 42 to 45 days after inoculation (DAI) or at harvest by visual evaluation of the roots. For this, the plants were carefully uprooted, and the roots were washed with tap water and visually examined for gall formation. In the case of the field experiment, the plants were carefully uprooted at the end of flowering and visually examined for galls. The plants were scored on a 0 to 3 scale as described by Buczacki et al. (1975), where 0 = no galls, 1 = one or a few small galls on the lateral roots, 2 = several small to medium galls on the lateral roots and 3 = large galls on the

main and lateral roots (Figure 2.2). A disease severity index (DSI) was calculated for each family using the following formula (Strelkov et al., 2006):

$$\text{DSI}(\%) = \frac{\sum(n_0 \times 0 + n_1 \times 1 + n_2 \times 2 + n_3 \times 3)}{N \times 3} \times 100$$

where n_0, n_1, n_2 and n_3 is the number of plants included in the disease severity classes 0, 1, 2 and 3, respectively, and N is the total number of plants evaluated.

Figure 2.2 Disease severity classes for clubroot disease in *Brassica napus* based on gall development (Score 0 = no visible gall; Score 1= one or few small galls on lateral roots; Score 2 = several small to medium galls on lateral roots; Score 3= large galls on main and lateral roots).

2.2.6 Evaluation for plant fertility

The F_5 population was grown in greenhouse in 6.5 cm × 6.5 cm × 8 cm (L × W × D) pots and in 12 cm × 12 cm × 15 cm (L × W × D) pots, and the F_7 population was grown in 12 cm

× 12 cm × 15 cm (L × W × D) pots, and the two populations were evaluated for fertility based on silique set and seed weight per plant (g). For silique set, plants were visually scored on a 0-9 scale, where 0 = no silique produced, 1-3 = about 1-30% silique produced, 4-5 = about 50% silique produced, 6-8 = about 70-90% silique produced, and 9 = fully fertile plant (Figure 2.3); in this evaluation, the *B. napus* parent A04-73NA was considered fully fertile. The plants were harvested at maturity, dried at room temperature for about 10 days, and threshed manually for seed weight per plant. The *B napus* parent A04-73NA was also grown together with these populations as check.

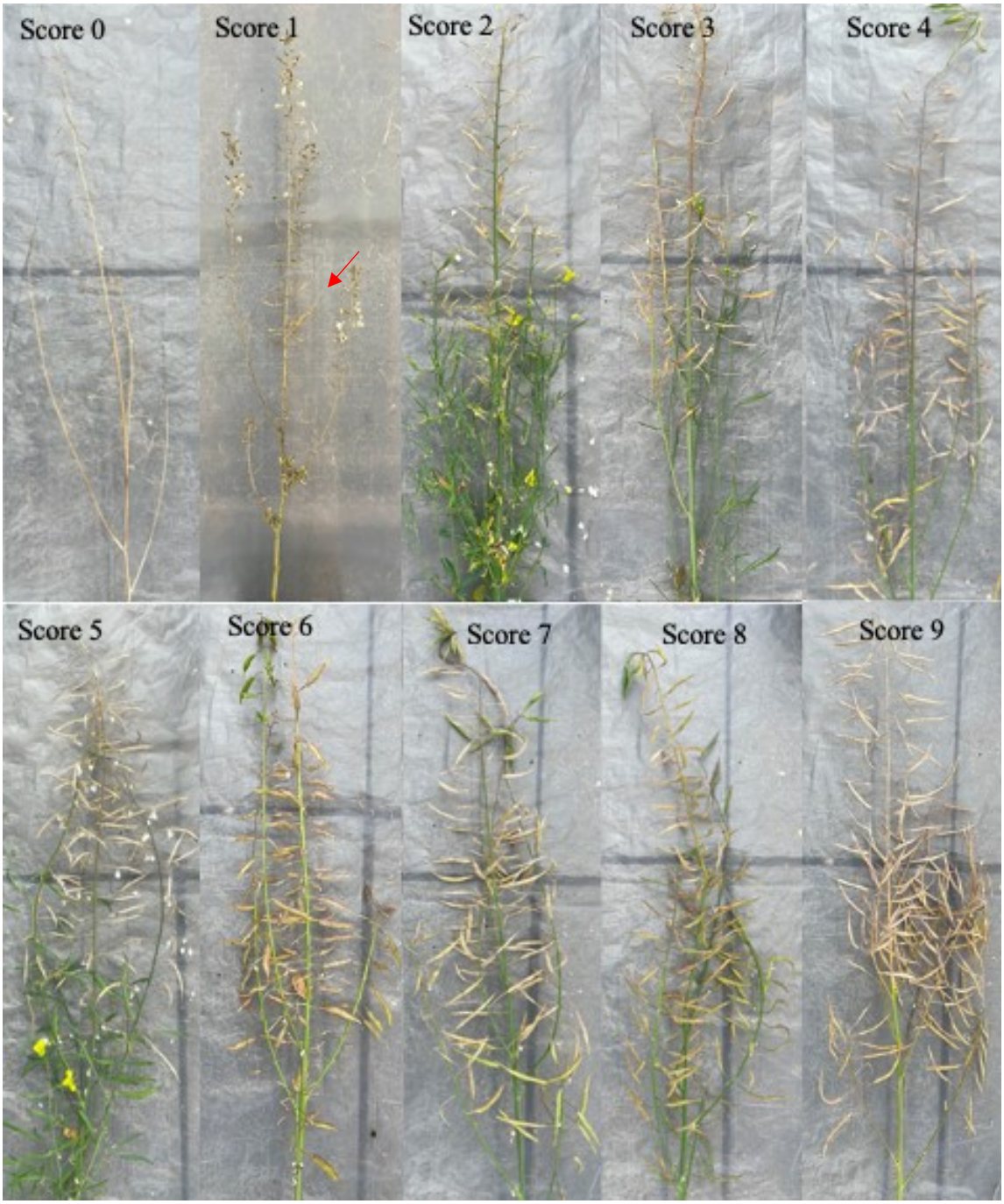

Figure 2.3 The measurement of seed set score on a 0-9 scale (score 0 = no silique produced, score 1-3 = about 1-30% silique produced, score 4 and 5 = about 50% silique produced, score 6-8 = about 70-90% silique produced and score 9 = more than 90% silique produced.

2.2.7 Field evaluation for days to flowering and seed quality traits

The F_6 population was grown in field plots at Edmonton Research Station (South Campus) of the University of Alberta for evaluation of days to flowering and seed quality traits. Seeding of 193 F_6 families was done in single row 3-meter-long plots with 50 cm space between the rows and with one replication. The *B. napus* parent A04-73NA was seeded at every 20th plot as check. Standard crop management practices were followed for growing a good crop. Days to flowering data was recorded when about 50% plants in a plot had at least one open flower. Open-pollinated seeds harvested from the plots were used for estimation of oil, protein and GSL contents by near-infrared spectroscopy (NIR Systems, Model 6500, Foss North America, Eden Prairie, MN). For this, 2.5 to 4.0 g bulk seed was used. Oil and protein contents are reported as percentage of the whole seed on a dry weight basis while GSL content was calculated on 8.5% moisture basis and reported as μmol/g seed (Daun et al., 1994 cited by Rahman and Kebede, 2012).

2.2.8 Erucic acid analysis

Self-pollinated F_6 seeds harvested from F_5 plants were used for estimation of fatty acid contents of the seed oils. For this, 0.10 to 0.25 g seed of each plant was used. Seeds were placed in 50 ml conical tube containing 1.25 ml methylating solution and 1.25 ml hexane solvent, and were crushed using a glass rod and the samples were vortexed at high speed for 30 min for extraction of the oil and conversion it into methyl esters. After that, 1.5 ml of 1M NaCl solution was added to the tube and waited for 10 min to maximize the recovery of the short chain fatty acids. Tubes were then centrifuged at 1500 rpm for 10-15 min and the supernatant (containing methyl esters) was transferred to a 10 × 75 mm autosampler vial for analysis by a Gas Chromatograph (Agilent, model 7890A) for fatty acid profile of the oil. The following fatty acids were estimated and the contents reported as percent of the total fatty acids: Lauric acid (C12:0),

myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), arachidic acid (C20:0), behenic acid (C22:0), erucic acid (C22:1), and lignoceric acid (C24:0).

2.2.9 Ploidy analysis

The F_6 and F_7 generation plants and their parents were analyzed for relative nuclear DNA content (Partec value) following flow-cytometric technique. For this, approximately 0.5 cm^2 of young leaf from each plant was chopped with razor blade in 400 µl extraction buffer using 55 mm petri dish, and the samples were incubated for 1 to 2 min. After incubation, samples were filtered through a 50 µm Partec CellTrics disposable filter, and 1.6 ml CySTain® UV Precise staining buffer (Sysmex Partec GmbH, Görlitz, Germany) was added to the sample, and the samples were incubated for 30 to 60 sec. The samples were analysed using a Partec ploidy analyzer (Partec GmbH, Münster, Germany) to estimate relative nuclear DNA content of the plants (Partec values). The Partec values were used to estimate similarity of the plants to the *B. napus* or *B. oleracea* parents for nuclear DNA content.

2.2.10 Statistical analysis

Statistical analysis of the data was carried out using Microsoft Excel (Version 16.56) and RStudio Software (Version 1.3.1073). Tests for significant difference between different generation populations for clubroot resistance were conducted by one-way analysis of variance (ANOVA) in RStudio (Version 1.3.1073) (RStudio Team, 2020). Welsch's *t*-test with an alpha set to 0.05 was performed in excel (Version 16.56) for significant difference between populations or between population and the *B. napus* parent for different traits, such as days to flowering, seed quality traits, erucic acid content, and ploidy level. Chi-square (χ^2) test for goodness of fit to a segregation ratio was carried out using the following formula: $\chi^2 = \sum(O - E)^2/E$, where O is the observed number and E is the expected number.

2.3 Results

2.3.1 Evaluation of clubroot resistance

A total of 707 F_5 plants belonging to 21 families (disease score of the F_4 plants: 0 to 3; mean = 2.50 ± 0.04) of *B. napus* A04-73NA × *B. oleracea* var. *acephala* cv. Winterbor (AM114) cross were evaluated for resistance to *P. brassicae* pathotype 3H at harvest stage where 581 (82.2%) plants were observed resistant (disease score 0 to 1; mean = 0.78 ± 0.02) while 126 (17.8%) plants were susceptible (disease score 2 to 3; mean = 2.59 ± 0.04). The DSI of the F_5 families varied from 25.0% to 100.0% with a mean of 42.54 ± 3.38% (Table 2.1).

A total of 3,159 F_6 plants belonging to 256 families were evaluated for resistance to pathotype 3H at 45 DAI. This included 2,632 F_6 plants belonging to 211 families derived from 211 resistant F_5 plants (disease score 0 to 1; mean = 0.55 ± 0.03), and 527 F_6 plants belonging to 45 families derived from 45 susceptible F_5 plants (disease score 2 to 3; mean = 2.56 ± 0.07). In case of the progeny of the resistant F_5 plants, 43.0% (1,132/2,632) plants were resistant (disease score 0 to 1; mean = 0.79 ± 0.01) while 57.0% (1,500/2,632) plants were susceptible (disease score 2 to 3; mean = 2.53 ± 0.01). On the other hand, the majority of the F_6 plants (80.3%; 423/527), descendent of the susceptible F_5 plants, were susceptible (disease score 2 to 3; mean = 2.56 ± 0.02) where only 19.7% (104/527) plants were resistant (disease score 0 to 1; mean = 0.94 ± 0.02). Thus, selection for resistance was found to be effective in this population. This was also evident from the variation of DSI in this population. The DSI of the F_6 families derived from the resistant F_5 plants varied from 11.1% to 100.0% with a mean of 58.84 ± 1.52%, while the DSI of the F_6 families derived from the susceptible F_5 plants varied from 40.0% to 100.0% with a mean of 75.54 ± 2.28%.

In addition to the evaluation of the F_6 families in greenhouse, 257 families were also evaluated for clubroot resistance in a *P. brassicae*-infested field in Spruce Grove, Alberta. This population included 215 families derived from resistant F_5 plants (disease score 0 to 1; mean = 0.69 ± 0.03) and 42 families derived from susceptible F_5 plants (disease score 2 to 3; mean = 2.62 ± 0.08). Majority of the F_6 families, whether derived from the resistant or susceptible plants, did not show disease symptoms under field conditions (Figure 2.4). The DSI of the families derived from the resistant plants varied from 0.0% to 100.0% with a mean of 7.56 ± 0.93%, while DSI of the families derived from the susceptible plants varied from 0.0% to 50.0% with a mean of 7.56 ± 2.04% (Table 2.1; Figure 2.4). This low incidence of disease under field condition can also be seen from the proportion of the total 2,742 plants scored for visible disease symptoms; only about 9.2% (251/2,742) has clubbed root (disease score = 2 and 3) while 90.8% (2,491/2,742) of the lack disease symptoms (disease score = 0 and 1).

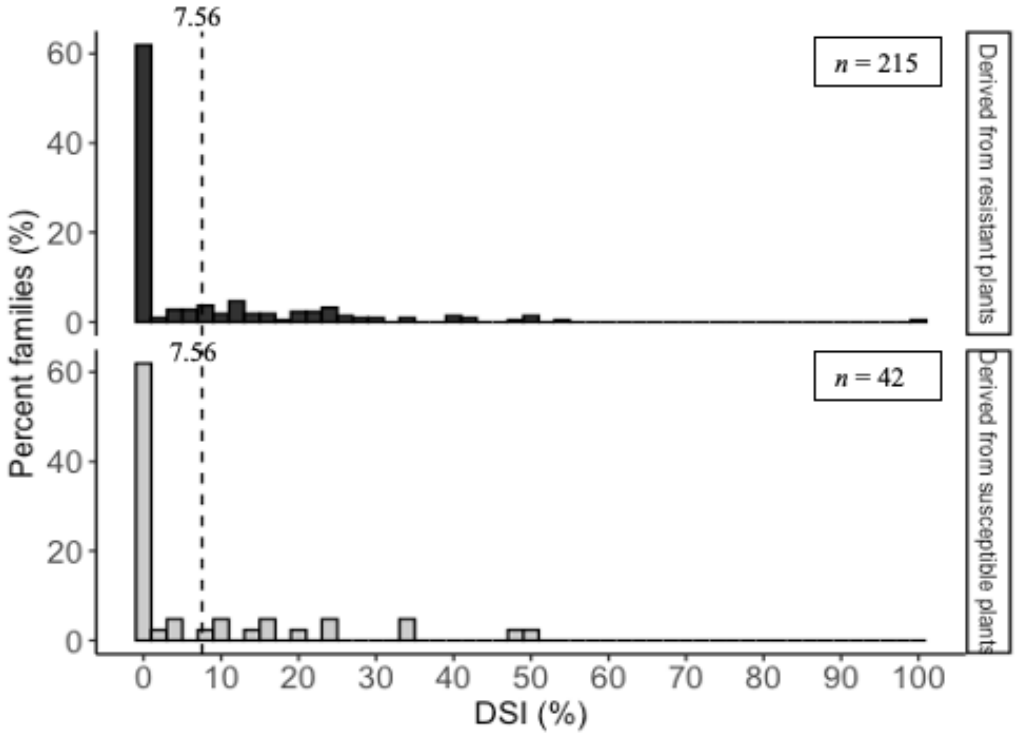

Figure 2.4 Frequency distribution of 257 families of F_6 field trial for clubroot evaluation in Spruce Grove. *n* = the number of families evaluated.

Table 2.1 Evaluation of different generation populations of *Brassica napus* A04-73NA × *B. oleracea* var. acephala cv. Winterbor (AM114) interspecific cross for resistance to *Plasmodiophora brassicae* pathotypes 3H in greenhouse, and in a clubroot infested field.

Pedigree	Generation	Test conditions	No. families	Total plants	No. res. plants	No. sus. plants	Disease severity index (%) of the families	
							Range	Mean±S.E.
B. nap. A04-73NA × *B. ole.* var. *acephala* cv. Winterbor (AM114)	**F_5**	**Greenhouse**	**21**	**707**	**581**	**126**	**25.0-100.0**	**42.54 ± 3.38**
B. nap. A04-73NA × *B. ole.* Var. *acephala* cv. Winterbor (AM114)	F_6 (F_5 Res.)	Greenhouse	211	2632	1132	1500	11.1-100.0	58.84 ± 1.52
B. nap. A04-73NA × *B. ole.* var. *acephala* cv. Winterbor (AM114)	F_6 (F_5 Sus.)	Greenhouse	45	527	104	423	40.0-100.0	75.54 ±2.28
Total	**F_6**	**Greenhouse**	**256**	**3159**	**1236**	**1923**	**11.1-100.0**	**61.78 ± 1.38**
B. nap. A04-73NA × *B. ole.* var. *acephala* cv. Winterbor (AM114)	F_6 (F_5 Res.)	Field	215	2388	2168	220	0.0-100.0	7.56 ± 0.93
B. nap. A04-73NA × *B. ole.* var. *acephala* cv. Winterbor (AM114)	F_6 (F_5 Sus.)	Field	42	354	323	31	0.0-50.0	7.56 ± 2.04
Total	**F_6**	**Field**	**257**	**2742**	**2491**	**251**	**0.0-100.0**	**7.56 ± 0.85**
B. nap. A04-73NA × *B. ole.* var. *acephala* cv. Winterbor (AM114)	F_6 (F_6 field Res.)	Greenhouse	43	294	141	153	4.8-95.2	55.23 ± 4.52
B. nap. A04-73NA × *B. ole.* var. *acephala* cv. Winterbor (AM114)	F_6 (F_6 field Sus.)	Greenhouse	5	35	1	34	85.7-100.0	93.32 ± 3.23
Total	**F_6**	**Greenhouse**	**48**	**329**	**142**	**187**	**4.8-100.0**	**59.20 ± 4.39**

Note: Disease scores 0 and 1 were considered as resistant, and scores 2 and 3 were considered as susceptible.
The F_5 and F_6 populations grown in greenhouse were evaluated for resistance at maturity and 45 days after inoculation, respectively, while the F_6 population grown in field were evaluated at the end of flowering (about 70 days after seeding). Analysed data of the whole population are bolded.

Based on field evaluation of the F_6 families, a total of 48 families were selected for test in greenhouse for resistance pathotype 3H at mature stage and to harvest F_7 generation seeds. These 48 families included 43 families with DSI 0% and five families with DSI 45.89 ± 2.73% (range: 40.35 - 54.55%). A total 294 plants belonging to the 43 families of the resistant group were evaluated, where about 48% (141/294) plants were resistant (disease score 0 to 1; mean = 0.55 ± 0.04) and 52% (153/294) were susceptible (disease score 2 to 3; mean = 2.67 ± 0.04); the DSI of this population was 55.23 ± 4.52% (range: 4.8 - 95.2%). On the other hand, of the 35 plants belonging to the susceptible group, only one plant (2.9%; disease score 0) showed resistance while 97.1% of the plants (34/35; disease score 2 to 3; mean = 2.88 ± 0.06) were susceptible with DSI of 93.32 ± 3.23% (range: 85.7 - 100.0%) (Table 2.1). Thus, the efficiency of selection for clubroot resistance under field condition was also evident in this interspecific cross-derived population, despite low incidence of disease in the field.

2.3.2 Days to flowering and seed quality traits

A total of 193 F_6 families were grown in field plots together with their spring *B. napus* parent for flowering time and seed quality traits. These families required 43 to 65 days to flower with a mean of 54.7 ± 0.3 days (Table 2.2). Most of the F_6 families required 49 to 60 days to flower; however, about 2% of the families flowered at about the same time (43-45 days) as the *B. napus* parent (44 days) (Figure 2.5). Four of the 193 families failed to produce any seed that could be harvested with a plot combine. Seed yield in the remaining 189 families varied from 0.05 to 47.20 g per plot with a mean seed yield of 13.54 ± 0.65 g per plot, which was significantly lower than the *B. napus* parent (32.16 ± 0.51 g per 3-meter row plot) ($t = 2.145$, $p < 0.001$).

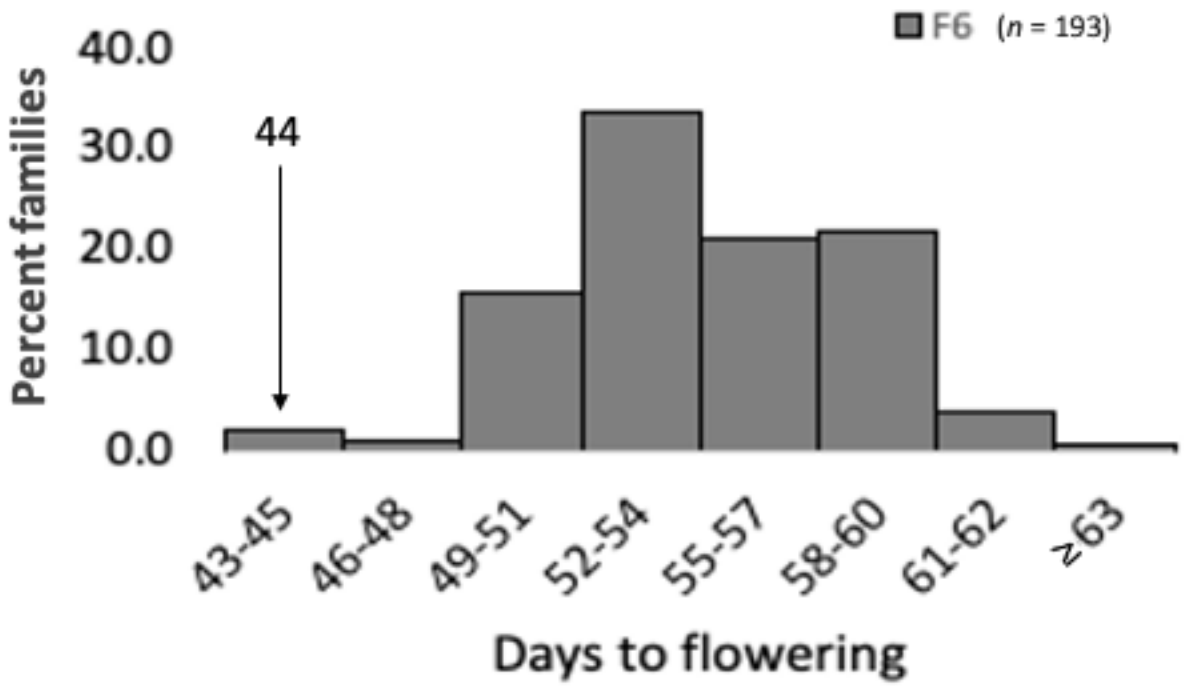

Figure 2.5 Frequency distribution of the F_6 families (n = 193) of *Brassica napus* A04-73NA × *B. oleracea* var. acephala cv. AM-114 interspecific cross for days to flowering. Days to flowering of the *B. napus* parent A04-73NA shown with a vertical arrow.

Table 2.2 Days to flowering and seed yield of the F_6 families of *Brassica napus* A04-73NA × *B. oleracea* var. acephala cv. AM-114 interspecific cross.

Population	No. families	Days to flowering		No. of fertile fam.	% fertile family[1]	Seed weight (g)/3-m row	
		Range	Mean ± S.E.			Range	Mean ± S.E.
F_6	193	43-65	54.7 ± 0.3	189	96%	0.05 - 47.20	13.54 ± 0.65
B. napus A04-73NA	3	44	44.0	3	100%	31.48 - 33.15	32.16 ± 0.51

[1]Plants having at least one viable seed were considered as fertile

The seeds harvested from 182 F_6 families were used for estimation of oil, protein and glucosinolate contents; this analysis could not be performed on the remaining seven families due to lack of sufficient quantity seeds. A continuous distribution of the F_6 population was found for seed oil and protein contents, while almost a bi-modal distribution was found for seed glucosinolate content (Figure 2.6). The average oil, protein and glucosinolate contents of this population was 43.51 ± 0.15%, 26.81 ± 0.11% and 41.09 ± 1.31 μmol/g seed, respectively. Seed oil (46.67 ± 0.87%) and protein (28.01 ± 0.39%) contents of the *B. napus* parent A04-73NA was about 3% and 1% higher than the F_6 population, respectively; however, these differences were not significant (for oil, t = -3.56, p = 0.07; for protein, t = -3.01, p = 0.09). Seed glucosinolate content of the F_6 population was significantly higher than the *B. napus* parent (41.09 ± 1.31 vs. 17.09 ± 0.99 μmol/g seed; t = 14.64, $p = 7.01\times10^{-10}$) (Table 2.3; Figure 2.6).

Table 2.3 Seed quality traits of the F_6 families of *Brassica napus* × *B. oleracea* interspecific cross.

Population	No. families	Seed oil (%)		Seed protein (%)		Glucosinolates (μmol/g seed)	
		Range	Mean ± S.E.*	Range	Mean ± S.E.*	Range	Mean ± S.E.*
F_6	182	37.1-48.5	43.51 ± 0.15[a]	24.1-31.1	26.81 ± 0.11[a]	12.3-87.7	41.09 ± 1.31[a]
B. napus A04-73NA	3	45.0-47.9	46.67 ± 0.87[a]	27.5-28.8	28.01 ± 0.39[a]	15.5-18.9	17.09 ± 0.99[b]

* Comparison made between the F_6 families and the *B. napus* parent A04-73NA grown in multiple plots. Mean ± S.E. following the same letter are not significantly different

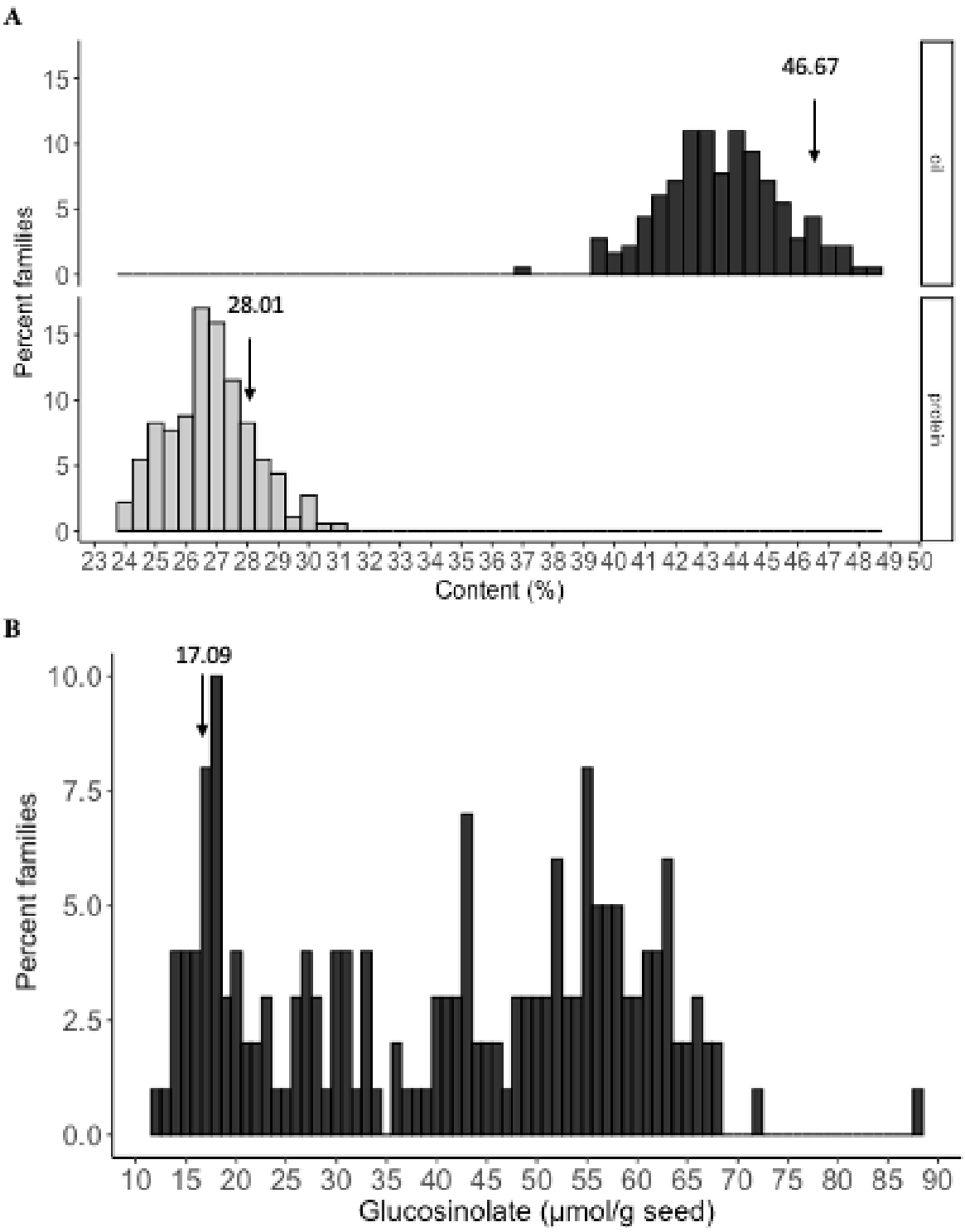

Figure 2.6 Frequency distribution of the F_6 (n = 182) families of *Brassica napus* × *B. oleracea* interspecific cross for (A) seed oil (%) and protein (%), and (B) glucosinolate (µmol/g seed) contents. The value of the *B. napus* parent A04-73NA for these three seed quality traits are shown with vertical arrows.

2.3.3 Erucic acid content

Self-pollinated F_6 seeds harvested from 140 F_5 plants were used for estimation of erucic fatty acid content in oil. The content of this fatty acid in this population varied from 5.25 % to 32.32 % with a mean of 22.06 ± 0.43%. Frequency distribution of the F_6 population for erucic acid presented in Figure 2.7. Of the 140 F_6 seed families, only one (0.7%) family had about 5% erucic acid; the content of this fatty acid in the remaining families (99.3%) varied from more than 5% to 32.32% (Figure 2.7).

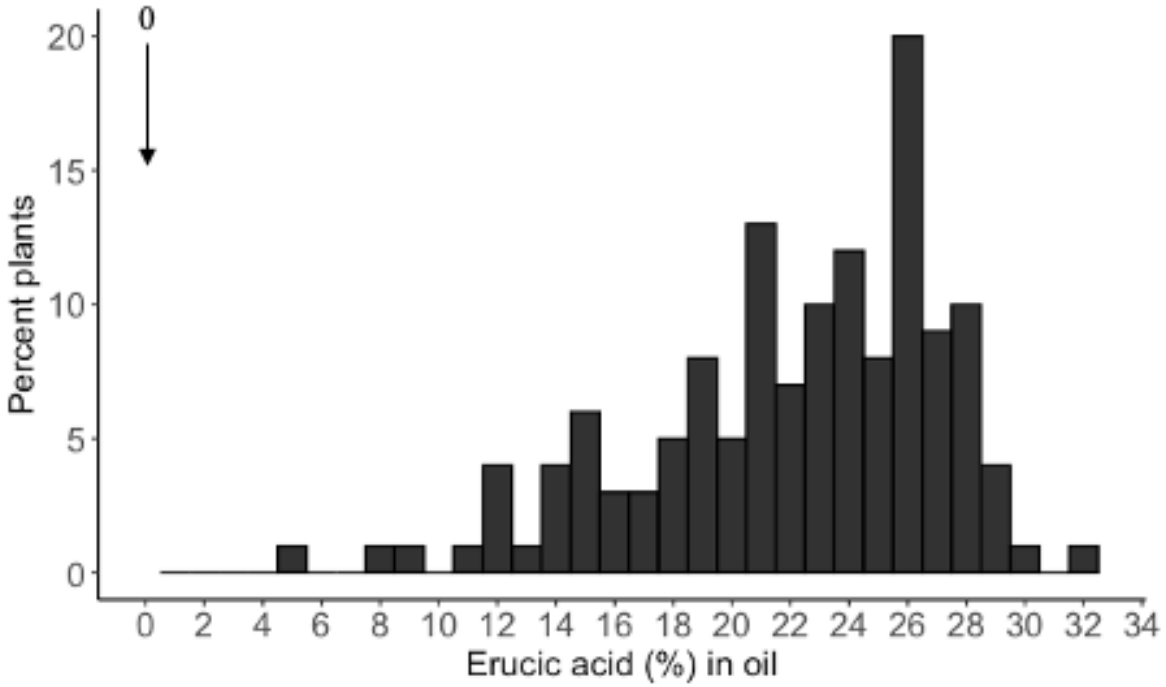

Figure 2.7 Frequency distribution of the selected F_6 (n = 140) plants of *Brassica napus* × *B. oleracea* interspecific cross for erucic acid content (%) in seed oil. The value of erucic acid content of the *B. napus* parent A04-73NA was shown with a vertical arrow.

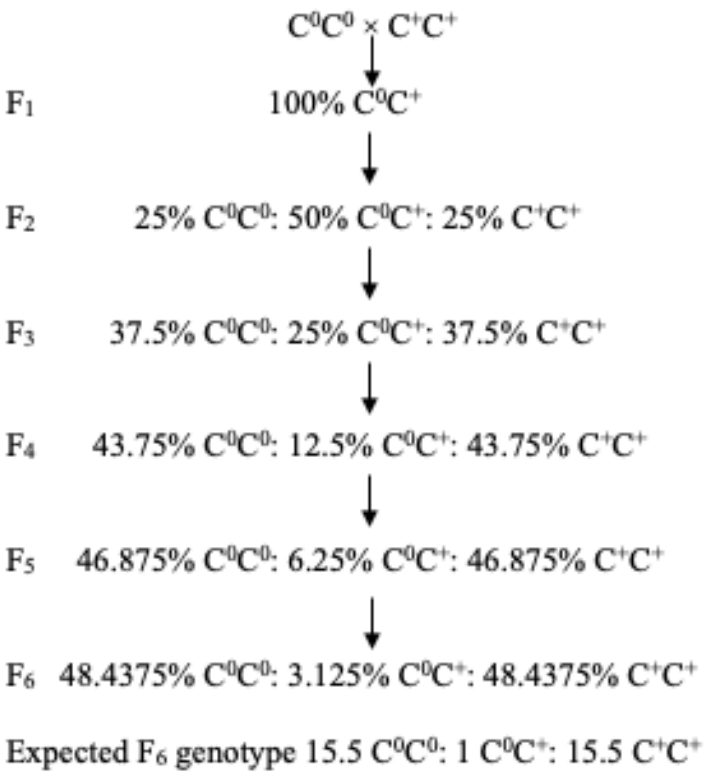

Figure 2.8 Expected genotype frequency in F_6 generation segregating for a single locus in the C-genome erucic acid allele.

2.3.4 Ploidy analysis

A total of 208 F_6 and 291 F_7 plants were analysed together with their *B. napus* A04-73NA and *B. oleracea* var. *acephala* cv. AM-114 for nuclear DNA content (Partec value). Almost a normal distribution was found for the Partec values in the F_7 population; however, a wider distribution without any sharp peak was found in the F_6 population (Figure 2.9). The Partec values of the F_6 plants varied from 174.9 to 633.0 with a mean of 415.53 ± 7.96 S.E., when the value of the *B. napus* parent A04-73NA was 401.93 ± 0.51 SE and of *B. oleracea* AM114 was 230.96 ± 11.83 S.E. (Table 2.4). In the case of F_7, the Partec values varied from 239.1 to 608.4 with a mean of 420.49 ± 3.72 S.E., while the value of A04-73NA was 413.92 ± 7.23 S.E. and AM114 was 228.46 ± 14.32 S.E. (Table 2.4). The mean Partec values of the F_6 and

F_7 populations were not significantly different from *B. napus*; however, the values were significantly different from the *B. oleracea* parent ($p < 0.05$) (Table 2.4).

Table 2.4 Relative nuclear DNA content (Partec value) of the F_6 and F_7 plants of *Brassica napus* × *B. oleracea* interspecific cross along with their parents.

Population	No. plants	Partec value	
		Range	Mean ± S.E.[1]
F_6:			
B. nap A04-73NA × *B.ole* AM114	208	174.9-633.0	415.53 ± 7.96 [a]
B. napus A04-73NA	4	400.5-402.7	401.93 ± 0.51 [a]
B. oleracea AM114	2	219.1-242.8	230.96 ± 11.83 [b]
F_7:			
B. nap A04-73NA × *B.ole* AM114	291	239.1-608.4	420.49 ± 3.72 [a]
B. napus A04-73NA	4	400.3-427.4	413.92 ± 7.23 [a]
B. oleracea AM114	2	214.1-242.8	228.46 ± 14.32 [b]

[1] While comparing the F_6 or F_7 populations with their parents, the mean ± S.E. values followed by same letter indicate no significant difference according to Welsch's *t*-test ($p > 0.05$).

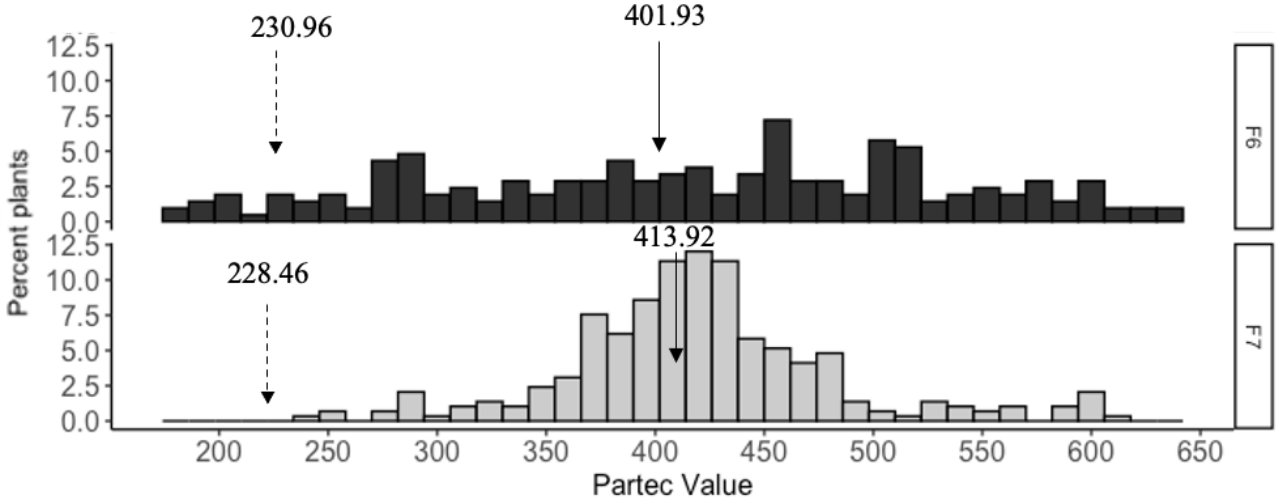

Figure 2.9 Frequency distribution of the F_6 (n = 208) and F_7 (n = 291) plants of *Brassica napus* × *B. oleracea* interspecific cross for relative nuclear DNA content (Partec value). The mean Partec value of the F_6 population was 415.53 while that of the F_7 population was 420.49. The Partec values of the *B. napus* and *B. oleracea* parents are shown with vertical solid and dashed arrows, respectively.

2.3.5 Seed set

A total of 707 F_5 plants grown in small pots in greenhouse for clubroot test were evaluated for fertility based on seed set. Of the 707 plants, 43.1% (305/707) plants received seed set score of 0, while 3.5% (25/707) plants were fully fertile (seed set score of 9). When the same F_5 population (n = 470) were grown in large pots for seed increase, 26.8% (126/470) of the plants received seed set score of 0 and only 0.6% (3/470) plants were fully fertile (seed set score of 9). Evaluation of 683 F_7 plants grown in large pots for seed increase also showed a similar level of fertility (24.7% plants received seed set score 0, and 3.4% received score of 9) (Figure 2.10). Thus the advancement of generation from F_5 to F_7 through self-pollination did not increase the fertility of the plants significantly.

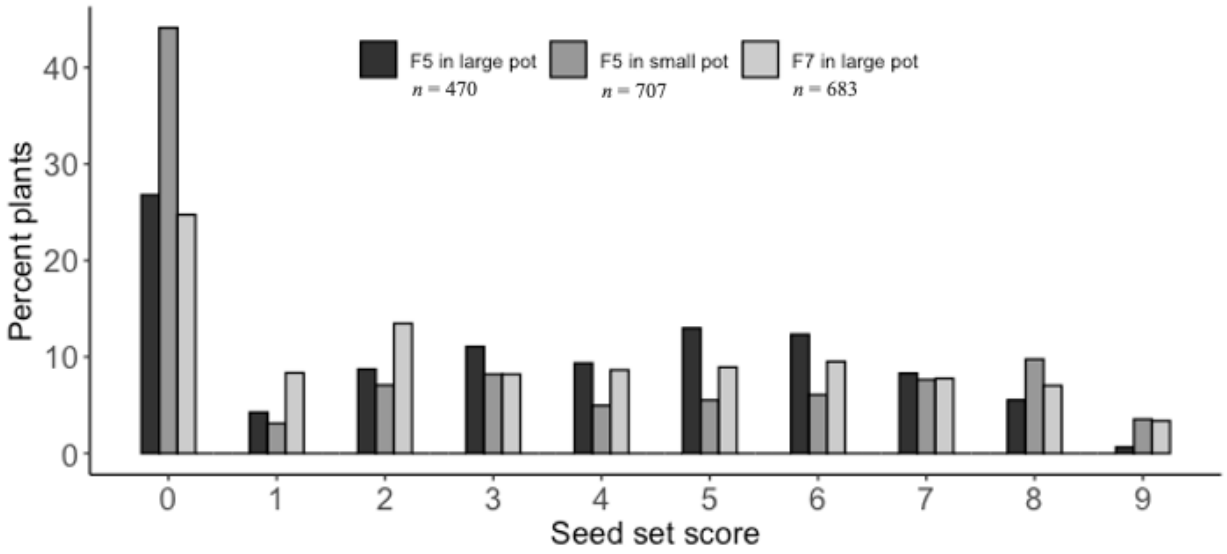

Figure 2.10 Frequency distribution of the F_5 and F_7 populations of *Brassica napus* × *B. oleracea* interspecific cross for fertility, which was estimated based on silique and seed set. The F_5 plants were grown in large pots for seed increase (n = 470) and in small pots for clubroot test (n = 707), and the F_7 plants were grown in large pots for seed increase (n = 683). A 0 to 9 scale was used for this (Figure 2.3), where 0 = no silique or seed produced, and 9 = silique and seed set similar to the *B. napus* parent.

Based on clubroot disease score, the F_5 population grown in small pots were grouped into four Classes I to IV based on disease score of 0, 1, 2 and 3, respectively, and seed yield of the plants presented in Figure 2.11. A similar trend for seed set was found in all classes where majority of the plants (90.1%; 637/707) produced less than 0.4 g seeds per plant. Only 4 plants (0.57% of the total 707) belonging to Class I and 1 plant (0.14% of the total 707) belonging to Class II produced more than 1.2 g seeds per plant. Plants belonging to Class I produced 0.0 - 1.9 g seeds per plant with a mean of 0.24 ± 0.03 g; plants belonging to Class II produced 0.0 - 2.3 g seeds per plant with a mean of 0.10 ± 0.01 g; plants belonging to Class III produced 0.0 - 0.7 g seeds per plant with a mean of 0.11 ± 0.02 g; and the plants belonging to Class IV produced 0.0 - 0.9 g seeds per plant with a mean of 0.08 ± 0.02 g. The seed yield of Class I was significantly different than that of other three Classes (t = 5.04, p <0.001; t =3.53, p <0.001; t =5.01, p <0.001).

The F_5 population grown in large pots for seed increase was derived from the F_4 plants with disease score of 0 to 3. In this population, 26.8% (126/470) plants produced no seed under self-pollination and 40.2% (189/470) plants produced more than 1.2 g seed per plant (Figure 2.11).

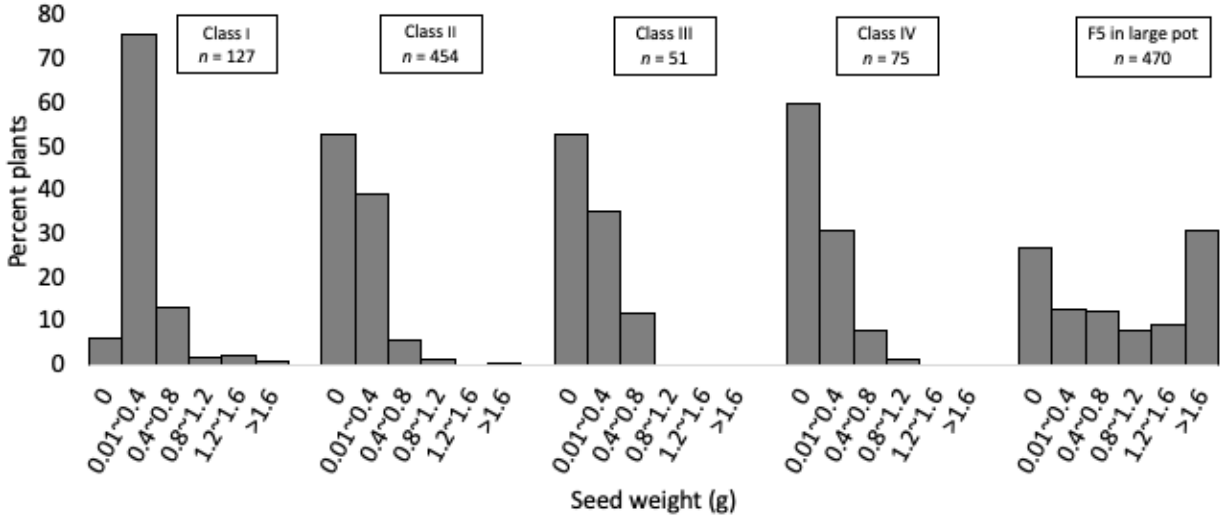

Figure 2.11 Seed yield (g) per plant of the F_5 population of *Brassica napus* × *B. oleracea* interspecific grown in small (class I-IV) and large pots. Class I = F_5 in small pot with disease

score 0; Class II = F_5 in small pot with disease score 1; Class III = F_5 in small pot with disease score 2; Class IV = F_5 in small pot with disease score 3; *n* is the number of plants.

F_7 population grown in large pots were grouped into four classes on the basis of clubroot disease score (0, 1, 2 and 3) in their earlier generation (F_6). A similar trend for seed yield per plant was observed in the four groups (Figure 2.12). Like the F_5 population, the great majority of the F_7 plants (64.6% of the total, i.e. 441/683) produced 0.0 to 0.4 g seeds per plant, while 15.2% (104/683) produced more than 1.2 g seeds per plant. The F_7 plants derived from the F_6 plants with disease score of 0 produced 0.0 - 2.4 g seeds per plant with a mean of 0.29 ± 0.04 g; the plants which had disease score of 1 in F_6 produced 0.0 - 4.4 g seeds per plant with a mean of 0.48 ± 0.06 g; the plants which had disease score of 2 in F_6 produced 0.0 - 0.3 g seeds per plant with a mean of 0.11 ± 0.06 g; the plants which had disease score of 3 in F_6 produced 0.0 - 5.7 g seeds per plant with a mean of 0.74 ± 0.06 g. The seed yield of the plants derived from the F_6 plants with disease score of 0 was significantly different from plants derived from F_6 with disease score of 1 or 3 (t = -2.66, p <0.05; t = -6.34, p <0.001), however, this difference was not significant with the plants that derived from F_6 with disease score of 2 (t = 2.46, p = 0.057).

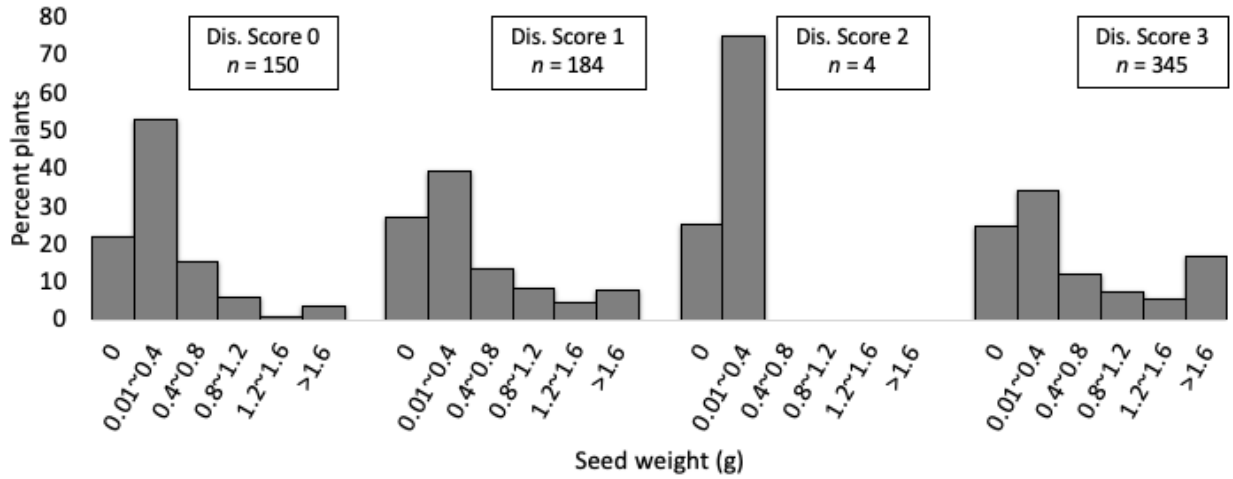

Figure 2.12 Seed yield (g) per plant of the F_7 population of *Brassica napus* × *B. oleracea* interspecific cross grown in large pots. Disease score (Dis. score) of 0 to 3 indicates the score of the F_6 generation plants. *n* is the number of plants.

The F_5 population grown in large pot for seed increase produced 0.0 - 7.0 g seeds per plant with a mean of 1.19 ± 0.06 g (Figure 2.11), while the F_7 population grown in large pot produced 0.0 - 5.7 g seeds per plant with a mean of 0.57 ± 0.04 g (Figure 2.12). It was surprising to see that seed yield of the F_7 population was significantly lower than that of the F_5 population (t = 8.73, p <0.001), which might be due to the difference in environmental conditions in the greenhouse.

2.3 Discussion

Screening of *Brassica* germplasm for resistance to clubroot disease demonstrated the potential of using resistances of the A genome of *B. rapa* (Hasan et al., 2012) and the C genome of *B. oleracea* (Farid et al., 2020) in the breeding of clubroot resistant *B. napus* canola. Several researchers introgressed the resistances of *B. rapa* into *B. napus* through interspecific cross between these two species (e.g. Hirani et al., 2016; Hasan et al., 2021; Kaur et al., 2022; Zhan et al., 2022). In case of the resistances of *B. oleracea*, several researchers identified clubroot resistance loci in the C genome of *B. oleracea* (Rocherieux et al., 2004; Nagaoka et al., 2010; Peng et al., 2018; Dakouri et al., 2018; Farid et al., 2020). However, efforts for introgression of this resistance into *B. napus* cannot be found in literature, which might be, primarily, for the difficulty of producing hybrids of the *B. napus* × *B. oleracea* interspecific cross (Bennett et al., 2008; Iftikhar et al., 2018). Given the fact that several accessions of *B. oleracea* carry excellent resistance to different *P. brassicae* pathotypes (Rocherieux et al., 2004; Farid et al., 2020), introgression of this resistance into *B. napus* will not only increase the pool of clubroot resistance genes in *B. napus*, but this will also allow pyramiding the A and C genome resistances in *B. napus* for a stronger and durable resistance to this disease in this crop. The present study was,

therefore, undertaken to investigate the prospects of introgression of the C genome resistance into *B. napus* through *B. napus* × *B. oleracea* interspecific cross.

In the present study, clubroot resistant plants was found in different generation populations; however, their resistance did not hold true in the next generation. For example, less than 50% of the F_6 plants derived from resistant F_5 plants were found to be resistant (Table 2.1); furthermore, clubroot resistant plants were also found in the progeny of the susceptible plants (Table 2.1). While working with the earlier generation populations of this interspecific cross, Zhang (2022) also reported similar difficulties in achieving a homozygous resistant line. Zhang reported that the progeny generation (F_4) of the resistant F_3 plants yielded only 20% resistant plants, and progeny generation of the susceptible F_3 plants yielded 12.5% resistant plants as well. This is in contrast to introgression of major resistance genes from *B. rapa* into *B. napus* (Kaur et al., 2022) where a clubroot resistant homozygous line could be achieved after self-pollination of heterozygous plants for one generation (e.g. in BC_1F_2 generation). Therefore, meiotic anomalies of chromosomes in the progeny of *B. napus* × *B. oleracea* interspecific cross cannot be considered affecting the resistance. It is highly likely that the resistance of the kale accession that has been used in the present study is controlled by multiple gene loci where recessive genes might also play a role in resistance. Despite this, selection for resistance was found quite effective in this study. For example, about 48% of the F_6 plants of the F_6 families selected based on their resistance under field conditions showed resistance to pathotype 3H under greenhouse conditions (Table 2.1).

The F_6 generation population of the *B. napus* × *B. oleracea* interspecific cross was studied for days to flowering and seed quality traits. Days to flowering (Long et al., 2007; Mei et al., 2009), seed oil (Fu et al., 2017; Chao et al., 2017; Rahman and Kebede, 2021) and protein

content (Schatzki et al., 2014; Chao et al., 2017; Rahman and Kebede, 2021) in *Brassica* are generally considered as quantitative traits controlled by multiple gene loci, while glucosinolate content is controlled by far less number of loci (five to seven) (Uzunova et al., 1995; Schatzki et al., 2014; Rahman et al., 2014a; Rahman and Kebede, 2021). A wide variation of days to flowering was observed in the F_6 generation population where two families flowered one day earlier (43 days) than the *B. napus* parent (44 days). This further support the perspectives of Rahman et al. (2011b) that *B. oleracea* carry alleles which can improve the earliness of *B. napus*. As expected for a quantitative trait, the seed oil and protein contents showed a continuous variation and the mean values were lower than that of the *B. napus* parent (Table 2.3). This result was not surprising given the fact that *B. oleracea* has never been subjected to breeding for the improvement for these traits. It is highly likely that undesirable alleles for these two seed constituents have been introgressed from *B. oleracea* into this population; however, improvement of these traits would be possible through cross-breeding these lines with canola. A bi-modal distribution for seed glucosinolate content observed in this study indicates that a few gene loci to be involved in the control of this trait (Uzunova et al., 1995; Schatzki et al., 2014; Rahman et al., 2014a; Rahman and Kebede, 2021). Based on glucosinolate content of this population, it can be inferred that high-glucosinolate alleles of *B. oleracea* has been introgressed into this population; however, plants with about 12 µmol glucosinolate per g seed was found in this population.

Erucic acid content in *B. napus* is known to be controlled by two major gene loci, one from each the A- and C-genomes (Rahman et al., 2008). The *B. napus* A04-73NA parent used in this study was a canola quality type (zero erucic acid in oil and <15 µmol glucosinolates per g/seed in seed meal) while the *B. oleracea* var. *acephala* parent was a non-canola quality type.

Therefore, it was expected that the F_6 population will be segregating only for the C-genome erucic acid alleles. Based on this genetic background of the parents and in the absence of selection for erucic acid in earlier generations, theoretically, it was expected that about 48.44% of the F_6 families will be zero erucic acid type (less than 1% erucic acid) while the remaining 51.56% families will have a variable content of this fatty acid (Figure 2.8). However, none of the F_6 families was zero erucic acid type; only one family had a low content (5.25%) of this fatty acid. This result suggests that segregation distortion for the erucic acid alleles occurred in this population. However, about 2.17% (3/138) of the F_6 families found to have less than 10% erucic acid in oil; this provide evidence that a zero erucic acid *B. napus* line can be obtained from this population through selection for low content of this fatty acid.

The genome composition of the F_1 of the *B. napus* × *B. oleracea* cross was expected to be ACC (di-genomic triploid). In meiosis, the diploid set of C-genome chromosomes were expected to produce bivalents and follow normal chromosome segregation, while haploid set of the A-genome chromosomes were expected to segregate randomly and incorporate in the gametes. Due to this behaviour of the chromosomes, the number of chromosomes in the advanced generation population was expected to vary from $2n = 18$ (CC) to $2n = 38$ (AACC). Attri and Rahman (2018) and Iftikhar et al. (2018) provided compelling evidence in support that the advanced generation populations of *B. napus* × *B. rapa* (AAC) and *B. napus* × *B. oleracea* (ACC) interspecific cross stabilizes into *B. napus* (AACC, $2n = 38$) type. Analysis of the ploidy level of the F_6 and F_7 populations showed that the majority of the plants were close to the *B. napus* parent for chromosome number (Partec value). However, a wide variation for Partec values was found in both F_6 and F_7 generations where variation in the F_6 population (174.9-633.0) was greater than that in the F_7 population (239.1-608.4) (Table 2.4). This indicated that the F_7 population was

closer to the *B. napus* parent for chromosome number than the F_6 population. However, a few plants had Partec value greater than the *B. napus* parent indicating that these plants carry in excess of 38 chromosomes. Cui et al. (2012) provided evidence that abnormal or unreduced gametes can be produced in the progeny of *Brassica* interspecific hybrids, and this could result plants with a greater number of chromosomes than expected. The results from the present study provided further evidence of the occurrence of unreduced gametes in the progeny of *Brassica* interspecific hybrids.

Seed set in the population derived from the *B. napus* × *B. oleracea* interspecific cross was generally low. The parent *B. oleracea* var. *acephala* used in this study was self-incompatible. According to Nasrallah et al. (1988), self-incompatibility in *B. oleracea* is controlled by multiple S-alleles, and Rahman (2005) found that resynthesized *B. napus* lines inherit S-alleles from its progenitor species *B. rapa* and *B. oleracea* and exhibit a strong self-incompatibility phenotype resulting in poor seed set. Therefore, in addition to meiotic anomalies of chromosomes, self-incompatibility might have played a role in poor seed set in the F_5 and F_7 populations that has been investigated in the present study.

Thus, the results from this thesis research demonstrated the possibility of introgression of clubroot resistance from the C genome of *B. oleracea* into *B.* napus, and developing a genetically stable canola quality clubroot resistant *B. napus* line from *B. napus* × *B. oleracea* interspecific cross. This study provided further evidence that the advanced generation populations of *B. napus* × *B. oleracea* interspecific cross stabilizes into *B. napus* type, and thus provided further evidence of the possibility of using *B. oleracea* in the breeding of *B. napus* canola.

Chapter 3 Mapping of clubroot resistance in the C genome using the population derived from *B. napus* × *B. oleracea* interspecific cross

3.1 Introduction

Brassica napus canola (AACC, 2*n* = 38) is grown in temperate regions of the world as an oilseed crop. Its oil is used for food purposes, and the protein-rich seed meal is used in animal feed (Canola Council of Canada, 2022). Canola oil can also be used for production of biodiesel (Ge et al., 2017), and the dried stem of this crop can be used for production of textile fiber (Shuvo et al., 2019).

The world production of canola in 2020/21 was 25.1 million tonnes, where Canada was the largest producer (4.49 million tons), followed by Germany (3.78 million tons), China (2.97 million tons), and India (2.52 million tons) (www.fao.org/faostat, retrieved in April 2023). In Canada, canola is one of the top cash crops, which currently, make an annual contribution of about $29.9 billion to the economy of this country (LMC International, 2020). However, several abiotic and biotic stresses pose threat to canola production in Canada; among these, clubroot disease caused by *Plasmodiophora brassicae* is currently the most important one (Tewari et al., 2005; Pageau et al., 2006; Strelkov et al., 2007; Dixon, 2009; Hwang et al., 2011; Strelkov et al., 2020).

The soil-borne pathogen *P. brassicae* infects the roots and disrupts the uptake of water and nutrients (for review, see Javed et al., 2022). In Canada, more than 80% yield loss has been reported under extreme infestation (Pageau et al., 2006; Hwang et al., 2011). The traditional agronomic practices found not to be effective to manage the clubroot disease (for review, see Peng et al., 2014); therefore, the development of clubroot-resistant canola cultivars has been considered the most important strategy to manage this disease (Rahman et al., 2011, 2014).

Recently, new virulent *P. brassicae* pathotypes evolved in Canada which overcome the resistance of the commercial canola cultivars carrying a single dominant gene (Strelkov et al., 2016). Therefore, broadening the genetic base of *B. napus* canola for clubroot resistance genes is required.

Most of the commercial clubroot-resistant *B. napus* canola cultivars grown in Canada carry one or two clubroot resistance genes. Hasan et al. (2012) identified several accessions of *B. rapa* and *B. oleracea* carrying resistance to different *P. brassicae* pathotypes. Similarly, Farid et al. (2020) found resistance to pathotypes 3A and 5X (L-G2) in several *B. oleracea* accessions, and identified QTL on chromosomes C03, C04, C06, C07, C08, and C09 contributing to this resistance. Thus, the two parental species of *B. napus* (*B. rapa* and *B. oleracea*) could be the valuable resources for increasing the pool of clubroot resistance genes in *B. napus*. To date, the majority of the clubroot-resistant canola cultivars grown in Canada carry resistance in the A-genome derived from *B. rapa* (Rahman et al., 2011; Hirani et al., 2016; Kaur et al., 2022; Hasan et al., 2021; Zhan et al., 2022). In this regard, introgression of the C-genome resistance from *B. oleracea* into *B napus* would broaden the genetic base of resistance in this crop, and this can be achieved through *B. napus* × *B. oleracea* interspecific cross (Bennett et al., 2012; Rahman et al., 2015; Zhang, 2022).

Nowadays, molecular markers are extensively used in genetic studies to identify alleles for a particular trait of interest. Molecular markers are categorized as hybridization-based markers, such as restriction fragment length polymorphism (RFLP) markers (Botstein et al., 1980), and PCR-based markers such as randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) (Vos et al., 1995), and simple sequence repeat (SSR) markers (for review, see Agarwal et al., 2008; Vos et al., 1995; Zietkiewicz et al., 1994).

Among the different PCR-based markers, the SSR markers are widely used in mapping and genetic diversity studies due to their co-dominance behaviour and abundance in the genome. For example, Hasan and Rahman (2016) mapped the clubroot resistance locus on chromosome A08 by using SSR markers. Iftikhar et al. (2018) used SSR markers to demonstrate the prospects of developing genetically diverse *B. napus* lines carrying genome contents of *B. oleracea* from *B. napus* × *B. oleracea* interspecific crosses.

The objectives of this study were map the clubroot resistance of *B. oleracea* introgressed into *B. napus*, and to identify molecular markers associated with this resistance.

3.2 Materials and Methods

3.2.1 Plant materials

Sixty-nine F_7 families derived from an interspecific cross between a clubroot-susceptible *B. napus* line A04-73NA and the clubroot-resistant *B. oleracea* var. *acephala* cv. Winterbor (AM114), reported in Chapter 2, were used in this study. Plants of each family were grown in a greenhouse set at 21/15 ± 2 °C (day/night), 16 h photoperiod, and light intensity of 450 μmol/m^2s for extraction of DNA.

3.2.2 DNA extraction

Young and healthy leaves of 99 F_7 plants belonging to 8 homozygous resistant and 11 homozygous susceptible F_6 families were collected from three to four-week-old plants in 1.5 ml Eppendorf tubes and placed in liquid nitrogen for 1-2 min. Afterwards, leaf samples were ground with plastic pestle (Argos Technologies Inc., Dundee, IL, USA) and 500 μL DNA extraction buffer (Invitrogen, Carlsbad, CA, USA) was added to each of the tubes. The mixtures were incubated in a water bath at 65 °C for 30 minutes with gentle inversions and 3 μL of RNase solution (Invitrogen, Carlsbad, CA, USA) was added to each tube, which was inverted 2 to 5

times and incubated at 37 °C for 25 minutes. After that, 150 µL of protein precipitation solution was added to each sample and the samples were placed on ice for 5 minutes. After adding 500 µL of chloroform to each sample followed by several inversions, the mixtures were centrifuged at 10,000 rpm for 15 minutes at 4 °C. The clumped debris was carefully discarded, and the aqueous layer was placed in new 1.5 mL tube. 500 µL of ice-cold isopropanol was added to the tubes and mixed well with mild inversions and centrifuged at 10,000 rpm for 10 minutes at 4 °C. Next, the supernatant was removed, the pellets were washed (3 times) with 300 µL of 70% cold ethanol and centrifuged at 10,000 rpm for 3 minutes and the ethanol was discarded. The tubes were kept open to air-dry at room temperature for 10 minutes.

Following the manual of the DNA extraction kit (Invitrogen, Carlsbad, CA, USA), the dried pellets were dissolved with 50 µL of nuclease-free water (Life Technologies, Austin, USA) for resuspension of the DNA. After that, the quantity and quality of the DNA was determined by a NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The DNA samples were diluted to a concentration of 25 ng/µL and stored at -20°C for polymerase chain reaction (PCR). The purity of the DNA samples was determined by the ratio of absorbance of the ultraviolet light at 260 and 280 nm; the DNA samples with 260/280 ratio ranging from 1.80 to 2.00 were considered as high-quality DNA (Desjardins and Conklin, 2010).

The DNA quality of 99 F_7 plants was determined where 39 were discarded due to poor quality. Of the 60 samples, 56 were used for preparation of bulks. The following four DNA bulks, based on resistance phenotype of the F_6 plants reported in Chapter 2 (Supplementary Table 3.1), were prepared for genotyping:

RB = Bulk of 26 plants (belonging to 5 F_6 families) resistant to pathotype 3H with disease scores of ‘0’ and ‘1’;

SB = Bulk of 30 plants (belonging to 7 F_6 families) susceptible to pathotypes 3H with disease score of '3'.

Plants with disease scores of '0' and '1' were considered as resistant and '2' and '3' as susceptible (as described in Chapter 2).

In addition to this, the following two DNA samples of the parents were used:

RP = Resistant parent *B. oleracea* var. *acephala* cv. Winterbor (AM114); resistant to pathotype 3H (DSI < 25 %);

SP = Susceptible parent *B. napus* A04-73NA; susceptible to pathotypes 3H (DSI = 100%);

3.2.3 Simple sequence repeat (SSR) markers

Two-hundred forty publicly available SSR markers (Zhang, 2022; Parkin et al., 2014) and markers designed by the Canola Breeding Program of the University of Alberta from the nine C-genome chromosomes were used to identify the polymorphic markers by using the above-mentioned parental and bulk DNAs: RP, SP, RB and SB. The markers producing clear polymorphic bands were used for genotyping the 60 individual plants which included 27 resistant (belonging to 6 F_6 families) and 33 susceptible (belonging to 10 F_6 families) F_7 plants (Supplementary Table 3.1).

3.2.4 PCR amplification

PCR amplification of the genomic DNA was performed using SimpliAmp Thermal Cycler (Life Technologies Holdings Pte Ltd., Singapore). The PCR reaction mixture was prepared in a total volume of 12 µl, containing 2 µl of 25 ng/µl genomic DNA, 2.4 µl of 5X colorless GoTaq buffer, 1.2 µl of 50 mM $MgCl_2$, 2.4 µl of 10 mM dNTPs (Life Technologies, Carlsbad, USA), 0.3 µl of 25 nM fluorescent dye-labeled M13 primer (FAM, VIC, NED and

PET; Applied Biosystem, Foster City, USA), 1 µl of each forward and reverse primer, 1.575 µl of nuclease-free water, and 0.125 µl of GoTaq DNA polymerase enzyme (Promega Corporation, Madison, USA). The PCR cycles included a single cycle of initial denaturation at 95 °C for 3 minutes followed by 42 cycles of denaturation at 95 °C for 30 seconds, annealing at 56 °C for 30 seconds and extension at 72 °C for 45 seconds, and one cycle of final elongation at 72 °C for 10 minutes and then a hold stage at 4 °C (Kebede et al., 2010). The melting temperature (Tm) of the primers chosen for PCR amplification was used to calculate the annealing temperature. The PCR products were stored at -20 °C until used for genotyping.

3.2.5 ABI (Applied Biosystem Instruments) sequencing

The Schuelke's (2000) M13 primer labeling technique was used to visualize the PCR products. For this, the universal M13 sequence 5'-CACGACGTTGTAAAACGAC-3' labelled with four fluorescent dyes FAM, VIC, NED and PET (Applied Biosystem, Foster City, USA) was appended to the 5' end of the forward primer of each SSR marker. For sequencing using a ABI sequencer, 1.5 µl of each fluorescent-labelled amplified PCR product was added to a mixture of 7.9 µl highly deionized (Hi-Di) formamide (Thermo Fisher Scientific, Carlsbad, USA) and 0.1 µl GeneScan-500 LIZ size standard (Applied Biosystems, Warrington, UK). The amplified products were detected by a capillary electrophoresis system using ABI sequencer No. 3730 analyzer (Applied Biosystems, Foster City, CA) and the genotypic data was analyzed by GeneMarker software (version 2.6.3) (Soft Genetics LLC, USA).

3.2.6 Data analysis and linkage map construction

To visualize the genotypes of the bulks and individual plants from ABI sequencing, the presence of a *B. napus* allele was denoted as 'A' while the presence of a *B. oleracea* allele as 'B'. The clear and sharp peaks were counted as marker amplicons. The mapping population, which

included 27 resistant and 33 susceptible F_7 plants was used to calculate the frequency of the occurrence of *B. oleracea* alleles in the mapping population. This calculation was carried out using Microsoft Excel (Version 16.56) separately for the resistant and susceptible groups based on their genotypic and phenotypic data.

For linkage map construction, genotypic data were coded by number. Allele 'A' and 'B' were coded as '0' and '2' respectively, while the missing values of markers were coded as '-1'. The coded data was analyzed using the software program QTL IciMapping version 4.2 (Meng et al., 2015) with a minimum logarithm of odds (LOD) score of 3.0. Kosambi mapping function (Kosambi, 1944) was applied to calculate the recombination frequency, and the recombination frequency was converted to centi-Morgan (cM).

To identify the SSR markers associated with the QTL conferring resistance to pathotype 3H and their additive effects, the genotypic and phenotypic data (DSI %; results described in Chapter 2) were analyzed using Single Marker Analysis (SMA) and Inclusive Composite Interval Mapping-Additive (ICIM-ADD) methods in QTL IciMapping version 4.2 (Meng et al., 2015). The parameters were, a walking speed of 0.1 cM and a stepwise regression probability of 0.001 (Manichaikul et al., 2009). The LOD threshold with a *p*-value lower than 0.05 was obtained from 1,000 permutations to declare a QTL (Churchill & Doerge, 1994). A QTL with LOD score of 3.0 or higher and explained more than 10% of the total phenotypic variance were considered as major QTL while QTL with less than 10% variance were considered as minor (Lander & Kruglyak, 1995).

3.3 Results

3.3.1 Molecular marker analysis of the F_7 population

A total of 240 SSR markers (Supplementary Table 3.2) from the nine C-genome

chromosomes were screened for polymorphism of which 12 (5%) were polymorphic (Table 3.1). Of the 12 polymorphic markers, eight (66.7%) producing clear fragments with a difference of more than 10 bp were used for genotyping the 27 resistant and 33 susceptible F_7 plants. Three of the eight markers were from chromosome C04, three from C05, and two from C08 (Table 3.1).

Table 3.1 List of 12 polymorphic SSR markers used to genotype the clubroot resistant and susceptible F_7 plants of *Brassica napus*× *B. oleracea* var. acephala interspecific cross.

Marker name[1]	Chr.	Forward primer (5'-3')	Reverse primer (5'-3')	Source
C04_3772*	C04	GGACACCGAAAGTTCCTCCA	TATACTCAGCGCCGCAACAT	Zhang (2022)
C04_3779*	C04	TTGAGAGTCGCACACCAGAT	GCCGGAATCCAATAGAGCGA	Zhang (2022)
C04_3784*	C04	TTTTTGAGAGTCGCACACCAG	TCCTTAAACGTTGTGCCGGA	Zhang (2022)
C05_5458*	C05	TTGACAGGAGGCGAAGAGAG	GGCGACTTAACCAATGACGG	*B. napus* cv. Darmor v10.0
C05_5461	C05	AGCATCCATTCCACATTACTGA	TATATAACGTTCAGGCCGGC	*B. napus* cv. Darmor v10.0
C05_5463*	C05	ACTCTGTTCCTGTTCTTGTGT	CCATCCTGGCCCATAGAAGA	*B. napus* cv. Darmor v10.0
C05_5466*	C05	ATTGGAACCATTGCCCACAG	GTTTCTGAACCGAGCATGGA	*B. napus* cv. Darmor v10.0
C05_5468	C05	ATTCTGAGCTGGAACCGAGG	CCTTGTTGACTTTGACCTTGACT	*B. napus* cv. Darmor v10.0
C05_5470	C05	TGGCTTCATTTGGATTACGGG	GCAACAAGATTGGCTGATCAA	*B. napus* cv. Darmor v10.0
C08_4001*	C08	CGAGCACTCGCGTTAAAAGT	TGGAGCAGTTATCGTTCGCA	Parkin et al. (2014)
C08_4002*	C08	TCGAGCACTCGCGTTAAAAG	GAGCAGTTATCGTTCGCAGC	Parkin et al. (2014)
C08_4004	C08	CGAGCACTCGCGTTAAAAGTC	AGCAGTTATCGTTCGCAGCA	Parkin et al. (2014)

*Eight polymorphic SSR markers showing clear fragments (>10 bp) were used for genotyping the clubroot resistant and susceptible F_7 plants.

[1]Markers C05_5458, C05_5461, C05_5463, C05_5466, C05_5468 and C05_5470 were designed by the Canola Program of the University of Alberta based on the genome sequence information of *B. napus* cv. Darmor whole genome assembly v10.0.

3.3.2 Frequency of the *B. oleracea*-specific alleles

Of the eight polymorphic markers, six detected *B. oleracea* allele in both resistant and susceptible plants, while the markers C05_5458 and C05_5466 detected *B. oleracea* allele only in the resistant plants (Table 3.2). The frequency of occurrence of *B. oleracea* allele for the six markers in the susceptible plants varied from 3.4% to 27.6%, while in the resistant plants it varied from 87.5% to 100.0% (Table 3.2); however, no clear linkage association of these markers with resistance to pathotype 3H could be established. In case of the marker C05_5458, 92.6% (25/27) of the resistant plants carried the *B. oleracea* allele and this allele could not be found in the susceptible plants. For the marker C05_5466, 100% (24/24) of the resistant plants carried the *B. oleracea* allele; this allele could not be found in the susceptible plants (Table 3.2).

3.3.3 Linkage map and QTL mapping of clubroot resistance

Genetic linkage maps of C04, C05 and C08 chromosomes were constructed by using the genotypic data to locate the clubroot-resistance loci of *B. oleracea* introgressed into *B. napus* (Figure 3.1). Only two to three markers from each of these three chromosomes were used to genotype the mapping population; therefore, the linkage maps captured only a small segment (about 4 to 24 cM) of each of these three chromosomes. While focusing on one chromosome at a time, the QTL mapping identified two loci on chromosome C04. One of the two loci was detected between the SSR markers C04_3779 (0.00 cM) and C04_3784 (14.11cM) with LOD score of 7.33; this QTL explained 18.86% of the total phenotypic variance for resistance to pathotype 3H and exerted an additive effect of -25.94% DSI (Table 3.3; Figure 3.1). The second locus on C04 was detected between the markers C04_3784 (14.11cM) and C04_3772 (24.52cM) with LOD score of 37.65; this locus explained 39.55% of the total phenotypic variance and exerted an additive effect of -37.58% DSI (Table 3.3). In case of chromosome C05, one QTL

was found which was flanked by the markers C05_5458 and C05_5466 (Figure 3.1); this QTL was detected with LOD score of 47.38, explained 97.47% of the total phenotypic variance, and exerted additive effect of -37.58% DSI (Table 3.3). In the case of the chromosome C08, one QTL was detected between the markers C08_4001 and C08_4002 (Table 3.3; Figure 3.1) with LOD score of 42.98; this QTL explained 85.58% of the total phenotypic variance and exerted an additive effect of -37.58% DSI (Table 3.3). While analyzing genotypic data of all three chromosomes together, QTL mapping identified one major QTL on chromosome C05 between the markers C05_5458 and C05_5466; this QTL was detected with LOD score of 47.35, explained 97.37% of the total phenotypic variance, and exerted additive effect of -37.58% DSI (Table 3.4). In all cases, *B. oleracea* allele reduced disease severity.

Single marker analysis (SMA) was carried out using genotypic data of all three chromosomes together to confirm the above-mentioned QTL analysis results. The eight SSR markers identified the QTL regions of C04, C05 and C08 with LOD scores of 6.23 to 47.38, and these QTL exerted an additive effect of -23.48% to -37.58% DSI (%). However, based on SMA analysis, these genomic regions explained only 6.79% to 17.40% of the total phenotypic variance for resistance to pathotype 3H, which was much lower than the total phenotypic variance detected using inclusive composite interval mapping-additive (ICIM-ADD) method (Table 3.4). The SMA analysis also showed that the *B. oleracea* alleles reduced DSI in *B. napus*.

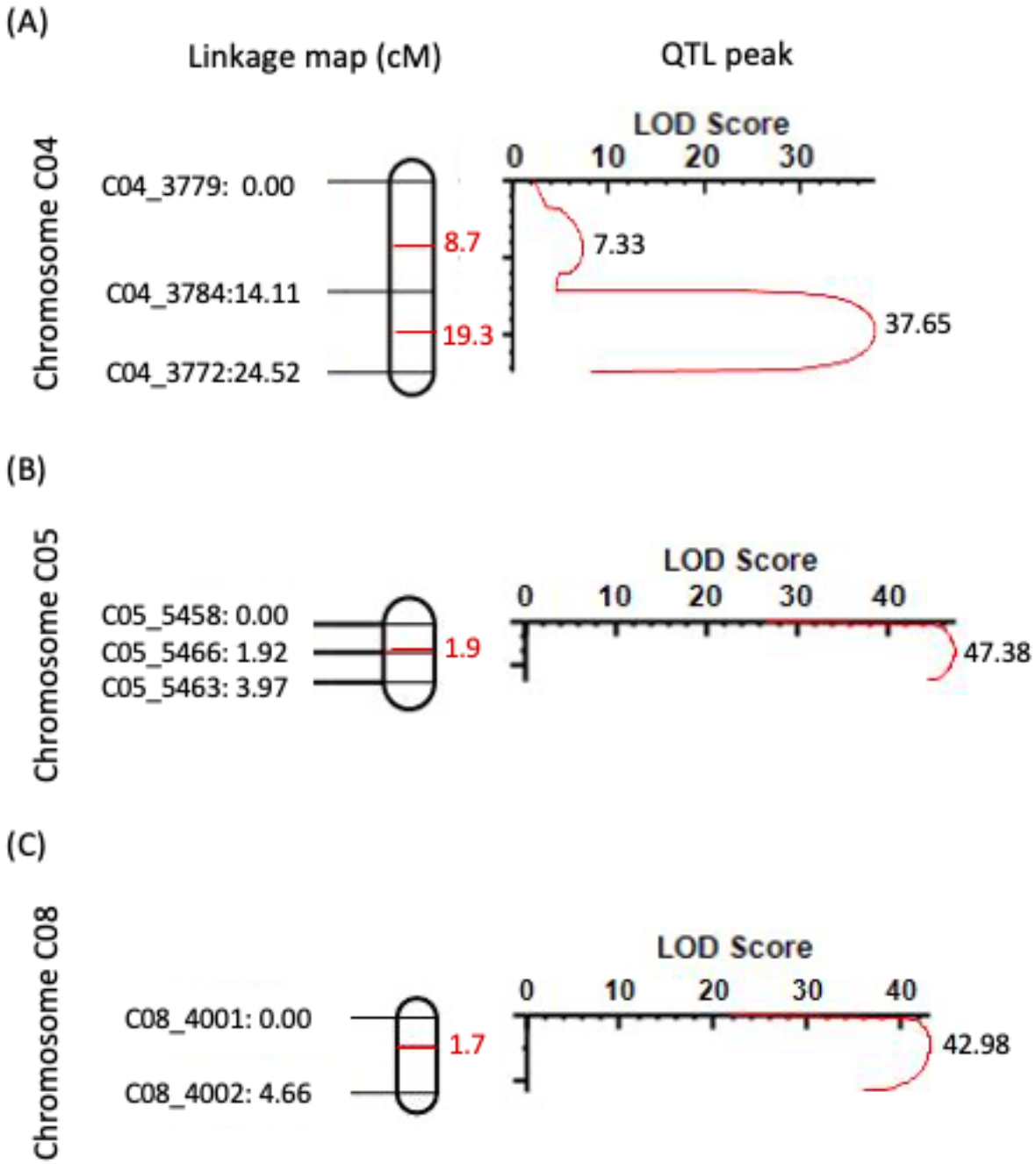

Figure 3.1 Construction of genetic linkage maps and QTL analysis by considering one chromosome at a time. (A) Chromosome C04 map was constructed using 3 SSR markers, (B) C05 map was constructed using 3 SSR markers, and (C) C08 map was constructed using 2 SSR markers. For QTL likelihood profiles, the x-axis represents the LOD score and y-axis represent map distance (cM). Marker names and their genetic position (cM) are shown on left side of the linkage map; QTL positions (cM) are shown on right side of the linkage map.

Table 3.2 Occurrence of SSR marker alleles of *Brassica oleracea* in 27 clubroot resistant and 33 susceptible F_7 plants of *Brassica napus* × *B. oleracea* var. acephala interspecific cross.

Marker name[1]	Chr.	Total no. of plants used (no. S and R plants)	Frequency of Ole. allele in S plants[2] (%)	Frequency of Ole. allele in R plants[3] (%)
C04_3772	C04	54 (30S+24R)[4]	16.7 (5S)	87.5 (21R)
C04_3779	C04	53 (29S+24R)	27.6 (8S)	91.7(22R)
C04_3784	C04	52 (29S+23R)	17.2 (5S)	100.0 (23R)
C05_5458	C05	60 (33S+27R)	0.0 (0S)	92.6 (25R)
C05_5463	C05	55 (29S+26R)	3.4 (1S)	96.2 (25R)
C05_5466	C05	54 (30S+24R)	0.0 (0S)	100.0 (24R)
C08_4001	C08	57 (31S+26R)	6.5 (2S)	92.3 (24R)
C08_4002	C08	50 (28S+22R)	7.1 (2S)	95.5 (21R)

[1] Eight polymorphic SSR markers detecting clear bands of more than 10 bp fragment size difference were used.
[2] The occurrence of *B. oleracea* alleles in the susceptible plants.
[3] The occurrence of *B. oleracea* alleles in the resistant plants.
[4] The number in bracket is the number of susceptible and resistant plants detected SSR marker allele
Note: Chr. = chromosome; S = susceptible; R = resistant; Ole. = *B. oleracea*

Table 3.3 Quantitative trait loci (QTL) for resistance to *Plasmodiophora brassicae* pathotype 3H detected in a *Brassica napus* population of 60 F_7 plants carrying clubroot resistance of *Brassica oleracea* var. acephala cv. Winterbor (AM114); QTL analysis carried out using inclusive composite interval mapping-additive (ICIM-ADD) method and considering one chromosome at a time.

Chr.	Flanking marker		Peak of QTL (cM)	Confidence interval (cM)[1]	LOD[2]	PVE (%)[3]	ADD[4]
	Left	Right					
C04	C04_3779	C04_3784	8.7	5.35 - 11.55	7.33	18.86	-25.94
C04	C04_3784	C04_3772	19.3	16.75 - 21.85	37.65	39.55	-37.58
C05	C05_5458	C05_5466	1.9	0.65 - 3.35	47.38	97.47	-37.58
C08	C08_4001	C08_4002	1.7	0.45 - 3.45	42.98	85.58	-37.58

[1] Confidence interval: the region with LOD >3.0
[2] LOD: logarithm of the odds score. LOD score of 3 or higher indicates significant linkage.
[3] PVE (%): proportion of the phenotypic variance explained by the QTL.
[4] ADD: estimated additive effect of the QTL; negative sign indicates *B. oleracea* var. *acephala* allele reduces disease severity index (DSI %).
Note: Chr. = chromosome

Table 3.4 Quantitative trait loci (QTL) for resistance to *Plasmodiophora brassicae* pathotype 3H detected in a *Brassica napus* population of 60 F_7 plants carrying clubroot resistance of *Brassica oleracea* var. acephala cv. Winterbor (AM114); QTL analysis carried out using inclusive composite interval mapping-additive (ICIM-ADD) method and considering all chromosomes in the analysis.

Chr.	Flanking marker		Peak of QTL (cM)	Confidence interval (cM)	LOD	PVE (%)	ADD
	Left	Right					
C05	C05_5458	C05_5466	1.9	0.65 - 3.35	47.35	97.37	-37.58

Table 3.5 List of SSR markers associated with clubroot resistance to *Plasmodiophora brassicae* pathotype 3H detected in *Brassica napus* population of 60 F_7 plants carrying clubroot resistance of *Brassica oleracea* var. acephala cv. Winterbor (AM114); QTL analysis carried out using single marker analysis (SMA) method

Marker name	Chr.	Genetic position (cM)[1]	LOD[2]	PVE (%)[3]	ADD[4]	*p*-value
C04_3779	C04	0.00	6.23	6.79	-23.48	5.89×10^{-7}
C04_3784	C04	14.11	13.17	11.37	-30.38	6.76×10^{-14}
C04_3772	C04	24.52	6.90	7.36	-24.32	1.26×10^{-7}
C05_5458	C05	0.00	26.68	15.57	-35.87	2.09×10^{-27}
C05_5466	C05	1.92	47.38	17.40	-37.58	4.17×10^{-48}
C05_5463	C05	3.97	24.31	15.10	-35.01	4.90×10^{-25}
C08_4001	C08	0.00	18.00	13.38	-32.96	1.00×10^{-18}
C08_4002	C08	4.66	11.80	10.65	-29.32	1.59×10^{-12}

[1] Position of the marker located on genetic linkage maps of the chromosomes C04, C05 and C08 (Figure 3.1)
[2] LOD: logarithm of the odds score. LOD score of 3 or higher indicates significant linkage.
[3] PVE (%): proportion of the phenotypic variance explained by the QTL.
[4] ADD: estimated additive effect of the QTL; negative sign indicates *B. oleracea* var. *acephala* allele reduced disease severity index (DSI).

3.4 Discussion

In this study, 240 SSR markers from nine C genome chromosomes were tested on the parents and bulks of F_7 plants derived from *B. napus* × *B. oleracea* var. *acephala* cv. Winterbor (AM114) interspecific cross where only 12% of the markers were found to be polymorphic. The polymorphic markers identified *B. oleracea* alleles in this population providing evidence that the genomic regions of this progenitor species were introgressed in the re-constituted *B. napus* F_7 population. While working with F_3 population derived from the same interspecific cross but using different SSR markers, Zhang (2022) found a wide variation for the occurrence of *B. oleracea* alleles; it ranged from as low as 0% to as high as 100%. However, by working with polymorphic markers and an advanced generation population (F_7), I found a high frequency (87.5-100%) for the occurrence of *B. oleracea* alleles. The SSR markers that I used to genotype the population were selected based on polymorphism between the resistant and susceptible plants. Therefore, it is highly likely that these makers to be associated with resistance, which derived from *B. oleracea*, and this might be one of the reasons for the occurrence of *B. oleracea* alleles at a high frequency in this population.

The clubroot resistance in *B. oleracea* known to be controlled by genes located on several chromosomes. For example, Peng et al. (2018) identified 23 QTL on chromosomes C01, C02, C03, C04, C06, C07, and C08, where an individual locus contributed about 6.1% to 17.8% of the total phenotypic variance. Nagaoka et al. (2010) reported five QTL on C02 (two regions), C03, C05, and C07; these QTL exerted an additive effect of -0.54 to 1.31 disease severity on a 0 to 5 scale and explained 3% to 47% of the total phenotypic variance. However, Dakouri et al. (2018) reported a locus (*Rcr7*) on C07 contributing about 56-73% of the total phenotypic variance. Previous studies (e.g. Nagaoka et al., 2010; Peng et al., 2018) reported both negative and positive

additive effect QTL alleles suggesting that alleles contributing to clubroot resistance can be found in both resistant and susceptible parents. The additive effect of the QTL that have been detected in the present study was negative in all cases implying that *B. oleracea* allele reduced disease severity in *B. napus*. In the present study, inclusive composite interval mapping by including genotypic data of all three chromosomes together identified a clubroot resistance QTL on C05 flanked by the markers C05_5458 and C05_5466; this QTL explained 97.37% of the total phenotypic variance for resistance to *P. brassicae* pathotype 3H. However, single marker analysis showed that the C05 QTL explained only 15.10-17.4% of the total phenotypic variance. The large difference for contribution of the QTL detected following two methods could be due to the accuracy of two methods. Inclusive composite interval mapping analysis tests QTL every 0.1 cM between the adjacent markers and this method is more accurate than the single marker analysis method.

Zhang (2022) also used the three SSR markers (C04_3772, C04_3779 and C04_3784) that I used in the present study; however, Zhang was not able to establish linkage association of the markers with clubroot resistance. However, I was able to identify two clubroot resistance loci on chromosome C04 by genotyping the F_7 population with these three markers. The F_3 population that was used by Zhang (2022) was highly heterozygous and segregating for clubroot resistance, while the population that I used in this study was almost homozygous for resistance, and this might be the reasons for the difference between these two studies.

Multiple factors including marker density and population size can exert significant effect on the accuracy of QTL detection. Based on a computer simulation study considering one linkage group at a time only, Su et al. (2016) suggested that at least 20 markers is needed in an additive effect model when recombination frequency between two adjacent markers is 0.056. In

this study, only two to three markers per chromosome were used, which is extremely small number for mapping a trait; therefore, the QTL and markers identified in this study are indeed suggestive. Small population size can also result an inaccuracy in QTL mapping (Vales et al., 2005) where the number of QTL detected can be underestimated and their genetic effect could be overestimated. In this study, it was not possible to detect any minor QTL. This is apparently due to the use of a small population and very limited number of markers, and this might be one of the reasons for the high estimates of phenotypic variance, especially for the C05 and C08 QTL. The *B. oleracea* resistance introgressed in *B. napus* is very novel; further research using a large mapping population and high-density markers will be needed to map this resistance and to develop molecular markers for use in breeding.

Chapter 4 Inheritance of clubroot resistance in *Brassica napus* canola

4.1 Introduction

Clubroot disease, caused by *Plasmodiophora brassicae* Woronin, exerts a negative effect on yield and quality of the crops belonging to the Brassicaceae family. This disease was first reported in canola in the province of Alberta, Canada in 2003 (Tewari et al., 2005) and later rapidly spread across the country (Howard et al., 2010; Hollman et al., 2021). The pathogen attacks the roots of the host plant and disrupts the transportation of water and nutrients (for review, see Javed et al., 2022, and Hasan et al. 2021a) resulting in significant yield loss in canola (Pageau et al., 2006; Botero-Ramírez et al., 2021). Different cultural practices, biological control methods, and use of chemicals and fungicides were evaluated for the control of this disease (Hwang et al., 2015; Hwang et al., 2017; Liu et al., 2018; Liao et al., 2022; Fox et al., 2022). Some of these practices found to be effective; however, they are not efficient under large-scale production of canola (Fox et al., 2022). In contrast, growing of clubroot-resistant canola cultivars has been considered to be most effective and efficient way of controlling this disease (for review, see Rahman et al. 2013).

To date, several researchers introgressed clubroot resistance from related species and exotic germplasm into canola and mapped the resistances (Dakouri et al., 2018; Zhu et al., 2019; Huang et al., 2019; Karim et al., 2020; Fredua-Agyeman et al., 2020; Farid et al., 2020; for review, see Hasan et al., 2021a; Hasan et al., 2021b, 2021c; Wang et al., 2022). For example, Hirani et al. (2016), Hasan et al. (2021) and Kaur et al. (2022) introgressed the resistance from *B. rapa* into *B. napus*. Rahman et al. (2014), Hasan and Rahman (2016), Fredua-Agyeman and Rahman (2016) and Hasan et al. (2021b, 2021c) developed spring *B. napus* canola with resistance derived from winter *B. napus* canola or rutabaga. Introgression of clubroot resistance

from *B. oleracea* to *B. napus* is not common due to complex genetic control of resistance in the C-genome (Farid et al., 2020) and the difficulties of producing a *B. napus* × *B. oleracea* interspecific hybrid (Bennett et al., 2012; Iftikhar et al. 2018; Zhang, 2022).

Several clubroot resistant canola cultivars, such as '45H29', 'L135C', PV585GC, and 'D3152', were developed by different seed companies where almost all of them carry RoundUp® or LibertyLink® herbicide tolerance trait. Canola cultivars carrying these traits considered as genetically modified (GM) crop. There is a demand for non-GM canola oil in different countries. The imidazoline herbicide tolerant canola was developed using mutagenesis (Swanson et al. 1989) and this trait is considered non-GM. The traditional canola, which does not carry RoundUp® or LibertyLink® herbicide tolerance trait, also considered as non-GM. The ultimate objective of this part of my thesis research is to develop non-GM clubroot resistant canola germplasm by crossing clubroot resistant and susceptible non-GM canola. As part of this breeding goal, the inheritance of clubroot resistance was studied in segregating F_2 and backcross populations to understand the genetic control of this trait in this breeding population for efficient development of non-GM clubroot resistant canola germplasm.

4.2 Materials and Methods

4.2.1 Parental materials

One clubroot resistant *B. napus* canola line (CR *B. napus*) and four clubroot susceptible *B. napus* canola lines/cultivars viz., UA19-4 (UA CountyGold), Cougar and A04-73NA, were used in this study. All parental cultivars and lines are spring growth habit type and were developed by the Canola Program of the University of Alberta. The parents Cougar and UA19-4 are Clearfield® herbicide tolerant type, and A04-73NA and CR *B. napus* are conventional type; all are non-GM canola.

4.2.2 Development of different segregating populations

The clubroot resistant canola line was grown together with the four clubroot susceptible canola lines/cultivars in a growth chamber and were crossed using the resistant canola line as female: CR *B. napus*× UA19-4, CR *B. napus* × Cougar, and CR *B. napus* × A04-73NA (Supplementary Table 4.1). For this, mature unopened flower buds of the female parent at about one day before anthesis were emasculated and pollinated with fresh pollen from the male parent, and the cross-pollinated buds were bag–isolated to prevent further cross-pollination.

The F_1 plants of the three crosses were evaluated for resistance to *P. brassicae* pathotype 3H and were self-pollinated to produce F_2 seeds. The F_1 plants were also backcrossed to both parents to produce backcross (BC) populations ($B_1 = F_1 \times P_1$, and $B_2 = F_1 \times P_2$) (Supplementary Table 4.1). The F_1, F_2, B_1 and B_2 populations evaluated for resistance to pathotype 3H. The F_2, B_1 and B_2 plants were also self-pollinated for use of the harvested seeds in breeding.

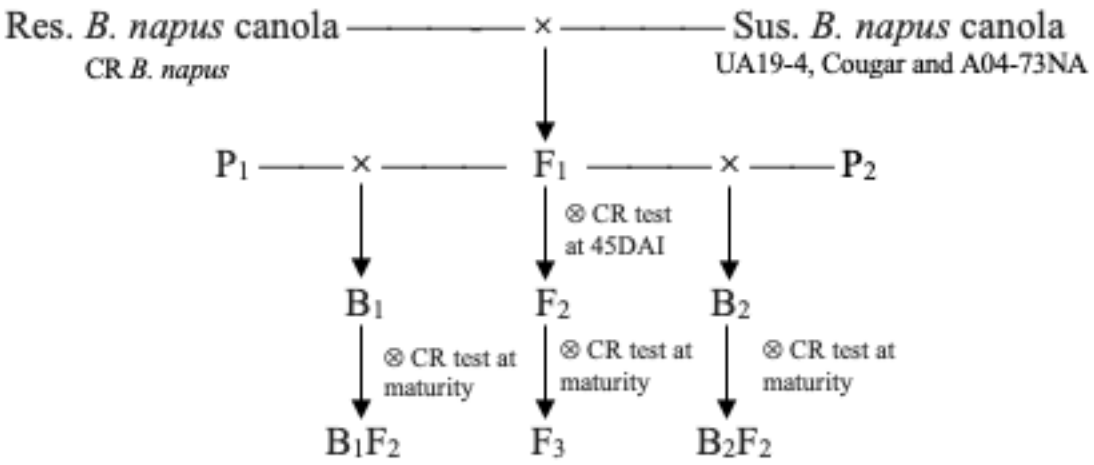

Figure 4.1 Flow diagram showing the development of different *Brassica napus* canola populations segregating for resistance to *Plasmodiophora brassicae* pathotype 3H.

4.2.3 Inoculum preparation and inoculation of seedlings

Single spore isolate of *P. brassicae* pathotype 3H as per Canadian Clubroot Differential (CCD) set (Strelkov et al., 2018) was used in this study. The inoculum was prepared one day before inoculation from preserved galls of the clubroot susceptible *B. napus* cv. Hi-Q and spore concentration was adjusted to 1×10^7 spores/ml as described in Section 2.2.3 of Chapter 2. The inoculum was stored at 4 °C overnight for inoculation in the next day. The seedlings were inoculated at 7-10 days after germination by pipetting 1 ml inoculum at the base of each seedling, and the inoculation was repeated on the following day as described in Section 2.2.4 of Chapter 2 for successful infection.

4.2.4 Screening for clubroot resistance

The evaluation for clubroot resistance was carried out at 45 days after inoculation (DAI) for the F_1 plants and at harvest stage for the F_2 and backcross populations. The reason for scoring the F_2 and backcross populations at harvest stage was that the Canola program could use the seeds of the resistant plants in breeding. For evaluation of resistance, the seedlings or mature plants were carefully uprooted and the roots were washed with tap water and visually examined for the severity of galls or clubbed roots on a 0 to 3 scale; the details of scoring have been reported in Section 2.2.5 of Chapter 2. The DSI value for each of the F_2 and backcross populations was calculated following Strelkov et al. (2006). Plants with disease score of 0 and 1 were classified as resistant while those with disease score of 2 and 3 were classified as susceptible.

4.2.5 Statistical analysis

The clubroot resistance data was analyzed for mean and standard error using Microsoft Excel (Version 16.56) and RStudio Software (Version 1.3.1073). For segregation analysis, Chi-

square (χ^2) test was carried out for fit of the observed segregation to the expected segregation by using the following formula: $x^2 = \sum(O - E)^2/E$ (Pearson, 1900; McHugh, 2013), where O is the observed number and E is the expected number.

4.3 Results

4.3.1 Resistance in F_1 populations

The F_1 hybrids of all three crosses were resistant to *P. brassicae* pathotype 3H, except for one plant of the cross CR *B. napus* × UA19-4 (Table 4.1). The mean DSI was 0.93 ± 0.69%, 0.20 ± 0.19% and 0.69 ± 0.49% for the F_1's of *B. napus* × UA19-4, CR *B. napus* × Cougar and *B. napus* × A04-73NA, respectively. The DSI value of the four F_1 populations was not significantly different (Table 4.1).

4.3.2 Resistance in F_2 populations

A total of 240 plants of CR *B. napus* × UA19-4 were evaluated for resistance to pathotype 3H where 169 (70.42%) plants were resistant and 71 (29.58%) susceptible; the DSI of this population was 29.44 ± 2.18%. Of the 238 plants of CR *B. napus* × Cougar, 162 (68.07%) were resistant and 76 (31.93%) susceptible with DSI of 32.54 ± 4.42%. In the case of the *B. napus* × A04-73NA cross, 161 (67.93%) plants were resistant and 76 (32.07%) susceptible from 237 F_2 plants; the DSI of this population was 32.30 ± 3.32% (Table 4.1). The DSI of the F_2 populations of the three crosses were statistically similar (Table 4.1).

The distribution of the F_2 populations for resistance to pathotype 3H was almost bi-modal for all three crosses (Figure 4.2). Of the total number of plants tested, 70%, 66% and 67% plants received disease score 0, and 29%, 32% and 32% received disease score 3 of the CR *B. napus* × UA19-4, CR *B. napus* × Cougar and CR *B. napus* × A04-73NA crosses, respectively (Figure 4.2).

Chi-square test for goodness of fit to a 3:1 (resistant : susceptible) segregation ratio was done on the three F_2 populations. Among these, a 3:1 segregation was found in the population of CR *B. napus*× UA19-4 cross (χ^2= 2.68, P = 0.1-0.2). Segregation in the other two populations deviated significantly from the 3:1 ratio (CR *B. napus* × Cougar: χ^2= 6.10, $P < 0.05$; CR *B. napus* × A04-73NA: χ^2= 6.31, $P < 0.05$) (Table 4.1).

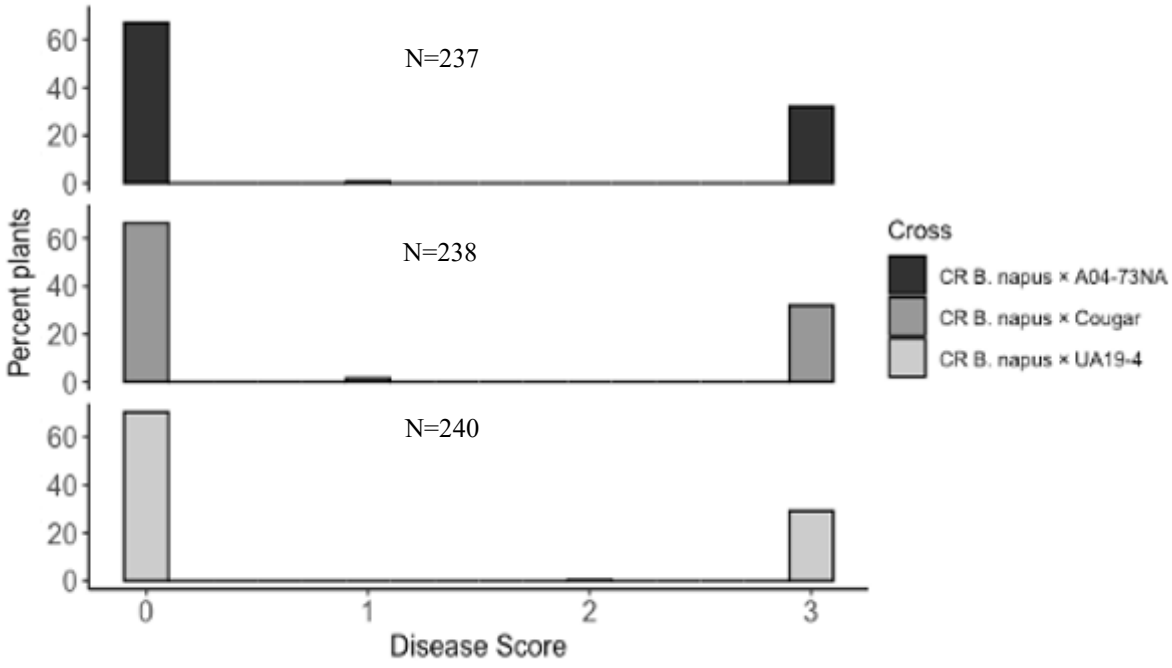

Figure 4.2 Distribution of the F_2 populations of three *Brassica napus* crosses for resistance to *Plasmodiophora brassicae* pathotype 3H. Plants with disease score of 0 and 1 were considered as resistant and the plants with disease score of 2 and 3 were considered as susceptible. N = total number of plants.

4.3.3 Resistance in backcross populations

Backcross populations were developed by crossing the F_1's to both resistant and susceptible parents; these two backcross populations were designated as B_1 (F_1 × Resistant) and B_2 (F_1 × Susceptible) populations, respectively.

***B_1 (F_1× resistant*):**

A total of 317 B_1 plants of the three crosses were evaluated for resistance to pathotypes 3H. Of the 104 plants of (CR *B. napus* × UA19-4) × CR *B. napus,* 94 (90.38%) were resistant and 10 (9.62%) susceptible; the DSI of this population was 11.92 ± 6.68%. Of the 104 plants of (CR *B. napus* × Cougar) × CR *B. napus*, 89 (85.58%) were resistant and 15 (14.42%) susceptible with DSI of 15.97 ± 3.83%. Among 109 plants of (CR *B. napus* × A04-73NA) × CR *B. napus*, 104 (95.41%) were resistant and 5 (4.59%) susceptible; the DSI of this population was 5.33 ± 2.00% (Table 4.1). The DSI of (CR *B. napus* × A04-73NA) × CR *B. napus* was statistically different from the DSI of the other two crosses (Table 4.1).

The majority (86%, 80% and 94%) of the B_1 plants of all three crosses received disease score of 0, i.e. were resistant. Considering a major dominant gene is involved in the control of resistance, it was expected that 100% of the B_1 plants will be resistant; however, a small fraction of the total plants (6 - 20%) were scored as susceptible (Figure 4.3).

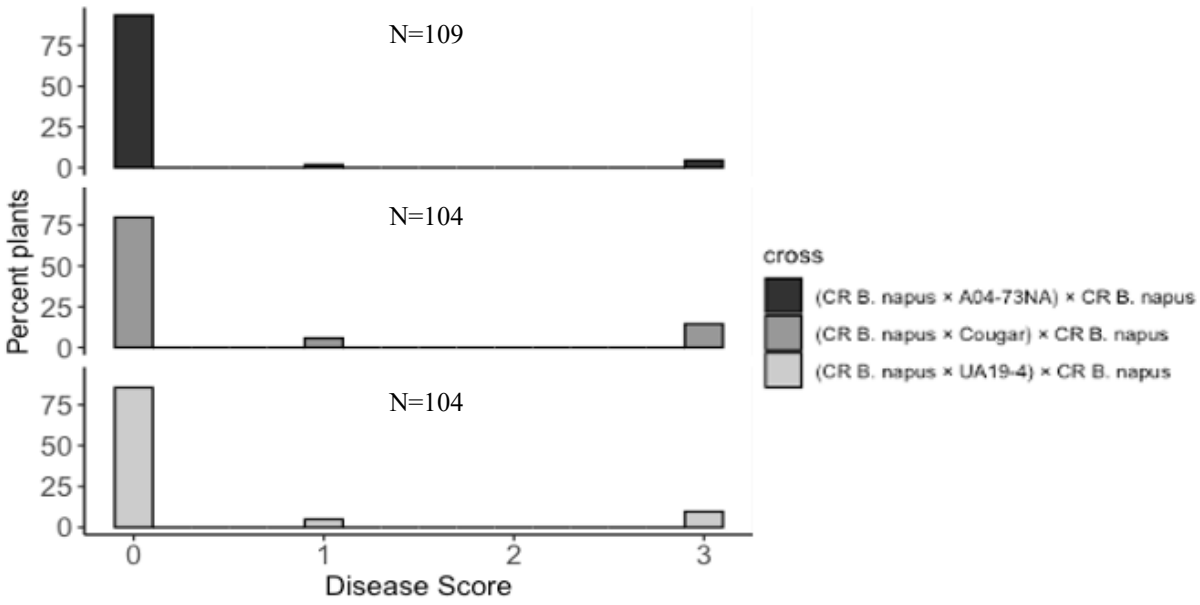

Figure 4.3 Distribution of the B_1 (F_1 × Resistant) populations of three *Brassica napus* crosses for resistance to *Plasmodiophora brassicae* pathotype 3H. Plants with disease score of 0 and 1 were

considered as resistant and the plants with disease score of 2 and 3 were considered as susceptible. N = total number of plants.

***B_2 (F_1× Susceptible*)**:

A total of 337 B_2 plants of the three crosses were evaluated for resistance to *P. brassicae* pathotypes 3H. As expected, the B_2 populations had higher DSI as compared to the B_1 populations for resistance to pathotype 3H. In the case of (CR *B. napus* × UA19-4) × UA19-4, 115 plants were evaluated where 59 (51.3%) were resistant and 56 (48.7%) susceptibile; the DSI value of this population was 46.72 ± 6.46%. Of the 111 plants of (CR *B. napus* × Cougar) × Cougar, 59 (53.15%) were resistant and 52 (46.85%) susceptible, and the DSI of this population was 49.14 ± 4.56%. Among the 111 plants of (CR *B. napus* × A04-73NA) × A04-73NA, 43 (38.74%) were resistant and 68 (61.26%) susceptible; the DSI of this population was 60.89 ± 7.31% (Table 4.1). The three B_2 populations were statistically similar for the DSI (Table 4.1).

The distribution of the B_2 populations for resistance to pathotype 3H was bi-modal for all three crosses (Figure 4.4). Of the total number of B_2 plants of the three crosses, 51%, 49% and 37% plants received disease score of 0, and 49%, 47% and 61% plants received score 3 for the (CR *B. napus* × UA19-4) × UA19-4, (CR *B. napus* × Cougar) × Cougar and (CR *B. napus* × A04-73NA) × A04-73NA crosses, respectively (Figure 4.4).

Chi-square test for goodness of fit to a 1:1 (resistant : susceptible) segregation ratio was done on three B_2 populations. A 1:1 segregation was found in the populations of (CR *B. napus* × UA19-4) × UA19-4 (χ^2= 0.08, P = 0.7 - 0.9) and (CR *B. napus* × Cougar) × Cougar (χ^2= 0.44, P = 0.5 - 0.7). However, segregation in (CR *B. napus* × A04-73NA) × A04-73NA population was significantly different from the 1:1 ratio (χ^2= 5.63, $P < 0.05$) (Table 4.1).

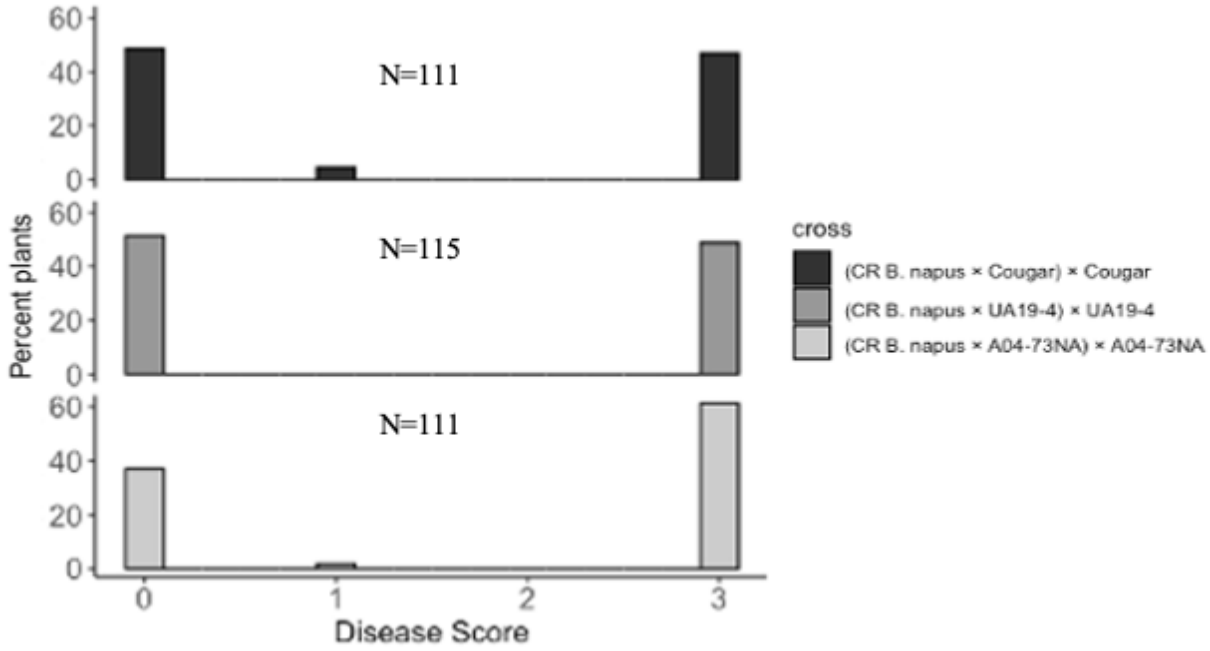

Figure 4.4 Distribution of the B_2 (F_1 × Susceptible) populations of three *Brassica napus* crosses for resistance to *Plasmodiophora brassicae* pathotype 3H. Plants with disease score of 0 and 1 were considered as resistant and the plants with disease score of 2 and 3 were considered as susceptible. N = total number of plants.

Table 4.1 Segregation for resistance to *Plasmodiophora* brassicae pathotypes 3H in different generation populations of *Brassica napus* canola crosses.

Cross[1]	No. families	Total plants	No. res. plants	No. sus. plants	DSI Mean±S.E.[2]	Chi-square test[3]	
						χ^2	*p*
F_1							
CR *B. napus* × UA19-4	17	111	110	1	0.93±0.69 [a]		
CR *B. napus* × Cougar	21	154	154	0	0.20±0.19 [a]		
CR *B. napus* × A04-73NA	20	145	145	0	0.69±0.49 [a]		
F_2							
CR *B. napus* × UA19-4	6	240	169	71	29.44±2.18 [a]	2.68	0.1-0.2
CR *B. napus* × Cougar	6	238	162	76	32.54±4.42 [a]	6.10	<0.05
CR *B. napus* × A04-73NA	6	237	161	76	32.30±3.32 [a]	6.31	<0.05
B_1 (F_1 × Resistant parent)							
(CR *B. napus* × UA19-4) × CR *B. napus*	6	104	94	10	11.92±6.68 [a]		
(CR *B. napus* × Cougar) × CR *B. napus*	6	104	89	15	15.97±3.83 [a]		
(CR *B. napus* × A04-73NA) × CR *B. napus*	6	109	104	5	5.33±2.00 [b]		
B_2 (F_1 × Susceptible parent)							
(CR *B. napus* × UA19-4) × UA19-4	6	115	59	56	46.72±6.46 [a]	0.08	0.7-0.9
(CR *B. napus* × Cougar) × Cougar	6	111	59	52	49.14±4.56 [a]	0.44	0.5-0.7
(CR *B. napus* × A04-73NA) × A04-73NA	6	111	43	68	60.89±7.31 [a]	5.63	<0.05

[1] The F_1 population was evaluated for resistance at 45 DAI, while the F_2, B_1 and B_2 populations were evaluated at harvest stage.
[2] Comparison made between the crosses of the same generation population; mean ± S.E. values followed by the same letter are not significantly different according to Welsch's *t*-test ($p > 0.05$).
[3] χ^2 test was carried out considering the plants with disease score 0 and 1 as resistant and plants with disease score 2 and 3 as susceptible.

4.4 Discussion

The CR *B. napus* line used in this study was homozygous for resistance to pathotype 3H, as none of the 131 plants tested for resistance was susceptible, and the F_1 plants of all CR *B. napus* × susceptible canola crosses were resistant. Considering a single locus is involved in resistance, a 3 resistant : 1 susceptible segregation was expected in the F_2 populations and a 1 resistant : 1 susceptible segregation was expected in the B_2 populations. The F_2 population of CR *B. napus* × UA19-4 followed a 3:1 segregation, and the B_2 populations of (CR *B. napus* × UA19-4) × UA19-4 and (CR *B. napus* × Cougar) × Cougar followed a 1:1 segregation. Segregation in the other two F_2 populations and one B_2 population deviated significantly from the 3:1 and 1:1 segregation, respectively. However, the distribution of the F_2 and B_2 populations in all cases was a bimodal. Considering a bimodal distribution of the populations and a fit to the expected segregation in three of the six F_2 and B_2 populations, it is highly likely that a major locus is involved in the control of this resistance. The deviation from the expected segregation in some of the populations might have resulted from the involvement of minor genes in the control of clubroot resistance. Apart from this, segregation distortion resulting from the occurrence of a greater number of susceptible plants cannot be ruled out. Distorted segregation for simple Mendelian trait has also been reported by Rahman (2001).

The backcross populations were obtained by crossing the F_1's with their respective resistant or susceptible parents. The B_1 populations of F_1 × resistant parent was expected to be genetically closer to their resistant parent, while the B_2 populations of F_1 × susceptible would be closer to their susceptible parent which are elite lines and cultivars. This implies that the B_1 population would have a greater level of resistance than the B_2 population; however, the B_2 population will be closer to the elite lines and cultivars. The mean DSI of the B_1 populations of

three different crosses (11.92 ± 6.68%, 15.97 ± 3.83% and 5.33 ± 2.00%) was much lower than that of the B_2 populations (46.72 ± 6.46%, 49.14 ± 4.56% and 60.89 ± 7.31%). For the development of clubroot resistant non-GM canola that are close to the elite lines and cultivars (UA19-4, Cougar and A04-73NA) for agronomic and seed quality traits, it would be preferred to focus on the B_2F_2 plants of (CR *B. napus* × UA19-4) × UA19-4, (CR *B. napus* × Cougar) × Cougar and (CR *B. napus* × A04-73NA) × A04-73NA crosses. In case of (CR *B. napus* × UA19-4) × UA19-4 and (CR *B. napus* × Cougar) × Cougar, a greater proportion of the B_2F_2 plants would be tolerant to imidazoline herbicide, while the population derived from (CR *B. napus* × A04-73NA) × A04-73NA cross expected to be conventional type; all are expected to be genetically close to the elite lines and cultivars as compared to the F_2 and B_1 populations. Imidazoline herbicide tolerance is controlled by two gene loci, designated as P_1 and P_2 (Swanson et al. 1989). For a high level of resistance to this herbicide, the presence of both loci is needed in a plant. Therefore, it would easier to develop imidazoline herbicide tolerant canola lines from the B_2 populations of (CR *B. napus* × UA19-4) × UA19-4 and (CR *B. napus* × Cougar) × Cougar as compared to the F_2 populations of CR *B. napus* × UA19-4 and CR *B. napus* × Cougar crosses.

The F_3, B_1F_2 and B_2F_2 populations developed in this study can be used in breeding to develop clubroot resistant non-GM canola cultivars. A better understanding of the genetic control of this resistance including the development of molecular markers for this resistance would benefit the use of this material in the next cycle of breeding.

Chapter 5 General discussion and conclusion

5.1 General discussion

Canola (*Brassica napus*) that evolved through interspecific hybridization between *Brassica rapa* and *Brassica oleracea* (Morinaga, 1934) is extensively cultivated worldwide for its oil-rich (45%) seed (Canola Council of Canada, 2019). With a low level of saturated fatty acids (6-7%), canola oil is considered as a healthy oil compared to other edible oils such as soybean (14.9%) and sunflower oil (11.3%) (Eskin et al., 2020). Canola oil is also an important source of essential fatty acids, linoleic acid (21-22%) and α-linolenic acid (10-11%), that human body cannot generate itself (Di Pasquale, 2009). All the above-mentioned features make canola oil valuable for human nutrition. However, the crop canola is vulnerable to different threats, of which, clubroot disease caused by *Plasmodiophora brassicae* is the most destructive. *Plasmodiophora brassicae* is able to infect both host and non-host plants from all genera of the Brassicaceae family and result in a global yield loss of approximately 10-15% of *Brassica* crops (for review, see Dixon 2009). Many strategies have been used to manage the clubroot disease, and these approaches include crop rotation (Ernst et al., 2019), adjustment of soil pH (Niwa et al., 2008; Fox et al., 2022), and application of fungicides (Liao et al., 2022), soil fumigants (Hwang et al., 2017) and use of ultraviolet light (Zahr et al., 2021); however, they were proved not to be feasible or economically viable. Therefore, developing clubroot resistant canola cultivars and implementing proper management practices is considered the best approach of controlling this disease.

Currently, the available clubroot-resistant canola cultivars are encountering a challenge due to the evolution of new virulent *P. brassicae* pathotypes (Strelkov et al., 2016, 2018). Therefore, identifying new sources of clubroot resistance and introgression into canola is

imperative. To date, the A-genome clubroot resistances found in winter canola cv. Mendel, rutabaga and *B. rapa* have been utilized to develop spring *B. napus* canola (Rahman et al., 2011a; Hirani et al., 2016; Hasan et al., 2021; Kaur et al., 2022; Zhan et al., 2022). Clubroot resistance loci in the C-genome of *B. oleracea* have also been identified (Rocherieux et al., 2004; Nagaoka et al., 2010; Peng et al., 2018; Dakouri et al., 2018; Farid et al., 2020), but not much effort has been paid to introgress this resistance into canola. In this regard, introgressing the resistance from *B. oleracea*'s C-genome into *B. napus* may increase the diversity of the genetic resistance in canola. In this MSc thesis research, by working with a segregating population of *B. napus* × *B. oleracea* interspecific cross and developing clubroot resistant advanced generation families, I demonstrated the prospects for transfer of the C-genome clubroot resistance from *B. oleracea* into *B. napus*.

Beside phenotypic evaluation of the population derived from *B. napus* × *B. oleracea* interspecific cross for clubroot resistance, I also carried out molecular analysis of the resistance to confirm whether *B. oleracea* alleles were introgressed in the re-constituted *B. napus* population. The clubroot resistance of *B. oleracea* was reported to be controlled by multiple loci (Peng et al., 2018; Nagaoka et al., 2010). In this study, four loci on chromosomes C04 (two loci), C05 and C08 were found to be contributing to resistance to pathotype 3H; in all cases, the QTL allele of *B. oleracea* reduced disease severity in *B. napus*. SSR markers were used to identify the *B. oleracea* alleles. A high frequency (87.5-100%) for the occurrence of *B. oleracea* alleles was found in the re-constituted F_7 population when compared with the frequency (0-100%) of *B. oleracea* alleles in the F_3 population of this interspecific cross studied by Zhang (2022). Zhang (2022) was not able to establish an association of the C04 SSR markers with clubroot resistance. However, I found two C04 SSR markers that Zhang used to be associated with clubroot

resistance in the F_7 population. It is highly likely that the F_3 population that was used in Zhang's study was highly heterozygous and segregating for clubroot resistance, while the F_7 population that I used in this study was nearly homozygous for resistance.

Following the genetic control of agronomic and seed quality traits (Long et al., 2007; Mei et al., 2009; Chao et al., 2017; Rahman and Kebede, 2021), a continuous variation was found for days to flowering, seed oil and protein contents in the segregating population of this *B. napus* × *B. oleracea* interspecific cross. The population derived from this interspecific cross has a lower seed oil and protein contents; however, the mean values were not significantly different from the value of the *B. napus* parent. In case of days to flowering, I identified two families exhibiting earlier flowering than the *B. napus* parent. Earliness of flowering and maturity are one of the most important traits of canola. In this regard, the development of early-flowering *B. napus* germplasm carrying the flowering allele of *B. oleracea* var. *acephala* is an important contribution to canola breeding. Rahman et al. (2011b) also demonstrated the prospect of the introgression of early flowering alleles from *B. oleracea* var. *alboglabra* into *B. napus*.

The seed glucosinolate content of the interspecific cross-derived population was significantly higher than the *B. napus* parent indicating that undesirable alleles for this trait has been introgressed into this population from *B. oleracea*. A bi-modal distribution for glucosinolate content was observed in this study, which is apparently due to a few loci involved in the control of this trait. According to several researchers (Uzunova et al., 1995; Schatzki et al., 2014; Rahman and Kebede, 2021), seed glucosinolate content in *B. napus* is controlled by about five to seven loci where the C genome carry about the half of the loci. Based on this genetic control of the trait, several plants with low glucosinolate content in the seed could be identified.

This demonstrates the prospect of developing a low glucosinolate line from this interspecific cross.

In case of erucic fatty acid content, segregation for this trait in the canola × kale cross was expected to involve only the single locus of the C-genome. According to Rahman et al. (2015), a segregation distortion for erucic acid alleles occurs in the population derived from *B. napus* × *B. oleracea* interspecific cross where this distortion occur in favour of the high erucic acid allele originating from *B. oleracea*. This might be one of the reason that none of the F_6 families were zero erucic acid type; however, 2.17% of the families had less than 10% erucic acid in the oil demonstrating the prospect of obtaining a zero-erucic plant from this population. Thus, it is apparent that some of the undesirable alleles of *B. oleracea* have been introgressed into the population derived from *B. napus* × *B. oleracea* interspecific cross, however, these alleles can be eliminated through cross-breeding of the clubroot resistant lines with canola.

According to Iftikhar et al. (2018), the advanced generation of *B. napus* × *B. oleracea* interspecific cross stabilizes into *B. napus* ($2n = 38$) type. In this study, I also find similar results. The genome composition of the F_6 and F_7 populations based on nuclear DNA content was close to *B. napus*; however, a wide variation for DNA content was found in both populations where some of the plants has a greater content of DNA than the *B. napus* parent. This indicates that some of the F_6 and F_7 generation plants may carry more than 38 chromosomes. According to Cui et al. (2012), unreduced gametes can be produced in the progeny of *Brassica* interspecific hybrids, and this might be the reason for the occurrence of plants with greater than 38 chromosomes in the progeny of *B. napus* × *B. oleracea* interspecific cross. Thus, the results from this study provided further evidence of the occurrence of unreduced gametes in the progeny of *Brassica* interspecific cross.

The inheritance of clubroot resistance was also examined in this MSc thesis research by evaluation of the segregating F_2 and backcross ($B_1 = F_1 \times$ Resistant parent; $B_2 = F_1 \times$ Susceptible parent) populations derived from three clubroot resistant canola × susceptible canola (UA19-4, Cougar and A04-73NA) crosses. Considering the involvement of a single locus in clubroot resistance, a 3 resistant : 1 susceptible segregation was expected in the F_2 populations and a 1 resistant : 1 susceptible segregation was expected in the B_2 populations. However, not all F_2 population followed a 3:1 segregation and not all B_2 population followed a 1:1 segregation. It indicates that a major locus and some minor loci might be involved in the control of clubroot resistance in these populations. A single major gene control of clubroot resistance have been reported by several researchers (Dakouri et al., 2018; Huang et al., 2019; Hasan et al., 2021b). The ultimate aim of this part of this thesis research was to develop clubroot resistant non-GM canola. Based on the backcrossing concept, the B_2 population will be genetically close to their susceptible elite canola parents as compared to the F_2 and B_1 populations. Therefore, the B_2-derived (B_2F_2 and subsequent generations) populations from (CR *B. napus* × UA19-4) × UA19-4 and (CR *B. napus* × Cougar) × Cougar can be used to develop clubroot resistant non-GM canola carrying imidazoline herbicide tolerance. With the understanding of the genetic control of clubroot resistance in these populations, clubroot resistant non-GM canola carrying other favorable traits can be developed efficiently.

5.2 Conclusion

The following conclusions can be drawn from my MSc thesis research:

- It's possible to develop clubroot resistant spring growth habit *B. napus* plants from a *B. napus* × *B. oleracea* interspecific cross.

- Homozygous clubroot resistant lines can be achieved in advanced generation population of the *B. napus* × *B. oleracea* interspecific cross.
- Four QTL conferring resistance to *P. brassicae* pathotype 3H were identified on chromosomes C04 (two loci), C05 and C08 of the re-constituted *B. napus* F_7 population.
- Segregation analysis of the F_2 and backcross populations demonstrated the prospects of developing non-GM clubroot resistant canola germplasm/cultivars.

5.3 Future research

- Cross-breeding of the clubroot resistant lines of the *B. napus* × *B. oleracea* interspecific cross with elite canola lines/cultivars need to be carried out to achieve the canola quality traits (zero erucic acid and <15 glucosinolates per g/seed) with favorable oil and protein contents.
- A mapping population need to be developed by crossing the clubroot resistant lines derived from *B. napus* × *B. oleracea* interspecific cross with clubroot susceptible lines and high-density molecular markers need to be used to accurately map the resistance and develop molecular markers for use in marker-assisted breeding.
- The clubroot resistant lines carrying the resistance of *B. oleracea* need to be crossed with canola lines carrying resistance in the A-genome for pyramiding the A- and C-genome resistances to broaden the genetic base of clubroot resistance in canola for durable resistance in this crop.
- Further breeding on the populations derived from resistant canola × susceptible need to be carried out to develop clubroot resistant non-GM canola carrying.

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Appendix

Supplementary Table 3.1 List of F_7 plants derived from *Brassica napus* A04-73NA × *B. oleracea* var. *acephalla* (AM114) interspecific cross used for genotyping

Sample#	F_6 Reg name	CR score	F_7 Reg name
R1	1CA2525.765-A1261 p1	0	1CA2525.1099-A1271 p1
R2	1CA2525.765-A1261 p1	0	1CA2525.1099-A1271 p2
R3	1CA2525.765-A1261 p2	0	1CA2525.1100-A1271 p1
R4	1CA2525.765-A1261 p3	0	1CA2525.1101-A1271 p5
R5	1CA2525.765-A1261 p4	0	1CA2525.1102-A1271 p2
R6	1CA2525.766-A1261 p2	0	1CA2525.1249-A1271 p1*
R7	1CA2525.777-A1261 p1	1	1CA2525.1110-A1271 p1
R8	1CA2525.777-A1261 p1	1	1CA2525.1110-A1271 p2
R9	1CA2525.777-A1261 p6	0	1CA2525.1278-A1271 p2
R10	1CA2525.777-A1261 p7	0	1CA2525.1253-A1271 p1
R11	1CA2525.794-A1261 p1	1	1CA2525.1275-A1271 p1
R12	1CA2525.794-A1261 p3	0	1CA2525.1276-A1271 p1
R13	1CA2525.794-A1261 p3	0	1CA2525.1276-A1271 p2
R14	1CA2525.794-A1261 p5	1	1CA2525.1277-A1271 p1
R15	1CA2525.794-A1261 p5	1	1CA2525.1277-A1271 p2
R16	1CA2525.794-A1261 p7	1	1CA2525.1279-A1271 p2
R17	1CA2525.799-A1261 p1	0	1CA2525.1119-A1271 p1
R18	1CA2525.799-A1261 p1	0	1CA2525.1119-A1271 p2
R19	1CA2525.799-A1261 p1	0	1CA2525.1119-A1271 p3
R20	1CA2525.799-A1261 p1	0	1CA2525.1119-A1271 p4
R21	1CA2525.799-A1261 p6	0	1CA2525.1120-A1271 p2
R22	1CA2525.938-A1261 p1	1	1CA2525.1292-A1271 p3
R23	1CA2525.938-A1261 p2	0	1CA2525.1199-A1271 p2
R24	1CA2525.938-A1261 p3	1	1CA2525.1293-A1271 p1
R25	1CA2525.938-A1261 p5	1	1CA2525.1294-A1271 p2
R26	1CA2525.938-A1261 p6	1	1CA2525.1295-A1271 p1
R27	1CA2525.938-A1261 p7	1	1CA2525.1296-A1271 p1
S1	1CA2525.833-A1261 p1	3	1CA2525.1135-A1271 p3
S2	1CA2525.833-A1261 p3	3	1CA2525.1137-A1271 p1
S3	1CA2525.833-A1261 p5	3	1CA2525.1139-A1271 p1
S4	1CA2525.833-A1261 p6	3	1CA2525.1140-A1271 p1
S5	1CA2525.850-A1261 P4	3	1CA2525.1159-A1271 p2*
S6	1CA2525.872-A1261 p6	3	1CA2525.1172-A1271 p1*
S7	1CA2525.961-A1261 p1	3	1CA2525.1201-A1271 p1

S8	1CA2525.961-A1261 p2	3	1CA2525.1202-A1271 p1
S9	1CA2525.961-A1261 p3	3	1CA2525.1203-A1271 p1
S10	1CA2525.961-A1261 p5	3	1CA2525.1204-A1271 p1
S11	1CA2525.961-A1261 p7	3	1CA2525.1206-A1271 p1
S12	1CA2525.1015-A1261 P5	3	1CA2525.1309-A1271 p1*
S13	1CA2525.1046-A1261 p1	3	1CA2525.1212-A1271 p1
S14	1CA2525.1046-A1261 p2	3	1CA2525.1213-A1271 p1
S15	1CA2525.1046-A1261 p5	3	1CA2525.1214-A1271 p1
S16	1CA2525.1046-A1261 p7	3	1CA2525.1215-A1271 p1
S17	1CA2525.1071-A1261 p2	3	1CA2525.1316-A1271 p1
S18	1CA2525.1071-A1261 p4	3	1CA2525.1226-A1271 p1
S19	1CA2525.1071-A1261 p5	3	1CA2525.1227-A1271 p1
S20	1CA2525.1073-A1261 p2	3	1CA2525.1229-A1271 p1
S21	1CA2525.1073-A1261 p3	3	1CA2525.1318-A1271 p1
S22	1CA2525.1073-A1261 p4	3	1CA2525.1230-A1271 p1
S23	1CA2525.1073-A1261 p5	3	1CA2525.1319-A1271 p1
S24	1CA2525.1073-A1261 p6	3	1CA2525.1231-A1271 p1
S25	1CA2525.1075-A1261 p1	3	1CA2525.1232-A1271 p1
S26	1CA2525.1075-A1261 p1	3	1CA2525.1232-A1271 p2
S27	1CA2525.1075-A1261 p1	3	1CA2525.1232-A1271 p3
S28	1CA2525.1075-A1261 p3	3	1CA2525.1233-A1271 p1
S29	1CA2525.1075-A1261 p3	3	1CA2525.1233-A1271 p2
S30	1CA2525.1088-A1261 p3	3	1CA2525.1235-A1271 p1
S31	1CA2525.1088-A1261 p3	3	1CA2525.1235-A1271 p2
S32	1CA2525.1088-A1261 p7	3	1CA2525.1236-A1271 p1
S33	1CA2525.1088-A1261 p7	3	1CA2525.1236-A1271 p3

*Samples with star sign were not included in the bulks for bulk segregant analysis, however, they were included while genotyping the individual samples.

Supplementary Table 3.2 List of 240 SSR markers and their sequences

Marker ID	Chr.	Forward Sequence (5'-3')	Reverse Sequence (5'-3')
3460	C4	GGCTAACCTTGTGAGCCCAT	TTGAAACCGAGGGACGTGAG
3461	C4	CGGAGCGGCTTGAGATGTAT	ACAGTCACAGGCACCAAACA
3462	C4	GCCCATCCTGCGATCACATA	GAAACCGAGGGACGTGAGTT
3463	C4	ATACCCTGCTTGACCAGTGC	AGCTGGGTATGACCAGCAAC
3464	C4	TGTTTGGTGCCTGTGACTGT	GGTGGGCGACTAACCTTTGA
3465	C4	AGAACACTTGCCGAGACCAG	AACCGAGCCCTAAAGTCTGC
3466	C4	GCAGACTTTAGGGCTCGGTT	TATGTGATCGCAGGATGGGC
3467	C4	CAGAAGAGTAACCGCCAGCA	GCACTGGTCAAGCAGGGTAT
3468	C4	GAACCGATGGCAACTCCAGA	ACCGATCCGAACTGTTCACC
3469	C4	TGAACAGTTCGGATCGGTGG	TCGCTCTTCGTCAAGCACAT
3470	C6	GATGGCGAATGGTTGTCAGC	ATTAGAGCAAGCGCAGTGGT
3471	C6	GTCAGCTCGAAAGCGTATGG	GCAAGCGCAGTGGTGATTTTA
3472	C6	TGGCGAATGGTTGTCAGCTC	AATTAGAGCAAGCGCAGTGG
3473	C6	TTCGATGGCGAATGGTTGTC	AGCAAGCGCAGTGGTGATTT
3474	C6	TCAGCTCGAAAGCGTATGGAA	GCAAGCGCAGTGGTGATTT
3475	C6	GGTTGTCAGCTCGAAAGCGT	TTAGAGCAAGCGCAGTGGT
3476	C6	GGAAATGTCAGCCGGTTCAT	GAGACCCTTTTCCACCTGACA
3477	C6	ATGGAAATGTCAGCCGGTTC	AGACCCTTTTCCACCTGACA
3478	C6	GCGAATGGTTGTCAGCTCG	GAGCAAGCGCAGTGGTGATT
3479	C6	GTCAGCTCGAAAGCGTATGGA	AGCAAGCGCAGTGGTGATT
3480	C7	TCCATGGAGGCGTGTTGAAG	AACCAGTTCCCCAGCATCAG
3481	C7	AAGACCATCGGTCAAGCCAG	ATACCACCTTCAACACGCCT
3482	C7	TAAGCCGTTTGGACCGTACC	CGTTCCAGTGTGAAGAACCC
3484	C7	GATCGGGCCGAAAAGACTGA	ATACCACCTTCAACACGCCTC
3485	C7	AAAGACTGATTGGGCCGTGT	ACGTTCCAGTGTGAAGAACCC
3486	C7	GAGTAGAACCCGGAACCGAC	TCAACACGCCTCCATGGAAAA
3487	C7	GGGTTCTTCACACTGGAACG	AAACCAGTTCCCCAGCATCA
3488	C7	GGGATGAGCTTTCGGCTACA	CAACACGCCTCCATGGAAAAT
3489	C7	CCATGGAGGCGTGTTGAAGG	AACTTGGCGCAAAAACCAGT
3490	C8	ACGTGGAGCCTTCTCATTGG	AGGAAATCAGCTCCAGCCAC
3491	C8	GGGTGATAGGTAGCTGGTGC	AGCAGACGGCAAGGAAATCA
3492	C8	GTCTTTCCAGACCCGACGAG	ACCAATGAGAAGGCTCCACG
3494	C8	AGGTAGCTGGTGCATTGACG	CAGCGAGGAAATCAGCTCCA
3495	C8	GTAGCTGGTGCATTGACGTG	GACGGCAAGGAAATCAACCG
3496	C8	GTGGCCACCCAAGTCAAAAG	CCAGCTACCTATCACCCACG
3540	C9	ATGAGCGGCTACATGTCGAG	ACGGCAATTGTTCCCTCCTT
3541	C9	AAGGAGGGAACAATTGCCGT	TACCGGTTAACCTTTCCGGC

3542	C9	TCTCCGTGCTGTCTTGTGAC	GCTCTACGGCAATTGTTCCC
3543	C9	AGGAGGGAACAATTGCCGTA	CGGTTAACCTTTCCGGCTCT
3544	C9	GCTGAGGTGGACAAGAACGA	TGTTCCCTCCTTTGCTTGTGT
3545	C9	AGCTGAGGTGGACAAGAACG	CTACGGCAATTGTTCCCTCCT
3546	C9	TTGGTGTCTTCCCATGGTCG	TCTACGGCAATTGTTCCCTCC
3547	C9	AGGAGGGAACAATTGCCGTAG	AAAAAGTTGCGTTTCCCCCG
3548	C9	GGAGGGAACAATTGCCGTAGA	CTTTCCGGCTCTCATCCGTT
3549	C9	CAAAGGAGGGAACAATTGCCG	TTTCCGGCTCTCATCCGTTC
3716	C7	CGGAGTGGATAACCGAGACG	GGCCAGCTACCTGTATCGAC
3717	C7	ACCGGAGTGGATAACCGAGA	GGGCGAGCTAGGATGTTACC
3718	C7	AGACGACCAATGGTGGAACC	GGGGCGAGCTAGGATGTTAC
3719	C7	ACTTCGTGACGGAAGAACCC	TCACTGTCTTAGCCGCAAGG
3720	C7	CACCACTCGGTGCACTACTT	CCTCACTGTCTTAGCCGCAA
3721	C7	GTAACATCCTAGCTCGCCCC	CACGAAGTAGTGCACCGAGT
3722	C7	GGTAACATCCTAGCTCGCCC	GTCACGAAGTAGTGCACCGA
3723	C7	CCTTGCGGCTAAGACAGTGA	TTGCTTCCAGTGCCCATCAT
3724	C7	CGAGACGACCAATGGTGGAA	CGTAGCCAATGTCGACCGTA
3725	C7	ATGTCCCGGAGGAGACTACC	CGAGTGGTGGTGTTCCTTGA
3726	C7	AGAGCTACGCATACGAACGG	CCACAACCAAGTCGGACTGA
3727	C7	GCACAGAGCTACGCATACGA	GTAAGGTCCCGACGAATGCT
3728	C7	GCGACGTATGCACAGAGCTA	ACACCTAACCGAGCCACAAC
3729	C7	TAGCGACGTATGCACAGAGC	CCTAACCGAGCCACAACCAA
3730	C7	GGTCCAATGGTGTGGAACGA	TTCTCACCGTAAGGTCCCGA
3731	C7	CGTCATGAAGCATTCGTCGG	CGACAGGACCGTTCATCGTT
3732	C7	GCGTCATGAAGCATTCGTCG	TAACCGAGCCACAACCAAGT
3733	C7	GAAGCATTCGTCGGGACCTT	TACTCTGTTGCGACAGGACC
3734	C7	ATGAAGCATTCGTCGGGACC	TTGCGACAGGACCGTTCATC
3735	C7	TCAGTCCGACTTGGTTGTGG	AATCCCCGGAGGATCATAGC
3736	C7	ATCCAAAGACTGCGCCCTAC	CCTGACGTGACCACATCCAA
3737	C7	CGCAGGGTGGCTCAAATCTA	GTGACCACATCCAACTCGGT
3738	C7	GGTCCAATGGTGTGGGACTT	ACATCCAACTCGGTGCGATT
3739	C7	CAAACTAGCTGCCTTCGCAC	TAGATTTGAGCCACCCTGCG
3740	C7	GCCCTACAGCATTCGACAGA	GCGGCTATATTCTCGCCGTA
3741	C7	GATCCAAAGACTGCGCCCTA	CGGACCGCTCATTGATTCCT
3742	C7	AAACTAGCTGCCTTCGCACA	TCGGACCGCTCATTGATTCC
3743	C7	GGAATCAATGAGCGGTCCGA	ACGTGACCACATCCAACTCG
3744	C7	CCTTCGCACAGAACTACGCT	CGATCGGACCGCTCATTGAT
3745	C7	TTGGTCCACAATCGGGTTCG	GTAGGGCGCAGTCTTTGGAT
3746	C8	ACACCAATGTCTATGTCAGCCT	GCGTCAACCCATGTAGTCCA
3747	C8	AACACCAATGTCTATGTCAGCCT	TTGGAGCTGGTCGTAGAGGA

3748	C8	CACCAATGTCTATGTCAGCCT	TATCGAAAGGCGGCAGTCTC
3749	C8	AACACCAATGTCTATGTCAGCC	ACATGATGTCCCCGCAGAAG
3750	C8	AGGAAGAATGAGAGGGCAAGC	AGGCTGACATAGACATTGGTGT
3751	C8	GGAAGAATGAGAGGGCAAGC	TCAGGCTGACATAGACATTGGT
3752	C8	TGCTGTACACACAAAGTGATTGA	GCTGCTCCAGGATTCCTACC
3753	C8	TAACACCAATGTCTATGTCAGCCT	GGCTGCTCCAGGATTCCTAC
3754	C8	GGAAGGAAGAATGAGAGGGCA	CAGGCTGACATAGACATTGGTG
3755	C8	TAACACCAATGTCTATGTCAGCC	CGGCAGTCTCTTGTCGTGAT
3756	C7	TGGACCTTACGGCGAAAACA	TGTAGTGCCCGCAAACATCT
3757	C7	TCCAACCGGAACAAACGCTA	TGTTTTCGCCGTAAGGTCCA
3758	C7	TGAAAGCTTCGTCCTAGCCC	CGAATCTCGTTGTGAACCGC
3759	C7	CCTTACGGCGAAAACATCGC	AGTGTAGTGCCCGCAAACAT
3760	C7	CAAGCTAGCCAAAGCTGGGA	TCGGACCGCTCATTTGATCC
3761	C7	GCCTAACTACGACCATGCCA	TACGAGCCACATCCGAGACT
3762	C7	GATGTTTGCGGGCACTACAC	TATTCGCATCACCCACAGGC
3763	C7	AGCTAGCCAAAGCTGGGAAA	GGCATGGTCGTAGTTAGGCT
3764	C7	AAGCTAGCCAAAGCTGGGAA	GAGCCACATCCGAGACTGAA
3765	C7	ATGTTTGCGGGCACTACACT	GCATCACCCACAGGCATAGG
3766	C4	AAAAGGAGACCTCCGCAGTG	CCCTTCCAACGAGTCGAACA
3767	C4	CACCGAAAGTTCCTCCACCA	AGTCGGCAACTCCACGATTT
3768	C4	CACCCACTACGTCTACGCTC	ACCAACCCTTCCAACGAGTC
3769	C4	TGTTGCGGCGCTGAGTATAA	GCGTAGACGTAGTGGGTGAG
3770	C4	CGTCATCCTCACCCACTACG	ACGAGTCGAACAACGCAAGA
3771	C4	TCTCATGGTGAGGACACCGA	CGTAGTGGGTGAGGATGACG
3772	C4	GGACACCGAAAGTTCCTCCA	TATACTCAGCGCCGCAACAT
3773	C4	GGTGGCTTCTCATGGTGAGG	GTAGTGGGTGAGGATGACGC
3774	C4	GCGTCATCCTCACCCACTAC	CGAATCTAGCCATGGAGGCA
3775	C4	ATGTGAGACTGAGACGAGCG	TCGGCAACTCCACGATTTCT
3776	C4	GTAAAGATCTCACCGGCGCA	ACATCTGGTGTGCGACTCTC
3777	C4	CGCACACCAGATGTTCGCTA	CGACCTGAGCATAGCCAGAG
3778	C4	TTTGAGAGTCGCACACCAGA	GAATCCGGACAGCCTAGCTC
3779	C4	TTGAGAGTCGCACACCAGAT	GCCGGAATCCAATAGAGCGA
3780	C4	TTTTGAGAGTCGCACACCAGA	ACTTGAAGCCCTGTCTCACG
3781	C4	TTGAGAGTCGCACACCAGATG	CCTATGCAACCTGCAGGGAA
3782	C4	GAGAGTCGCACACCAGATGTT	CAGGGCTTTTGCAATGTGCT
3783	C4	TTGGATCGGTTCTGCATCACA	AACATCTGGTGTGCGACTCT
3784	C4	TTTTTGAGAGTCGCACACCAG	TCCTTAAACGTTGTGCCGGA
3785	C4	CCTGAGAAAATTCTGCTGCCG	ATCTGGTGTGCGACTCTCAA
3786	C6	TGCTTGTAGGGTCAACATCTGA	AGGATCCAGCGAGGTAGGTT
3787	C6	TGGGTTTCCGAAGGAAGACG	TCAGATGTTGACCCTACAAGCA

3788	C6	AAGCGATGGGGAAGGTGAAG	CCACTCAATCAGATGTTGACCC
3789	C6	GGGTCAACATCTGATTGAGTGG	ACGTCCCATTTCTGAGCAGG
3988	C6	ACCTAGCTTGGGTAACACGC	GCGCCGGATAAACTGGATCT
3989	C6	CCTAGCTTGGGTAACACGCA	GTTCGCCCTTTGGTTCAACG
3990	C6	CGTTGAACCAAAGGGCGAAC	AGAGGGAGGTTCATCGGGAA
3991	C6	CCAAAGGGCGAACTCTCTCA	GATGCGAGACAGAGCCATGA
3992	C6	TGAACCAAAGGGCGAACTCT	AACAGGATGCGAGACAGAGC
3993	C6	AGCTTGGGTAACACGCAGAT	TGAGAGAGTTCGCCCTTTGG
3994	C6	AAAGGGCGAACTCTCTCAGG	TGCGAGACAGAGCCATGAAG
3995	C6	GCTTGGGTAACACGCAGATG	GAGAGAGTTCGCCCTTTGGT
3996	C6	GCGAACTCTCTCAGGCACTA	AATAGGCTCAAGCCTTCCCG
3997	C6	CTCGTTGAACCAAAGGGCGA	CAGCCAGAGGGAGGTTCATC
3998	C8	GCAGTTGCTGCGAACGATAA	CTCGCTGTGTTGAGTCGGAT
3999	C8	ATGGACGAAGACCCCACAAC	AGTGGATTGTCGAGGCATGT
4000	C8	CATGGACGAAGACCCCACAA	AAGCTTCGCAGTCTGCATTC
4001	C8	CGAGCACTCGCGTTAAAAGT	TGGAGCAGTTATCGTTCGCA
4002	C8	TCGAGCACTCGCGTTAAAAG	GAGCAGTTATCGTTCGCAGC
4003	C8	GTGACCATCGTAGCGCCTAT	CGTCGGGGAGTGGATTGTC
4004	C8	CGAGCACTCGCGTTAAAAGTC	AGCAGTTATCGTTCGCAGCA
4005	C8	TGGTCGACGGAAGTTTACCC	GTCGCTGGCATTGGTCACAT
4119	C9	CACGACGTTGTAAAACGACGGCAAA GAGGAGAGCAACAG	ACCGTGAAAACATGCCTCTC
5375	C1	TCGACCGTAAAGGGCATATTCGTCA	TCGCTAGATCCTGGTCCGACAC
5376	C1	GGATCGCGTCGTTCTCCGACAT	ACTGCCACTTGGTTCACTCTCCA
5377	C1	GCATGCATATTTGGGGGATA	GGGTTGAAAGCTTACGCAGT
5378	C1	CGTCGAAGAGAGGTTTCGAG	GTGGCCAGGTGTCAAGAAAT
5379	C1	AGGATCCAAACATGCGAAAC	TTCAAGGACCATGCTTCTCC
5380	C1	GAATCCAAACATGCCAAACC	TTCCCCAACTGATAGGAGGA
5381	C1	TTGACTCTAACTGAAACCACATCAA	GTTTCCTCCGCCCATTATTT
5382	C1	TGTACTGAGGCAACTTGCTTTT	TCAAGGATTTTTGCCTCTGA
5383	C1	CAAAAACCAATCATTGTTGTGAA	GATGTCCATAACCAGCCCATA
5384	C1	AAATCCCAAAACCCAACCTC	CACACCAAGGACACTCCAAA
5385	C1	GGGGTTCAGTACTTCGGTGA	TGTTGAAATAAGCGGGAAAAA
5386	C1	TTGGATTCGTCACAGTTAGGC	ATGGGCCTTTCCCATTAGTC
5387	C1	TGAGGAAGATACGGCAGTGA	GCCTAACTGTGACGAATCCAA
5388	C1	CTGAATGAAACGCATTACGC	GTGGCAATATTCACGCAAAG
5389	C1	CGTTAACTCTTCCCCAACGA	CTTCAGAGATGGAGCCGAAC
5390	C1	GCGTCGTTCTCCGACATTAT	TGCCACTTGGTTCACTCTCC
5391	C1	GCCCTCTGCAGTGTCTTCTC	CAGCAGTCTGCCTCTCTTGA
5392	C1	TGGAAGATTTGTGTGGATGG	ACGCAATACACTGCAACGAG

5393	C1	AAGGCTTTCTTCTGGCACCT	CGTTTACATCCTCCTCGTCTC
5394	C1	CAAGAATGCCAGCTGAAACA	TCCGCCCATAAATACAAAGG
5395	C1	AGCTACTTCGGCTTCAATGC	TCCGCCCATAAATACAAAGG
5396	C1	GGTTGGCAAGTAATGCCACT	GACCCTGGAAAACAGACCAA
5397	C1	GCAGTCAAACCCATACGCAA	GAAACAGCCTCTCACGATGC
5398	C1	CGCAATTTTAGGGTTCTTCTTCG	GCACGAGTTGGTAAAACTAGCA
5399	C1	ATCTTAGCTCCTACGATGCATT	ACCTTCTTATCCTGTCGTTTGTC
5400	C1	GAGTGATGAGCTGGCAGTTG	TCAAGAGCCTCCCAGTTGAG
5401	C1	GACTCATCGTGCAATCCATATTT	AGTGTGTCAGTGGCTACAGA
5402	C1	AGATCGGGCACAAGTTGAGA	CGATCAGCTTGTTCCAGTCG
5403	C1	ACCGCTCCTCACATGTTGTT	GCCTCTCTAATGGGACCTCA
5404	C1	GAGGTCCCATTAGAGAGGCA	TGTTCTGCTCTTGTCCCCAT
5405	C1	GGTATCCTAAGTCTAAGCCGC	CCTTCACCATGGTTTTGTCCC
5406	C1	CCGAAACCAACACTCGTCTC	TCGTTCCAAGCCTCTTCGTA
5407	C1	ACCTTTCCCTGATTGCTCCA	GTCACGGAATCTGAGGAGGT
5408	C1	TCATCGGTTCTGTGTCGGAA	CCCTTGTTTTAGCCGCCATT
5409	C1	CCACGTGTCATTCATCGGTT	CTTCCCTTGTTTTAGCCGCC
5410	C1	CTGCCACGTGTCATTCATCG	CGGTTGTGTTATCGAGCTAAGC
5411	C1	CGCATTCATGGACGAGGAAG	GCAGTGAAGCAACCTTACAAGA
5412	C1	AAAATGAGTTGGTGGTGGGC	GGCTGTTTCCGACTATTCCG
5413	C1	CCTTCCTCTCCAATCTCTTCAC	CTTCTGCTTGATTCGGCTGG
5414	C1	GAGACAAAGACTGGGACCCA	TGGCGAAATGATCACAGTCA
5415	C1	ACGGAAAGAAGATGCTGCTG	TAAGTGGGAAGATAGGCGGC
5416	C1	GGTTCGATGTAGGAGGTGAAAC	GCAGCCTTTGGAGATGAACA
5417	C1	ACTCTTCCTCCCTTAGCCAC	TGATGATGGGTGACGAGGAG
5418	C1	GATCGTCTTCCTGATTGTTGCA	AGTGAAGAGATTGGAGAGGAAGG
5419	C1	TGATCACCATTGCCCTTACCT	GGAGTTCCGATCAGTTCTTGG
5420	C1	ACATGGACACGTTCTTGAATGA	CGTCTTAGGGATTGGGTTTCC
5421	C1	TACCAGAATCCTCCTCCCCA	AATGATGCAGGCTCCAGACA
5422	C1	CCTCCCCATCATCCTCTTCC	GCATGGAGTACGGCTTTGTG
5423	C1	AAACGAACAACAACCTGCGA	CCTTGGTGGTGGAAATTGTAGT
5424	C1	AAGAACACCAGCCTTCCATG	CCGTTCCATGTTTTCCTGCA
5425	C1	GCGGACGTAAAAGAAGAGCC	TCCTGCAACTAGAAATCTCCTTC
5426	C1	GGTGATGATGAAACCTGGCT	GCTGGTCCATCTACTCTCCC
5427	C1	TGCATTGACTTCACCCAGTT	CCAGTCCTGCAACACTTAGC
5428	C1	AATCAGAGGAGGCGAGACAG	GCCTCCCATCCCATTGCTTA
5429	C1	GGCTCCAAAGTCGATCACAC	GACCGCCAAAGAAGACGAAA
5430	C1	GAAGAGGAATCGCGGGAAAA	TTGACCGCCAAAGAAGACG
5431	C1	GCTCTATGTCTCCGTCGTGA	CCGTGGATCTATTCGCTCCT
5432	C1	GTCGTGACAAGGCGTTTCC	CGACGGTGAAGATTGCGAAA

5433	C1	GCGCTACACAATTTCATTCGT	TTCCATCGCTCTATCACCGG
5434	C1	AAGAAGGAGACGAAGGTGGG	CTTATGCAAAGCTTCCATCGC
5435	C1	ATCTCCCCATTCACCCGATC	ACGAAGGATATGTGCCGATG
5436	C1	TGAAAGTGATGAGTGGGGAAC	TCCATGGCTTCTCTGAATCTCA
5437	C4	GGAGCAGATGTGTCCTAGGT	TCCCTCTTTCCCTCTTCTTTGT
5438	C4	CGGAGAAAGAGGGAATGAGGT	CCCTTCCCTGTCCTCCAAT
5439	C4	TGCGAGTTTGGCTTCTTCTT	ATTCGTTGACCCTTCCCTGT
5440	C4	TGTCGTGAAACCAGAGAATAGAT	TCTGGTTCATAACTGGCCCA
5441	C4	AGCAACCGTCTTTTGTCGTT	TGCGTATTGGTGAGTACTTTTGT
5442	C4	TGACAGACATGACCACCTCA	GGAAGATTGGGGTCACGGAT
5443	C4	AAATTTCGCCGATCCGTGAC	CAGAATCCTAGCCTGGTGTGA
5444	C4	CGTGACCCCAATCTTCCAGA	CGGGATCGGGACTGATTCG
5445	C4	AGCTGAGATCGGGTACAAGT	ATAAGCCTGTTCCAGTCCGT
5446	C4	GGGGACTGCAAAATCATCCA	TCCGGCGAGAAGAATAGACC
5447	C4	GGGGACTGCAAAATCATCCATAA	CGAGAAGAATAGACCGTCGC
5448	C4	TGAACCAAACATGAACCGGA	CCAAAGGTGTGTGTCCGTAT
5449	C4	TTGGACGGAACTTGCCTTCT	GAAGCAAGTCAACGTGGGT
5450	C4	CGATTGGACGGAACTTGCC	TCTACTTAGATCGGGTCAGCAC
5451	C4	TGTCTTAAAAGTGAACCCGAGG	ACCAAAGCAAGCCACTTAACA
5452	C4	CAACAAGACAATGATGATGCTGT	GTGACTAAGCCAACCAAAGCA
5453	C4	AAGGCACCCAATAGATCCAAA	TCAATAGGATTGATTCGCATGCA
5454	C5	GAAGGAGCAAGCACATAGGC	GGGACGTCGACTGTTTATGC
5455	C5	AATCAGCAGGAAGGAGCAAG	ACGTCGACTGTTTATGCATCA
5456	C5	TCTTCAAAGAGTGCAAGGAGAT	GCTTAGTTTGGGCCTATGGG
5457	C5	GGAATCAGGAGGAGGAGGAG	TGGTTGGAAGTTGAGAATTACGT
5458	C5	TTGACAGGAGGCGAAGAGAG	GGCGACTTAACCAATGACGG
5459	C5	TCTAGGAAGAAGCAGCAGGAG	GCAGAGCAGTTGAAGAAGCA
5460	C5	GCGTCCTCAATTAAGCAGCT	CCGAGGGAGGCAGGAATAAT
5461	C5	AGCATCCATTCCACATTACTGA	TATATAACGTTCAGGCCGGC
5462	C5	CTGCACCCATTCGTTAACCC	CATGTGCTTGATCGTCTGGG
5463	C5	ACTCTGTTCCTGTTCTTGTGT	CCATCCTGGCCCATAGAAGA
5464	C5	ACCCTGTTCTTAACCTTGTTCC	GCTACCACCATCTCCTTTCC
5465	C5	TCAAGAGATCGTAAAGACCAGAA	TCGCTTAGGTTTAGAAATGGCTG
5466	C5	ATTGGAACCATTGCCCACAG	GTTTCTGAACCGAGCATGGA
5467	C5	CCCACAGCTAACTAAGATTTTGG	GCCACACCACCTCTTCTCTT
5468	C5	ATTCTGAGCTGGAACCGAGG	CCTTGTTGACTTTGACCTTGACT
5469	C5	GGAGTGAAGACAGTGAGGAGA	TCTTAACTTGACTACTCGCTCCT
5470	C5	TGGCTTCATTTGGATTACGGG	GCAACAAGATTGGCTGATCAA
5471	C5	CCTTGCGCGGGATTAAGTTA	GGCTGATCAATTTAGTCGTCCA
5472	C5	TCAAGTGTGATCCCTGGAGAC	TGTCTGCTCCTCCTCCATTC

5473	C5	TCTCCACATCAGAAGGACGC	GGGTTTGGAAGCAAGGAAGG
5474	C5	GAGGCCCGTCTCCACATC	CTTCCTCCATTGATCACGGC
5475	C5	ATCGGCTTCCACCAGTATCC	ATCTTCCTCCTCCCAATCCG
5476	C5	TAGTTGAAGACGAAGGCTGC	TCAAGTCATAACGGTGGGCA

Supplementary Table 4.1 List of F_1, F_2 and backcross *Brassica napus* populations segregating for clubroot resistance

Pedigree
F_1 [a]
A1363.342× UA19-4
A1363.362× UA19-4
A1363.364× UA19-4
A1363.371× UA19-4
A1363.340× Cougar
A1363.348× Cougar
A1363.349× Cougar
A1363.350× Cougar
A1363.340× A04-73NA
A1363.348× A04-73NA
A1363.349× A04-73NA
A1363.350× A04-73NA
F_2 [b]
A1363.364× UA19-4
A1363.371× UA19-4
A1363.340× Cougar
A1363.349× Cougar
A1363.340× A04-73NA
A1363.348× A04-73NA
BC_1 (= F_1 × resistant parents)
(A1363.364× UA19-4) × A1363.364
(A1363.371× UA19-4) × A1363.371
(A1363.340× Cougar) × A1363.340
(A1363.349× Cougar) × A1363.349
(A1363.340× A04-73NA) × A1363.340
(A1363.348× A04-73NA) × A1363.348
BC_2 (= F_1 × susceptible parents)
(A1363.364× UA19-4) × UA19-4
(A1363.371× UA19-4) × UA19-4
(A1363.340× Cougar) × Cougar
(A1363.349× Cougar) × Cougar
(A1363.340× A04-73NA) × A04-73NA
(A1363.348× A04-73NA) × A04-73NA

[a] A1363.342, A1363.362, A1363.364, A1363.371, A1363.340, A1363.348, A1363.349 and A1363.350 are resistant inbred F_9 *B. napus* derived from the *B. napus* and *B. oleracea* interspecific cross, while UA19-4, Cougar and A04-73NA are susceptible *B. napus*; and F_1 is the combinations of eight resistant parents and four susceptible parents.

[b] F_2 is self-pollinated from six selected F_1 hybrids based on the clubroot resistance evaluation.