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Introgression of Resistance to Multiple Pathotypes of *Plasmodiophora brassicae* from Turnip (*Brassica rapa* ssp. *rapifera*) into Spring *B. napus* Canola

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Abstract: Clubroot disease resistance has been introgressed from *Brassica rapa* into canola following different approaches including a *B. napus* × *B. rapa* interspecific cross; however, the details of this cross are not available. To extend our knowledge of this cross for the introgression of resistance to multiple pathotypes, we crossed a clubroot-resistant turnip with a susceptible canola and backcrossed the F_1′s to canola. The backcross population was subjected to pedigree breeding with selection for clubroot resistance and canola-quality traits; selection for resistance to pathotype 3H and 3A was performed in the early and advanced generations, respectively. The advanced generation lines were also evaluated for resistance to 3H, 3A, 2B, and 5x, and for field resistance to clubroot, and agronomic and seed quality traits. Following this, we developed canola lines carrying resistance to multiple pathotypes and nuclear DNA content similar to *B. napus*. Resistance to 3H showed a weak correlation with other pathotypes whereas resistance to 3A showed a strong positive correlation with 5x indicating that resistance to these pathotypes was under a similar genetic control. Thus, the results demonstrate that resistance to multiple pathotypes can be introgressed from a turnip into a canola even when selection for resistance to single pathotype is performed in early generations.

Keywords: canola; turnip; *Plasmodiophora brassicae*; multiple pathotypes; introgression; interspecific cross

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1. Introduction

Clubroot, a soil-borne disease, caused by *Plasmodiophora brassicae* Woronin, has been known since the 13th century in cruciferous oilseed and vegetable crops such as *Brassica napus* (2*n* = 38, AACC), *Brassica rapa* (2*n* = 20, AA), and *Brassica oleracea* (2*n* = 18, CC) [1]. *P. brassicae* is an obligate biotrophic protist which attacks root hair and results in abnormal cell division, and thus, forms club shaped roots or 'galls'. These clubbed roots inhibit the uptake of water and nutrients and result in stunted growth, wilting, chlorosis, and even the death of the plant [2]. This disease is known to be present in about 60 countries and causes 10–15% yield loss of *Brassica* crops on a global scale [1]. In the case of canola, yield loss due to this disease has been reported in many countries including Canada [3,4], China [5], India [6], Europe [7,8], and Australia [9]. This disease can result in about 30% yield loss in canola [4]; however, under extreme conditions, complete failure of the crop has been reported [10]. Apart from yield loss, this disease can result in about a 6% decrease in seed oil [11].

The resting spores of *P. brassicae* can survive in soil for more than 15 years [12] and various pathotypes of this pathogen generally occur in soil [13–15]. The structure of the pathotype population of *P. brassicae* in soil can change in a short period of time [15]. For example, in the initial stage of the occurrence of this disease in Canada, pathotype 3 or 3H was the most virulent and prevalent. However, new virulent pathotypes, such as 3A, 2B, 3D, and 5x evolved in canola fields after growing resistant cultivars for about 10 years rendering some of the resistances ineffective [15,16]. Strelkov et al. [17] reported that 3H was the most

common pathotype in the Peace Country of Canada in 2017, and this pathotype was almost absent in 2018; however, novel pathotypes such as 8C were detected in this region. Control of this disease by crop management practices such as cultural, chemical, and agro-technical treatments was not sustainable [18]; therefore, the deployment of resistant cultivars has been considered the most effective, economic, and environment-friendly approach for the long-term management of this disease [3].

To date, a total of 24 clubroot-resistant loci conferring resistance to different pathotypes have been mapped on the A01, A02, A03, A05, A06, A07, and A08 chromosomes of the A genome of *B. rapa* and *B. napus* [18]. The majority of the loci including *CRa*/*CRb* [19–24] *CRd* [25], *CRk* [26], *Crr3* [27,28], *CRq* [29], *Rcr1* [30], *Rcr2* [31], *Rcr4* [32], and *Rcr5* [33] have been mapped at the 15.5–16.3 Mb and 23.8–26.8 Mb regions of A03 of the reference genome *B. rapa* 'Chiifu-401' [18]. Among the other chromosomes, A08 carries five loci while the remaining chromosomes carry one or two loci. These loci often confer race-specific resistance to pathotypes, such as *Rcr1*, *Rcr2*, and *Rcr5* which confer resistance to pathotype 3, *Crr3* and *CRq* which confer resistance to pathotype 2, and *CRb* which confers resistance to pathotypes 2, 3, 4, and 8 [18]. On the other hand, clubroot resistance in the C genome of *B. oleracea* is often under polygenic control with the involvement of recessive to dominant gene loci with a small to large effect [18,34]. Of the major clubroot-resistance loci of the A genome, only a few of them have been used to develop *B. napus* canola cultivars in Europe and Canada [3,18]. For example, in Canada, the major clubroot resistance genes were obtained from the European winter oilseed *B. napus* cv. 'Mendel' [35], and *B. rapa* subsp. *chinensis* cv. Flower Navina [36] and Chinese cabbage (*B. rapa* ssp. *pekinensis*) [37,38]; among these, resistance of the cv. 'Mendel' has been used extensively in breeding. The cv. 'Mendel' carries a race-specific resistance originating from a resynthesized *B. napus*, which was developed from a partially resistant cabbage (*B. oleracea* ssp. *capitata*) accession 'ECD 15' and a resistant turnip (*B. rapa* ssp. *rapifera*) accession 'ECD 04' [39]. However, the major resistance gene of this cultivar conferring resistance to pathotype 3H is located on A03 [40]; this gene is apparently derived from the turnip accession ECD 04. The European turnips were also used to develop race- or pathotype-specific clubroot-resistant rutabaga (*B. napus* var. *napobrassica*) cultivars [41,42]. The clubroot resistance of rutabaga cv. Brookfield, located on A08, has been introgressed into Canadian canola [43]. A cultivar carrying a single major clubroot-resistance gene has been reported to become ineffective only after a few years of cultivation; this has been reported in several countries including Canada [15] and Japan [27]. This is primarily due to the change in the population structure of this pathogen in soil and the evolution of new virulent pathotypes [15,16,44,45]. As mentioned above, the A-genome of *B. rapa* carries several clubroot-resistance loci [18]; in this regard, introgression of additional loci from this species into canola will broaden the genetic base of clubroot resistance in this crop.

As discussed above, a few clubroot-resistance loci have been introgressed into *B. napus* from *B. rapa* [36,37,46]. However, detailed information of this including the breeding behavior of the *B. napus* × *B. rapa* interspecific cross progeny and the prospect of the introgression of resistance to multiple pathotypes cannot be found in the literature. The objectives of this study were to investigate the feasibility of the introgression of resistance to multiple pathotypes from a turnip into a spring *B. napus* canola, and the development of a canola-quality *B. napus* line carrying resistance to these pathotypes. The knowledge from this research could be used for the efficient introgression of additional resistances from the A genome of *B. rapa* into *B. napus* canola.

2. Materials and Methods

2.1. Plant Material and Population Development

The parents used in this study include a clubroot-susceptible spring *B. napus* ($2n = 38$, AACC) canola-quality (zero erucic acid in oil and low glucosinolate (GSL) in seed meal) line A04-73NA and a clubroot-resistant *B. rapa* var. *rapifera* turnip accession of the European Clubroot Differential (ECD) set (accession ECD 01 or cv. Debra). ECD 01 was completely

resistant to multiple pathotypes including pathotype 3H and 3A (disease score = 0, disease severity index or DSI = 0%). The *B. napus* × *B. rapa* interspecific cross was made by using A04-73NA as the female, and F_1 seeds (>2 seeds/pollination) of this cross could be obtained without the application of the cell and tissue culture technique [47]. High meiotic anomalies were expected in the allotriploid (AAC) F_1 plants; therefore, the F_1 plants were backcrossed to A04-73NA to achieve canola-quality clubroot-resistant euploid ($2n$ = 38) *B. napus* lines. The BC_1 plants were self-pollinated for seven generations with selection for spring growth habit, canola-quality traits, and resistance to the *P. brassicae* pathotype 3H in the early generations, and resistance to 3A and other pathotypes in later generations.

2.2. Greenhouse Assay for Clubroot Resistance

Single spore isolates of *P. brassicae* pathotypes 3H, 3A, 2B, and 5x used in this study were obtained from Dr. Stephen Strelkov, University of Alberta. These isolates were preserved in galls of a susceptible *B. napus* cv. Hi-Q at −18 °C until use. The inoculum of the resting spores was prepared following a modified version of Williams [48] technique as described in Hasan et al. [49]. In brief, approximately 37 g galls were soaked in 1000 mL distilled water for 2 h and homogenized in a blender (Ninja Professional Blender 1100 W) at a medium speed for 2 min. The homogenate was filtered through cheesecloth and the concentration of the spore suspension was adjusted to 1×10^7 spores/mL and stored at 4 °C overnight before inoculation.

The BC_1F_2 to BC_1F_7 generation plants of the (*B. napus* × *B. rapa*) × *B. napus* interspecific cross were grown together with the susceptible check *B. napus* cv. Hi-Q in a greenhouse of the University of Alberta in 32-cell (7 cm × 7 cm × 9 cm, L × W × D) trays filled with Sunshine Professional Growing Mix (Sunshine Horticulture, 15831 N.E., Bellevue, WA, USA); the greenhouse conditions were 20–22/15 °C day/night, 16 h photoperiod, and light intensity of 450 µE (mV) m^2 s^{-1}. The BC_1F_2 to BC_1F_6 generation populations were tested for resistance to pathotype 3H, while the BC_1F_5 to BC_1F_7 populations were tested for resistance to pathotype 3A. The BC_1F_8 generation population was also tested for resistance to pathotypes 3A, 2B, and 5x in 2 replications (8 plants per replication). Inoculation was carried out at 7–10 days after germination with 1 mL inoculum following the pipette method [50]. In this method, the inoculum was pipetted at the base of the seedling, and the inoculation was repeated the following day to ensure successful infection. After inoculation, soil in the cells was kept well saturated for 14 days to ensure sufficient moisture for infection; afterwards, the inoculated seedlings were watered daily and fertilized once a week. During the advancement of generation from BC_1F_2 to BC_1F_7, five to ten buds of the main raceme of the plants were self-pollinated by bag isolation and the plants were scored for resistance at maturity; seeds were harvested for growing the next generation population. The BC_1F_8 generation seeds were produced by growing the BC_1F_7 plants in 5-inch size pots and through self-pollination of individual plants by bag isolation.

Scoring of the plants for clubroot disease severity was carried out using a 0–3 scale (for details, see [14,51]), where 0 = no gall, 1 = small galls on less than 1/3rd of the roots, 2 = small to medium-sized galls on 1/3rd to 2/3rd of the roots, and 3 = severe galling with medium to large-sized galls on more than 2/3rd of the roots. The disease severity index (DSI) of different generation families was calculated using the following formula:

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

where, n is the number of plants in each disease severity class and 0, 1, 2, and 3 are the disease symptom severity classes, and N is the total number of plants [49]. A DSI of less than 20% was considered resistant (R) and more than 70% was considered susceptible (S) while DSIs of >20 to 40% and >40 to 70% were considered as moderately resistant (MR) and moderately susceptible (MS), respectively. While growing the BC_1F_2 to BC_1F_7 generation plants in the greenhouse, selection was performed for clubroot resistance and zero erucic acid in seed oil.

2.3. Field Evaluation for Clubroot Resistance

A total of 341 BC_1F_8 lines exhibiting resistance to pathotype 3H and containing zero erucic acid in seed oil were tested in a *P. brassicae*-infested field in Spruce Grove, Alberta in 2020 for clubroot resistance. This field carries multiple *P. brassicae* pathotypes including 5x (Alberta Agriculture and Forestry, personnel communication). For this, a 0.5 g seed was seeded in a 3 m long single-row plot in one replication with 70 cm space between the rows; the susceptible check cv. Hi-Q was seeded at every 10th row as a check. Clubroot resistance was assessed at the end of flowering by uprooting 25 plants from each row and scoring the plants on a 0–3 scale, as described above.

2.4. Field Trials for Agronomic and Seed Quality Traits

A total of 452 BC_1F_8 lines, including the 341 lines tested in a *P. brassicae*-infested field, were also evaluated in field nursery plots in 2020 at the South Campus Research Station of the University of Alberta for agronomic and seed quality traits. For this, seeding was performed in 3 m long single row plots in one replication as described above. The canola line A03-73NA was seeded at every 20th row as a check. Open-pollinated seeds harvested by hand were used for chemical analysis.

Of the above-mentioned BC_1F_8 lines, 112 selected lines (BC_1F_9) were tested in replicated field trials in 2021 together with a recently released open-pollinated canola cultivar UA CountyGold for agronomic and seed quality traits. These trials were placed at three locations: the ERS-Michener, ERS-West 240, and St. Albert research farms of the University of Alberta within a 30 km radius of Edmonton, Alberta. Seeding was performed with a plot seeder in 7.5 × 1.8 m plots using 8 g seeds per plot, and the plots were trimmed to 5.0 × 1.8 m after emergence. All trials were laid in a modified randomized complete block design where each replication was divided into four blocks; the number of replications for each trial was two.

The following agronomic and seed quality traits were recorded: days to flowering, days to maturity, seed yield (kg/ha), seed oil (%), protein (%), and GSL (μmol/g seed) contents. Days to flowering data were recorded when about 50% of the plants in a plot had at least one open flower. Days to maturity data were collected when the silique color changed from green to light yellow or brown and the seed color in the silique of the main branch changed to brown or black. All yield trial plots were harvested using a plot combine and plot yield data were converted to kilograms per hectare.

2.5. Chemical Analysis

The fatty acid profiles of the seed oil of the BC_1F_5 and BC_1F_6 generation plants grown in greenhouse and of the seeds harvested from field trials were estimated by the gas chromatographic technique. Bulk seeds harvested from the field plots were analyzed for oil, protein, and GSL contents using the near-infrared spectroscopy (NIRS) technique (FOSS NIRSystems, Model 6500). About 0.5 g seed of each sample was used for the fatty acid analysis while 2.5 g seed was used for NIRS analysis. Erucic fatty acid and saturated fatty acids, which include lauric acid, myristic acid, palmitic acid, stearic acid, arachidic acid, behenic acid, and lignoceric acid, were reported as a percent of the total fatty acids; GSL content was reported as μmol g^{-1} seed at 8.5% moisture. All these analyses were carried out in the Analytical Laboratory of the Canola Program of the University of Alberta; this laboratory has been certified by the Canadian Grain Commission for these analyses.

2.6. Ploidy Analysis

Nuclear DNA contents of the clubroot-resistant BC_1F_6 generation plants grown in the greenhouse were estimated using a flow cytometer (Partec GmbH, Munster, Germany) to assess their approximate chromosome number. For this, two leaf samples (each of about 0.5 cm^2) were collected from each plant at 3–4 weeks after seeding and the samples were chopped in an extraction buffer (Partec GmbH, Munster, Germany). The contents were filtered through a 50 μm Partec CellTrics disposable filter and a 1.6 mL nuclear fluorochrome

DAPI staining buffer (4,6- diaminido-2-phenylindole, Sigma, product number D-9542) was added to each sample. After incubation for 1–2 min, the samples were analyzed by a Partec Ploidy Analyzer (for detail, see Iftikhar et al. [52]). The mean value of the two samples was used for the estimation of the approximate chromosome number of the plants.

2.7. Statistical Analysis

Analysis of variance (ANOVA) was carried out using the software program 'R' [53]. In this, the lmer function of the 'lme4' package was used to fit a linear mixed-effects model for each trait. Least-square mean (lsmean) values were calculated using the 'lsmeans' package of R, and Tukey's test was carried out to compare the mean values for significant difference. Broad-sense heritability (*H*) was estimated using the following formula: $H = \delta^2 g / (\delta^2 g + \delta^2 ge/E + \delta^2 \varepsilon/ER)$, where $\delta^2 g$, $\delta^2 ge$ and $\delta^2 \varepsilon$ are the variance components for genotype, genotype × environment interaction and residual, respectively, and E and R are the number of environments and replications, respectively; the variance components were calculated using 'R'. Other statistical analyses, such as mean and standard error, were calculated using Microsoft Excel. Pearson's correlation, principal component analysis (PCA), and genotype by trait biplot (GT biplot) analysis for the relationship between the lines for resistance to different pathotypes were carried out using the software program 'R'. The significance of correlations was tested using *t*-tests [54].

3. Results

3.1. Development of B. napus Lines Carrying Resistance to Pathotype 3H

Thirty-five BC_1F_1 plants were grown in the greenhouse of which fourteen were spring growth habit type and produced seeds under bag isolation; the remaining plants were either winter growth habit type (require vernalization for flowering) or failed to produce seeds under self-pollination. A total of 358 BC_1F_2 plants belonging to nine families (harvested from nine BC_1F_1 plants) were evaluated where 213 (59.5%) plants were found to be resistant and 145 (40.5%) susceptible (Table 1a). Self-pollinated seeds (BC_1F_3 seeds) of all 213 resistant BC_1F_2 plants were harvested. The BC_1F_3 and subsequent generation populations were grown from self-pollinated seeds harvested from the resistant plants only. In total, 594 BC_1F_3, 930 BC_1F_4, 767 BC_1F_5, and 286 BC_1F_6 plants, respectively, of 44, 108, 191, and 46 families were grown and evaluated for resistance to pathotype 3H. A decline in DSI from 41.4% in BC_1F_2 to 2.1% in the BC_1F_6 generation was found demonstrating the effectiveness of the selection for resistance to this pathotype in this interspecific population.

Table 1. Occurrence of plants resistant to *Plasmodiophora brassicae* (a) pathotype 3H and (b) pathotype 3A in different generation populations of the (*B. napus* × *B. rapa*) × *B. napus* interspecific cross.

Generation	Total Families	Total Plants	No. Segr. Families	No. Non-Segr. Families	No. Plants with Disease Score 0 to 3, and Disease Severity Index (DSI)					Percent Plants *	
					0	1	2	3	DSI (%)	Resistant	Susceptible
(a) Selection for resistance to pathotype 3H:											
BC_1F_2	9	358	5	4	173	40	30	115	41.4	59.5	40.5
BC_1F_3	44	594	23	21	436	43	35	80	19.8	80.6	19.3
BC_1F_4	108	930	53	55	587	96	57	190	27.9	73.4	26.5
BC_1F_5	191	767	20	171	725	9	1	32	4.6	95.6	4.3
BC_1F_6	46	286	0	46	280	0	0	6	2.1	97.9	2.1
(b) Selection for resistance to pathotype 3A:											
BC_1F_5	217	725	59	158	183	64	91	387	64.6	34.0	66.0
BC_1F_6	168	1109	53	115	90	24	36	959	89.3	10.3	89.7
BC_1F_7	91	1151	58	33	976	49	36	90	11.3	89.0	11.0
BC_1F_8	233	1561	11	222	1485	55	11	10	2.3	98.6	1.4

* Plants with disease scores 0 and 1 were classified as resistant while plants with scores 2 and 3 were classified as susceptible.

3.2. Development of B. napus Lines Carrying Resistance to Pathotype 3A

Due to the high sterility in early generations, the evaluation for resistance to pathotype 3A was delayed until the BC_1F_5 generation when sufficient seeds could be harvested from a good number of plants for the evaluation of resistance to pathotype 3H as well as 3A. A

total of 725 BC_1F_5 plants belonging to 217 families were tested for resistance to pathotype 3A; these families were found to be resistant (disease score 0) to pathotype 3H. Of the 217 families, 59 (27.2%) were segregating for resistance and 158 (72.8%) were completely susceptible to this pathotype (Table 1b). Of the total BC_1F_5 population, 247 (34.0%) plants were found to be resistant to pathotype 3A. Thus, despite the selection performed in the earlier generations for resistance to pathotype 3H, several BC_1F_5 plants were found to carry resistance to pathotype 3A. In BC_1F_5 as well as in subsequent generations, the resistant plants were self-pollinated to grow the next generation population. A total of 1,109 BC_1F_6 plants belonging to 168 families were evaluated where only 114 (10.3%) plants were found to be resistant and the remaining 995 (89.7%) were susceptible. Thus, the proportion of resistant plants in BC_1F_6 generation declined as compared to the BC_1F_5 generation. However, in BC_1F_7 and BC_1F_8, respectively, 89.0% (1025/1151) and 98.6% (1540/1561) plants were found to be resistant (Table 1b). Thus, repeated selection for resistance to pathotype 3A reduced the DSI from 64.6% in BC_1F_5 to 2.3% in the BC_1F_8 generation.

3.3. Evaluation for Resistance to Clubroot under Field Conditions

A total of 341 BC_1F_8 lines, descended from 46 BC_1F_6 families homozygous for resistance to pathotype 3H, were evaluated in a clubroot-infested field (carrying pathotype 5x) in Spruce Grove, Alberta. However, resistance data from 315 lines were obtained; the number of plants for the remaining lines was less than the threshold number of plants needed for evaluation. Of these 315 lines, 168 (53.3%) were R, 10 (3.2%) MR, 6 (1.9%) MS, and 130 (41.3%) S (Figure 1A). Thus, the results demonstrated that the inbred *B. napus* lines derived from this interspecific cross carry resistance to multiple pathotypes including 5x.

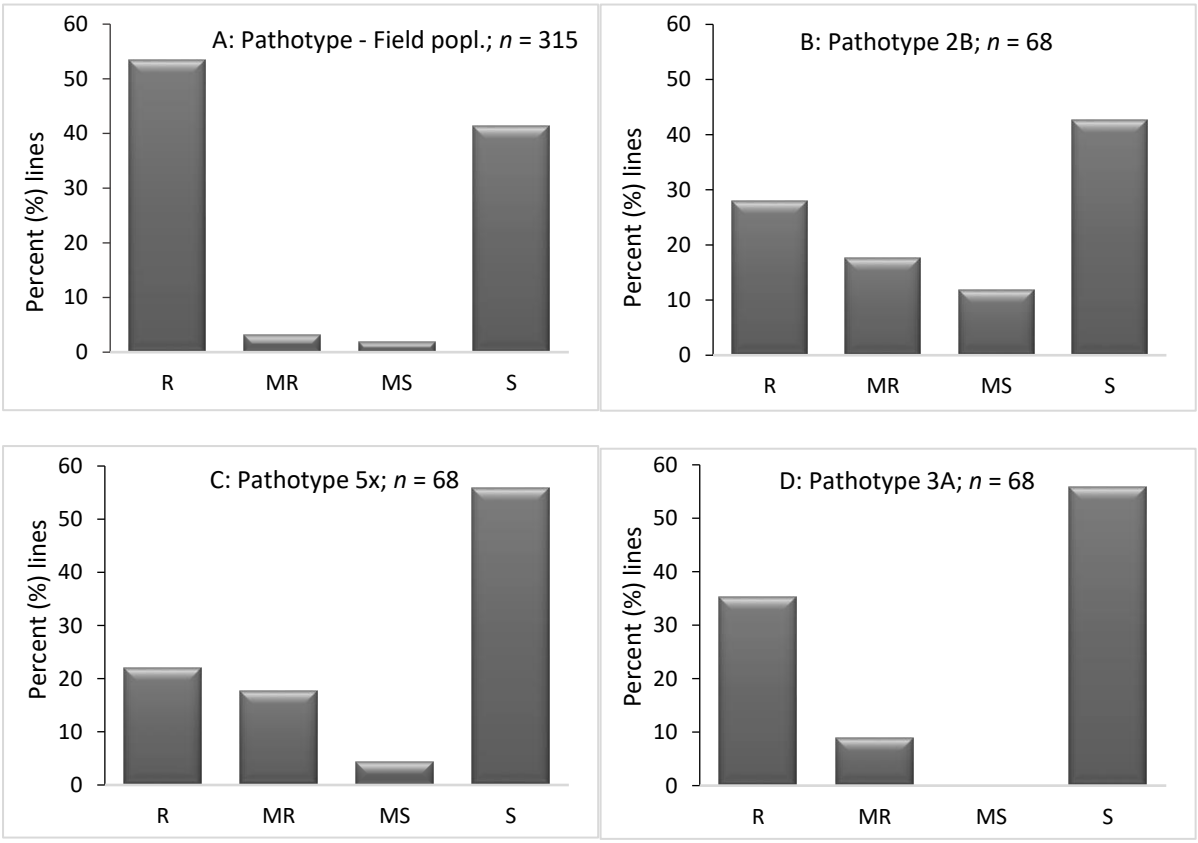

Figure 1. *Cont.*

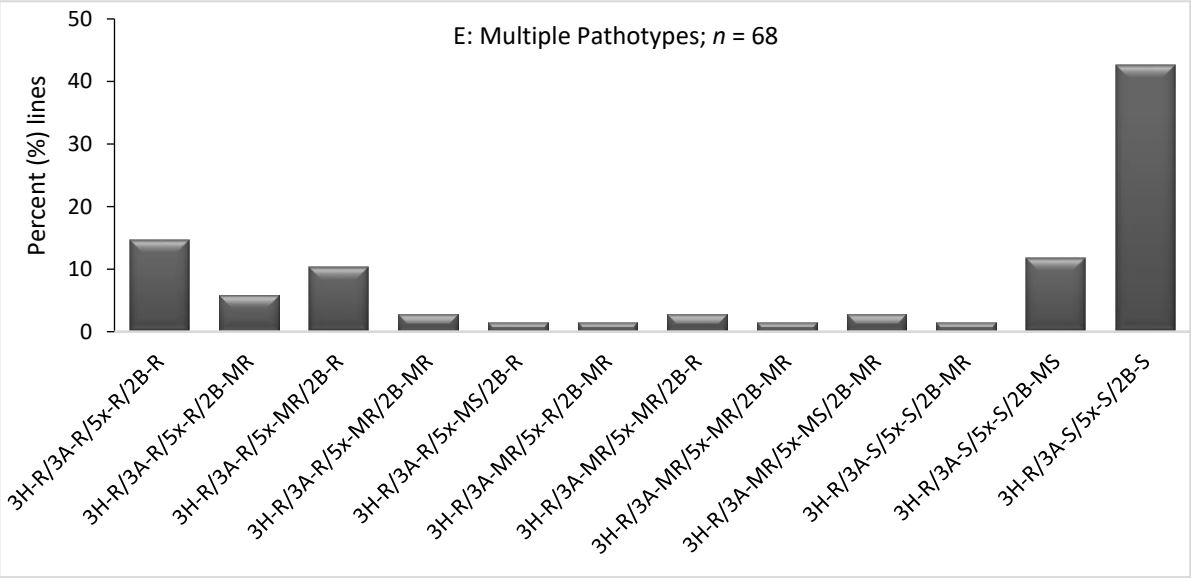

Figure 1. Distribution of the BC_1F_8 generation *Brassica napus* lines derived from the (*B. napus* × *B. rapa*) × *B. napus* interspecific cross exhibiting clubroot resistance under field conditions and to different *Plasmodiophora brassicae* pathotypes under greenhouse conditions: (**A**) Field condition in Alberta, (**B**) pathotype 2B, (**C**) pathotype 5x (L-G1), (**D**) pathotype 3A, (**E**) multiple pathotypes (*x*-axis: 3H-R/3A-R/5x-R/2B-R = resistant to all pathotypes (3H, 3A, 5x and 2B); 3H-R/3A-R/5x-R/2B-MR = resistant to pathotypes 3H, 3A and 5x while moderately resistant to pathotype 2B; 3H-R/3A-R/5x-MR/2B-R = resistant to pathotypes 3H, 3A and 2B while moderately resistant to pathotype 5x; 3H-R/3A-R/5x-MR/2B-MR = resistant to pathotypes 3H and 3A while moderately resistant to pathotypes 5x and 2B; 3H-R/3A-R/5x-MS/2B-R = resistant to pathotypes 3H, 3A and 2B while moderately susceptible to pathotypes 2B; 3H-R/3A-MR/5x-R/2B-MR = moderately resistant to pathotypes 3A and 2B while resistant to pathotypes 3H and 2B; 3H-R/3A-MR/5x-MR/2B-R = moderately resistant to pathotypes 3A and 5x while resistant to pathotypes 3H and 2B; 3H-R/3A-MR/5x-MR/2B-MR = moderately resistant to pathotypes 3A, 5x, and 2B while resistant to 3H; 3H-R/3A-MR/5x-MS/2B-MR = resistant to 3H, moderately resistant to pathotypes 3A and 2B while moderately susceptible to pathotypes 5x; 3H-R/3A-S/5x-S/2B-MR = resistant to 3H, susceptible to pathotypes 3A and 5x while moderately resistant to pathotype 2B; 3H-R/3A-S/5x-S/2B-MS = resistant to 3H, susceptible to pathotypes 3A and 5x while susceptible to pathotype 2B; 3H-R/3A-S/5x-S/2B-S = resistant to 3H and susceptible to other three pathotypes). R = resistant (DSI $\leq$ 20%); MR = moderately resistant (DSI $\geq$ 20–40%); MS = moderately susceptible (DSI $\geq$ 40–70%); S = susceptible (DSI > 70%). '*n*' indicates the number of lines.

3.4. Evaluation for Resistance to Multiple P. brassicae Pathotypes in the Greenhouse

Sixty-eight BC_1F_8 lines, homozygous for resistance to pathotype 3H, were evaluated for resistance to pathotype 2B, 5x and 3A, where nineteen (27.9%) lines were classified as R, eleven (17.6%) as MR, eight (11.8%) as MS and twenty-nine (42.7%) as S for resistance to pathotypes 2B (Figure 1B). In case of resistance to pathotype 5x, 15 (22.1%) were R, 12 (17.6%) were MR, 3 (4.4%) were MS, and 38 (55.9%) were S (Figure 1C); while for resistance to pathotype 3A, 24 (35.3%) were R, 6 (8.8%) were MR and 38 (55.9%) were S (Figure 1D). A joint analysis of the data of the 68 lines for resistance to multiple pathotypes showed that 10 (14.7%) lines were resistant to all four pathotypes; 29 (42.6%) were resistant to pathotype 3H but susceptible to pathotypes 2B, 3A, and 5x; and the remaining 29 (42.6%) lines were resistant to pathotype 3H but moderately resistant or susceptible to pathotypes 2B, 3A, or 5x (Figure 1E). This demonstrates that resistance to multiple pathotypes can be introgressed from a turnip into a *B. napus* canola through a *B. napus* × *B. rapa* interspecific cross.

Principal component analysis (PCA) was carried out to understand the nature of genetic variability among the BC_1F_8 lines for resistance to different *P. brassicae* pathotypes.

The genotype by trait biplot analysis explained 98.2% of the total variance, where PC1 explained 96.4% of the total variance caused by genotype and PC2 explained 1.8% of the total variance which was due to the resistance to different pathotypes (Figure 2). The size of the vector for resistance to pathotype 3A was relatively longer as compared to the other pathotypes, suggesting that this pathotype was the major discriminator of the BC_1F_8 population. The vectors for resistance to pathotype 3A and 5x had an angle of nearly zero ($r = 0.97$, $p < 0.01$) indicating that these two pathotypes accounted for a similar type of variation. The trait pairs, resistance to pathotype 3A vs. 2B ($r = 0.94$, $p < 0.01$), and resistance to 5x vs. 2B ($r = 0.95$, $p < 0.01$) had an acute (<90°) angle suggesting that their variation was positively correlated. The trait pairs' resistance to pathotype 3H vs. 3A ($r = 0.02$, $p < 0.01$), 3H vs. 5x ($r = 0.08$, $p < 0.01$) and 3H vs. 2B ($r = 0.08$, $p < 0.01$) had a near-right angle, indicating that resistance to pathotype 3H was weakly or not correlated with resistance to the other pathotypes.

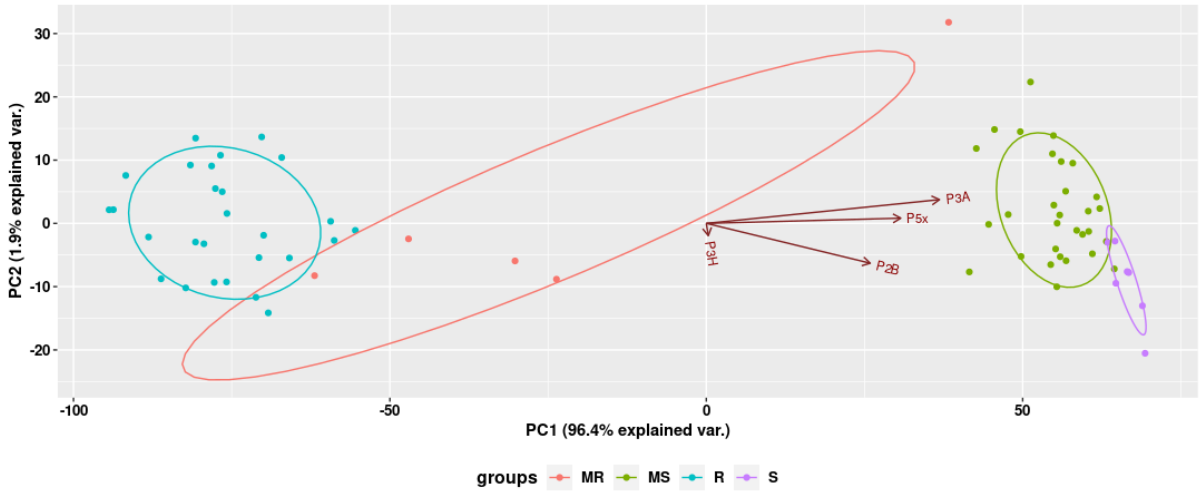

Figure 2. Principal component analysis (PCA) of the BC_1F_8 *B. napus* population ($n = 68$) derived from the (*B. napus* × *B. rapa*) × *B. napus* interspecific cross for resistance to *Plasmodiophora brassicae* pathotypes 2B (P2B), 3A (P3A), 3H (P3H), and 5x (P5x). This analysis placed the population into four groups based on disease severity index (DSI%) for these pathotypes.

3.5. Flow Cytometric Analysis

A total of 150 BC_1F_6 plants belonging to 46 families which showed resistance to pathotype 3H were analyzed for relative nuclear DNA content (Partec value); the parents *B. rapa* ECD 01 and *B. napus* A03-73NA were used as controls. Nuclear DNA content of the *B. rapa* parent was 161.5 ± 2.0 S.E. while it was 404.2 ± 1.2 S.E. for the *B. napus* parent (Table 2, Figure S1). Relative nuclear DNA content of the BC_1F_6 population varied from 351.4 to 409.2 with a mean of 383.0 ± 0.9 S.E. which was close to the *B. napus* parent. This suggests that the majority of the BC_1F_6 plants had a chromosome number similar to the *B. napus* parent (Table 2).

Table 2. Partec values for the relative nuclear DNA content in the BC_1F_6 population of the (*B. napus* × *B. rapa*) × *B. napus* interspecific cross.

Population	Generation	No. Plants	Range	Mean ± S.E.
(*B. napus* × *B. rapa*) × *B. napus*	BC_1F_6	150	351.4–409.2	383.0 ± 0.9
B. napus (A04-73NA)	Parent	6	400.3–408.2	404.2 ± 1.2
B. rapa (ECD 01)	Parent	6	154.3–169.2	161.5 ± 2.0

3.6. Agronomic and Seed Quality Traits

Erucic acid content in the seed oil of the BC_1F_5 plants grown in a greenhouse varied from 0.01 to 30.9% with a mean of 18.1 ± 0.6% S.E. (Figure S2). Of the total 240 plants evaluated, 17% (41/240) plants had <1% erucic acid in the seed oil, i.e., were of the zero-erucic acid type. All these zero-erucic acid plants were selected, and selection for additional zero erucic acid plants was performed in the progeny of the families containing <10% erucic acid in oil. All BC_1F_8 lines tested in the field nursery plots in 2020 were of the zero erucic acid type.

Days to flowering in the BC_1F_8 lines tested in the field nursery in 2020 varied from 46 to 60 days with a mean of 51.0 ± 0.2 days, which was about three days later than the spring *B. napus* parent A04-73NA (48.0 ± 0.2 days) (Table S1a, Figure S2). About 17% of the BC_1F_8 lines (76/455) flowered earlier than the *B. napus* parent. This population, on average, took 105.8 ± 0.2 days to mature (range 99 to 115 days); however, about 18% of the BC_1F_8 lines (83/455) matured earlier than A04-73NA. The oil content in the seeds of the BC_1F_8 lines varied from 40.6 to 51.5% with a mean of 46.2 ± 0.1% which was lower than the *B. napus* parent A04-73NA (48.7 ± 0.3%). However, the best BC_1F_8 line contained 51.5% oil, which was about 2.8% higher than the *B. napus* parent. In contrast, the mean seed protein of this population was about 1.6% higher (range 19.2 to 30.0%; mean 24.9 ± 0.1 S.E. %) than the *B. napus* parent (23.4 ± 0.3 S.E. %) (Table S1a, Figure S2). Glucosinolates in this population varied from 7.8 to 31.2 µmol/g seed (Table S1a, Figure S2) with a mean of 14.4 ± 0.6 S.E. The confidence limits of GSL content for the *B. napus* canola parent were 11.6–12.4 µmol/g seed (n = 24; range 10.2–14.2 and mean = 12.0 ± 0.2 S.E. µmol/g seed). Based on this, about 31% of the BC_1F_8 lines had a GSL content either similar to or lower than the *B. napus* parent.

Based on clubroot resistance under field conditions in Spruce Grove, and the agronomic and seed quality data from the 2020 nursery plots, 112 lines were selected for tests in replicated yield trials in 2021. Analysis of variance of the 112 BC_1F_9 lines tested in the replicated field trials showed the existence of significant variation ($p < 0.001$) for seed yield, days to flowering, and days to maturity. The variation due to locations and due to the interaction between genotypes and locations (G × L) was significant ($p < 0.05$) for days to flowering; however, no significant variation was found between the locations for seed yield and days to maturity (Table S2). Broad sense heritability for seed yield, days to flowering, and days to maturity were 0.67, 0.96 and 0.87, respectively. In the case of the seed quality traits, significant variation was found for seed oil and GSL contents ($p < 0.001$) (Table S2); G × L was significant ($p < 0.001$) for seed protein and GSL. Broad sense heritability for seed oil, protein and GSL were 0.81, 0.69, and 0.95, respectively.

Days to flowering in the BC_1F_9 population varied from 42 to 55 days with a mean of 48.7 ± 0.2 days when the check cultivar UA CountyGold took 49.0 ± 0.6 days to flower. About 50% of the lines flowered earlier than UA CountyGold and a large majority of the lines also matured earlier than this check. Seed yield of the lines varied from 1008 to 3626 kg/ha with a mean of 1,749 ± 17.8 kg/ha, which was similar to mean yield of the check (1744 ± 93.9 kg/ha); about 20% of the lines yielded similar or greater than the check. On average, the seed oil content in the BC_1F_9 population was lower, and seed protein, GSL, and saturated fatty acid contents were similar to the check (Figure 3); indeed, all 112 lines were of the zero erucic acid type. Thus, several canola-quality lines with acceptable agronomic and seed quality traits and exhibiting resistance to multiple *P. brassicae* pathotypes could be obtained from this interspecific cross.

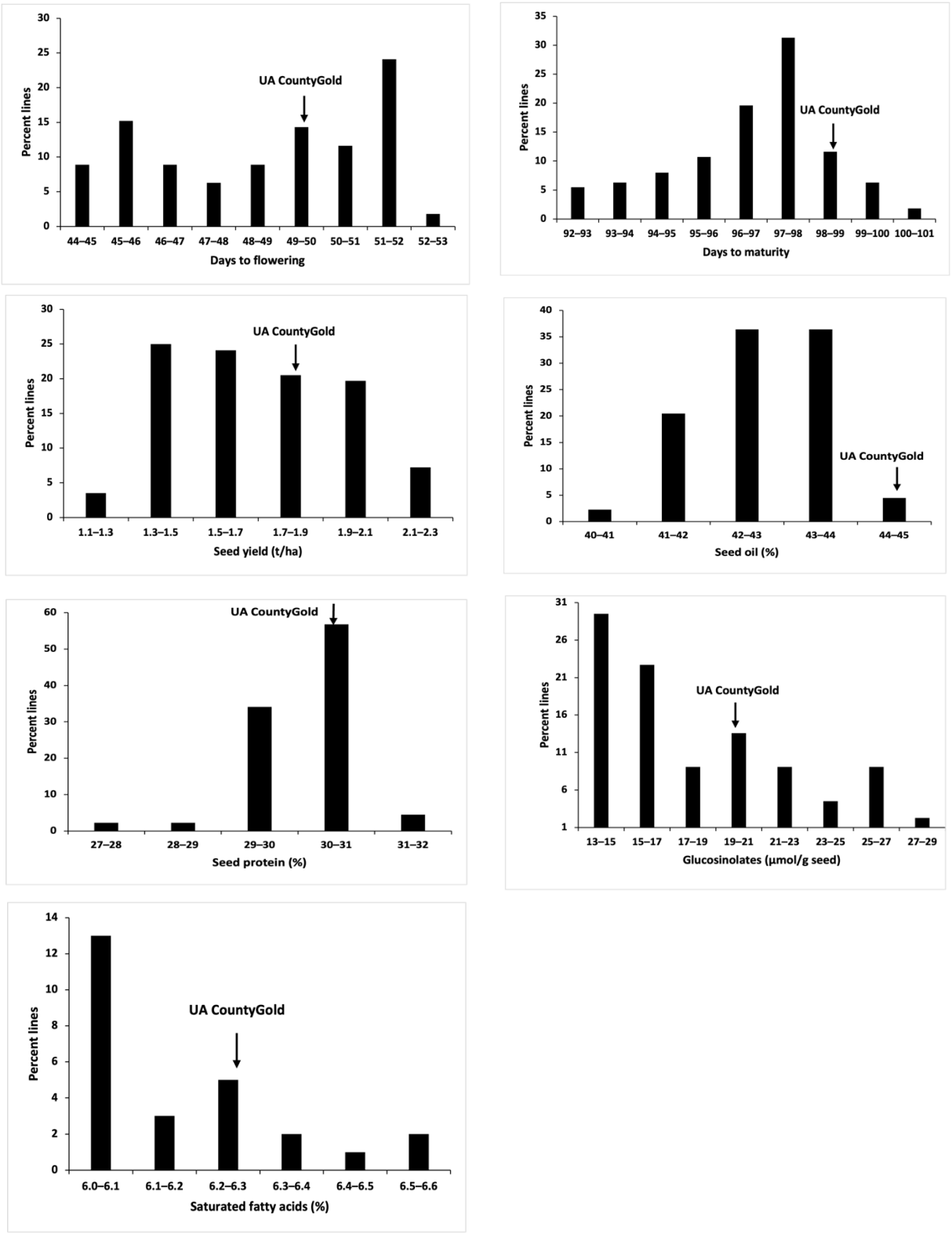

Figure 3. Distribution of the BC_1F_9 lines of the (*B. napus* × *B. rapa*) × *B. napus* interspecific cross grown in replicated field trials in 2021, for days to flowering (n = 112 lines), days to maturity (n = 112), and seed yield (n = 112), and seed oil (n = 44), protein (n = 44), glucosinolate (n = 44), and saturated fatty acids (n = 44). The value for the check cultivar UA CountyGold is indicated by the vertical arrow.

4. Discussion

Interspecific crosses between *B. napus* and *B. rapa* have been carried out by several researchers for different purposes [47,55] including the introgression of clubroot resistance from *B. rapa* into *B. napus* [36–38]. Among the different forms of *B. rapa*, several accessions of European turnips (*B. rapa* ssp. *rapifera*) have been reported to carry clubroot resistance [49,56]. Some of the European turnips have been used to develop clubroot-resistant Chinese cabbage (*B. rapa* ssp. *chinensis/pekinensis*) [27], oilseed *B. napus* [39], and rutabagas (*B. napus* var. *napobrassica*) [41,42]. Molecular mapping of clubroot resistance using turnip accessions identified several loci conferring resistance to different *P. brassicae* races or pathotypes. For example, Suwabe et al. [57,58] mapped the loci *Crr1* and *Crr2,* conferring resistance to race 2 and race 4 by using the *B. rapa* ssp. *rapifera* cv. Siloga. Similarly, by using the turnip cv. 'Debra' two clubroot-resistant loci, *CRk* and *CRc*, conferring resistance to the isolates M85 and K08, and K08, respectively, were identified by Sakamoto et al. [26]. QTL analysis using resistance derived from the turnip cv. Milan White, Hirai et al. [27] identified the locus *Crr3* conferring resistance to isolate Ano-01 [28]. The turnip accession ECD 04 carries five clubroot-resistant loci where they mostly provide race-specific resistance to the *P. brassicae* strains *Pb2*, *Pb4*, *Pb7*, and *Pb10* [59]. Hirani et al. [60] reported that four turnip accessions of the ECD set, viz. ECD 01, ECD 02, ECD 03, and ECD 04, carry resistance to the Canadian field isolates. Similarly, Fredua-Agyeman et al. [61] reported two major dominant loci, *CRa*/*CRb*^{Kato} and *Crr1* in ECD02 exhibiting resistance to pathotypes 5x and 5G. Zhan et al. [62] fine-mapped the clubroot-resistant locus *PbBa8.1* of ECD 04. Thus, it is apparent that several turnip accessions including the cv. Debra (ECD 01) may carry multiple clubroot-resistant loci and confer resistance to multiple pathotypes including 3H and 3A [49,63] and the present study]. However, the possibility of the introgression of resistance to multiple pathotypes from a turnip into a *B. napus* canola through a *B. napus* × *B. rapa* interspecific cross has not been investigated so far. The results from our study demonstrate that resistance to multiple *P. brassicae* pathotypes can be introgressed from the turnip into the canola even when selection for resistance to a single pathotype is performed in an early segregating population.

According to Attri and Rahman [47], a loss of about 55% of the *B. rapa* alleles can occur in the progeny of the *B. napus* × *B. rapa* interspecific cross by the F_4 generation. This is apparently due to a high meiotic anomaly in the early generation populations of this interspecific cross. Therefore, we backcrossed the di-genomic triploid F_1 plants (AAC) to the *B. napus* (AACC) canola parent to reduce meiotic anomalies and thus, to recover canola- quality *B. napus* plants without much difficulty. We applied selection for resistance to pathotype 3A first in the BC_1F_5 generation and were still able to develop *B. napus* lines carrying resistance to this pathotype. In fact, following this approach of selection for resistance to pathotype 3H in the early generations and for resistance to pathotype 3A in BC_1F_5, we were able to develop 10 BC_1F_8 canola lines carrying resistance to four pathotypes (2B, 3A, 3H, and 5x). The reconstituted clubroot-resistant lines derived from this *B. napus* × *B. rapa* interspecific cross were of the *B. napus* type as evident from the flow cytometric analysis for nuclear DNA content (Table 2). This agrees with the results reported by Attri and Rahman [47] that most of the plants derived from the *B. napus* × *B. rapa* interspecific cross stabilize into the *B. napus* type.

In the present study, the early generation interspecific population was selected for resistance to pathotype 3H; however, some of the lines also exhibited resistance to other pathotypes such as 3A, 2B, and 5x. This could have resulted from the pleiotropic effect of the 3H resistance to resistance to other pathotypes; the co-localization of multiple clubroot-resistant genes in the same genomic region conferring resistance to multiple pathotypes cannot be ruled out. Bi-plot and principal component analyses of the lines carrying resistance to multiple pathotypes revealed that the resistance to pathotypes 3A and 5x was under a similar genetic control, while a different genetic control was involved in the control of resistance to pathotype 3H. This was also evident from a strong correlation (r = 0.94–0.97) between the resistance to the 3A and 5x pathotypes, while there was a weaker

correlation (r = 0.01–0.08) between the resistance to the 3H and 3A or 5x pathotypes. Thus, it is highly likely that, even though selection for resistance to pathotype 3H was performed in an early segregating generation, additional clubroot-resistant loci were introgressed into the inbred population derived from this interspecific cross.

The genetic control of the two canola-quality traits, erucic acid in seed oil and GSL in seed meal, is relatively simple compared to the genetic control of many other quantitative traits, such as days to flowering and maturity [64] and seed oil and protein contents [65]. Erucic acid content in *B. napus* is controlled by two major loci located on A08 and C03 [66] and these loci act in an additive [67] or partly dominant manner [68]; a homozygous recessive condition at both loci results in the zero erucic acid phenotype. Seed GSL content in *B. napus* is controlled by at least four gene loci [69] where about half of the loci are located in the A genome [70]; a homozygous recessive condition at all loci results in the low GSL phenotype. This genetic control of the two canola-quality traits and the breeding technique applied in this study, viz. backcrossing the F_1 to the canola-quality parent ($A^cA^tC^c \times A^cA^cC^cC^c$, where the superscripted *c* indicates the alleles for the canola-quality traits, and *t* indicates the turnip (non-canola quality) alleles), facilitated the achievement of canola-quality clubroot-resistant lines from this interspecific cross.

5. Conclusions

The results reported in this paper demonstrate that a canola-quality *B. napus* line carrying resistance to multiple *P. brassicae* pathotypes can be achieved from a (*B. napus* × *B. rapa*) × *B. napus* interspecific cross through selection for resistance to a single pathotype in the early generation population. The occurrence of resistance to multiple pathotypes in the advanced generation population and the strong correlation between resistance to some of these pathotypes indicate that a common genetic mechanism might be involved in the control of resistance to multiple pathotypes. A pleiotropic effect of a resistance gene conferring resistance to multiple pathotypes also cannot be ruled out; however, all these hypotheses need to be confirmed experimentally. Nevertheless, the knowledge gained from this study could be used by Brassica researchers and breeders for the introgression of clubroot resistance as well as other traits from *B. rapa* into *B. napus* canola.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12051225/s1, Figure S1: DNA histogram showing the relative nuclear DNA content in the *Brassica rapa* and *B. napus* parents and in a representative BC_1F_6 plant. The X-axis shows the relative quantity of DNA in a cell and the Y-axis shows the number of cells counted, Figure S2: Distribution of the BC_1F_8 lines of the (*Brassica napus* × *B. rapa*) × *B. napus* interspecific cross, grown in nursery plots in 2020, for days to flowering (n = 452 lines), days to maturity (n = 452), and seed oil (n = 450), protein (n = 450), and glucosinolate (n = 450) contents. Erucic acid content in the seed oil of the BC_1F_5 plants (n = 240) grown in the greenhouse also presented. The value for the *B. napus* parent A04-73NA are indicated by the vertical arrow, Table S1: Performance of the BC_1F_8 lines (n = 450–452; see Figure S2) derived from the (*B. napus* × *B. rapa*) × *B. napus* interspecific cross and their spring *B. napus* parent A04-73NA tested in nursery plots in 2020, and the BC_1F_9 lines (n = 112) and an open-pollinated cultivar UA CountyGold tested in replicated field trials in 2021 for different agronomic and seed quality traits, Table S2: Analysis of variance for the agronomic and seed quality traits of the BC_1F_9 lines derived from the (*B. napus* × *B. rapa*) × *B. napus* interspecific cross. Data from three trials conducted in 2021 were used in the analysis.

Author Contributions: H.R. conceived the research idea and supervised the experiments. K.K. carried out the experiments, collected and analyzed the data, and drafted the manuscript. Y.L. carried out the experiment for resistance to multiple pathotypes and PCA analysis. H.R. provided critical feedback throughout the experiments and helped in data analysis and finalizing the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare there is no conflict of interest.

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