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Response of *Brassica napus* lines containing all possible combinations of three clubroot resistance genes to infection by *Plasmodiophora brassicae*

by

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A creative component submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

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Program of Study Committee:
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Iowa State University

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Table of Contents

ABSTRACT 4
INTRODUCTION 4
MATERIALS AND METHODS 10
Plant materials 10
Pathogen inoculum 11
Clubroot resistance tests 11
Molecular Markers 12
Statistical Analysis 12
RESULTS 13
DISCUSSION 23
CONCLUSIONS 26
REFERENCES 27

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ABSTRACT

Canola (*Brassica napus* L.) has become a major field crop in Canada. One of the largest threats to canola production is the disease of clubroot caused by the soilborne pathogen *Plasmodiophora brassicae*. This disease can have devastating effects on canola yield and quality. Long-lived resting spores make this disease difficult to manage with few strategies proving to be effective. Currently, the most effective management tool is the development and deployment of host plant genetic resistance. We studied a double haploid population developed from crossing a male parent containing clubroot resistance genes *PH1* and *PH2* to a female parent containing clubroot resistance gene *PH3*. Molecular profiles for each of the three genes in the DH lines was determined. Planned crosses among a subset of the DH lines were then made to obtain 108 F1s with all possible homozygous and heterozygous combinations of *PH1*, *PH2*, and *PH3*.These 108 F1s 27 genotypes were tested in a greenhouse setting against *P. brassicae* pathotypes 3H, 3A, and 5X to phenotype their clubroot reaction. Disease index was compared between the 27 combinations of *PH1*, *PH2*, and *PH3* represented within the 108 F1s. Results found evidence of an epistatic effect between *PH2* and *PH3* that improved disease resistance to a greater extent than was observed when either gene was in single heterozygous form. The results highlight the importance of verifying gene reactions through gene stacking to identify epistatic effects. Utilizing gene stacking could produce more durable and broad-spectrum clubroot resistant canola varieties.

INTRODUCTION

Rapeseed (Oilseed rape OSR) comprises three species of oilseed: *Brassica rapa*, *Brassica napus*, and *Brassica Juncea* (L.). Oilseed rape was introduced to Canada in 1936 by Fred Solvonik who imported *B. rapa* seed from Poland (Bell 1982). *B. napus* seed was later introduced to Canada from Argentina. *B. napus* is an allotetraploid resulting from hybridization events of diploid progenitors *B. rapa* and *Brassica oleracea*. *B. napus* contains an A subgenome from *B. rapa* and a C subgenome from *B. oleracea* (Parkin et al., 1995). Rapeseed (*B. napus* and *B. rapa*) in Canada was originally grown for its oil to be used as a marine engine lubricant due to its special properties associated with the high erucic acid content (Bell 1982). This created large demand and incentive for farmers to grow rapeseed during World War II (Busch et al., 1994). When the war ended demand for rapeseed dropped while Canada was left with significant production capability and infrastructure in place (Bell 1982, Busch et al., 1994). At this time, rapeseed oil for human consumption was not common due to negative health effects of erucic acid. The meal was also not well suited for animal feed due to high glucosinolates. Research began to transition rapeseed oil from an engine lubricant to a desirable edible oil that could compete with other existing vegetable oils on the market.

Efforts to diversify crops of the Canadian prairies and create new demand for rapeseed, lead to the development of canola from genotypes of oilseed rape at the Universities of Manitoba and Saskatchewan in the 1970s (Rempel et al., 2014). In the early

1970's *B. rapa* was the dominant species in western Canada due to its early maturity. *B. napus* production increased in the 1980's as maturity was improved and was higher yielding. By the 1990's *B. rapa* production area had decreased to about 15 to 20% of canola production area. Currently in Canada, canola varieties grown are most commonly of *Brassica napus* (Government of Canada, 2014). *B. napus* is favored for its higher yields (Karim et al., 2014). However, due to *B. rapa* better withstanding of spring frosts and fewer frost-free days than *B. napus*, the remaining *B. rapa* hectares are typically grown north of *B. napus* producing areas (CFIA, 2014). Canola must meet internationally regulated standards for oil and dry matter composition. First, seeds must contain oil with less than 2% erucic acid in the fatty acid profile. In addition, the oil-free solid component must contain less than 30 micromoles of 3-butenyl glucosinolate, 4-pentenyl glucosinolate, 2-hydroxy-3 butenyl glucosinolate, and 2-hydroxy- 4-pentenyl glucosinolate or any combination thereof per gram (Canola Council of Canada, 2020). These requirements ensure seed designated as canola will uphold the reputation and the health benefits represented by this label. Due to low levels of saturated fat in combination with substantial amounts of monounsaturated fats and polyunsaturated fats, canola is considered a healthy vegetable oil option. Canola oil also contains plant sterols and tocopherols which contribute to cardiovascular health (Rempel et al., 2014). In addition, the canola meal has come to be known as an excellent source of Vitamin B and E as well as a protein source to be used in animal feed (Rempel et al., 2014). Due to these benefits, consumer demand for canola oil has grown and a large canola market has developed in Canada and around the world.

Canola is currently the second most grown crop by acreage and number one in farm gate receipts in Canada (Statistics Canada 2020). Its export adds 26.7 billion dollars and 250 thousand jobs to Canada's economy each year (Canola Council of Canada 2016). Roughly 8.5 million hectares of canola were grown in Canada in 2019. The majority of these hectares were planted in the prairie provinces of Saskatchewan (4.7 million), Alberta (2.4 million), and Manitoba (1.3 million), with few hectares in British Columbia (34.7 thousand), Ontario (18.9 thousand), Quebec (10.4 thousand), New Brunswick (600), and Prince Edward Island (365) (Statistics Canada 2019).

One of the largest threats to canola production in the Canadian prairies and to *Brassica* crops worldwide is the disease of clubroot. Clubroot is a soilborne disease of the *Brassicaceae* family and is caused by the obligate parasite *Plasmodiophora brassicae.* Highly infested clubroot fields have been reported in Canada (Tewari et al., 2005; Rahman, 2014). They have also been reported in other countries including China (Chai 2014) and India (Bhattacharya et al., 2014), as well as an increase of infestations in Europe (Diederichsen et al., 2014; Wallenhammar et al., 2014) and Australia (Donald and Porter 2014). Clubroot in Canada was traditionally found in *Brassica* vegetable crops in Ontario, Quebec, British Columbia, and the Atlantic Provinces (Howard et al., 2010). Clubroot infection was first identified within Canadian canola crops during the 2003 growing season. Multiple infected fields were found in the surrounding areas of Edmonton, Alberta (Tewari et al., 2005). The disease has since spread throughout the province and has been found on canola fields of Saskatchewan and Manitoba, as well as in the state of North Dakota (Chapara et al., 2019, Froese et al., 2019, McLaren et al., 2019, Strelkov et al., 2019,

Ziesman et al., 2019). Yield loss of severely infected fields has been estimated from 30% to 100% (Strelkov et al., 2007; Hwang et al., 2011a).

P. brassicae goes through three distinct lifecycle stages: resting spore stage, root hair infection stage, and cortex infection stage (Kageyama and Asano, 2009; Schwelm et al., 2016). Primary inoculum is the result of rotten host tissue dispersing resting spores into the surrounding soil (Kageyama and Asano, 2009). *P. brassicae* resting spores germinate in the presence of a susceptible host, releasing bi-flagellate motile zoospores. These primary zoospores initiate infection of a susceptible host via penetration of the cell walls of root hairs or wounds. This is the primary infection stage. Primary plasmodia are formed within the root hairs. In the plasmodia, several cycles of nuclear division occur followed by cleaving into zoosporangia, containing 4-16 secondary zoospores (Howard et al., 2010). The secondary zoospores are released into the soil and penetrate the host, invading cortical tissue of main roots where secondary plasmodia form (Howard et al., 2010). Secondary plasmodia translocate endophytically, infecting root cells and inducing rapid cell division which results in the formation of galls (Ingram and Tommerup, 1972; Voorrips 1995). Upon maturity, secondary plasmodia will develop into resting spores that are released back into the soil matrix as root tissue decays or is broken apart. Galls inhibit water and nutrient uptake by the plant. Severe infection will stunt the overall plant growth as well as cause wilting, stunted growth, and premature chlorosis of above ground plant organs. As a result, there may be significant loss of crop yield and quality (Howard et al., 2010).

Managing clubroot via cultural, chemical, or mechanical methods has proven to be very difficult. A number of management strategies have been previously proposed including, liming, nutrient supplements, fungicides, and crop rotation. Infections are most common when soil pH is below 7, though it is possible but rare for them to occur around 8 (Colhoun 1953), suggesting that liming to increase soil pH could be useful in clubroot management. Micronutrients such as calcium and boron can also influence disease development (Webster and Dixon 1991a, 1991b). However, soil amendments including liming and micronutrient application can be costly with variable efficacy (Colhoun 1953; Deora et al., 2011; Gossen et al., 2014). Synthetic fungicides and microbial biofungicides have been explored as control options but have been found to be ineffective and uneconomical (Peng et al., 2014). Improved drainage could reduce the movement of zoospores and subsequent infestations (Howard et al., 2010) but should only be considered a minor part of an integrated management strategy. Resting spores may have a half-life of approximately four years and can survive in the soil at detectable levels up to 20 years without a host (Wallenhammar 1996, Dixon 2009). Also, zoospores do not spread rapidly in the soil due to limited motility, therefore the majority of disease spread is through the movement of soil and plant material infected with resting spores (Howard et al., 2010). The longevity of the resting spores is a major constraint for efficient control of this disease. Minimizing spread of infested soil by reduced tillage and thorough equipment sanitation may help manage clubroot (Howard et al., 2010), however, adoption of these techniques by producers may be limited due to the time, effort, and cost involved. Furthermore, rotation can function as a means to reduce resting spore loads but due to the longevity of inoculum and economics of canola production in Canada, clubroot will inevitably persist (Wallenhammar 1996, Ernst et al., 2019). In

summary, cultural practices, biological and chemical treatments, as well as integrated systems have been studied for control of clubroot but have been found to be relatively ineffective or not economically feasible (Donald and Porter, 2009; Peng et al., 2011, 2014).

The development and deployment of host plant genetic resistance is currently the most effective management tool for minimizing crop loss (Diederichsen et al., 2014; Donald and Porter 2009). Multiple sources of clubroot resistance (CR) have been identified from *B. rapa* (AA, n=10), *B. oleracea* (CC, n=9), and *B. napus* (AACC, n=19) (Diederichsen et al., 2009). Between the two progenitor species of *B. napus*, CR has been more frequently found in *B. rapa* (A genome) (Hirai 2006). These tend to be dominant, pathotype-specific resistance genes (Hirai 2006). *B. oleracea* (C genome) has been found to have CR genes that tend to act quantitatively and pathotype-independent (Hirai 2006). In canola, major CR genes are found predominantly in the A genome of *B. napus,* sourced from *B. rapa* (Diederichsen et al., 2009, Neik et al., 2017) or winter *B. napus* (Rahman et al., 2011, Fredua-Agyeman and Rahman 2016). Currently there are 4 main clusters of CR genes identified and mapped on the A genome, including one cluster on the A1 chromosome, two clusters on the A3 chromosome, and one cluster on the A8 chromosome (Hirai 2006).

A plant's primary level of defense against a pathogen is pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI). When a pathogen infects the plant, the plant recognizes the invading pathogen by pattern recognition receptors (PRRs). The recognition causes the activation of PTI which induces the expression of defense genes (Mehraj et al., 2020). The pathogen, however, delivers virulence molecules called effectors that suppress the PTI. If the plant contains a resistance (R) gene that matches and recognizes the effectors (Avr proteins), an effector-triggered immunity (ETI) is activated causing a hypersensitive response (HR) (Balint-Kurti, 2019). The recognition between an R and Avr is called gene-for-gene resistance (Balint-Kurti, 2019). The HR is a defense response characterized by rapid cell death at the point of pathogen penetration to halt the spread of the infection (Balint-Kurit, 2019). Such host-pathogen interactions tend to result in evolutionary arms races causing relatively rapid rates of evolution (Balint-Kurit, 2019). Due to quickly evolving pathogens, single major gene resistance can be broken down over a small number of generations (Strelkov et al., 2018).

The physiological specialization of *P. brassicae* complicates efforts to breed for clubroot resistance. Existing cultivars typically incorporate single CR genes (Diederichsen et al., 2009). Such cultivars have proven unstable as resistance erodes with changing pathogen race structure (LeBoldus et al., 2012, Strelkov et al., 2016a, Strelkov et al., 2018). Multiple clubroot pathotypes exist and their capacity to infect different host-differential genotypes varies by isolate (Williams 1966, Strelkov et al., 2018). A few differential systems have been proposed to identify pathotypes of the clubroot pathogen, Williams (1966) pathotype designations being most common globally. Prevalent pathotypes based on the Williams (1966) differential in Canada include pathotypes 2, 5, 6, 8, and predominantly 3 (Deora et al., 2012). Various Canadian spring *B. napus* cultivars with excellent resistance to these five pathotypes have been developed. However, in 2013, virulent clubroot field isolates were identified within fields cultivating CR canola cultivars. These isolates were confirmed to

produce a new virulence phenotype (Strelkov et al., 2016a). These populations were classified as pathotype 5 on the differentials of Williams (1966), but due to their increased virulence they were distinguished from the original pathotype 5 by referring to the isolates as ‘5X’. New, highly virulent Canadian isolates were not fitting well into existing differential sets, therefore a new Canadian Clubroot Differential (CCD) Set was developed to classify 5X and other new pathotypes (Strelkov et al., 2018). Canadian isolates determined as Williams (1966) pathotype 3 have been further differentiated by the CCD set. Field isolates from Canada remain predominantly Williams pathotype 3 or CCD pathotype 3H. Williams pathotype 3 isolates with increased virulence on the CCD set include, for example, CCD pathotype 3A among others (Strelkov et al., 2018).

Fredua-Agyeman and Rahman (2016) found through genetic mapping that there were at least 2 regions of clubroot resistance on the A3 chromosome. One region included the *CRa* locus (Ueno et al., 2012), CRb^{kato} locus (Kato et al., 2012, 2013), and the *CRb* locus (Piao et al., 2004; Zhang et al., 2014). The *CRa* locus and the CRb^{kato} showed linkage association with the CR locus derived from ‘Mendel.’ Fredua-Agyeman and Rahman (2016) concluded that *CRa* and CRb^{kato} may be allelic forms of the same gene or two different genes that are closely linked. However, the molecular marker data distinguished the *CRb* locus (Piao et al., 2004; Zhang et al., 2014) as separate from CRa/Crb^{kato} locus. The second region included the *CRk* locus (Matsumoto et al., 2012) and the *Crr3* locus (Hirai et al., 2004; Saito et al., 2006). The markers for these loci showed no linkage association with the CR locus derived from cv. ‘Mendel.’ Fredua-Agyeman and Rahman (2016) concluded that their molecular marker mapping had demonstrated that CR in the winter canola cv. ‘Mendel’ is conferred by the CRa/CRb^{kato} located on the A3 chromosome of the *Brassica* A genome. Based on internal unpublished data the gene referred to as *PH1* in this study has been mapped to the same region as the CRa/CRb^{kato} locus, while the gene designated as *PH3* is located in the second region of the A3 chromosome in which *CRk* and *Crr3* are located. The *B. rapa* line ECD 04 has been shown to possess resistance in the *Crr1* region of the A8 chromosome (Fredua-Agyeman et al., 2018) that may have been introgressed into cv. ‘Mendel’. ‘Mendel’ originates from a resynthesized *B. napus* form from a cross of *B. rapa* ECD-04 and *B. oleracea* ECD-15, with the aid of ovule culture (Diederichsen and Sacristan 1996). Based on internal unpublished data, the gene referred to as *PH2* in this study has been mapped to the *Crr1* genetic position.

The objective of this study is to determine what resistance phenotypes can be attributed to all combinations of the three CR genes discussed (*PH1*, *PH2*, *PH3*). We currently have an understanding of the resistance phenotypes of the three CR genes as they work individually (Internal unpublished data, Table 1). These were determined through club root screens of single heterozygous CR gene hybrid varieties. The clubroot reactions of these hybrids has been well characterized over time. The primary goal is to determine the nature of the three CR genes in question. Namely, do they function in a pure gene-for-gene resistance model or are there unknown epistatic effects (Table 2) between combinations of resistance genes at these three loci. It has been shown that pyramiding major resistance genes can confer high resistance against multiple pathotypes (Matsumoto et al., 2012). Using

differentiating pathotypes 3H, 3A, and 5X (CCD) it is possible to verify the following hypotheses:

H_o: Each CR gene in question will respond based on a typical gene-for-gene model (Table 2).

H_a: Genes at these CR loci may not function along a strict gene-for-gene model, potential epistatic outcomes (Table 2).

Table 1: Previously determined resistance phenotypes (Internal unpublished data).

	Pathotype [a]		
	3H	3A	5X
Genotype[b]	**Reaction**[c]		
Aabbcc [d]	-	+	+
aaBbcc [e]	-	-	-/+
aabbCc [f]	-/+	+	+

[a] Pathotype designations are based on the systems of the Canadian Clubroot Differential (CCD) Set (Strelkov et al., 2018).
[b] (A = *PH1* present, a = *PH1* absent; B = *PH2* present, b = *PH2* absent; C = *PH3* present, c = *PH3* absent).
[c] A plus (+) sign indicates a susceptible host reaction, a minus (-) sign a resistant reaction, and a minus/plus (-/+) sign an intermediate reaction.
[d] Reaction based on internal data (Rahman et al., 2011; Fredua-Agyeman and Rahman, 2016; Fredua-Agyeman et al., 2018; Strelkov et al., 2018)
[e] Reaction based on internal data (Fredua-Agyeman et al., 2018; Fredua-Agyeman et al., 2020a; Fredua-Agyeman et al., 2020b; Strelkov et al., 2018)
[f] Reaction based on internal data

Table 2: Null and Alternative Hypotheses reactions

		H_O[a]			H_A[b]		
		Pathotype[c]			**Pathotype**[c]		
		3H	3A	5X	3H	3A	5X
Genotype[d]	**Reaction**[e]						
AaBbcc		-	-	-/+	-	-	-
AabbCc		-	+	+	-	-	-
aaBbCc		-	-	-/+	-	-	-
aabbCC		-/+	+	+	-	-	-

[a] Null Hypothesis reactions, hypothetical reaction of stacked CR genes following a strict gene-for-gene model.
[b] Alternative Hypothesis reactions, hypothetical epistasis resulting in resistance genotypes and phenotypes.
[c] Pathotype designations are based on the systems of the Canadian Clubroot Differential (CCD) Set (Strelkov et al., 2018).
[d] (A = *PH1* present, a = *PH1* absent; B = *PH2* present, b = *PH2* absent; C = *PH3* present, c = *PH3* absent).
[e] A plus (+) sign indicates a susceptible host reaction, a minus (-) sign a resistant reaction, and a minus/plus (-/+) sign an intermediate reaction.

MATERIALS AND METHODS

Plant materials

Plant material used in this study was selected from *Brassica* germplasm of Corteva Agriscience™ (Des Moines, IA, USA). In 2014, a cross was made between two *B. napus* parental lines, one containing the *PH3* gene and another containing both the *PH1* and *PH2* genes. From the F1 generation, a doubled haploid (DH) population was created. In 2020, 111 lines from this DH population were grown under controlled greenhouse conditions with temperatures of 20-25/15-18°C day/night and 16 h photoperiod. Leaf samples of all 111 lines were taken and molecular markers were used to detect what combination of the *PH1*, *PH2*, and *PH3* genes each line contained. From within the DH population, 60 DH lines were used as parents for a total of 108 crosses providing a minimum of 3 F1s representing each of the 27 possible heterozygous and homozygous combinations of *PH1, PH2,* and *PH3*. All 108 F1s were planted with two technical replicates of six plants each per inoculum type (3A, 3H, 5X). The universally clubroot susceptible *B. rapa* var. *pekinensis* 'Granaat' (ECD 05) was planted as well as the *B. napus* hybrid 45H29 (Pioneer Hi-bred Production Ltd.) which is known to be susceptible to the newer virulent pathotypes 3A and 5X but resistant to the older and more prevalent pathotype 3H.

Pathogen inoculum

Inoculum preparation generally followed methodology outlined in Strelkov et al. (2006). Briefly, clubroot galls representing enriched pathogen populations of pathotypes 3A, 3H, and 5X as per the CCD (Strelkov et al., 2018) were used. Galls were derived or propagated during previous clubroot inoculations of the universally susceptible ECD 05. These galls were stored at -20°C then removed and thawed at room temperature on the day of inoculum preparation. The galls were ground via mortar and pestle with distilled water and filtered through 8 layers of cheesecloth. The spore concentration was adjusted to 1×10^7 resting spores mL^{-1}. A single batch of inoculum was prepared for each pathotype and each batch was used within 24 h of preparation.

Clubroot resistance tests

The seed was planted into 2.92 x 4.04 x 5.72 cm (WxLxD) pots filled with Sunshine Mix #4 Aggregate Plus Soil (Sungro Horticulture Canada Ltd.) that had been compacted and saturated with water. They were kept in a greenhouse at the University of Alberta at 20-25/15-18°C day/night and 16 h photoperiod. The pots were irrigated daily from above the soil for the first 10 days. Thinning and transplanting occurred 10 days after planting to ensure one plant per pot. Inoculation occurred concurrently 10 days after planting. Plants were inoculated via the pipette method (Lamers and Toxopeus, 1977 as cited by Voorrips and Visser, 1993) with 1 mL of inoculum dispensed into the soil surrounding each seedling. Watering was switched to bottom watering for a week post inoculation to allow establishment of infection. Subsequent irrigation returned to above soil watering.

Six weeks after inoculation, the plants were gently removed from the potting medium, washed in water, and assessed for clubroot severity. A 0-3 scale was used, where: 0 = no galls; 1 = a few small galls on <1/3 of the roots; 2= medium galls on 1/3-2/3 of the roots, and 3 = large galls on >2/3 of the roots (Figure 1. Kuginuki et al., 1999). A disease index was calculated using the following formula (Strelkov et al. 2006)

$$\text{ID (\%)} = \frac{\sum (n \times 0 + n \times 1 + n \times 2 + n \times 3)}{N \times 3} \times 100\%$$

where Σ is the sum total; *n* is the number of plants in a class; *N* is the total number of plants; and 0, 1, 2, and 3 are the symptom severity classes.

Figure 1. Clubroot rating system based on a 0-3 scale adapted from Kuginuki et al. (1999). 0 = no clubs, 1 = few small clubs, 2 = moderate clubbing, 3 = severe clubbing.

Molecular Markers

Fresh leaf tissue samples of one 6.35 mm disc were taken from each plant of all experimental lines using a *Tinker Tooling Puncher*. Each sample went into a 1.2 mL polypropylene bullet tube and the bullet tubes went into 96 well plates. These 96 well plates were packaged with ice packs into coolers and sent to the Corteva molecular lab in Ontario, Canada. Previously developed polymorphic Taqman® SNP markers tightly linked to the three genes of interest (*PH1*, *PH2*, and *PH3*) were selected for the DH population to be genotyped using an array tape (Douglas Scientific, Alexandria, MN). These markers were used again to screen all plants in the clubroot resistance test to confirm each line contained the expected genotype.

Statistical Analysis

Disease index was calculated for all replicates representing the 27 unique molecular profiles (Table 3). Mean disease index was calculated for each genotype under each pathotype environment of 3H, 3A, 5X and a heat map was produced (Microsoft Excel version 1808). Mean disease indices were not normally distributed, therefore the non-parametric Kruskal-Wallis Rank Sum Test was conducted in R version 3.6.2 (R Core Team, 2020) for each of the three *P. brassicae* pathotypes tested comparing disease index by genotype. Dunn's Test of multiple comparisons using rank sums (package = "dunn.test";

Dinno, 2017) was used to conduct pairwise comparisons among all 27 genotypes for disease index reaction to detect median differences. This was conducted for each of the three pathotype conditions. Boxplots were created in R for each pathotype environment and overlaid with a compact letter display (package = “rcompanion”; Mangiafico, 2020) based on Dunn’s test of significant differences (α=0.05). Designations of susceptible, resistant and intermediate reactions were assigned to each genotype under each pathotype condition. A high disease index indicates a susceptible reaction and designations were made based on significant differences as determined by the Dunn’s Tests (α = 0.05). A genotype that had a reaction not significantly different than the susceptible genotype (*aabbcc*) was designated as susceptible (+). A genotype that did not share any letters of the compact letter display with a susceptible genotype was considered resistant (-). Genotypes that shared letters of the compact letter display with both susceptible and resistant genotype reactions were designated as having an intermediate reaction (+/-).

RESULTS

The universally susceptible line, EDC05, showed full clubroot disease symptoms under the same conditions as experimental lines. The 45H29 hybrid, known to be susceptible to pathotypes 3A and 5X also showed full clubroot disease symptoms under 3A and 5X inoculum conditions.

Overall, average disease index induced across all genotypes was lowest under pathotype 3H conditions (DI=11.1%), followed by pathotype 5X (DI=28.3%), while pathotype 3A produced the highest average disease index (DI=37.5%). Mean disease index was calculated for each genotype and compared within each pathotype environment (Table 3). A Kruskal-Wallis test was run for each pathotype condition and each pathotype condition resulted in significant differences in disease index between genotypes (Figure 2, 3, 4). The Dunn’s test results were used to determine resistance or susceptibility designations for the main genotypes of interest for which the original hypotheses were made, under each pathotype condition (Table 4) as well as for each of the full set of 27 genotypes tested (Table 3). Notably, heterozygous *PH1* (*Aabbcc*) resulted in a resistant reaction against 3H and a susceptible reaction against 3A and 5X, while homozygous *PH1* (*AAbbcc*) elicited the same response. Heterozygous *PH2* (*aaBbcc*) resulted in a susceptible reaction across all three pathotypes 3H, 3A and 5X, however, homozygous *PH2* (*aaBBcc*) was resistant across all three. Heterozygous *PH3* (*aabbCc*) and homozygous *PH3* (*aabbCC*) both resulted in an intermediate reaction against pathotype 3H and a susceptible reaction against pathotypes 3A and 5X. The *PH1* and *PH2* stack (*AaBbcc*) against pathotype 3A and 5X resulted in susceptible reactions. Reactions of all other genotype combinations of *PH1*, *PH2* and *PH3* are also represented within Table 3.

Dunn’s multiple pairwise test provided significance groupings that are reported as a compact letter display overlaid on boxplots in Figures 2-4 as a visual comparison between all genotypes. Figures 5, 6, and 7 illustrate the change in disease index against 3H with changing combinations of homozygosity and heterozygosity of the *PH1* and *PH2* genes when *PH3* is

in a state of recessive homozygosity, heterozygosity, and dominant homozygosity, respectively. This has also been illustrated by figures 8, 9, and 10 for the 3A pathotype, and figures 11, 12, and 13 for the 5X pathotype.

Table 3. Mean disease index (DI) and resistance designations for 27 genotypes against *P. brassicae* pathotypes 3H, 3A, and 5X

	Pathotype [a]					
	3H		3A		5X	
Genotype[b]	Mean DI (%)	Resistant Reaction[c]	Mean DI (%)	Resistant Reaction[c]	Mean DI (%)	Resistant Reaction[c]
aabbcc	92.2	+	84.3	+	71.3	+
aabbCc	43.3	-/+	74.3	+	57.0	+
aabbCC	18.1	-/+	81.9	+	71.3	+
aaBbcc	67.2	+	63.1	+	34.5	+
aaBbCc	20.5	-	23.7	-	4.2	-
aaBbCC	2.2	-	1.4	-	0.0	-
aaBBcc	11.2	-	3.7	-	0.0	-
aaBBCc	3.7	-	2.8	-	0.0	-
aaBBCC	2.3	-	0.0	-	1.1	-
Aabbcc	10.4	-	100.0	+	100.0	+
AabbCc	3.1	-	75.0	+	79.1	+
AabbCC	1.1	-	48.5	-/+	26.7	-/+
AaBbcc	3.8	-	68.0	+	26.3	+
AaBbCc	2.6	-	9.3	-	5.1	-
AaBbCC	0.6	-	3.3	-	1.0	-
AaBBcc	1.9	-	7.6	-	1.1	-
AaBBCc	0.6	-	5.1	-	0.5	-
AaBBCC	0.0	-	0.0	-	0.0	-
AAbbcc	6.7	-	96.7	+	83.3	+
AAbbCc	1.1	-	87.0	+	92.2	+
AAbbCC	3.6	-	75.6	+	65.3	+
AABbcc	0.0	-	64.7	+	28.5	+
AABbCc	2.4	-	13.7	-	7.6	-
AABbCC	0.0	-	3.1	-	1.7	-
AABBcc	0.0	-	15.0	-	2.0	-
AABBCc	0.0	-	1.9	-	1.1	-
AABBCC	0.0	-	3.3	-	1.9	-

[a] Pathotype designations are based on the systems of the Canadian Clubroot Differential (CCD) Set (Strelkov et al., 2018).

[b] (A = *PH1* present, a = *PH1* absent; B = *PH2* present, b = *PH2* absent; C = *PH3* present, c = *PH3* absent).

[c] A plus (+) sign indicates a susceptible host reaction, a minus (-) sign a resistant reaction, and a minus/plus (-/+) sign an intermediate reaction. Host reactions were classified as +, –, or -/+ based on the disease index (DI) 6 weeks after inoculation; reactions were considered resistant based on significant difference (Dunn's Test, α = 0.05) from the susceptible genotype (*aabbcc*).

[d] Heat map generated through Microsoft Excel with red representing high disease index and green representing low disease index.

Table 4. Resistance designations of the main genotypes of interest from clubroot screen

	Pathotype [a]		
	3H	3A	5X
Genotype[b]	**Reaction[c]**		
AaBbcc	-	+	+
AabbCc	-	+	+
aaBbCc	-	-	-
aabbCC	-/+	+	+

[a] Pathotype designations are based on the systems of the Canadian Clubroot Differential (CCD) Set (Strelkov et al., 2018).
[b] (A = *PH1* present, a = *PH1* absent; B = *PH2* present, b = *PH2* absent; C = *PH3* present, c = *PH3* absent).
[c] A plus (+) sign indicates a susceptible host reaction, a minus (-) sign a resistant reaction, and a minus/plus (-/+) sign an intermediate reaction. Host reactions were classified as +, –, or -/+ based on the disease index (DI) 6 weeks after inoculation; reactions were considered resistant based on significant difference (Dunn's Test, $\alpha = 0.05$) from the susceptible genotype (*aabbcc*).

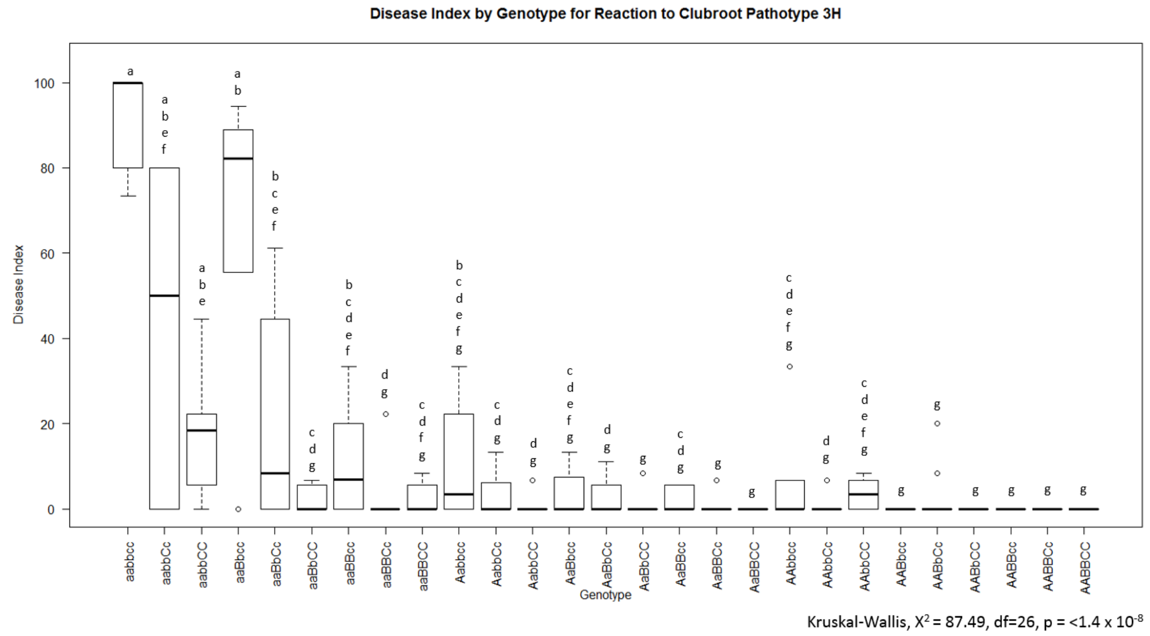

Figure 2. Clubroot disease index by genotype against *P. brassicae* pathotype 3H (CCD; Strelkov et al., 2018). Compact letter display was generated by significant differences as determined by the Dunn's test (α = 0.05). Genotypes sharing the same letter indicates no significant difference between them.

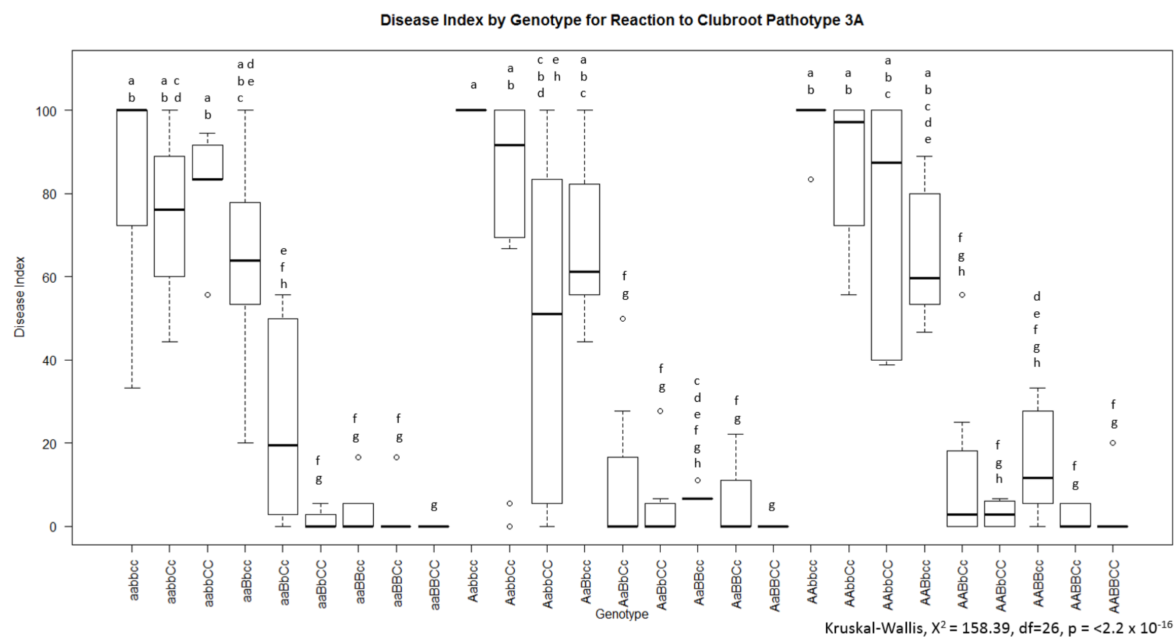

Figure 3. Clubroot disease index by genotype against *P. brassicae* pathotype 3A (CCD; Strelkov et al., 2018). Compact letter display was generated by significant differences as determined by the Dunn's test ($\alpha = 0.05$). Genotypes sharing the same letter indicates no significant difference between them.

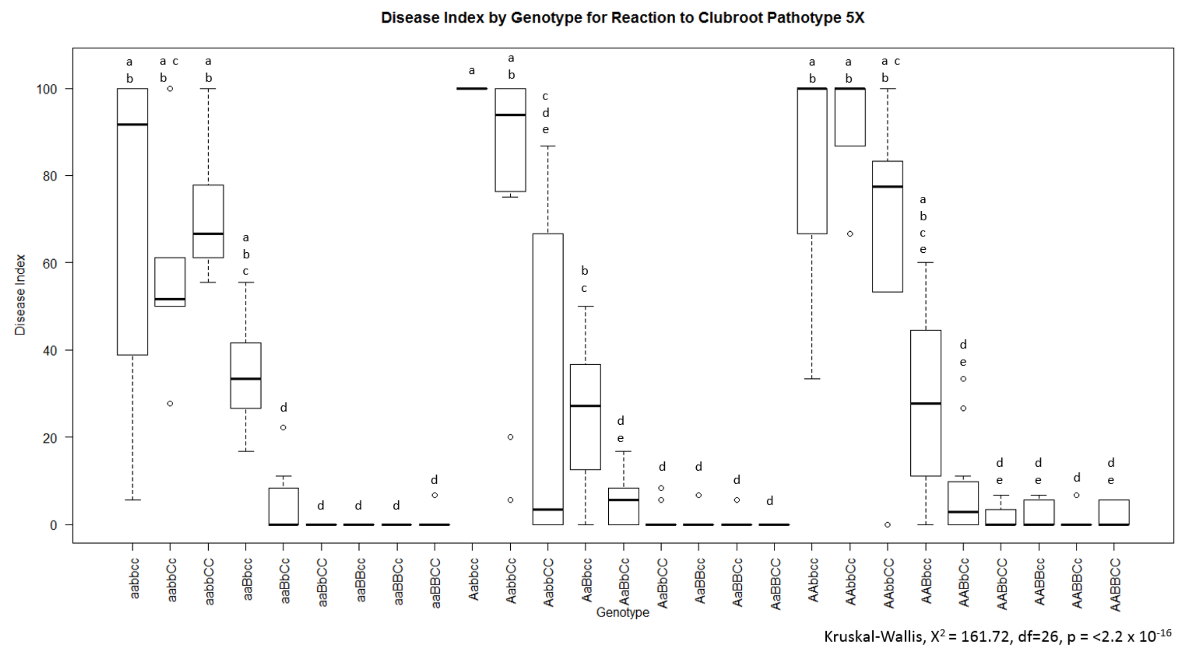

Figure 4. Clubroot disease index by genotype against *P. brassicae* pathotype 5X (CCD; Strelkov et al., 2018). Compact letter display was generated by significant differences as determined by the Dunn's test ($\alpha = 0.05$). Genotypes sharing the same letter indicates no significant difference between them.

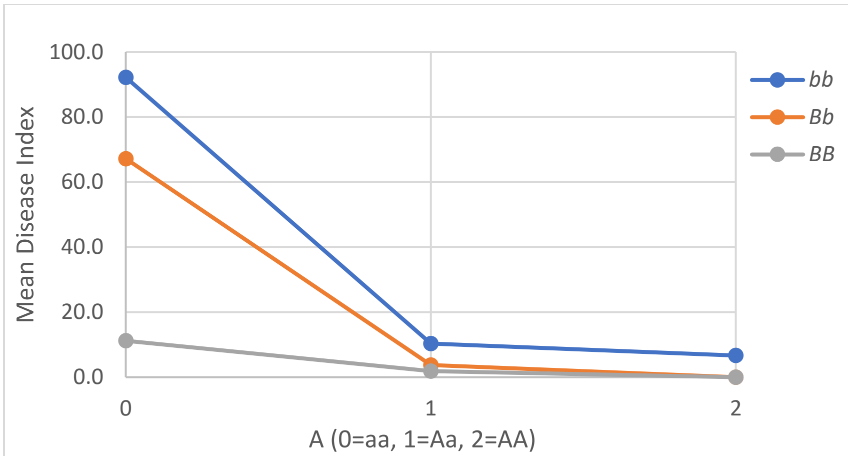

Figure 5. Mean disease index of each genotype combination of homozygosity/heterozygosity of genes A and B when gene C is cc against 3H (A = *PH1* present, a = *PH1* absent; B = *PH2* present, b = *PH2* absent; C = *PH3* present, c = *PH3* absent).

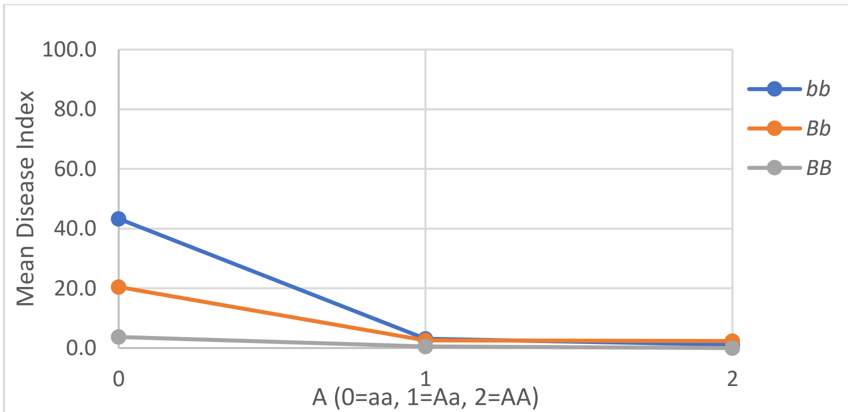

Figure 6. Mean disease index of each genotype combination of homozygosity/heterozygosity of genes A and B when gene C is Cc against 3H (A = *PH1* present, a = *PH1* absent; B = *PH2* present, b = *PH2* absent; C = *PH3* present, c = *PH3* absent).

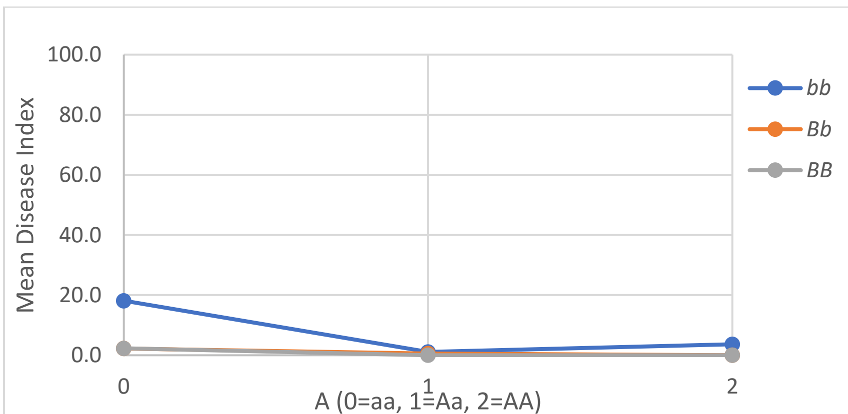

Figure 7. Mean disease index of each genotype combination of homozygosity/heterozygosity of genes A and B when gene C is CC against 3H (A = *PH1* present, a = *PH1* absent; B = *PH2* present, b = *PH2* absent; C = *PH3* present, c = *PH3* absent).

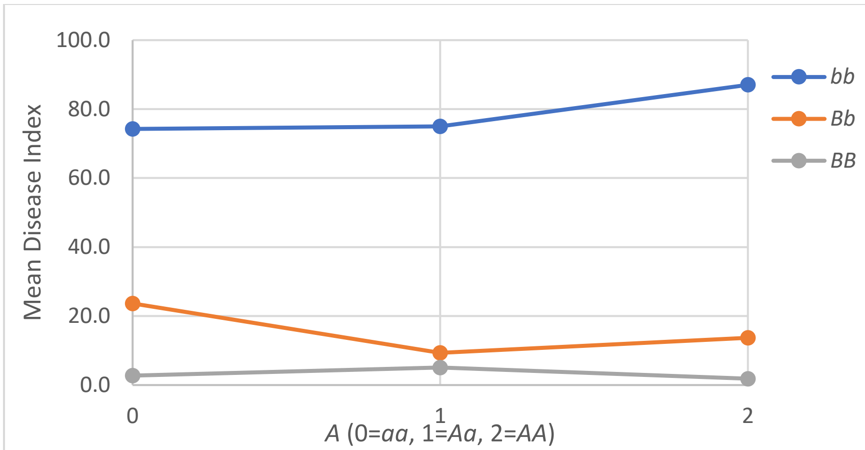

Figure 8. Mean disease index of each genotype combination of homozygosity/heterozygosity of genes A and B when gene C is cc against 3A (A = *PH1* present, a = *PH1* absent; B = *PH2* present, b = *PH2* absent; C = *PH3* present, c = *PH3* absent).

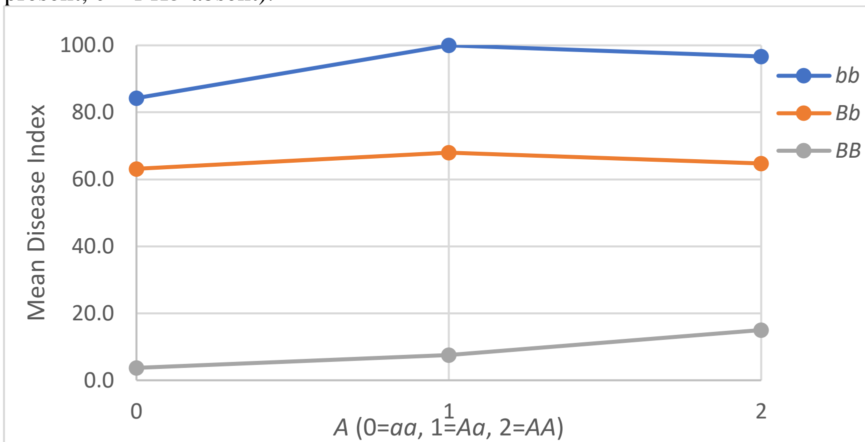

Figure 9. Mean disease index of each genotype combination of homozygosity/heterozygosity of genes A and B when gene C is Cc against 3A (A = *PH1* present, a = *PH1* absent; B = *PH2* present, b = *PH2* absent; C = *PH3* present, c = *PH3* absent).

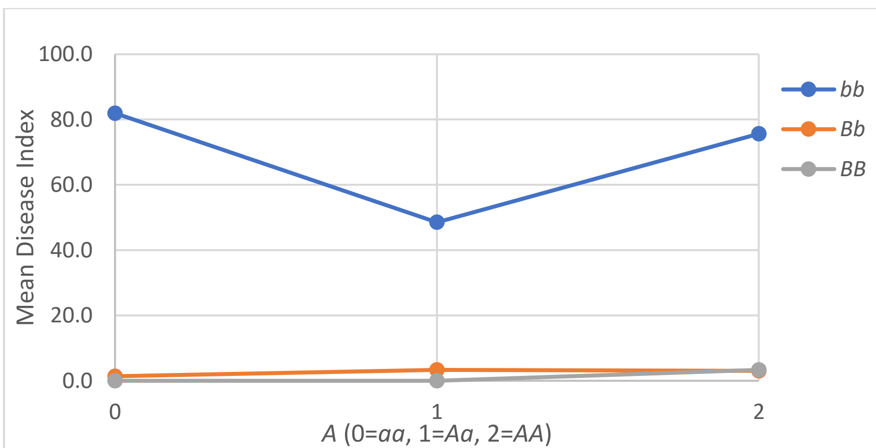

Figure 10. Mean disease index of each genotype combination of homozygosity/heterozygosity of genes A and B when gene C is CC against 3A (A = *PH1* present, a = *PH1* absent; B = *PH2* present, b = *PH2* absent; C = *PH3* present, c = *PH3* absent).).

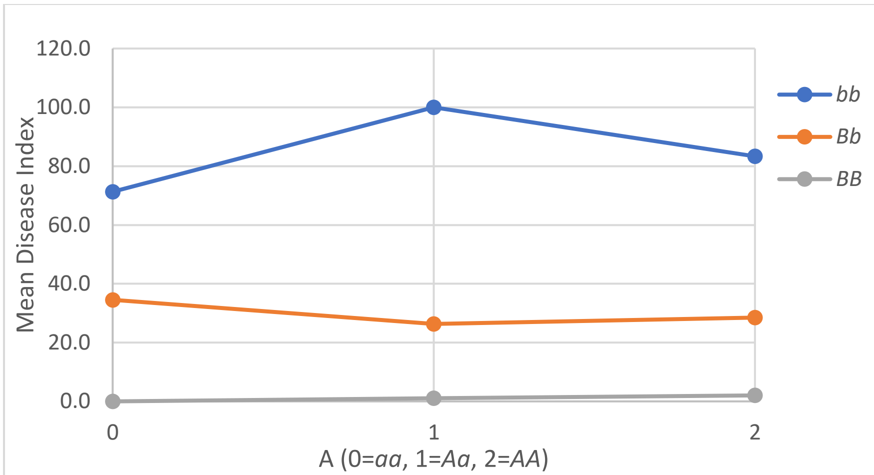

Figure 11. Mean disease index of each genotype combination of homozygosity/heterozygosity of genes A and B when gene C is cc against 5X (A = *PH1* present, a = *PH1* absent; B = *PH2* present, b = *PH2* absent; C = *PH3* present, c = *PH3* absent).

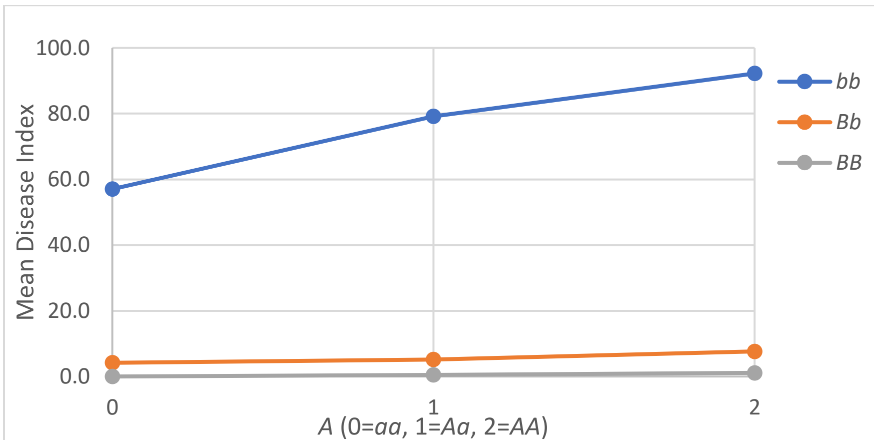

Figure 12. Mean disease index of each genotype combination of homozygosity/heterozygosity of genes A and B when gene C is Cc against 5X (A = *PH1* present, a = *PH1* absent; B = *PH2* present, b = *PH2* absent; C = *PH3* present, c = *PH3* absent).

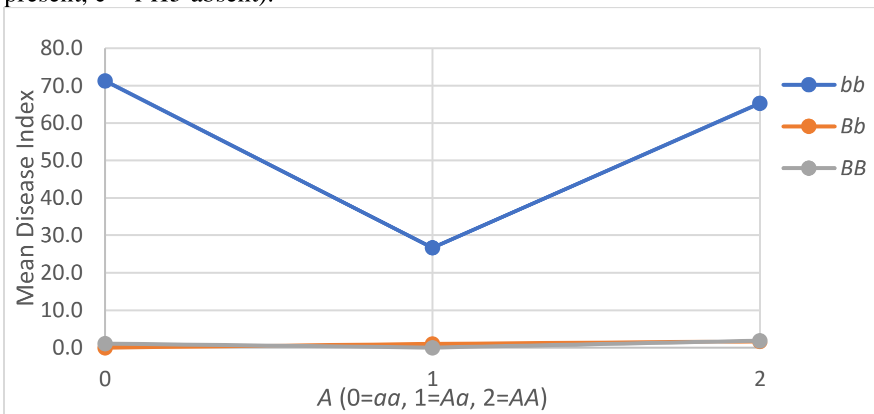

Figure 13. Mean disease index of each genotype combination of homozygosity/heterozygosity of genes A and B when gene C is CC against 5X (A = *PH1* present, a = *PH1* absent; B = *PH2* present, b = *PH2* absent; C = *PH3* present, c = *PH3* absent).

DISCUSSION

Breeding clubroot resistance genes into commercial varieties is currently the best known clubroot management option available. Clubroot can reduce the quality and quantity of canola yield, also inoculating the soil with long lasting *P. brassicae* resting spores. Previous studies have determined that other management strategies including cultural, chemical, and mechanical methods are not efficient at reducing the disease (Donald and Porter, 2009; Peng et al., 2011, 2014). Although genetic resistance currently plays the most important role in clubroot management (Diederichsen et al., 2014; Donald and Porter 2009), CR varieties should be used as part of an integrated management plan. CR genetics may be overcome by shifting composition and therefore virulence of *P. brassicae* populations. This may occur when CR canola is grown in a field over multiple consecutive years or in short rotation (LeBoldus et al., 2012; Strelkov et al., 2016a; Strelkov et al., 2018). Short rotation promotes propagation of virulent spores which may appear due to mutation or emergence of rare, pre-existing pathotypes within the population. Highly virulent pathogen field populations can overcome current CR varieties. This leads to an increase of virulent inoculum in the soil. Such practices may render a previously resistant variety ineffective in an infested field. Integrating a crop rotation of ≥2-year break from a susceptible host reduces field inoculum (Ernst et al. 2019) which in turn reduces available virulent inoculum load.

Another strategy to prevent pathotype virulence shifts is the use of multiple CR genes to provide resistance. Stacking multiple CR genes into a single variety increases the number of CR genes the virulent spores must overcome, reducing the chances of successful inoculation. However, gene stacking has the risk of increasing highly virulent spore loads capable of overcoming multiple CR genes. This could result in an accelerated shift to very high virulence. Another option is to rotate between CR varieties that each contain a differing single CR gene. CR gene rotation may reduce build-up of virulent pathotypes capable of overcoming any single specific CR gene. Ideally, multiple CR genetic stacks could be used in rotation to best reduce chances of virulence shifting and CR breakdown. These genetic strategies integrated with crop rotation may slow down and ideally halt buildup of virulent spores, reduce virulence shift and reduce overall *P. brassicae* spore load in the soil.

In this study we were interested in stacking three known clubroot genes to determine if they followed a pure gene-for-gene resistance model or if synergistic interactions occur between the different combinations. Inoculations were successful for all three pathogen types (3H, 3A, 5X) as confirmed by the symptoms on ECD05 and 45H29, therefore we can be confident in the phenotype observed on the experimental lines.

Previous data from Corteva Agriscience had determined that *PH1* has a dominant resistant reaction to pathotype 3H but was fully susceptible to the more virulent pathotypes 3A and 5X (Internal unpublished data). These reactions were confirmed in our study by the heterozygous *PH1* genotype (*Aabbcc*; Table 3). The *PH1* gene appears to be a dominant major gene effective against pathotype 3H but susceptible to 3A and 5X. *PH1* does not appear to have any epistatic effect with *PH2* or *PH3* as is apparent based on heterozygous

PH1/*PH2* (*AaBbcc*) and heterozygous *PH1*/*PH3* (*AabbCc*) being susceptible to pathotype 3A and 5X.

One of the most interesting findings was that *PH2* did not appear to act as a dominant resistance gene as we had previously believed. The genotype of heterozygous *PH2* (*aaBbcc*) gave a susceptible reaction to all three pathotypes (Table 3). When a second copy of *PH2* was present (*aaBBcc*), a fully resistant reaction was observed against all three pathotypes (Table 3). These results indicate that the *PH2* gene reaction does not fit a dominant gene model that activates a hypersensitive response. Rather, it suggests that *PH2* acts in a recessive or codominant manner.

The *PH3* gene was previously determined to have an intermediate reaction to the 3H pathotype and a susceptible reaction to 3A and 5X in the heterozygous form (*aabbCc*; Internal unpublished data) which was confirmed in this study (Table 3). However, it was expected that a genotype of homozygous *PH3* (*aabbCC*) would result in a full resistant reaction. Although, a second copy of *PH3* did appear to improve the mean disease index (Table 3) against pathotype 3A, the disease index was not significantly different than that of a single copy of *PH3* (Figure 2). We therefore, are not able to confirm an improved reaction by a homozygous *PH3* genotype over a heterozygous *PH3* genotype.

A gene stack of *PH1* and *PH3* (*AabbCc*) did not result in significant resistance beyond the gene-for-gene reactions demonstrated in *PH1* and *PH3* single gene heterozygous genotypes (*Aabbcc, aabbCc*). However, a gene stack of *PH2* and *PH3* (*aaBbCc*) provided full resistance to all three pathotypes. This suggests an epistatic or additive effect occurs between these two genes to induce clubroot resistance and further suggests that previous testing may not have properly characterized the true nature of *PH2* clubroot disease resistance.

Figures 5 through 7 demonstrate that against 3H, *PH1* is a very effective dominant resistance gene. Resistance is observed with one or two copies of *PH1* despite the state of *PH2* or *PH3*. *PH2* demonstrates a codominant resistance to 3H when *PH1* is absent. The resistance of *PH2* is improved by the presence of *PH3*, with two copies of *PH3* being more effective than one. Figures 8 through 10 demonstrate that *PH1* is ineffective against 3A, making no difference to the resistance reaction. *PH2* again appears to have a codominant resistance reaction, with a single copy of *PH2* reducing the disease index in the absence of *PH1* or *PH3*. However, two copies of *PH2* demonstrates an even greater resistance to 3A. The addition of a single copy of *PH3* did not appear to influence the resistance. The addition of two copies of *PH3* increased the resistance reaction of a single copy of *PH2* to be nearly equal to the reaction observed when two copies of *PH2* were present. Against 5X as illustrated in figures 11 through 13, *PH1* appears ineffective while *PH2* again demonstrates a codominant reaction. The addition of a single copy of *PH3* does appear to be effective at increasing resistance in combination with a single copy of *PH2*. The addition of two copies of *PH3* in combination with one or two copies of *PH2* reduces disease index to about 0. Future testing would be useful in determining the statistical significance of the interactions

demonstrated by these figures and strengthen our understanding of the potential epistatic effects at play.

The results of this study highlight the importance of verifying gene action with gene stacks and not rely solely on single gene reactions to fully understand the nature of resistance reactions. Testing gene stack combinations allows epistatic effects to be recognized where single gene testing may not have revealed any useful resistant gene reaction. *PH2* was previously believed to be a dominant gene that provided resistance in the heterozygous form. This study however, found *PH2* lacking a dominant response. *PH3* has shown little resistance reaction in the heterozygous form in this study as well as in past internal testing. We have demonstrated that combining these two genes can significantly improve the disease resistance reaction. This suggests other potential CR genes may have been previously overlooked due to a lack of dominant gene action. By testing for epistatic effects, we may open the door to many more gene combinations for use in building durable clubroot resistant varieties. Increasing the number of gene combinations that are available to breeders could assist in reducing virulence shift, thereby prolonging the resistance efficacy of all CR varieties. By identifying viable CR genes that work through epistatic interaction in numerous combinations we may improve efficacy over a larger range of pathotypes.

Examples of the use of gene stacks can be found in other crops for resistance to other diseases. Chukwu et al. (2019) found in their review of bacterial leaf blight disease in rice that gene stacking resistance genes to bacterial leaf blight disease in rice provided a greater broad-spectrum resistance to virulent bacterial leaf blight isolates compared to single resistance genes. Genetic resistance based on a single gene was also broken down more quickly, leading to susceptibility.

In canola, Leboldus et al. 2012, found that two clubroot resistant cultivars had an increased disease reaction after a small number of cycles of exposure to a *P. brassicae* pathogen population. They believed the major gene resistance was quickly eroded, resulting in an increased disease index. A slight disease reduction remained with little further change after additional cycles which they attributed to several minor resistance genes conferring partial resistance to *P. brassicae*. This study demonstrated the rapid pace in which major gene resistance can erode. There has also been previous evidence that stacking CR genes in *B. rapa* can result in reduced disease reaction due to genes working complimentarily (Matsumoto et al., 2012). A study in *B. napus* demonstrated that by stacking CR genes, the range of resistance across pathotypes could be increased (Peng, 2019). Results following multiple cycles of exposure to *P. brassicae* population, showed that this resistance from stacking CR genes remained more durable than a single CR gene (Peng, 2019).
This study also demonstrated that increased inoculum loads in the soil can accelerate the erosion of genetic resistance, further highlighting the importance of reducing soil inoculum levels by using CR genetics as well as regular crop rotation.

CONCLUSIONS

The use of genetic stacks will serve as an important tool for canola breeders to prevent CR varieties from failing and reduce shifting of disease virulence. We have demonstrated the importance of increased testing of stacked genes to identify CR genes that show little promise as a single gene but in combination with other CR genes may provide epistatic or additive effects. Through gene stacking we have gained evidence that the gene actions of *PH2* and *PH3* may not follow a pure gene-for-gene dominant gene reaction. This new understanding will allow us to more effectively integrate these CR genes into future CR canola varieties that may provide broader spectrum and robust clubroot protection.

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