Suppression of Canola (*Brassica napus*) Resistance by Virulent Isolates of *Plasmodiophora brassicae* (Clubroot) During Primary Infection

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Abstract

The planting of clubroot resistant (CR) canola (*Brassica napus*) is the most effective method to manage clubroot. Since 2013, many *Plasmodiophora brassicae* isolates capable of overcoming resistance have been detected, often in mixtures with avirulent isolates. To improve understanding of the effect of low concentrations of virulent isolates on host resistance, three CR canola cultivars (45H29, L135C, and L241C) were inoculated with pairs of isolates representing virulent/avirulent pathotypes (2*/2, 3*/3, and 5*/5) collected after or before the introduction of CR canola, respectively. Seven-day-old seedlings of each cultivar were incubated for 2 days in low concentrations (1×10^3 spores/ml) of the virulent isolates, followed by a second inoculation with a high concentration (1×10^7 spores/ml) of the avirulent isolates. Positive controls comprised seedlings inoculated with low concentrations of the virulent isolates followed by high concentrations of the virulent isolates (PC1)

or only with high concentrations of virulent isolates (PC2). Negative controls comprised seedlings inoculated only with high concentrations of the avirulent isolates (NC1) or only with low concentrations of the virulent isolates (NC2). Clubroot severity was significantly higher in all nine experimental treatments (low virulent plus high avirulent) than in the negative control NC1 (high avirulent) but was lower in the experimental treatments than in the positive controls (PC1 and PC2). Low concentrations of virulent isolates alone (NC2) caused moderate clubroot. Disease severity correlated well with *P. brassicae* biomass in canola as determined by quantitative PCR analysis 28 to 35 days after inoculation. This study revealed that low concentrations of virulent isolates compromised canola resistance for infection by avirulent isolates.

Keywords: compromised host defense, relative biomass, virulence

The life cycle of *Plasmodiophora brassicae* Woronin, the cause of clubroot of canola (*Brassica napus* L.) and other crucifers, includes three distinct stages: resting spore survival in the soil, primary (root hair) infection, and secondary (cortical) infection (Hwang et al. 2012). Under favorable conditions, which include high soil moisture, moderate temperature, and the presence of root exudates, the long-lived resting spores of the pathogen are stimulated to germinate and produce primary zoospores (Dixon 2014). The primary zoospores invade the root hairs of potential hosts and form primary plasmodia, which subsequently undergo nuclear divisions and cleavage into zoosporangia that contain 4 to 16 secondary zoospores each (Howard et al. 2010; Kageyama and Asano 2009). These secondary zoospores are released into the rhizosphere and reinfect the host, producing secondary plasmodia within the cortical tissues of the main roots. Proliferation of the secondary plasmodia results in hypertrophy and hyperplasia of the infected cortical cells and the characteristic clubbing of the roots (Howard et al. 2010; Kageyama and Asano 2009). Secondary plasmodia eventually are cleaved into large numbers of resting spores, which are released back into the soil as the galls degrade, serving as inoculum to start new cycles of infection (Hwang et al. 2012).

In the life cycle of *P. brassicae*, primary zoospores are produced before secondary zoospores, but both can sometimes exist

simultaneously in root hairs or epidermal cells. Varying lengths of time have been reported for the onset of primary infection and the development of secondary infection. Dobson and Gabrielson (1983) and Feng et al. (2013b) detected primary zoospores in the root hairs of Chinese cabbage (*B. rapa* L. var. *pekinensis*) and canola, respectively, 12 to 24 h after inoculation, whereas secondary zoospores were observed 2 to 3 days after inoculation (dai). Other researchers, however, have reported the observation of both primary and secondary infections much later after inoculation. Primary and secondary infections were observed after 3 to 4 days and 5 to 8 days, respectively, when turnip (*B. rapa* L. var. *rapifera*) root hairs and *P. brassicae* were cultured together under axenic conditions (Kageyama and Asano 2009). Devos et al. (2005) and Agarwal et al. (2009, 2011) observed primary and secondary infection between 4 to 7 dai and 10 to 19 dai, respectively, when Chinese cabbage and Arabidopsis were grown in soil. Therefore, the onset of primary and secondary infection varies with different host genotypes, the virulence of the *P. brassicae* isolates used, as well as their interactions with environmental factors such as temperature, humidity, pH, and nutrients.

Primary infection by *P. brassicae* occurs in both host and nonhost species (Feng et al. 2012; Friberg et al. 2006; Ludwig-Müller et al. 1999; MacFarlane 1952). Secondary infection of nonhost species, however, rarely leads to the development of typical clubroot symptoms (Hwang et al. 2015; Ludwig-Müller et al. 1999; MacFarlane 1952). It is difficult to estimate the contribution of primary and secondary zoospores to clubroot development, since these spore types cannot be distinguished from each other based on morphology or general biological function (Kageyama and Asano 2009). Nonetheless, in spite of the inextricable similarities in morphology, the two phases of the life cycle are distinct. Dobson and Gabrielson (1983) demonstrated by controlling soil water matric potential that the observation of secondary zoospores coincided with the initiation of cortical infection. The two types of zoospores also seem to have different roles in pathogenesis, based on the number of genes that are upregulated or downregulated in resistant cultivars during primary and secondary infection (Fei et al. 2016; Feng et al. 2013a).

The aim of this study was to test the hypothesis that when low resting spore concentrations of different virulent *P. brassicae* isolates are inoculated on seedlings of resistant canola cultivars for a short time,

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they infect the roots and suppress the resistance of the host to avirulent isolates. Challenging seedlings of the same cultivar independently with virulent and avirulent isolates of the pathogen permitted the comparison of the experimental treatments to the positive and negative controls. Finally, the inoculation of resistant seedlings first with virulent isolates followed by avirulent isolates provides a window into the possible damage that the emergence of even low concentrations of new *P. brassicae* pathotypes could cause canola and other *Brassica* species.

Materials and Methods

Plant materials and *P. brassicae* isolates. Three clubroot resistant (CR) canola cultivars (45H29, L135C, and L241C) were evaluated for their reaction to three pairs of avirulent/virulent isolates of *P. brassicae* obtained from infected roots collected before and after the introduction of the clubroot resistance trait in Alberta, Canada. The “avirulent” or “virulent” designations refer to the capacity of the isolates to cause disease on CR canola. The avirulent isolates (SACAN-ss3, SACAN-ss1, and ORCA-ss4) were single spores and classified as pathotypes 2, 3, and 5, respectively, on the differentials of Williams (1966) (Xue et al. 2008). The virulent isolates (F183-14, CDCN#4, and F-359-13) represent field isolates (collections of resting spores derived from single root galls) that were able to overcome the resistance(s) in 45H29, L135C, and L241C (Strelkov et al. 2016, 2018). They were classified as pathotypes 2, 3, and 5 on the differentials of Williams (1966) but are hereafter referred to as pathotypes 2*, 3*, and 5* to denote their increased virulence on CR canola. All six single-spore and field isolates were maintained as resting spores in frozen root galls on the universal clubroot-susceptible host Chinese cabbage (*B. rapa* var. *pekinensis* ‘Granaat’).

Inoculum preparation. Frozen galls of each pathotype were homogenized separately in sterile distilled water in a Waring LB10G blender (Cole-Parmer) as described by Fredua-Agyeman et al. (2018). The spore suspension was collected by passing the slurry through eight layers of cheesecloth into a conical flask. The

concentrations of resting spores were quantified with a hemocytometer (VWR, Mississauga, ON, Canada) and adjusted with sterile distilled water. Two spore suspensions of each of the virulent pathotypes 2*, 3*, and 5* were prepared at concentrations of 1×10^3 spores/ml (low) and 1×10^7 spores/ml (high). A single concentration (1×10^7 spores/ml [high]) of each of the avirulent pathotypes 2, 3, and 5 was prepared.

Clubroot tests. To carry out clubroot tests, ~2,000 to 2,400 seedlings each of 45H29, L135C, and L241C were produced per experiment by pregerminating seeds on moistened sterile filter paper in Petri dishes (100×15 mm) for 7 days at room temperature and a 12-h photoperiod. Each experiment consisted of five treatments comprising nine combinations (45H29, L135C, and L241C $\times$ 2*/2, 3*/3, and 5*/5) (Table 1).

The experimental treatments (ETs) comprised roots of seedlings of each cultivar in Petri dishes (100×15 mm) incubated for 2 days with low concentrations (50 ml; 1×10^3 spores/ml $\times$ 30 seedlings/Petri dish) of resting spores of each of the virulent pathotypes, followed by washing off the inoculum from the roots and a subsequent re-inoculation by dipping the washed roots into high concentrations (1×10^7 spores/ml) of resting spores of the corresponding avirulent pathotype. The seedlings were transplanted into 8×4 flat trays (cell size $7 \times 6 \times 6$ cm) filled with Sunshine Mix #4 Aggregate Plus growing mix (Sungro Horticulture, Seba Beach, AB, Canada).

Two positive controls were used for each of the three cultivars. The first positive control (PC1) comprised seedlings incubated for 2 days with low concentrations of each virulent pathotype followed by washing off the inoculum and a second inoculation with high concentrations of the corresponding virulent pathotypes. The second positive control (PC2) comprised seedlings inoculated only with high concentrations of the virulent pathotypes. The seedlings were transplanted into Sunshine Mix #4 Aggregate Plus growing mix as described above. Similarly, two negative controls were used in each experiment, which were composed of seedlings inoculated only with high concentrations of the avirulent pathotypes (NC1) or only with

Table 1. The effect of low concentrations of virulent *Plasmodiophora brassicae* pathotypes on disease severity index (DSI %) on clubroot resistant canola cultivars

Pathotype ^x	Treatment ^y	Inoculation method	Mean DSI % $\pm$ S.E. (range) on canola cultivar ^z		
			45H29	L135C	L241C
2*/2	ET	10^3 spores/ml of pathotype 2* plus 2 days later 10^7 spores/ml of pathotype 2	37.9 $\pm$ 8.4 b (22.0–50.3)	32.8 $\pm$ 6.9 b (19.4–42.3)	43.1 $\pm$ 15.0 b (14.2–64.4)
	NC1	10^7 spores/ml of pathotype 2	10.2 $\pm$ 4.0 c (3.5–17.3)	2.3 $\pm$ 1.7 c (0.0–5.7)	1.9 $\pm$ 1.1 c (0.0–3.9)
	NC2	10^3 spores/ml of pathotype 2*	21.9 $\pm$ 8.0 c (6.8–33.9)	15.0 $\pm$ 4.8 c (7.2–23.9)	39.3 $\pm$ 17.3 b (6.3–64.8)
	PC1	10^3 spores/ml of pathotype 2* plus 2 days later 10^7 spores/ml of pathotype 2*	91.9 $\pm$ 1.6 a (88.9–94.5)	91.1 $\pm$ 3.1 a (84.9–94.7)	89.7 $\pm$ 3.0 a (84.6–95.1)
	PC2	10^7 spores/ml of pathotype 2*	100.0 $\pm$ 0.0 a (100.0–100.0)	97.7 $\pm$ 1.9 a (93.8–100.0)	99.7 $\pm$ 0.3 a (99.1–100.0)
	ET	10^3 spores/ml of pathotype 3* plus 2 days later 10^7 spores/ml of pathotype 3	25.9 $\pm$ 5.6 e (14.8–32.5)	23.3 $\pm$ 8.7 e (13.6–40.6)	27.0 $\pm$ 8.9 e (17.1–44.8)
3*/3	NC1	10^7 spores/ml of pathotype 3	12.0 $\pm$ 3.8 f (4.3–16.3)	4.0 $\pm$ 3.1 f (0.0–10.1)	2.7 $\pm$ 2.1 f (0.0–6.9)
	NC2	10^3 spores/ml of pathotype 3*	2.3 $\pm$ 0.3 f (1.8–2.8)	2.0 $\pm$ 1.7 f (0.0–5.5)	1.3 $\pm$ 0.9 f (0.0–3.0)
	PC1	10^3 spores/ml of pathotype 3* plus 2 days later 10^7 spores/ml of pathotype 3*	94.4 $\pm$ 2.6 d (89.4–97.7)	88.0 $\pm$ 4.3 d (83.3–96.6)	89.7 $\pm$ 5.0 d (79.8–95.9)
	PC2	10^7 spores/ml of pathotype 3*	89.9 $\pm$ 2.8 d (85.5–95.1)	90.5 $\pm$ 3.6 d (83.3–94.7)	90.1 $\pm$ 4.5 d (83.3–98.7)
	ET	10^3 spores/ml of pathotype 5* plus 2 days later 10^7 spores/ml of pathotype 5	37.6 $\pm$ 3.7 i (30.3–41.4)	34.8 $\pm$ 3.7 i (27.6–40.0)	44.0 $\pm$ 13.7 i (16.7–58.3)
	NC1	10^7 spores/ml of pathotype 5	6.9 $\pm$ 1.6 j (4.8–10.0)	4.0 $\pm$ 3.1 g (0.0–10.1)	3.0 $\pm$ 2.4 j (0.0–7.9)
5*/5	NC2	10^3 spores/ml of pathotype 5*	20.1 $\pm$ 9.0 j (2.4–31.5)	17.4 $\pm$ 3.2 j (11.6–22.6)	30.3 $\pm$ 14.9 ij (0.8–48.5)
	PC1	10^3 spores/ml of pathotype 5* plus 2 days later 10^7 spores/ml of pathotype 5*	96.4 $\pm$ 0.2 h (95.9–96.7)	91.2 $\pm$ 3.8 h (83.7–95.6)	95.5 $\pm$ 1.0 h (94.4–97.6)
	PC2	10^7 spores/ml of pathotype 5*	98.5 $\pm$ 1.5 h (95.5–100.0)	95.2 $\pm$ 0.9 h (93.4–96.3)	97.0 $\pm$ 2.3 h (92.4–99.6)

^x Three *P. brassicae* pathotypes (2*, 3*, and 5*) used in the inoculation experiments could overcome resistance in the three canola cultivars 45H29, L135C, and L241C, whereas the three pathotypes (2, 3, and 5) could not.

^y Treatment designations are as follows: ET = experimental treatment, NC = negative control (NC1 and NC2), and PC = positive control (PC1 and PC2).

^z Data are presented as the mean DSI % $\pm$ S.E. for three repetitions with the DSI range for the three repeated experiments given in parentheses. For each genotype $\times$ pathogen treatment, the same letters next to the mean DSI values indicate no significant difference ($P < 0.05$) between treatments, whereas different letters indicate significant differences in treatments. Analysis of significance was conducted within pathotype pairs. DSI values in all ETs were significantly higher than in NC1; the DSI values in seven ETs were significantly higher than in NC2, whereas there was no significant difference in two others (2*/2 and 5*/5 $\times$ L241C). All PC1 and PC2 values were significantly higher than ET, NC1, and NC2.

low concentrations of the virulent pathotypes (NC2) before being transplanted into the growing mix.

The plants were kept in a greenhouse maintained at 20 to 25°C during the day and 15 to 18°C at night, with a 16-h photoperiod, and were watered daily with slightly acidified water (pH 6, adjusted with HCl). Fertilizer (N/P/K = 20:20:20) was applied once a week 3 weeks after transplanting. The inoculation experiments were arranged in a randomized complete block design with the different isolates as the main plots and the different cultivars as the subplots. Each inoculation treatment was carried out in triplicate with each repetition consisting of 120 to 160 seedlings. The trays were randomized twice in the second and the fourth weeks after the seedlings were transplanted into potting mix and each experiment was repeated three times.

Sampling and disease assessment. Root samples were collected 1, 3, 5, 7, 9, 11, 14, 17, 21, 28, and 35 dai for the five treatment combinations for each cultivar. Roots of three inoculated plants from each treatment were selected randomly, uprooted gently, and washed carefully in tubs containing water. The root samples (~4,455 = 11 time points $\times$ 5 treatments $\times$ 3 cultivars $\times$ 3 pathotypes $\times$ 3 plants $\times$ 3 replicates) were stored at -20°C until DNA extractions were carried out to determine the relative quantity (RQ) of pathogen biomass to the host by quantitative PCR (qPCR) analysis (see below).

The remaining plants (actual sample size of 25 to 97 plants per treatment per replication; not all seedlings survived transplantation) were dug out, washed with water, and rated for clubroot symptom development 6 weeks after inoculation. Clubroot symptoms were rated on a scale of 0 to 3 according to Kuginuki et al. (1999), where 0 indicates no galls, 1 indicates a few small or bead-sized galls on less than one-third of the roots, 2 indicates medium galls on one-third to two-thirds of the roots, and 3 indicates large galls on more than two-thirds of the roots. A disease severity index (DSI; 0 to 100%) was calculated for each replication using the formula of Horiuchi and Hori (1980) as modified by Strelkov et al. (2006):

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

where n is the number of plants in one class and N is the total number of plants in one biological replicate. The mean DSI for each treatment and for each experiment was calculated by averaging the DSIs of experiments 1, 2, and 3.

Disease incidence (DI) was calculated with the following formula:

$$\text{DI (\%)} = \frac{m}{N} \times 100$$

where m is the number of infected plants and N is the total number of plants in one biological replicate. The mean for each treatment and for each experiment was calculated by averaging the DIs of the three replicated experiments.

DNA extraction. Total genomic DNA (canola plus *P. brassicae*) was extracted from root galls of each of the three replicates for each treatment using the cetyltrimethyl ammonium bromide method (Sambrook and Russell 2001). The DNA concentration was quantified using an ND-2000c spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE) and the final concentration of each sample was adjusted to 20 ng of DNA/ μ l.

Real-time PCR and relative quantification of *P. brassicae* biomass. The comparative threshold cycle (Ct) or the delta-delta Ct ($\Delta\Delta$ Ct) qPCR method (Livak and Schmittgen 2001; Schmittgen and Livak 2008) was used to determine the RQs of *P. brassicae* DNA and canola DNA. The *P. brassicae*-specific primers (TC1F: 5'-GTGGTCTGAAGTTCATTAATTTGGGCTCTT-3'; and RTPbR1a: 5'-TCAGCACCGTTTCCGGCTGCTAAGGC-3') (Cao et al. 2014) and canola-specific β -actin (*BnAct2*) primers (F: 5'-ACGAGCTACCTGACGGACAAG-3'; and R: 5'-GAGCGACGGCTG- GAA GAGTA-3') (Yang et al. 2009) were used to confirm the similarity of the PCR efficiency of the pathogen and the host housekeeping genes, as well as reactions for the determination of the RQs of *P. brassicae* and canola DNA from the roots harvested over the time

course. The specificity of the primers was confirmed by melt curve analysis and also on 2% agarose gels.

To validate the efficiency of the PCR, two independent standard curves were generated by plotting cycle numbers against each *P. brassicae* DNA diluted to final concentrations of 40, 4, and 0.4 ng/ μ l and 40, 4, and 0.4 pg/ μ l and noninfected canola root DNA diluted to final concentrations of 100, 10, 1, and 0.1 ng/ μ l and 10 pg/ μ l (i.e., a 10-fold dilution per step). The Δ Ct ($C_{tP. brassicae} - C_{tcanola DNA}$) was calculated for each dilution. The suitability of the $\Delta\Delta$ Ct method was then determined from the absolute value of the slope of the plot of Δ Ct versus log DNA dilutions (Livak and Schmittgen 2001).

The qPCRs of the harvested samples were run in triplicate and also within each replicate three DNA repeats were used. SYBR Green was used as the fluorescent DNA binding dye on a QuantStudio 6 Flex Real-Time PCR System (Fisher Canada, Ottawa, ON, Canada). The qPCR conditions consisted of initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. After each run, a dissociation curve was obtained by heating the samples from 60 to 95°C.

The Δ Ct and $\Delta\Delta$ Ct were calculated using the formulae of Livak and Schmittgen (2001) and Schmittgen and Livak (2008) as follows:

$$\begin{aligned}\Delta\text{Ct}_1 &= C_{tP. brassicae \text{ gene}} - C_{t\text{noninfected canola gene}} \\ \Delta\text{Ct}_2 &= C_{t\text{canola } \beta\text{-actin gene}} - C_{t\text{noninfected canola gene}} \\ \Delta\Delta\text{Ct} &= \Delta\text{Ct}_1 - \Delta\text{Ct}_2\end{aligned}$$

where Ct is the cycle number when the fluorescence of the sample exceeds the background cycle. The noninfected canola gene was used as a calibrator to normalize two different qPCR experiments. The quantity of *P. brassicae* relative to the amount of plant tissue and normalized by the calibrator was then calculated as $2^{-\Delta\Delta\text{Ct}}$.

Statistical analyses. Data collected were analyzed by analysis of variance using the general linear model (GLM) in R software (R Development Core Team 2015). The GLM formula used was:

$$y_{ij} = \mu + \tau_i + \varepsilon_{ij}$$

where i indexed ETs in each combination and j indexed observations within the i th group. Comparisons among means of $\text{DSI} \pm \text{S.E.}$ and $\text{DI} \pm \text{S.E.}$ were conducted at $P = 0.05$ with Duncan's test (Steel and Torrie 1960). Statistical graphs in Excel were converted into box and whisker plots.

Results

Clubroot disease severity and incidence. The clubroot severity and incidence data (mean $\pm$ S.E. and range of three repeated experiments) of nine treatment combinations involving three pairs of virulent/avirulent pathotypes (i.e., 2*/2, 3*/3, and 5*/5) $\times$ three canola cultivars (i.e., 45H29, L135C, and L241C) are presented in Tables 1 and 2, respectively, and also as box and whisker plots in Supplementary Figures S1 and S2.

The inoculation of seedlings with high concentrations (1×10^7 spores/ml) of pathotypes 2, 3, and 5 (NC1) resulted in DIs that ranged from 5.8 ± 3.4 to $17.9 \pm 7.2\%$ on 45H29, L135C, and L241C. This confirms that the isolates representing pathotypes 2, 3, and 5 were avirulent on these three cultivars. In contrast, inoculation with high (1×10^7 spores/ml) concentrations of pathotypes 2*, 3*, and 5* (PC1 and PC2) resulted in significantly higher DIs of 94.2 ± 2.3 to $100.0 \pm 0.0\%$ for the three cultivars. This confirms that pathotypes 2*, 3*, and 5* were virulent on the three cultivars.

Inoculation with low (1×10^3 spores/ml) concentrations of pathotypes 2* and 5* (NC2) also resulted in significantly higher DIs that ranged from 24.9 ± 5.5 to $58.2 \pm 21.1\%$ on 45H29, L135C, and L241C (Table 2). Inoculation with a low concentration of the third virulent isolate representing pathotype 3*, however, resulted in DIs of 2.5 ± 1.5 to $3.4 \pm 1.0\%$ on 45H29, L135C, and L241C, which is similar to those obtained with high concentrations of the avirulent pathotypes (Table 2). Therefore, inoculation with 1×10^3 spores/ml of pathotypes 2* and 5* caused clubroot disease on 45H29, L135C, and L241C (in six of six treatment combinations), whereas inoculation with low

concentrations of 3* did not cause any significant clubroot symptoms on the three cultivars (three of three treatment combinations).

The mean DSI values for seedlings inoculated with the 2*/2 pathotype combination (low concentration of pathotype 2* plus high concentration of pathotype 2) were 37.9 ± 8.4 , 32.8 ± 6.9 , and $43.1 \pm 15.0\%$ for 45H29, L135C, and L241C, respectively (Table 1). Following inoculation with the 3*/3 and 5*/5 pathotype combinations, the DSI values were 25.9 ± 5.6 , 23.3 ± 8.7 , and $27.0 \pm 8.9\%$ and 37.6 ± 3.7 , 34.8 ± 3.7 , and $44.0 \pm 13.7\%$, respectively, for 45H29, L135C, and L241C (Table 1). In the case of NC1 (seedlings inoculated only with a high concentration of the avirulent pathotypes 2, 3, or 5), the DSI ranged from 1.9 ± 1.1 to $12.0 \pm 3.8\%$ on 45H29, L135C, and L241C (Table 1). Thus, the DSI values were significantly ($P = 0.05$) higher in all nine ETs compared with the NC1 in all pathotype–cultivar combinations.

The DSI values were significantly higher in seven of the nine ET combinations compared with NC2 in which seedlings were inoculated only with low concentrations of the virulent pathotypes 2*, 3*, and 5*. These seven ETs comprised all six 2*/2, 3*/3, and 5*/5 $\times$ 45H29 and L135C combinations (Table 1). The seventh pathotype–cultivar combination that showed significantly higher ($P = 0.05$) DSI values in the ET compared with NC2 was 3*/3 $\times$ L241C (Table 1). The 2*/2 and 5*/5 $\times$ L241C combination did not show any significant difference ($P = 0.05$) in DSI values in the ET compared with NC2 (Table 1).

There was no significant difference in the DSI values of PC1 (seedlings inoculated with low concentrations of pathotypes 2*, 3*, and 5* plus high concentrations of pathotypes 2*, 3*, and 5* 2 days later) and PC2 (seedlings inoculated with high concentrations of pathotypes 2*, 3*, and 5*). As expected, the DSIs were significantly higher in all nine PC1 and PC2 treatment combinations compared with the ETs, NC1s, and NC2s (Table 1).

In the case of mean DI, the ET values ranged from 40.3 ± 17.3 to $65.4 \pm 20.3\%$ compared with NC1 (5.8 ± 3.4 to $17.9 \pm 7.2\%$) for the nine treatment combinations (2*/2, 3*/2, and 5*/5 $\times$ 45H29, L135C, and L241C) (Table 2). Thus, the DI values (similar to the DSI trend) were significantly ($P = 0.05$) higher in all nine ETs compared with NC1 in all pathotype–cultivar combinations.

In addition, six of the nine treatments showed significant differences in DI values between the ET and NC2 (Table 2). The DI values of the six pathotype–cultivar combinations (3*/3 and 5*/5 $\times$ 45H29; 2*/2, 3*/3, and 5*/5 $\times$ L135C; 3*/3 $\times$ L241C) for the ETs ranged from 40.3 ± 17.3 to $57.9 \pm 8.7\%$ compared with 2.5 ± 1.5 to $31.4 \pm 14.1\%$ for NC2. Similar to the DSI, the DI values of the 2*/2 and 5*/5 $\times$ L241C combinations did not show any significant difference ($P = 0.05$) in the ET (55.6 ± 12.7 to $65.4 \pm 20.3\%$) compared with NC2 (40.0 ± 19.3 to $58.2 \pm 21.1\%$). In addition, the 2*/2 $\times$ 45H29 combination did not show any significant difference ($P = 0.05$) in the ET ($58.8 \pm 14.0\%$) compared with NC2 ($38.2 \pm 11.2\%$) (Table 2). Similarly, the DI values of PC1 and PC2 were significantly greater than that of the ETs, NC1s, and NC2s (Table 2).

Inoculation with low concentrations of pathotypes 2*, 3*, and 5* followed 2 days later by inoculation with high concentrations of pathotypes 2, 3, and 5 or 2*, 3*, and 5*, respectively, caused significant levels of clubroot (Tables 1 and 2). Thus, pathotypes 2* and 5* even at low concentrations caused significant clubroot on 45H29, L135C, and L241C, but the effect was elevated or magnified when the cultivars were subsequently inoculated with avirulent or virulent isolates. In contrast, pathotype 3* was virulent on the three CR canola cultivars at high concentrations, but at low concentrations inoculation with pathotype 3* needed to be followed by a high concentration of pathotype 3 or 3* to cause significant disease. Finally, there were no significant differences ($P = 0.05$) in the DSI and DI obtained for

Table 2. The effect of low concentrations of virulent *Plasmodiophora brassicae* pathotypes on disease incidence (DI %) on clubroot resistant canola cultivars

Pathotype ^x	Treatment ^y	Inoculation method	Mean DI % $\pm$ S.E. (range) on canola cultivar ^z		
			45H29	L135C	L241C
2*/2	ET	10^3 spores/ml of pathotype 2* plus 2 days later 10^7 spores/ml of pathotype 2	58.8 ± 14.0 b (30.9–75.4)	57.9 ± 8.7 b (43.0–73.1)	65.4 ± 20.3 ab (24.7–86.1)
	NC1	10^7 spores/ml of pathotype 2	17.9 ± 7.2 c (8.1–32.0)	7.0 ± 5.2 d (0.0–17.1)	5.8 ± 3.4 c (0.0–11.8)
	NC2	10^3 spores/ml of pathotype 2*	38.2 ± 11.2 bc (18.1–56.7)	30.0 ± 5.6 c (20.3–39.6)	58.2 ± 21.1 b (16.7–85.9)
	PC1	10^3 spores/ml of pathotype 2* plus 2 days later 10^7 spores/ml of pathotype 2*	100.0 ± 0.0 a (100.0–100.0)	99.6 ± 0.4 a (98.7–100.0)	97.1 ± 2.9 ab (91.3–100.0)
	PC2	10^7 spores/ml of pathotype 2*	100.0 ± 0.0 a (100.0–100.0)	99.0 ± 1.0 a (97.1–100.0)	100.0 ± 0.0 a (100.0–100.0)
	ET	10^3 spores/ml of pathotype 3* plus 2 days later 10^7 spores/ml of pathotype 3	46.9 ± 12.4 f (22.2–60.3)	40.3 ± 17.3 f (22.4–75.0)	44.2 ± 13.3 f (26.7–70.2)
3*/3	NC1	10^7 spores/ml of pathotype 3	15.0 ± 4.8 g (5.9–22.2)	11.9 ± 9.4 g (0.0–30.4)	7.6 ± 6.6 g (0.0–20.7)
	NC2	10^3 spores/ml of pathotype 3*	3.4 ± 1.0 g (1.8–5.1)	2.9 ± 2.0 g (0.0–6.8)	2.5 ± 1.5 g (0.0–5.2)
	PC1	10^3 spores/ml of pathotype 3* plus 2 days later 10^7 spores/ml of pathotype 3*	98.6 ± 0.8 e (97.2–100.0)	98.8 ± 0.6 e (98.1–100.0)	99.1 ± 0.9 e (97.4–100.0)
	PC2	10^7 spores/ml of pathotype 3*	95.2 ± 2.4 e (92.3–100.0)	96.5 ± 2.2 e (92.3–100.0)	94.2 ± 2.3 e (90.8–98.7)
	ET	10^3 spores/ml of pathotype 5* plus 2 days later 10^7 spores/ml of pathotype 5	56.5 ± 5.9 j (44.8–62.9)	52.8 ± 3.6 j (48.4–60.0)	55.6 ± 12.7 j (30.3–70.0)
	NC1	10^7 spores/ml of pathotype 5	7.9 ± 2.1 h (5.5–12.0)	11.1 ± 8.2 k (0.0–27.0)	8.4 ± 6.6 k (0.0–21.3)
5*/5	NC2	10^3 spores/ml of pathotype 5*	31.4 ± 14.1 k (3.7–50.0)	24.9 ± 5.5 k (14.1–32.1)	40.0 ± 19.3 jk (2.3–65.9)
	PC1	10^3 spores/ml of pathotype 5* plus 2 days later 10^7 spores/ml of pathotype 5*	99.6 ± 0.4 i (98.9–100.0)	100.0 ± 0.0 i (100.0–100.0)	100.0 ± 0.0 i (100.0–100.0)
	PC2	10^7 spores/ml of pathotype 5*	99.0 ± 1.0 i (97.0–100.0)	100.0 ± 0.0 i (100.0–100.0)	99.0 ± 1.0 i (97.1–100.0)

^x Three *P. brassicae* pathotypes (2*, 3*, and 5*) used in the inoculation experiments could overcome resistance in the three canola cultivars 45H29, L135C, and L241C, whereas the three pathotypes (2, 3, and 5) could not.

^y Treatment designations are as follows: ET = experimental treatment, NC = negative control (NC1 and NC2), and PC = positive control (PC1 and PC2).

^z Data are presented as the mean DI % $\pm$ S.E. for three repetitions with the DI range for the three repeated experiments given in parentheses. For each genotype $\times$ pathogen treatment, the same letters next to the mean DI values indicate no significant difference ($P < 0.05$) between treatments, whereas different letters indicate significant differences in treatments. Analysis of significance was conducted within pathotype pairs. DI values in all ETs were significantly higher than in NC1; the DI values in six ETs were significantly higher than in NC2, whereas three others (2*/2 $\times$ 45H29, 2*/2, and 5*/5 $\times$ L241C) showed no significant difference. All PC1 and PC2 values were significantly higher than ET, NC1, and NC2.

the different canola cultivars following inoculation with the same isolates (Tables 1 and 2).

Efficiency and specificity of primers for qPCR assay. The plot of Ct values versus log serially diluted DNA of *P. brassicae* and genomic DNA of canola confirmed the linearity of amplification over the dynamic range. The slope of the calibration curves was -3.18 ($R^2 = 0.999$) for *P. brassicae* (Supplementary Fig. S3) and -3.21 ($R^2 = 0.993$) for canola (Supplementary Fig. S4), which were very close to the theoretical slope of -3.32 required for qPCR assays with 100% efficiency. The qPCR assays generated a single peak in melting curve analysis, which indicated the specificity of the target. In addition, no amplified product was obtained when pure canola DNA was subjected to PCR with the *P. brassicae* primers.

Quantification of *P. brassicae* DNA relative to canola DNA by qPCR. In the qPCR assay, the RQs ($2^{-\Delta\Delta Ct}$ mean $\pm$ S.E. of three repeated experiments) of *P. brassicae* biomass to canola ranged from 0.1 ± 0.0 to 34.0 ± 12.3 , 0.6 ± 0.3 to 9.2 ± 3.1 , and 0.3 ± 0.1 to $16.7 \pm$

10.4 for the $2^*/2$, $3^*/3$, and $5^*/5 \times 45H29$ combinations, respectively, for the root samples taken from 1 to 28 dai (Supplementary Table S1). The RQs of *P. brassicae* biomass to canola for the $2^*/2$, $3^*/3$, and $5^*/5 \times L135C$ combinations ranged from 0.1 ± 0.0 to 14.7 ± 7.5 , 0.3 ± 0.2 to 26.5 ± 5.2 , and 0.3 ± 0.2 to 20.9 ± 0.3 , respectively, for the root samples taken over the same period (1 to 28 dai). In the case of $2^*/2$, $3^*/3$, and $5^*/5 \times L241C$, the RQs for the root samples ranged from 0.2 ± 0.1 to 13.2 ± 6.8 , 0.3 ± 0.1 to 18.7 ± 1.8 , and 0.4 ± 0.2 to 83.7 ± 75.6 , respectively. In general, the RQs for all nine combinations ($2^*/2$, $3^*/3$, and $5^*/5 \times 45H29$, L135C, and L241C) were not significantly different ($P = 0.05$) from each other until 35 dai (Fig. 1). Similarly, the RQs for NC1 and NC2 from 1 to 28 dai were not generally significantly ($P = 0.05$) different from the RQs for the ET over the same period (Fig. 1). The RQs of *P. brassicae* biomass to canola for NC1 and NC2 in the $2^*/2$, $3^*/3$, and $5^*/5 \times L135C$ and L241C combinations (range, 0.0 ± 0.0 to 31.2 ± 15.3) were in general only significantly different from the ET at 35 dai (range, 6.3 ± 4.1 to 106.8 ± 67.7).

Fig. 1. Clubroot disease progression in the canola cultivars **A**, 45H29, **B**, L135C, and **C**, L241C. Results were normalized by the $\Delta\Delta$ cycle threshold method with β -actin gene as the reference gene. All experiments were repeated three times. The y-axis represents the *Plasmodiophora brassicae* DNA amount relative to plant DNA in the root. Pathotype designations are as follows: P2, P3, and P5 denote *P. brassicae* isolates that are avirulent on clubroot resistant canola cultivars 45H29, L135C, and L241C, whereas P2*, P3*, and P5* represent virulent isolates causing elevated levels of clubroot on the three cultivars. Inoculum concentration designations are as follows: low = 1×10^3 spores/ml and high = 1×10^7 spores/ml. dai = days after inoculation.

In contrast, the RQs of *P. brassicae* biomass to canola for the two positive controls (PC1 and PC2) were generally significantly greater ($P = 0.05$) at 17 to 35 dai than the RQs of the respective ET, NC1, and NC2 over the same period (Fig. 1). In the 2*/2 × 45H29 treatments, the RQs following inoculation with low pathotype 2*/high pathotype 2* (PC1) at 21 to 35 dai ranged from 36.5 ± 27.9 to 177.7 ± 76.9 , whereas they ranged from 45.1 ± 16.0 to 580.3 ± 88.0 for the inoculation with high pathotype 2* (PC2). For the low pathotype 2*/high pathotype 2 (ET) combination, the RQs ranged from 3.0 ± 0.4 to 98.1 ± 66.6 , compared with a range of 0.5 ± 0.1 to 0.9 ± 0.1 for high pathotype 2 (NC1) and 0.0 ± 0.0 to 0.4 ± 0.2 for low pathotype 2* (NC2). In the case of 3*/3 × 45H29, the RQs following inoculation with low pathotype 3*/high pathotype 3* (PC1) and high pathotype 3* (PC2) ranged from 25.0 ± 21.3 to 236.2 ± 178.0 and 167.0 ± 79.9 to 798.5 ± 119.2 , respectively, compared with an RQ range of 6.8 ± 2.4 to 82.2 ± 78.1 for low pathotype 3*/high pathotype 3 (ET), 0.1 ± 0.0 to 0.9 ± 0.3 for high pathotype 3 (NC1), and 0.0 ± 0.0 to 0.4 ± 0.2 for low pathotype 3* (NC2). The RQs for the 5*/5 × 45H29 treatments ranged from 1.3 ± 0.7 to 160.8 ± 114.8 for low pathotype 5*/high pathotype 5* (PC1) and 93.7 ± 17.1 to 284.7 ± 49.4 for high pathotype 5* (PC2) at 21 to 35 dai, compared with RQs of 3.7 ± 1.4 to 23.2 ± 21.0 for low pathotype 5*/high pathotype 5 (ET), 0.7 ± 0.0 to 4.5 ± 2.7 for high pathotype 5 (NC1), and 0.0 ± 0.0 to 0.3 ± 0.1 for low pathotype 5* (NC2).

The RQs of *P. brassicae* biomass to canola for the 2*/2 × L135C combination at 21 to 35 dai ranged from 10.2 ± 4.8 to 162.2 ± 23.5 for the low pathotype 2*/high pathotype 2* inoculation (PC1) and 168.9 ± 69.8 to 563.5 ± 290.3 for the high pathotype 2* inoculation (PC2), compared with RQs of 5.4 ± 1.5 to 105.3 ± 50.0 for low pathotype 2*/high pathotype 2 (ET), 1.0 ± 0.3 to 1.4 ± 0.9 for high pathotype 2 (NC1), and 0.0 ± 0.0 to 1.3 ± 1.3 for low pathotype 2* (NC2). For the 3*/3 × 45H29 combination, the RQs for the low pathotype 3*/high pathotype 3* inoculation (PC1) and the high pathotype 3* (PC2) inoculation ranged from 47.0 ± 37.6 to 121.6 ± 51.1 and 53.6 ± 26.2 to 492.6 ± 174.6 , respectively, compared with 12.9 ± 5.3 to 56.0 ± 52.1 for low pathotype 3*/high pathotype 3 (ET), 0.1 ± 0.0 to 0.8 ± 0.1 for high pathotype 3 (NC1), and 0.1 ± 0.0 to 1.1 ± 1.1 for low pathotype 3* (NC2). The RQs for the 5*/5 × 45H29 combination ranged from 76.5 ± 73.3 to 143.9 ± 129.0 for the low pathotype 5*/high pathotype 5* inoculation (PC1) and from 40.3 ± 4.5 to 273.9 ± 40.3 for the high pathotype 5* inoculation (PC2), compared with 11.1 ± 3.5 to 23.2 ± 21.5 for low pathotype 5*/high pathotype 5 (ET), 1.7 ± 0.8 to 3.2 ± 1.2 for high pathotype 5 (NC1), and 0.0 ± 0.0 to 1.2 ± 0.3 for low pathotype 5* (NC2).

In the case of the 2*/2, 3*/3, and 5*/5 × L241C treatment combinations, the RQs of *P. brassicae* biomass to canola for PC1 ranged from 10.1 ± 2.7 to 305.2 ± 163.4 , whereas they ranged from 95.0 ± 36.2 to 944.3 ± 238.9 for PC2, compared with RQs of 4.3 ± 1.0 to 106.8 ± 67.7 for the ET, 0.8 ± 0.4 to 4.1 ± 2.2 for NC1, and 0.0 ± 0.0 to 3.3 ± 3.2 for NC2.

Overall, the qPCR data showed that 28 to 35 days was the optimum period at which there was sufficient *P. brassicae* biomass in the ET to be detected by the comparative Ct qPCR method. Nonetheless, *P. brassicae* biomass could be detected at 17 to 21 dai following inoculation of the seedlings with virulent pathotypes (PC1 and PC2). In contrast, *P. brassicae* could not be detected even at 35 dai following inoculation with low concentrations of virulent isolates (NC1) or high concentrations of avirulent isolates (NC2).

Discussion

The effects of low inoculum concentrations of virulent *P. brassicae* pathotypes on the resistance of three CR canola cultivars (45H29, L135C, and L241C) and the accumulation of pathogen biomass were studied. Although they are classified as CR, the three cultivars were not completely immune to the pathogen and developed mild symptoms of disease when inoculated with high concentrations of the avirulent pathotypes. Nonetheless, when the cultivars were first exposed to a low concentration of virulent inoculum prior to exposure to a high concentration of the avirulent inoculum, clubroot incidence (DI) and severity (DSI) were significantly greater than when they

were exposed only to the high concentration of avirulent inoculum (NC1) or only to the low concentration of virulent inoculum (NC2). This finding was observed across all three cultivars and virulent/avirulent pathotype combinations, and it suggests that prior exposure of a host to low concentrations of virulent inoculum may compromise resistance and make plants more susceptible to subsequent exposure to high concentrations of avirulent inoculum.

Two of the three virulent pathotypes (2* and 5*) caused moderate clubroot symptoms at low concentrations. However, the third virulent pathotype (3*) did not cause significant levels of disease when it was applied only at low concentrations. The virulent pathotypes were represented by field isolates, which may consist of mixtures of different (avirulent and/or virulent) pathotypes in various proportions. It is likely, therefore, that for the field isolate classified as pathotype 3*, the number of virulent resting spores in the mixture was not sufficient to cause significant clubroot symptoms on CR canola at low concentrations. Higher concentrations were needed to increase the spore density of the virulent component of this field isolate to a level sufficient to cause disease. Previous studies with virulent isolates found a positive correlation between clubroot severity and inoculum density (Hwang et al. 2017), and as expected, high inoculum concentrations of all three virulent pathotypes (PC1 and PC2) caused severe clubroot on the host cultivars. Notwithstanding the results with pathotype 3*, it appears that low or high concentrations of virulent isolates may cause clubroot and pose a significant threat to the production of canola. This is consistent with the findings of Strelkov et al. (2016, 2018) about elevated clubroot caused by the newly discovered *P. brassicae* isolates in Canada.

The most important finding of this study was the increased clubroot severity associated with previous exposure to low concentrations of virulent isolates, which may have compromised host immunity or induced susceptibility in the host to avirulent isolates. Based on the disease rating data (Tables 1 and 2), clubroot DSIs and DIs were significantly higher in the ET compared with NC1 and NC2 for all pathotype combinations examined. For example, the mean DSI and DI values of seedlings inoculated with only a low concentration of pathotype 3* were elevated when the seedlings were inoculated with low concentrations of pathotype 3* followed by inoculation with a high concentration of pathotype 3. This is similar to the induced susceptibility reported in barley and pea, where preliminary inoculation with a compatible pathogen induces susceptibility to an incompatible pathogen or nonpathogen (Akpa and Archer 1994; Kunoh et al. 1985; Ouchi 1983; Ouchi and Oku 1982; Ouchi et al. 1974). Therefore, the phenotypes resulting from these interactions may reflect the virulence of pathotypes, specific combinations of isolates, host genotypes, as well as environmental and other factors. Low concentrations of spores of virulent isolates causing moderate disease may also be explained by quorum sensing, which is a biological mechanism for microbial communication based on cell density. Quorum sensing has been reported to regulate the expression of virulence genes, which is a crucial step in pathogenesis (Albuquerque and Casadevall 2012). The presence of low concentrations of virulent spores may therefore inhibit the expression of virulence factors and multiplication of cells of the pathogen.

Relative quantification of *P. brassicae* DNA in relation to canola DNA in the 4,455 collected root samples was achieved with qPCR assays. The comparative Ct qPCR method offers a high-throughput assay for biomass quantification and has been used for the determination of pathogen biomass accumulation in many crops (Feng et al. 2010; Gachon and Saindrenan 2004; Gao et al. 2004; Marzachi and Bosco 2005; Weßling and Panstruga 2012). In contrast, microscopic examination requires cross-sectioning and staining of *P. brassicae*-infected roots as well as hours of observation and counting of spores under the microscope (McDonald et al. 2014), which can make it unsuitable for determining pathogen biomass in such a large number of infected root samples.

In a study of different pathogenic species and plant hosts, Gachon and Saindrenan (2004) reported that *Alternaria brassicicola* did not show significant variation in fungal DNA over the time course of

infection, whereas growth of *Botrytis cinerea* was rapid and increased steadily 72 h after inoculation of *Arabidopsis thaliana*. In a qPCR assay with the same primers used in this study and a TaqMan probe (TCPb1), Cao et al. (2014) observed that *P. brassicae* DNA increased significantly in susceptible and moderately resistant *Brassica* hosts from 5 to 42 dai. In contrast, the *P. brassicae* DNA concentration in the highly resistant host ECD 02 increased from 5 to 20 dai and then decreased to levels lower than the concentration of *P. brassicae* DNA in the susceptible or moderately resistant hosts. The results of our comparative Ct qPCR assay suggested that the RQs of *P. brassicae* biomass to canola of root samples taken 1 to 17 dai were in most cases not significantly different from each other in all nine pathotype–cultivar treatment combinations.

The comparatively longer time taken for *P. brassicae* biomass to be detected in the present study may be in part because of differences in the genetics of the hosts used in the different studies. The three canola cultivars used in this study (45H29, L135C, and L241C) are resistant to pathotypes 2, 3, and 5. In contrast, the canola cultivar Westar used by Feng et al. (2013a) is a universally clubroot-susceptible host, whereas the cultivar Zephyr used by McDonald et al. (2014) is resistant to pathotype 6 (mainly occurring in cruciferous vegetables in Canada) but highly susceptible to pathotype 3. In the ET, PC1, and PC2, the relative pathogen to canola biomass ratio increased steadily from 21 to 35 dai but remained mainly at nonsignificant levels in NC1 and NC2 throughout the time course of the experiment. It was not until 28 to 35 dai when there was sufficient *P. brassicae* biomass to be detected reliably by the comparative Ct qPCR method (Fig. 1). Sundelin et al. (2011) also identified 35 dai as the optimal time point at which *P. brassicae* was metabolically active both in the club and nonclubbed parts of the root, which is consistent with the findings of the current study.

In conclusion, this study found that inoculation with low concentrations of virulent *P. brassicae* isolates could prime or facilitate subsequent infection of the host by avirulent isolates. Additional studies are needed, however, to examine the impact of such inoculations on the defense response by the host. Moreover, the function of virulence genes during primary infection and the role of effectors in host defense are still unresolved and need to be investigated for a better understanding of clubroot pathogenesis.

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