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Effects of clubroot-resistant canola lines on soils infested by *Plasmodiophora brassicae*

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Introduction

Clubroot, caused by *Plasmodiophora brassicae* Woronin, poses a serious threat to the canola (*Brassica napus* L., *B. rapa* L.) industry in Alberta because of its long-lived spores, its ability to rapidly multiply, and its detrimental effect on canola yields. The pathogen induces the formation of galls on infected roots that impair growth and nutrient uptake (Dixon 2006), leading to wilting or death of the infected plants and yield losses of 30–50% or more (Wallenhammer et al., 1999; Strelkov et al., 2006). In Alberta, clubroot was first identified on canola in 2003 (Tewari et al., 2005), and ten years later the disease had spread to more than 1000 fields (Strelkov et al. 2013). The disease now presents a major threat to canola production in Alberta, and has recently been identified in the neighbouring province of Saskatchewan, suggesting that clubroot could eventually become widespread in the Canadian Prairies.

Once *P. brassicae* becomes established in a field, it builds up rapidly in the presence of susceptible crops via the formation of resting spores, which can persist in the soil for more than 15 years in the absence of a susceptible host (Wallenhammar, 1996). Therefore, crop rotations based on standard agronomic recommendations for Canada (one year of canola in four) will not significantly reduce resting spore populations (Strelkov *et al.*, 2006), and even substantially longer rotations may not be effective because several common and endemic weed species such as horseradish (*Armoracia rusticana* P.G. Gaertn., B. Mey. & Scherb, white mustard (*B. hirta* Moench), wild mustard (*B. kaber* (DC.) L.C. Wheeler), camelina/false flax (*Camelina sativa* L. Crantz), shepherd's purse (*Capsellabursa-pastoris* (L.) Medik.), and stinkweed (*Thlaspiarvense* L.) are also susceptible hosts (Howard *et al.*, 2010). Effective clubroot management in canola will largely depend on utilization of cultivar resistance and the reduction of viable resting spore populations in the soil. Ideally, the cropping of resistant cultivars should be accompanied by a reduction in the viable *P. brassicae* resting spore populations in the soil since stimulation of resting spore germination should reduce the viable spore population in the soil. However, clubroot resistant crops are not necessarily free of susceptible plants. Susceptible volunteers will continue to be present in infested fields for many years, susceptible weed species may also be present, and there may be a small percentage of susceptible canola off-types associated with hybrid production.The deployment of resistant cultivars may also create selection pressure on the pathogen to co-evolve and overcome resistance (Hwang *et al.*, 2011b; LeBoldus et al., 2012).

The life cycle of *P. brassicae* consists of two phases, a root hair infection phase, and a cortical (secondary) infection phase (Karling 1968; Tommerup and Ingram 1971). The resting spores germinate and release motile zoospores that are attracted to the root hairs. They penetrate the cell wall and form primary plasmodia. After a short interval, each primary plasmodium divides and forms numerous sporangia, which release secondary zoospores. Pairs of secondary zoospores fuse to form a dikaryon, then penetrate into root cells and form secondary plasmodia. The secondary plasmodia proliferate and induce host cellular hypertrophy and hyperplasia, leading to gall formation in the root tissues (Kageyama and Asano 2009). In resistant lines of some cruciferous species, the pathogen infects root hairs, develops primary plasmodia, and produces secondary zoospores, but seldom induces gall formation (Dekhuijzen 1979; Kroll et al. 1983; Yamagishi et al. 1986).

Rapid techniques to evaluate soil levels of *P. brassicae* inoculum are not yet available, although considerable research effort has been focussed on this area. Bait plants are used to test for the presence of viable resting spores, but testing can take up to 8 weeks and may not provide consistent results when inoculum levels are low. PCR-based techniques (standard and quantitative) have been developed, but the results can be skewed by non-viable inoculum and a range of other factors in soil (Strelkov, unpublished).Infection of the root hairs occurs much earlier than the development of visible symptoms so microscopic examination of root hair infection could provide more rapid results than bait plant assays. Samuel and Garrett (1945) and MacFarlane (1952) found a relationship between the log of the number of root hair infections observed and the log of the clubbing severity in older plants, but this correlation between root hair infection and clubroot severity has not always been clear. This may reflect host-pathogen interactions, environmental conditions or the timing of the assessment rather than the underlying relationship between root hair infection and spore density. Therefore, we examined the effects of cultivar resistance, inoculum density and seedling age on root hair infection in canola, with a view to developing additional methods for estimating resting spore densities in the soil. In addition, the relationship of root hair infection to clubroot disease severity, plant growth parameters and seed yield in canola was examined.

As with most diseases, clubroot development is regulated by the dynamic interaction of the host, the pathogen, and the environment (Browning et al. 1977; Crute 1986; Williamson 1987; Wallenhammar 1996). The influence of seedling age and inoculum density have been examined in Brassica vegetable

crops (Horiuchi and Hori 1980; Timila et al. 2008), there is little or no data available regarding their impact on the growth and yield of canola and other oilseed crops. To better understand this interaction in canola, the effect of seedling age and inoculum density on clubroot severity, plant height, and seed yield were investigated.

The objectives of this research are to study the effects of resistant crops, non-host crop rotations, repeated growth of a resistant cultivar, and mixtures of resistant and susceptible cultivars on soil pathogen population levels, vulnerability of roots to infection, and on the severity of clubroot in subsequent susceptible crops.

Materials and Methods

A. Effects of growing resistant cultivars on clubroot severity in subsequent crops

The canola cultivars 45H29 (resistant) and 45H26 (susceptible) were grown in soil-less mix (Sunshine Mix 4; pH 6.5; SUN GRO® Horticulture Canada Ltd., Seba Beach, AB) in plastic containers (35 cm × 23 cm ×13.5 cm), each containing 10 L of soilless mix, under greenhouse conditions (20° ± 2° C). The soilless mix was inoculated with *P. brassicae* resting spores collected from root galls and adjusted to a concentration of 1 x 10^6 spores/mL of soilless mix. Four rows of 25 seeds each were seeded in each container. The containers were placed on water-filled trays (water pH 6.5) for the first two weeks after seeding, in order to ensure high soil moisture, and were then watered as needed for four additional weeks, for a total growing period of six weeks. The plants were fertilized with a 0.1% solution of NPK (20-20-20) once a week (commencing at two weeks after seeding) until the end of each cycle of plantings. A fallow treatment (a container filled with clubroot-inoculated soilless mix but not seeded with any crop) was maintained alongside the canola-seeded treatments.

At the end of the 6-wk growth period, all canola plants were uprooted, the roots of the plants were macerated in water, filtered through eight layers of cheese cloth and re-incorporated back into the soil from which the plants came, after adjusting the water volume to 250 mL for each container. The water was poured evenly over the soil surface and then the spore suspension was thoroughly mixed throughout the soil in the container using a garden trowel. The soil was allowed to dry for 7 days. A new crop of the same cultivar was replanted into this soil. After three successive 6-wk cycles (Resistant-Resistant-Resistant; RRR or Susceptible-Susceptible-Susceptible; SSS), the same susceptible canola cultivar was grown in the soil from both cropping sequences, as well as in the soil used for fallow treatments (FFF). The inoculum potential in the re-inoculated soil-less mix was estimated by assessing root weight, plant height, clubroot incidence and severity, and by counting resting spore populations in the soil-less mix after each cycle. Disease severity was assessed on a 0–3 scale, where 0 = no galls, 1 = a few small galls, 2 = moderate galling and 3 = severe galling (Kuginuki et al. 1999). Severity ratings for each experimental unit were converted to an index of disease (ID) using the formula of (Strelkov et al. 2006):

$$ID\ (\%) = \frac{\Sigma\ (n_0 \times 0 + n_1 \times 1 + n_2 \times 2 + n_3 \times 3)}{N \times 3} \times 100 \qquad \text{(Eq.1)}$$

Where: n_0, n_1, n_2 and n_3 are the number of plants in each class, 0, 1, 2 and 3 are the symptom severity classes, and N is the total number of plants.

The experiments were conducted under greenhouse conditions with a 16-h photoperiod (20 ± 2° C day/16 ± 2° C nights). The experiment was set up in a RCB design with six replications. The experiment was conducted twice.

Isolation of resting spores from soil and DNA extraction

The inoculum potential was estimated by counting resting spores in the soil-less mix after each cropping cycle with a haemocytometer under a microscope. The resting spore extraction procedure was adopted from the method of Castlebury et al. (1994) as modified by Hwang et al. (2011a). Briefly, soil-less mix samples were collected after completion of each cropping cycle. Five cores of soil-less mix samples from each replicate tray of each of the treatments were collected with a cork borer (13mm dia.) and air-dried at room temperature (20 ± 2°C), thoroughly mixed and stored in a cold room (5°C) until use. For the extraction of resting spores, 0.5 g of soil-less mix sample from each replication of a treatment was added to 20 mL of water and mixed for 1 minute in a laboratory blender (Waring Commercial, Torrington, Connecticut, USA). The slurry was filtered through eight layers of cheese cloth. A 12 mL aliquot of the suspension was centrifuged at 3900 g for 15 minutes and the supernatant was discarded. The spores were suspended in 6 mL of 50% (w/v) sucrose solution, agitated in a vortex mixer for 2 minutes, and then centrifuged for 5 minutes at 1700 g. The supernatant was transferred into a 50 mL tube, and water was added to 45 mL, mixed with a vortex mixer and centrifuged at 3900 g for 15 minutes. The supernatant was discarded and the pellet was mixed with 5 mL of water using a vortex mixer, centrifuged at 3900 g for 15 minutes. The supernatant was again discarded and the pellet was suspended in 2 mL of water and refrigerated at -4°C until used for counting.

Prior to analysis of variance, all of the data sets were tested for homogeneity of variance using a normal probability plot. Analysis of variance of the data was performed separately for each cropping cycle using the GLM procedure of SAS (SAS Inc., Cary, NC, USA). Since there was no significant effect of repetition, the data from the two repeats of each cycle of cropping were combined for subsequent analyses. The treatment effects were compared using a protected LSD test at $P \leq 0.05$. In addition, repeated measures analysis of variance was performed to determine whether the changes in the

root mass, plant height, disease severity and resting spore populations resulting from the cropping cycles were significantly different.

An experiment to determine the effect of cultivar resistance and plant age on root hair infection was designed as a randomized complete block with five replicates. Five hybrid canola cultivars [resistant cultivars '45H29' and '73-77RR'; susceptible cultivars '34-65RR' (Monsanto), '45H26' and '45H73'(Pioneer Hi-Bred)] were grown planted in soilless mix in 9-cm-diam. (450 mL) cups (10 seeds/cup). After seeding, the cups were placed in water-filled trays to ensure high soil moisture. At each sampling date (4, 6, 8 and 10 days after sowing), all the seedlings per replicate cup were uprooted and washed gently with tap water. Immediately after harvest, half of the plants were fixed in FAA (5 mL of formalin, 5 mL of acetic acid, 90 mL of 50% ethanol), and maintained at room temperature (20 ± 2° C) until needed. The remainder of the seedlings were stored at -20° C for qPCR analysis. Root hair infection was assessed using the protocol described above.

A parallel experiment was conducted with inoculated and non-inoculated seedlings as the main plot treatments and canola cultivars as sub-plots. The plants were grown for 6 weeks, and plant height, root weight, and clubroot severity (0–3 scale, converted to ID) were assessed. Both experiments were conducted twice.

The amount of clubroot infection was also estimated using qPCR analysis of the roots. Roots of each sample were gently washed under tap water. Prior to DNA extraction, fresh root samples were washed with sterilized water and dried with a sterilized paper towel, and 200 mg subsamples were used for DNA extraction with a Fast DNA Spin Kit (Qbiogene Inc., Irvine, CA) as per the manufacturer's instructions. Quantitative PCR analysis was performed on undiluted DNA extracted from the root samples. Two *P. brassicae*-specific primer and TaqMan probe sets were used in the qPCR analysis. All qPCR amplifications were conducted using a StepOnePlus Real Time PCR System (Applied Biosystems, Warrington, UK) in a 20 μL volume containing 1.0 μL of template DNA solution, 10 μM of each primer, 2 μM of MGB-Probe and 10 μL TaqMan® Fast Universal PCR Master Mix (Applied Biosystems). All reactions were run at 95°C for 20 s, followed by 40 cycles at 95°C for 1 s and at 60°C for 20 s.

The homogeneity of variance of each data set was examined using a normal probability plot. Analysis of variance (GLM procedure, SAS) indicated a cultivar × sampling date interaction for both root hair infection and the amount *P. brassicae* DNA in infected roots, so the data were analyzed

separately by sampling date. Means comparison within each sampling date was performed using a LSMEAN T-test. Regression analysis was used to quantify the relationship between root hair infection and the amount of *P. brassicae* DNA in the infected roots. For this purpose, DNA and root hair infection data for each sampling date were pooled across canola cultivars. In addition, these data were also pooled for each cultivar across the sampling dates.

B. Effects of volunteer canola on clubroot severity in subsequent susceptible crops

The hybrid canola cultivars '45H29' (clubroot-resistant) and '45H26'(clubroot-susceptible) (Pioneer Hi-Bred, Caledon, ON, Canada) were grown together to simulate various proportions of susceptible plants (volunteer canola or *Brassica* weeds) growing within a stand of a clubroot-resistant canola cultivar. Seed of the susceptible and resistant cultivars were mixed together in ratios of 1:0, 3:1, 1:1, 1:3, and 0:1, respectively, and then four rows of 25 seeds each were planted in each experimental unit under greenhouse conditions (20 ± 2° C day/16 ± 2° C night, 16-h photoperiod). Each experimental unit consisted of a plastic tub (35 cm × 23 cm × 13.5 cm depth, with drainage holes) filled with soil-less mix consisting of peat moss, coarse perlite, gypsum, dolomitic limestone and starter nutrient (Sunshine Mix 4; pH 6.5; SUN GRO® Horticulture Canada Ltd., Seba Beach, AB). The soil-less mix was inoculated with resting spores of *P. brassicae* isolate SACAN-ss1, which was isolated by Xue et al. (2008) and characterized as pathotype 3 on the differentials of Williams (1966). The pathogen was maintained as frozen root galls, and the resting spores were extracted as previously described (Strelkov et al. 2006). The spore concentration was adjusted to 1×10^6 spores mL^{-1} of soil-less mix. After seeding, each tub was watered to saturation (water pH 6.5) and placed in a water-filled tray to maintain high soil moisture for the first 2 weeks, after which the plants were watered using an overhead sprinkler once each day for 4 weeks. At 6 weeks after seeding, the plants were pulled, the roots were washed under tap water, and data on plant height, root weight, and clubroot incidence and severity were assessed for 50 randomly chosen plants from each replication of a treatment, in order to provide an estimate of inoculum potential. Clubroot severity was assessed on a 0–3 scale, where: 0 = no galls, 1 = a few small galls (small galls on less than one-third of the roots), 2 = moderate galling (small to medium galls on one-third to two-thirds of the roots), and 3 = severe galling (medium to large galls on more than two-thirds of the roots) (Kuginuki et al. 1999). Severity ratings for each experimental unit were converted to an index of disease (ID) as described previously.

After recording the data, all of the roots representing a replication were pooled and macerated in a blender, and the slurry was filtered through eight layers of cheesecloth to separate the resting spores of *P. brassicae* from the plant material. The resting spores from each tub were returned to the growth substrate in that tub and mixed thoroughly into the substrate. The substrate was then sown with 100% clubroot susceptible canola ('45H26'). Post-seeding agronomic practices followed the procedure described earlier. Six weeks after seeding, data on plant height, clubroot incidence and severity were recorded. The experiment was laid out in a randomized complete block design (RCBD) with six replications, and the study was repeated once.

In a second experiment, the effect of comparatively low proportions (0, 3, 5, 7 and 10%) of susceptible plants ('45H26') in a stand of a resistant canola cultivar ('7145', Monsanto, Winnipeg, MB, Canada) was assessed. The experimental design, growing conditions, and agronomic practices were the same as in the initial experiment, except that the seeds of the resistant and susceptible cultivars were not mixed together. Instead, seeds of the susceptible cultivar were distributed at random in the four rows of 25 seeds, and marked with plastic tags to identify where seeds of the susceptible cultivar were sown. After 6 weeks, plant height, clubroot incidence and severity were recorded separately for plants of the resistant and susceptible cultivars. All of the roots of the susceptible plants (maximum of 3 – 10 roots, depending on the proportion of susceptible plants in each treatment) were collected. To make the number of roots equal (25 roots) for each replication, an additional 15 – 22 roots were randomly chosen from the resistant plants.

These 25 canola roots were macerated and filtered as described previously. The volume of the suspension for each tub was adjusted to 250 mL and the number of resting spores was estimated with a haemocytometer. The resting spores were returned to each tub, which was then planted with seed of the susceptible cultivar. Ten days after seeding, five seedlings per tub were uprooted and the roots were washed gently and then evaluated microscopically for root hair infection.

A 1.5-cm-long segment of the taproot at 2-3 cm below the hypocotyl (Murakami et al. 2000) was cut from two seedlings per replication and mounted on a slide with 5% (v/v) glycerine in water. The incidence of root hair infection in the mounted specimens was then examined with a compound

microscope at 100× magnification. A root hair was regarded as infected when a primary plasmodium could be discerned within it. At least 100 root hairs were examined per sample (Hwang et al. 2011a). In addition to root hair infection, secondary plasmodia were also counted below the epidermis and in the cortex of the roots, which were rated on a 1-5 scale, where 1 = few secondary plasmodia and 5= profuse secondary plasmodia. The remaining plants were allowed to grow to maturity. Plant height and clubroot severity were assessed at the flowering stage. The study was repeated once.

C. Effects of crop rotation using resistant canola on clubroot severity

Mini-plots were constructed, filled with naturally infested field soil collected near Edmonton, previously sown to canola (2010). Ten mini-plots were used for each of the six following treatments and were distributed on a concrete pad outside a greenhouse at CDC North in a randomized complete block. In 2011, treatments consisted of a) susceptible canola cultivar 45H26, b) pea, c, d and e) one barley and two oat treatments and f) fallow. In 2012, treatments consisted of a) susceptible canola cultivar 45H26, b and d) wheat, c and e) pea and f) fallow. In the 2013, all plots were sown to a susceptible variety of canola. Seedling emergence, fresh shoot and root weight, plant height, clubroot severity and yield were compared among the treatments after harvest in 2013.

A second experiment was conducted using 12 replicate mini-plots per treatment filled with naturally infested field soil. In 2010, Treatments were: a, b and c) Resistant canola cultivar 45H29 and d) barley. In 2011, the treatments were a and b) Resistant canola cultivar 45H29; c and d) barley. In 2012, the treatments were a) Resistant canola cultivar 45H29; b, c and d) barley. In 2013 all treatments were sown with susceptible cultivar 45H26. Clubroot severity were evaluated at the end of the growing season.

D. Effects of P. brassicae population density on clubroot level in canola

A trial was conducted under greenhouse conditions at the Crop Diversification Centre North. Canola cv. '34 SS 65' was seeded every week for 4 weeks into 450-mL Jiffy® cups (2 seeds/cup) filled with soil-less potting mixture to generate young plants of 1, 2, 3 and 4 weeks of age. The plants were thinned to one plant per cup after seedling establishment. In the fifth week, clubroot-infested soil (pH 6.73) collected from a commercial field near Leduc, AB, was mixed with sterilized soil to produce the inoculum density treatments, based on 1:0, 1:1, 1:2, and 0:1 ratios (v:v) of infested to sterile soil. The soil mixtures were distributed into 26-cm-diam. pots and seedlings that were 1, 2, 3 and 4 weeks of age

were transplanted into the pots (1 seedling/pot). Prior to transplanting, as much as possible of the soilless-mix associated with the plant roots was gently removed. Ungerminated seeds, representing a fifth seeding date, were planted directly into the pots at a rate of one seed per pot. The pots were maintained in a tray filled with water adjusted to pH 6.5 at the bottom for the first 2 weeks after planting. They were then transferred to a greenhouse bench, where they were watered (pH 6.5) daily from above to maintain a high level of soil moisture. Seven replicate pots for each seedling age were placed on the greenhouse bench in a split-plot design, where inoculum density was the main plot treatment and the sub-plots were seedling age. Trays of the different inoculum concentrations were separated by at least 30 cm to avoid cross-contamination during watering. The plants were fertilized once per week until flowering with a 0.1% solution of 20:20:20 (N: P: K). Plant height was assessed after flowering, while clubroot severity was assessed at maturity on a 0-3 scale, where: 0 = no galling, 1 = a few small galls, 2 = moderate galling and 3 = severe galling (Kuginuki et al. 1999). Seed yield was also recorded at this time. The experiment was conducted twice.

In a second greenhouse trial, soil from a field with high populations of resting spores was diluted with a soil-less mix as previously described to produce 0, 2, 4, 9, 17, 25, 33, and 50% of the resting spores present in the original infested soil. The soil mixtures were placed into ten pots (13-cm-diam.) per treatment, and five seeds of canola cv. '34 SS 65' were sown per pot. The pots were placed on trays with water on a greenhouse bench as described above, and arranged in a randomized complete block design with ten pots per treatment. Half of the replications were uprooted 6 weeks after sowing, and individual plants from each pot were scored for clubroot severity (0-3 scale) and plant height. The remaining replications were grown to maturity, and seed yield was recorded. The experiment was conducted twice.

To relate the results from the soil dilution experiments to specific spore densities, a trial was conducted using inoculated soil-less potting mixture. The soil-less mix was inoculated with a resting spore suspension prepared from air-dried canola galls as described by Strelkov et al. (2006), and adjusted to a concentration of 1×10^9 spores cm^{-3}. Aliquots of the spore suspension were added to the potting mix to produce final resting spore densities of 0, 1×10^5, 1×10^6, 1×10^7, and 1×10^8 spores cm^{-3}. The study was conducted in a greenhouse, as described previously, and arranged in a randomized complete block design with seven replications. Each treatment consisted of seven pots (replicates) of inoculated soil-less mix with five seeds of '34 SS 65' per pot. After 6 weeks, four pots of each treatment

were assessed for clubroot severity and plant height. The remaining pots were maintained until maturity to evaluate seed yield. The experiment was conducted twice.

E. Relationship between clubroot severity and yield

To determine the relationship between root hair infection and clubroot severity, canola growth and seed yield, a greenhouse trial was conducted using the infested soil dilution treatments described above; this experiment was carried out concurrently and in the same greenhouse as the soil dilution trial described above. Treatments were arranged in a factorial randomized complete block design, with the two cultivars as main plot treatments, soil dilutions as sub-plots, and five replications (cups). The plants were maintained under the same conditions as the soil dilution trial, except that they were fertilized with a 0.1% solution of NPK (20-20-20) once a week until pod filling. Seedling emergence was counted 7 days after sowing. The height of each plant was measured at the end of flowering. Each plant was assessed at harvest for clubroot symptom severity and seed yield. Disease severity was assessed on a 0–3 scale, where 0 = no galls, 1 = a few small galls, 2 = moderate galling and 3 = severe galling (Kuginuki et al. 1999). Severity ratings for each experimental unit were converted to an index of disease (ID) using the formula described previously (Strelkov et al. 2006).

F. Inoculum production and root hair infection

Inoculum was prepared from clubroot galls collected from several heavily infected canola crops near Edmonton, AB. The populations in these fields had been shown in previous research (Strelkov et al. 2006) to consist mainly of pathotype 3, as classified on the differentials of Williams (1966), or as ECD 16/15/12, as classified on the differentials of the European Clubroot Differential set (Buczacki et al. 1975). The galls were air dried and then ground to a fine powder in a blender. The concentration of resting spores in the powdered inoculum was estimated with a haemocytometer. The inoculum was added to a soil-less potting mix consisting of peat moss, coarse perlite, gypsum, dolomitic limestone and starter nutrient (Sunshine Mix 4; pH 6.5; SUN GRO® Horticulture Canada Ltd., Seba Beach, AB) to produce a range of spore densities.

The treatments consisted of factorial combinations of four spore concentrations (0, 1×10^3, 1×10^5, and 1×10^7 spores per mL of mix), the hybrid canola cultivars Pioneer ‘45H26’ (susceptible) and

Pioneer ‘45H29’ (resistant), and six sampling dates (4, 6, 8, 10, 12, and 14 days after sowing). Each experimental unit consisted of a 9-cm-diam. cup filled with 400 mL of infested soil-less mix and sown to canola at a density of five seeds per cup. After seeding, the cups were placed in water-filled trays to ensure high soil moisture. The study was conducted under greenhouse conditions (20 ± 2° C day/16 ± 2° C night) with a 16-h photoperiod At each sampling date, the seedlings were uprooted, washed gently with tap water, fixed in FAA (5 mL of formalin, 5 mL of acetic acid, 90 mL of 50% ethanol), and maintained at room temperature (20 ± 2° C) until needed.

A 1.5-cm-long segment was cut 2-3 cm below the hypocotyl (Murakami et.al. 2000) from the roots of two seedlings per replication and mounted on a slide with 5% (v/v) glycerin in water. The incidence of root hair infection in the mounted specimens was then examined using a compound microscope at 100× magnification. A root hair was rated as infected when a primary plasmodium could be seen within it. At least 100 root hairs were counted per sample. The experiment was conducted twice.

G. Contribution of clubroot galls to soil resting spore populations

Mini-plots consisting of plastic tubs (50 x 35 x 22 cm) were filled with 30 L of a black chernozemic soil that was naturally infested with *P. brassicae* (approximately 1 x 10^8 resting spores g^{-1}), collected from a commercial field, and diluted with soil-less mix (Sunshine Mix 4; pH 6.5; SUN GRO® Horticulture Canada Ltd., Seba Beach, AB) at ratios of 1:100, 1:50, 1:25, 1:10, 1:5, 1:3 and 1:2, resulting in an estimated resting spore gradient of 9.0 x 10^5, 1.9 x 10^6, 9.0 x 10^6, 1.6 x 10^7, 2.5 x 10^7, 3.3 x 10^7 and 5 x 10^7 spores mL^{-1}. Each tub was sown with a total of 40 seeds of the susceptible canola cultivar ‘45H26’ (Pioneer Hi-Bred, Caledon, ON), in eight rows across each tub. The tubs were placed outdoors at a field site near Edmonton, AB in a randomized complete block design (RCBD) with six replicates (tubs) on June 28, 2011. Irrigation was provided as required with an overhead sprinkler, in order to provide sufficient moisture for plant growth and clubroot development. Six weeks after planting, clubbed roots (galls) were collected from 10 of the 40 plants and weighed. In order to determine the amount of resting spores contributed to the soil, the remaining plants were harvested on September 21 (approximately 12 wk after planting), when the crop was fully mature and the galls were beginning to disintegrate. A 1 g aliquot of the gall from each replicate was placed in 20 mL of water and macerated in a laboratory blender. The resulting suspension was then sieved through eight layers of cheese cloth, and the resting spores were quantified with a haemocytometer under a microscope. Three

slides were observed for each replicate. The data were converted to resting spores contributed per mL of soil mix prior to conducting any statistical analysis. The experiment was conducted twice. Analysis of variance of the data was performed to determine the effect of inoculum concentration on gall mass and the amount of resting spores contributed to the soil after one crop of susceptible canola. Means separation was conducted using Duncan's Multiple Range Test ($P \leq 0.05$). In addition, regression analysis was performed to examine the relationships among the initial resting spore concentration in the soil, gall mass and the amount of resting spores contributed by one crop of susceptible canola.

Clubroot-resistant ('45H29') and susceptible ('45H26') canola cultivars were grown in a field near Edmonton, AB that was heavily infested with *P. brassicae* resting spores (1×10^8 spores g^{-1}). A single-factor experiment was set up in a randomized complete block (RCB) design with four replications. When the crop had matured and just prior to when the clubbed roots (galls) began to decay, on August 16, 20 plants in each plot were uprooted, washed gently under tap water, and the fresh weight of the galls was recorded. A small aliquot (1 g) of galls from each plot was macerated in a blender for 3–5 minutes and sieved through eight layers of cheesecloth. The resting spore concentration of the resulting suspension was estimated with a haemocytometer under a microscope, in order to determine the number of inoculum units contributed to the soil. The experiment was repeated in a separate field, also near Edmonton, AB.

Results

Effects of growing resistant cultivars on clubroot severity in subsequent crops and resting spore population

As expected, clubroot incidence and severity were higher in the susceptible than in the resistant cultivar. In the initial cycle of planting, plants grown in soil planted to the resistant cultivar were 25% taller but the root mass was about 400% greater in the susceptible cultivar compared to the resistant cultivar. Root mass was correlated to index of disease (ID) values calculated from clubroot severity. Root fresh weight, disease severity, disease incidence, and index of disease all declined with each successive cycle of planting for both resistant and susceptible cultivars. Root weight and the disease parameters were greater for the susceptible cultivar compared to the resistant cultivar in all three cycles. Plant height was greater for the resistant cultivar compared to the susceptible cultivar for the first cycle, but the height of plants was similar for the two cultivars in the second and third cycles.

In the assessment at the end of the cropping sequences, plants were smaller and root mass was larger following the SSS cropping sequence than following the RRR sequence, but plants were tallest and had the smallest root mass following the FFF sequence (Figure 1). The large root mass in the susceptible cultivar resulted from gall formation. At the end of the final assessment phase (fourth cropping cycle) with the susceptible cultivar, clubroot severity was 10-fold greater in the SSSS cropping sequence relative to the RRRS sequence, but there was no difference in severity between the RRRS and FFFS sequences (Figure 2)

In the qPCR analysis of the infested soilless mix after the first cycle of resistant and susceptible canola, the estimated resting spore number and Ct value and amount of *P. brassicae* were significantly greater with susceptible canola compared to the resistant canola and the unplanted control (Figure 3). The Ct values in the qPCR analysis ranged from 19.59 - 20.86 for the treatment in which a susceptible cultivar was grown and 23.57 - 26.49 for the treatment in which a resistant cultivar was grown; Ct values ranged from 24.73 - 25.92 in the control treatment. The soil resting spore concentration was higher following the SSSS sequence than the RRRS or FFFS sequences. The resting spore concentration increased after each cycle of cropping of the susceptible canola cultivar (S, SS, SSS and SSSS), but decreased in the FFFS and RRRS sequences.

The analysis of variance of the experiment on plant age and cultivar resistance indicated significant sampling date effects, cultivar effects, and a sampling × cultivar interaction for root hair infection and the amount of DNA extracted from clubroot-infected root hairs. Root hair infection was always significantly higher in the susceptible canola cultivars ('45H26', '34-65RR' and '45H73') than in the resistant cultivars ('45H29' and '73-77RR') at all sampling dates (Figure 4). The trend was similar for the DNA extracted from the root samples with the exception of one time-point; at 4 days, no significant differences in the amount of DNA were observed. Root hair infection and *P. brassicae* DNA concentration increased over time. However, increases in root hair infection and the amount of DNA extracted were greater in the susceptible cultivars relative to the resistant cultivars.

Regression analysis of root hair infection and the amount of pathogen DNA revealed a strong linear relationship between these two variables at all sampling dates (Figure 5). The r^2 values for the regressions were 80, 82, 90 and 99%, respectively, at 4, 6, 8 and 10 days. When regression analysis of root hair infection and *P. brassicae* DNA was conducted for each cultivar over the sampling dates, the linear relationships for the resistant cultivars ('45H29', r^2= 93%; and '73-77RR', r^2= 81%) was strongest. In contrast, the linear relationships between root hair infection and the amount of pathogen DNA in the susceptible cultivars was somewhat lower, with r^2 values of 71, 73 and 76% for '45H73', '34-64RR' and '45H26', respectively (Figure 6). Moreover, in the resistant cultivars, the increase in root hair infection and amount of pathogen DNA rose slowly and gradually and stabilized at 8 and10 days. In the susceptible cultivars, the increase in both root hair infection and amount of DNA rose sharply in two steps, first at 4-6 days and then at 8-10 days. Regression of the pooled data over the sampling dates and cultivars revealed a weak linear relationship between root hair infection and amount of DNA in *P. brassicae* infected roots (r^2 = 64%).

Results from the experiment to assess the effect of resistance on plant growth parameters and clubroot severity indicated that the height of both resistant and susceptible plants was reduced after inoculation with the pathogen, although this reduction was not significant for the susceptible cultivar '45H26'. The greatest reduction in plant height was observed for the susceptible cultivars '45H73' (58%) and '34-65RR' (37%). In both the resistant and susceptible canola cultivars, the root mass increased after inoculation with *P. brassicae*. However, in the susceptible cultivars, this increase was significantly higher than in the resistant cultivars (Figure 6). As expected, ID in the resistant cultivars was lower than

in the susceptible cultivars. Among the susceptible cultivars, ID was greater on '34-65RR' and '45H73' than on '45H26'.

Effects of volunteer canola on clubroot severity in subsequent susceptible crops

The fresh root weight of the plants increased with the proportion of susceptible canola plants. However, no significant difference in root mass was observed when the proportions of the susceptible cultivar were 25 to 50% (Figure 7). Plant height decreased and root weight, clubroot incidence and severity increased with increasing proportions of susceptible cultivar in the mixed populations. Fresh root weight, disease severity and disease incidence were significantly greater, and the plant height was the shortest in the soil where 100% susceptible cultivar was grown. These parameters generally trended in the same direction for the lower proportions of susceptible cultivars.

The study with lower proportions of susceptible canola indicated that the height of both resistant and susceptible canola plants was reduced relative to the control when inoculated with the clubroot pathogen (Table 1). The susceptible canola was normally shorter than the resistant canola, even in the absence of inoculation. There was no effect of treatment on clubroot severity in the susceptible cultivar grown in any proportion with the resistant cultivar. As expected, the total resting spore count in the clubroot galls was greater after growth of a higher proportion of susceptible plants (Table 2). Root hair infection in the successively grown susceptible cultivar also rose in treatments with a greater proportion of susceptible plants. Rates of secondary infection below the epidermis and in the cortex, as estimated by the number of secondary plasmodia, were also greater in the in treatments in which a higher proportion of susceptible plants were grown.

Effects of crop rotation using resistant canola on clubroot severity

Canola stand establishment was lower when preceded by three years of canola compared to all rotations except the Canola-Barley-Pea-Canola rotation (Table 3). The canola-barley- pea-canola rotation produced greater plant height compared to the other rotation treatments except the canola-fallow-fallow-canola and the canola-oat-pea-canola rotations; plant height was lower for continuous canola compared to all other treatments. Fresh shoot weights were greater and disease severity was lower for all other

treatments compared to continuous canola; fresh root weights were greater compared to continuous canola for all treatments except canola-fallow-fallow-canola.

In the second experiment, four years of continuous canola resulted in greater clubroot severity compared to any other rotation treatment (Table 4). Where a susceptible crop was preceded by three non-host crops, yield, plant height and shoot weight were greater and disease severity was lower compared to the same crop preceded by one, two or three resistant crops. Fresh root weight was lower where the susceptible crop was preceded by three years of non-host crops or three years of resistant crops compared to a mix of non-host and resistant crops. Clubroot severity was greater where the susceptible crop was preceded by three years of resistant crops, compared to a mixture of resistant and non-host crops or to three years of non-host crops. The canola-oat-pea-canola rotation produced a greater seed yield compared to any other treatment.

Effects of soil population density on clubroot of canola

As expected, canola plants grown in sterilized soil did not develop clubroot galls, grew taller, and produced more seed than those that were exposed to the pathogen. Inoculum density across the range of dilutions assessed (infested: sterilized soil at 1:0 to 1:2) had little or no impact on clubroot symptom severity or plant height, but seed yield declined as inoculum density increased (Figure 8). Plants seeded in infested soil undiluted with sterilized soil ripened prematurely and had lower yields than plants seeded in infested soil diluted with sterilized soil, irrespective of plant age.

Inoculum density across the range of dilutions assessed (infested: sterilized soil at 1:0 to 1:2) had little or no impact on clubroot symptom severity or plant height, but seed yield declined as inoculum density increased. Plants seeded in infested soil undiluted with sterilized soil ripened prematurely and had lower yields than other dilutions, irrespective of plant age. Plant height at 50% inoculum density (1 part infested soil: 1 part soil-less mix) was reduced by 56% relative to plant height at 2% inoculum density (1 part infested soil: 49 parts soil-less mix). Regression analysis indicated a strong linear and negative relationship between inoculum density and plant height. The maximum possible clubroot severity (ID of 100%) was obtained at 50% inoculum density; severity decreased as inoculum density declined and no clubroot symptoms were observed at 2% inoculum density. Seed yield declined dramatically with

increases in inoculum density from 2 to 10%, and dropped to zero at 50% density. Likewise, seed yield decreased with increased clubroot severity and reached zero at an ID of 100%.

Clubroot severity decreased ($P \leq 0.01$) as seedling age (at first exposure to the pathogen) increased (Figure 9). Symptoms were most severe in plants grown from seed planted directly into infested soil and in plants transplanted to infested soil as 1-week-old seedlings. Symptoms were intermediate in plants transplanted as 2-week-old seedlings and lowest in those transplanted as 3- and 4-week-old seedlings. Similarly, seed yield increased ($P \leq 0.01$) as seedling age at first exposure to the pathogen increased. Plant height was low for plants grown from seed sown directly into infested soil, higher in plants transplanted as 1-week-old seedlings, and intermediate for 2- to 4-week-old seedlings.

In the second greenhouse trial, the impact of inoculum density on clubroot severity, plant height, and seed yield was assessed using naturally infested soil diluted with soil-less mix as in the previous experiment (Figure 9). Plant height at 50% inoculum density (1 part infested soil: 1 part soil-less mix) was reduced by 56% relative to plant height at 2% inoculum density (1 part infested soil: 49 parts soil-less mix). Regression analysis indicated a strong linear and negative relationship between inoculum density and plant height ($y= 0.5888x + 58.356$; $R2= 0.90$). The maximum possible clubroot severity (ID of 100%) was obtained at 50% inoculum density; severity decreased with declining inoculum density ($y= 34.067\text{Ln}(x) – 29.936$; $R2= 0.95$) and no clubroot symptoms were observed at 2% inoculum density. Seed yield declined dramatically with increasing inoculum density from 2 to 10%, and dropped to zero at 50% density ($y= -0.6866\ \text{Ln}(x) + 2.584$; $R2= 0.89$). Plant height was 53% lower at an ID of 100% relative to an ID of 0%. Regression analysis revealed that plant height decreased with increasing clubroot severity, and the equation that best explained the model was $y= -0.005x2 + 0.2408x + 55.98$; $R2= 0.82$. Likewise, seed yield decreased with increasing clubroot severity and reached zero at an ID of 100% ($y= 0.0001x2- 0.0331x + 2.0361$; $R2= 0.92$).

In the study where known concentrations of resting spores were added to soil-less mix, plant height was not substantially reduced at 1 × 105 spores cm-3, but declined with further increases in spore density (Table 5). For example, there was a 47% reduction in height relative to the non-inoculated control at 1 × 108 resting spores cm-3. Clubroot severity increased as inoculum density increased($y= 0.3792\ \text{Ln}(x) + 0.044$; $R2= 0.99$), while plant height ($y= 0.6629- \text{Ln}(x2) + 1.5038\ \text{Ln}(x) + 64.028$; $R2=0.98$) decreased

with increasing inoculum density, although seed yield was not significantly different among the inoculated treatments. A similar trend of relationships was observed between ID (%) and plant height and between ID and seed yield, as in the previous trial.

Relationship between clubroot severity and yield

Analysis of variance indicated a significant effect of cultivar, inoculum density and cultivar × inoculum density interactions on seedling emergence, plant height, and clubroot index of disease and seed yield (Figure 10). Seedling emergence, plant height and seed yield were all higher in the resistant cultivar than in the susceptible cultivar, while clubroot severity was higher in the susceptible cultivar. Seedling emergence was reduced 3- to 4-fold in undiluted infested soil relative to treatments in which the infested soil had been diluted with uninfested potting mixture. In the susceptible cultivar, plant height and seed yield were reduced at every dilution relative to the control. In the resistant cultivar, only trace levels of clubroot, characterized by the rare occurrence of extremely small galls, were detected, and only in the 100% infested soil. Plant height, seedling emergence and seed yield often decreased numerically as the proportion of infested soil increased. Except for plant height, there were no significant differences in plants grown in undiluted soil and in a 1:1 dilution of infested soil. Regression analysis indicated a negative exponential relationship between inoculum density and seedling emergence ($y = 56.5*e^{-0.011x}$; $R^2 = 0.83$), plant height ($y = 104*e^{-0.01x}$; $R^2 = 0.96$) and yield per pot($y=2.7*e)^{-0.86x}$; $R^2 = 0.94$), and a cumulative distribution response between inoculum density and ID ($y =100-(0.379*e)^{-90.9x}$; $R^2 = 0.99$).

Root hair infection

Root hair infection was observed in both the resistant and the susceptible cultivar, although infection rates were significantly higher in the latter (Figure 11). Primary plasmodia were visible in the root hairs, while secondary plasmodia could be seen the inner and outer root cortex. A plasmodium-like structure was also visible just below the root epidermis of the susceptible cultivar. Some swollen cells could be seen emerging through the root epidermis of this cultivar prior to the formation of young galls.

Root infection rates in artificially infested potting mixture

The susceptible cultivar exhibited a higher level of root colonization and a different response to inoculum density than the resistant cultivar (Figure 12). Analysis of variance indicated significant effects of cultivar and inoculum density. The cultivar × inoculum density interaction was highly significant ($P \leq 0.01$). No root hair colonization was observed in either cultivar at 4 days after seeding. Root hair infection (formation of plasmodia) was observed at 6 days after seeding in both the resistant and susceptible cultivars (Figure 13). Significantly greater root hair colonization was observed in the susceptible cultivar relative to the resistant cultivar, at all inoculum densities tested. Root hair colonization in the susceptible cultivar was 2-3-fold higher than in the control. Nonetheless, root hair colonization increased with increasing inoculum density in both the resistant and the susceptible cultivar.

The root hair infection rate in the resistant cultivar increased over time from day 6 until day 12. By contrast, root hair infection in the susceptible cultivar was highest early in the sampling period (day 6 or 8) and declined over time. Root hair infection was significantly higher in the susceptible versus the resistant cultivar at all time-points except for day 12.

Infection rates in naturally infested soils

Root hair infection was detectable at 6 days after seeding. In the 11% dilution, the infection rates in both the resistant and susceptible cultivars were not significantly different ($P \leq 0.05$). When plants were grown in 50 and 100% infested soil, a significant difference was observed between the resistant and susceptible cultivars, with a higher rate of root hair infection observed on the susceptible cultivar at 6 days (Figure 14). The infection rate was still significantly greater in the susceptible cultivar at 10 days, but at 12 and 14 days, it was very similar in both cultivars (Figure 15). In general, root hair infection on the resistant cultivar increased slowly from 6 - 12 days and declined slightly at 14 days, while in the susceptible cultivar the infection rate declined throughout the course of the experiments. Regression analysis revealed a strongly positive cumulative distribution response between root hair colonization and ID in the susceptible canola cultivar '45H26' ($y= 100-(0.392e)^{-90x}$; $R^2 = 0.98$ (Figure 16).

Contribution of clubroot galls to soil resting spore populations

Cropping of the susceptible cultivar for a single cycle contributed 2×10^8 spores per g soil, while one cycle of the resistant cultivar contributed 1.2×10^7 resting spores per g soil, relative to the fallow control treatment (Table 6). The Ct values ranged from 19.59 - 20.86 for the treatment in which a susceptible cultivar was grown, and from 23.57 - 26.49 for the treatment in which a resistant cultivar was grown; Ct values ranged from 24.73 - 25.92 in the control treatment. The amount of *P. brassicae* DNA calculated from these Ct values was therefore significantly higher in the susceptible treatment. The Ct values and the amount of DNA in the resistant treatment compared to the control were not significantly different.

Summary

Clubroot spore populations increase dramatically with the successful formation of clubroot galls within cruciferous plants. In a study to determine the effects of resistant crucifer plants on spore populations, repeated introduction of both susceptible and resistant plants progressively increased spore populations in the soil. Introduction of a susceptible cultivar resulted in greater spore populations, higher disease levels and more root hair infection compared to the resistant cultivar. However, both disease parameters and plant height decreased with repeated cultivation of both susceptible and resistant cultivars.

Higher proportions of a susceptible volunteer led to higher root weights and disease severity and lower plant heights in a susceptible crop cultivated afterwards. Higher soil populations of viable spores tended to increase root weight in a susceptible crop due to clubroot galls, increase disease severity and reduce plant height and seed yield. At high inoculum densities, in naturally infested soil dilutions, variations in inoculum density did not affect clubroot severity but yield declined as inoculum density increased. Plant height was not affected by spore densities of 10^5 spores/cm^3, but at higher spore densities, plant height declined with increases in spore density. In artificially inoculated growth medium, clubroot severity increased with increasing inoculum density, but there was little effect on yield. High soil populations of *P. brassicae* not only reduce plant growth and yield but also interfere with seedling emergence.

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Table 1
Effect of 'volunteer' canola (proportion of crop that is susceptible) on plant height of resistant and susceptible canola plants grown under greenhouse conditions

		Plant height (cm)	
Susceptible plants (%)	Inoculation	Resistant cv	Susceptible cv
3%	Inoculated	25.7 b	22.5 b
	Control	35.0 a	31.3 a
5%	Inoculated	27.6 b	23.9 b
	Control	42.6 a	35.3 a
7%	Inoculated	28.8 b	22.1 b
	Control	41.2 a	34.8 a
10%	Inoculated	29.3 b	23.6 b
	Control	37.6 a	32.4 a

[1]Means followed by the same letter for inoculated vs. non-inoculated within a cultivar category do not differ at $P \leq 0.05$ based on LSMEAN t-test.

Table 2
Effect volunteer canola (proportion of susceptible canola as volunteer) on contribution of resting spores after one cycle of cropping susceptible canola, severity of clubroot, and seed yield under greenhouse conditions.

Susceptible plants (%)	DSI	Resting spore (x 10^9)	Root hair colonization[b] (%)	Secondary Plasmodium	Seed yield g/ 50 plants
0%	0 b	0 e	24 d	2.5 c	0.68
3%	78 a	0.6 d	34 c	3.4 b	0.54
5%	79 a	1.1 c	37 c	3.3 b	0.61
7%	84 a	1.4 b	53 b	4.0 a	0.52
10%	84 a	2.0 a	64 a	4.5 a	0.48

Means in a column followed by the same letter do not differ at $P \leq 0.05$ based on a LSMEAN t-test.
[a]Additional resting spore contributed to the soil-less mix after growing different proportion of volunteer canola.
[b]Root hair colonization (formation of primary plasmodium) was estimated 10 days after seeding susceptible canola (Pioneer Hi-Bred cv. 45H26) after growing different proportion of susceptible canola (Pioneer Hi-Bred cv. 45H26) as volunteer with resistant canola Monsanto cv. 71-45, and incorporation of resting spores from the galls formed on the volunteer canola after six weeks of first cycle of seeding.
[c]The secondary plasmodium was estimated using a qualitative scale 1-5 where 1= few and 5 profuse inside root epidermis and cortex.
The seed yield was obtained at maturity of the crop.

Table 3
Effect of crop rotation treatment on growth, clubroot severity and yield of a susceptible cultivar of canola (45H26) in clubroot-infested soils

Treatment	Yield (g)	Plant number	Fresh shoot weight (g)	Fresh root weight (g)	Plant height (cm)	Disease severity	DSI
1CCCC	0.52 c	25.00 c	9.11 b	8.28 b	20.65 c	2.74 a	91.25 a
2CPWC	3.20 b	35.30 ab	23.98 a	13.50 a	29.09 b	2.35 b	78.35 b
3COPC	5.43 a	32.20 ab	26.18 a	12.33 a	33.26 ab	2.10 b	70.06 b
4COWC	2.08 bc	37.30 a	21.22 a	13.17 a	27.39 b	2.03 b	67.71 b
5CBPC	3.03 b	29.60 bc	25.73 a	12.63 a	36.46 a	2.08 b	69.39 b
6CFFC	3.61 b	30.90 b	20.98 a	10.00 ab	30.92 ab	2.24 b	74.48 b

Data are the means of 10 replicate mini-plots
P = Pea cv. Midas
N = Oat cv. Derby
B= Barley cv. Harrington
W = Wheat cv. Lillian
F = Fallow

Table 4
Effect of resistant and non-host crop rotation treatment on growth, clubroot severity and yield of a susceptible cultivar of canola (45H26) in clubroot-infested soils

Treatment	Yield (g)	Plant number	Fresh shoot weight (g)	Fresh root weight (g)	Plant height (cm)	Disease severity	DSI
1RRRS	1.18 c	26.25 ab	11.35 c	7.37 b	18.67 c	2.74 a	91.22 a
2RRNS	6.71 b	30.42 a	28.84 b	16.75 a	30.95 b	2.25 b	74.83 b
3RNNS	3.60 bc	27.92 ab	27.76 b	15.66 a	36.50 b	2.09 b	69.59 b
4NNNS	11.73 a	22.75 b	51.75 a	10.78 b	48.77 a	1.13 c	37.76 c

Data are the means of 12 replicate mini-plots
N =Non-host crop (barley)
R =Clubroot-resistant canola cv. 45H29
S = Susceptible canola cultivar 45H26

Table 5

Effect of inoculum density of *Plasmodiophora brassicae* (based on dilution of infested soil with soil-less mix) on clubroot severity (index of disease), plant height and seed yield of canola in a greenhouse study.

Inoculum density[a]	Index of Disease[b] (%)	Plant height[b] (cm)	Seed yield g/plant[b]
0:1	0.0 f	53.4 ab	2.14 a
1:50	0.0 f	57.2 a	2.36 a
1:25	31.2 e	56.2 ab	1.70 b
1:10	52.1 d	48.2 bc	0.53 c
1:5	74.1 c	49.3 abc	0.49 c
1:3	81.2 bc	47.9 bc	0.40 cd
1:2	85.9 b	42.9 c	0.44 c
1:1	100.0 a	24.9 d	0.00 d

[a] Ratio of clubroot-infested field soil to commercial Sunshine Mix L4 soil-less mix (pH 6.5).
[b] Data are the mean of four replications; means with in a column followed by the same letters do not differ according to Fisher's protected least significant difference test at $P \leq 0.05$.

Table 6

Quantification of *Plasmodiophora brassicae* resting spores by conventional microscopy and quantitative PCR analysis in soil, after the cropping of resistant ('45H29') and susceptible ('45H26') canola cultivars under greenhouse conditions.

	Resting spores g^{-1} soil	DNA (ng-μL^{-1})
Resistant (canola cv. 45H29)	1.4×10^8 b	0.388 b
Susceptible (canola cv. 45H26)	2.0×10^8 a	6.248 a
Control[a] (fallow)	9.2×10^7 c	0.215 b

Note: data are the means of three replications and two trials. Columns in each concentration with the same letter do not differ at $P \leq 0.05$. Resting spores and DNA were extracted from 0.5 g of soil samples.
[a]Control = fallow treatment; no plants were grown

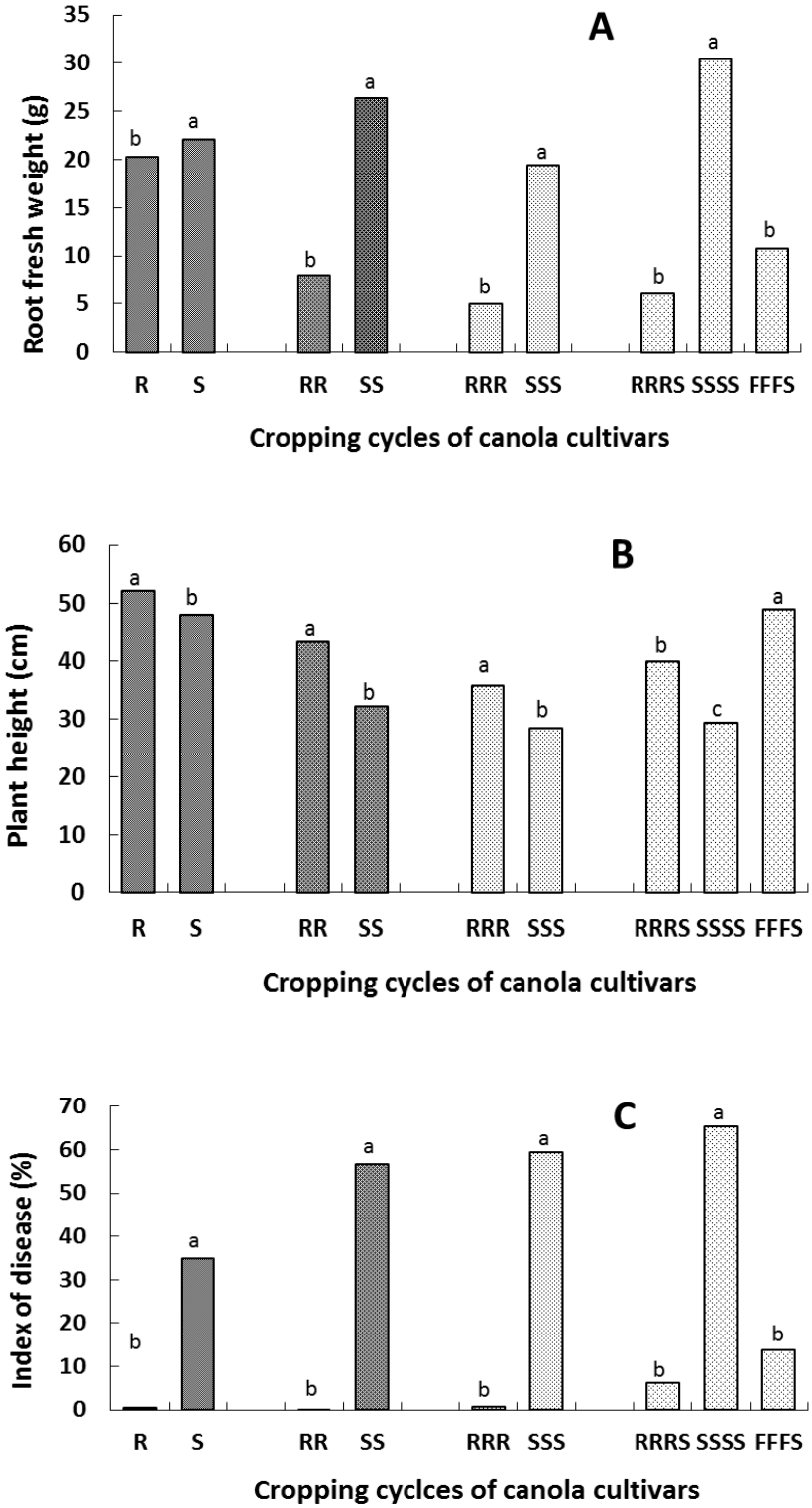

Figure 1
Effect of sequential cropping of resistant ('45H29', Pioneer Hi-Bred) and susceptible ('45H26', Pioneer Hi-Bred) canola cultivars on fresh root weight (A), plant height (B) and index of disease (C) caused by *Plasmodiophora brassicae* on a final susceptible canola crop. Bars in each cycle topped by the same letter do not differ at $P \leq 0.05$.

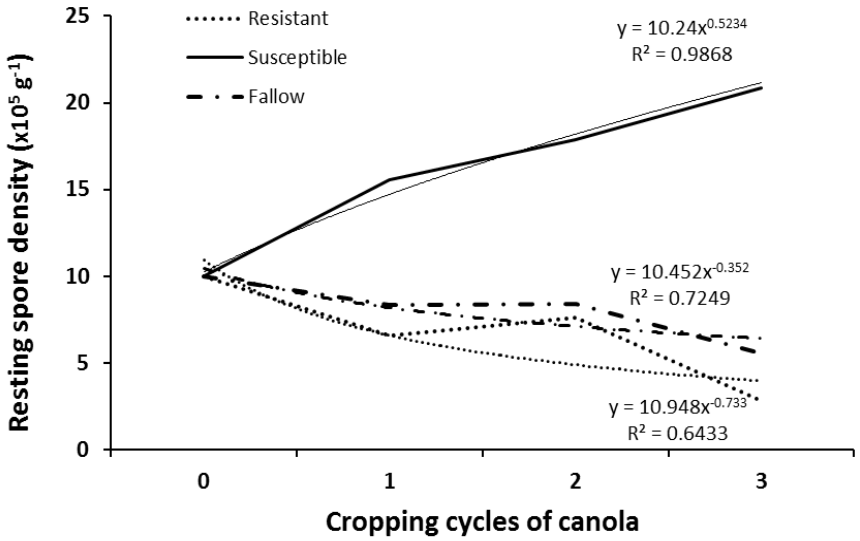

Figure 2
Changes in resting spore concentration over cropping cycles with resistant (R, '45H29', Pioneer Hi-Bred) or susceptible (S, '45H26', Pioneer Hi-Bred) canola cultivars, or a fallow (F) treatment. The fallow condition was maintained over the same period in which three cycles of resistant or susceptible canola was cropped.

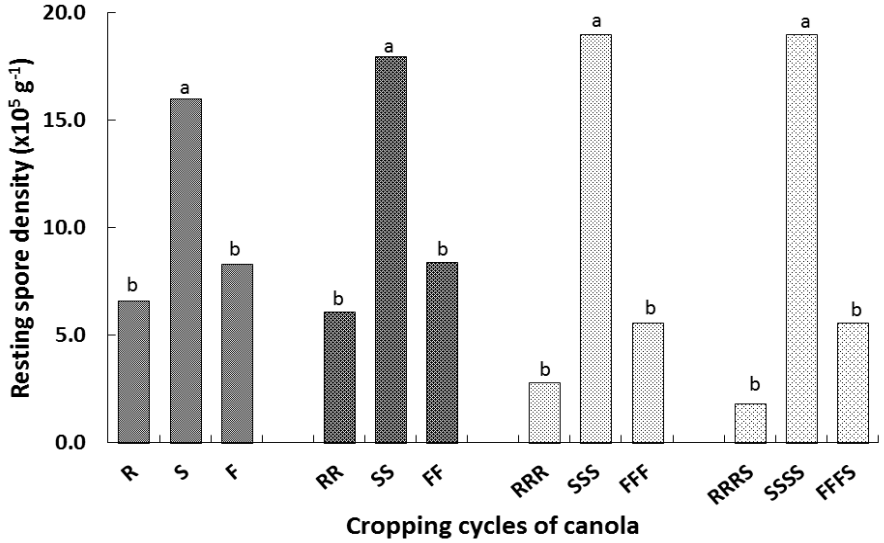

Figure 3
Effect of sequential cropping on resting spore concentration in soil growing resistant or susceptible canola cultivars or in a fallow condition. A different pattern of bars was used to distinguish each cycle denoted on the x-axis. Bars in each cycle topped with the same letter do not differ at $P \leq 0.05$ by Fisher's protected least significance difference test.

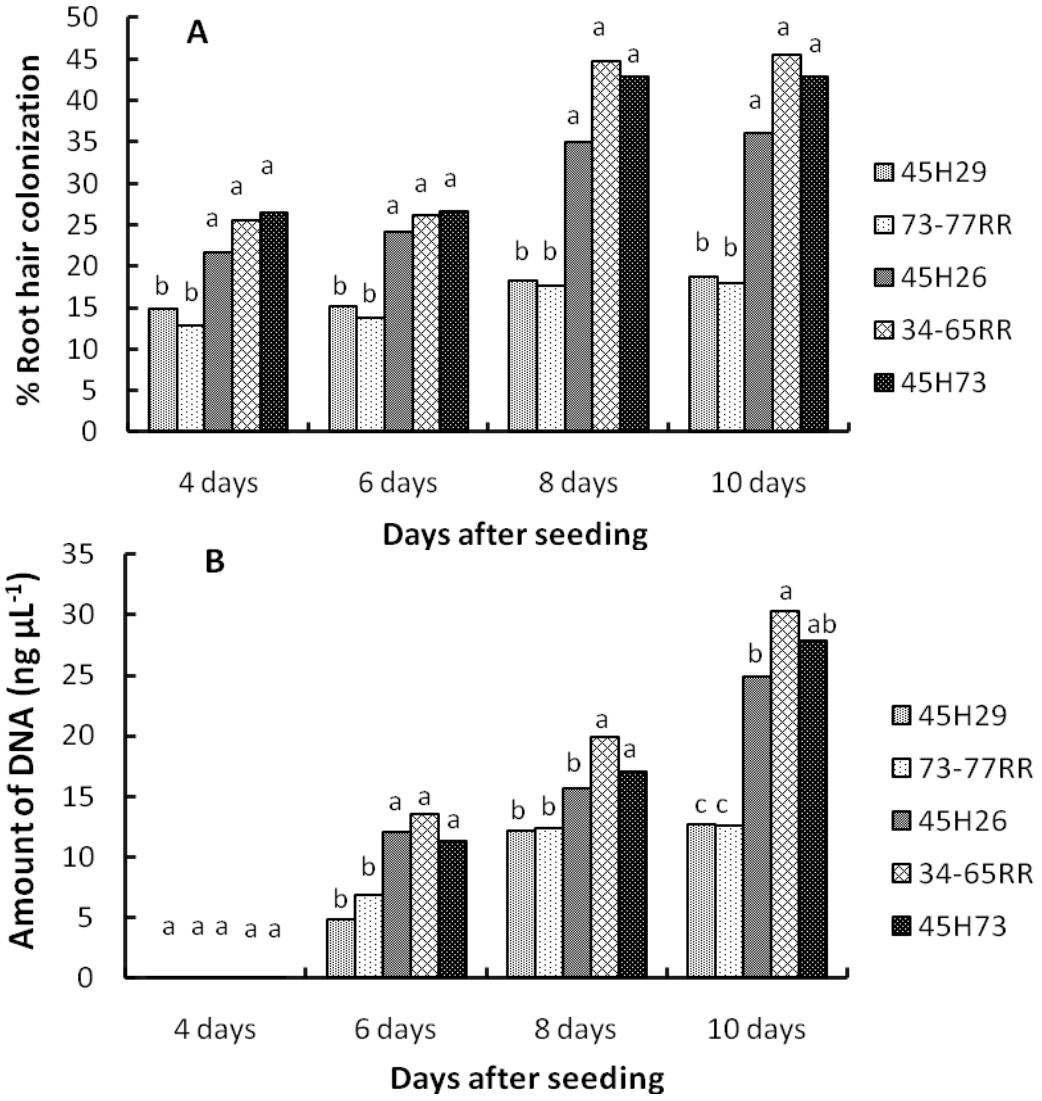

Figure 4
Root hair infection in resistant and susceptible canola cultivars at 4, 6, 8 and 10 days after seeding. (a) colonization of root hairs and (b) quantification of P. brassicae DNA via qPCR. Columns topped by the same letter do not differ at P £ 0Æ05 based on LSMEAN t-test. Each column represents the mean of six replications in each of two repetitions.

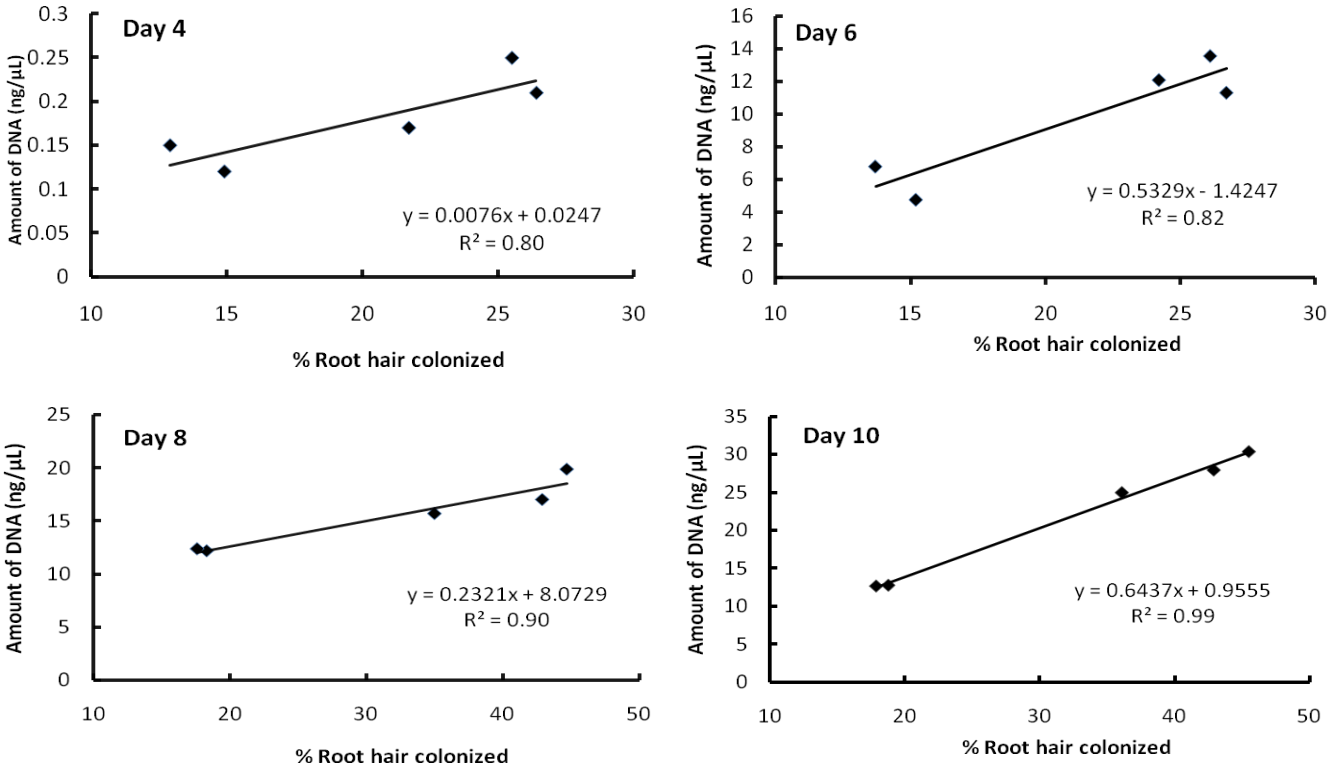

Figure 5
Relationship between root hair colonization and amount of *Plasmodiophora brassicae* DNA, as quantified by qPCR, in five canola cultivars (two resistant and three susceptible) at 4, 6, 8 and 10 days of inoculation under greenhouse conditions. Data points are the means of two trials × six replications of a canola cultivar.

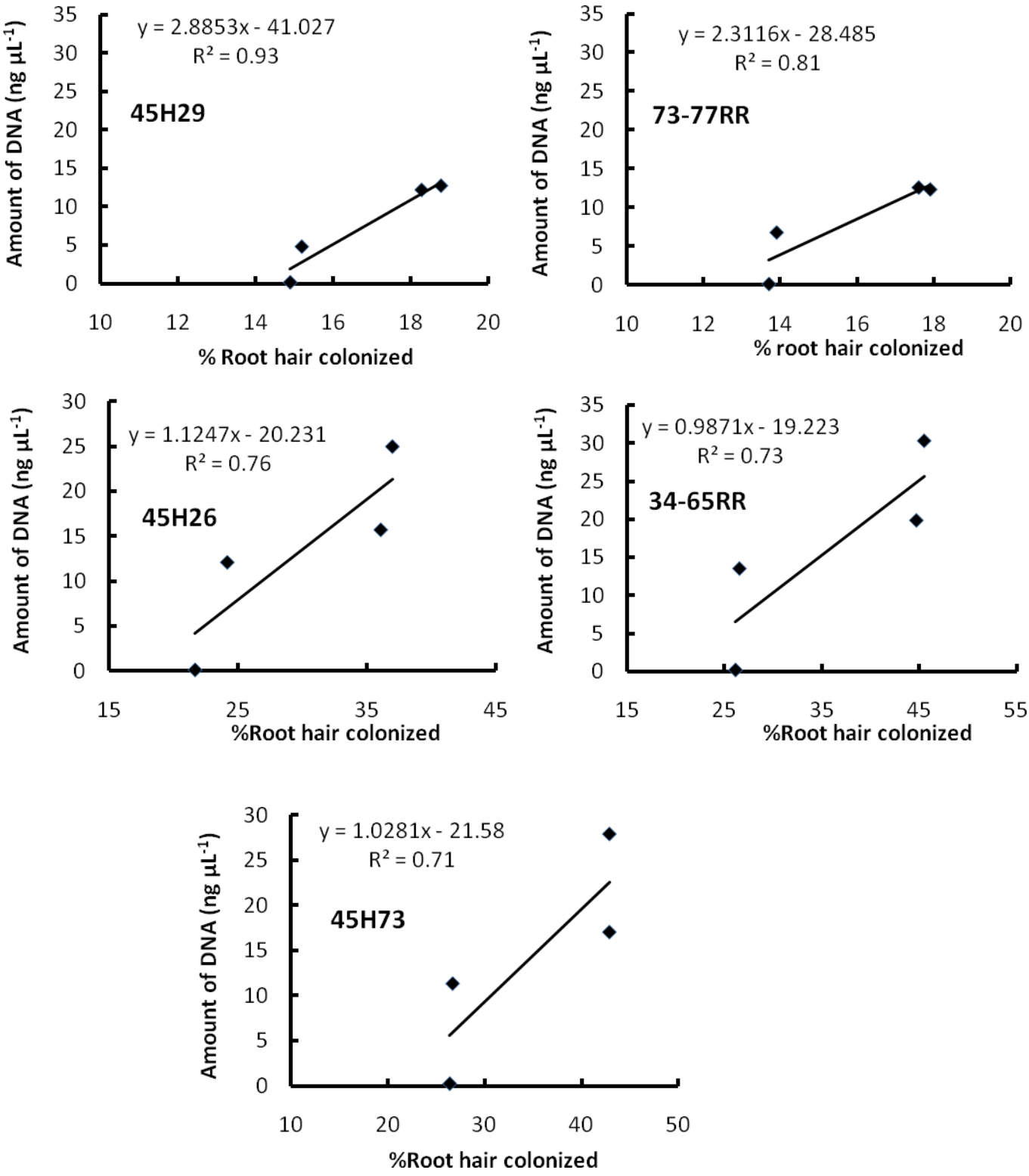

Figure 6
Relationship between root hair colonization and *Plasmodiophora brassicae* DNA, as quantified by qPCR, over time (4–10 days) in five canola cultivars (two resistant 45H29 and 73-77RR, and three susceptible 45H26, 34-65RR and 45H73). Data points are the means of two trials × six replications.

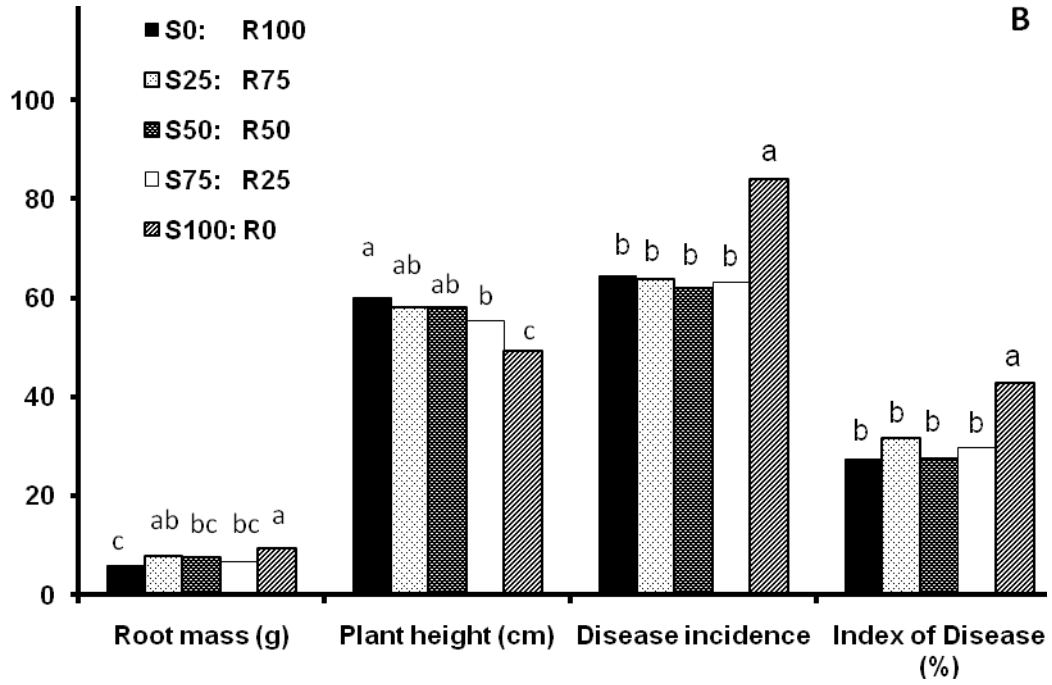

Figure 7
Plant growth and clubroot levels in a subsequent susceptible canola crop grown in *Plasmodiophora brassicae*-inoculated soil after the cropping of various ratios of resistant (R) and susceptible (S) canola plants

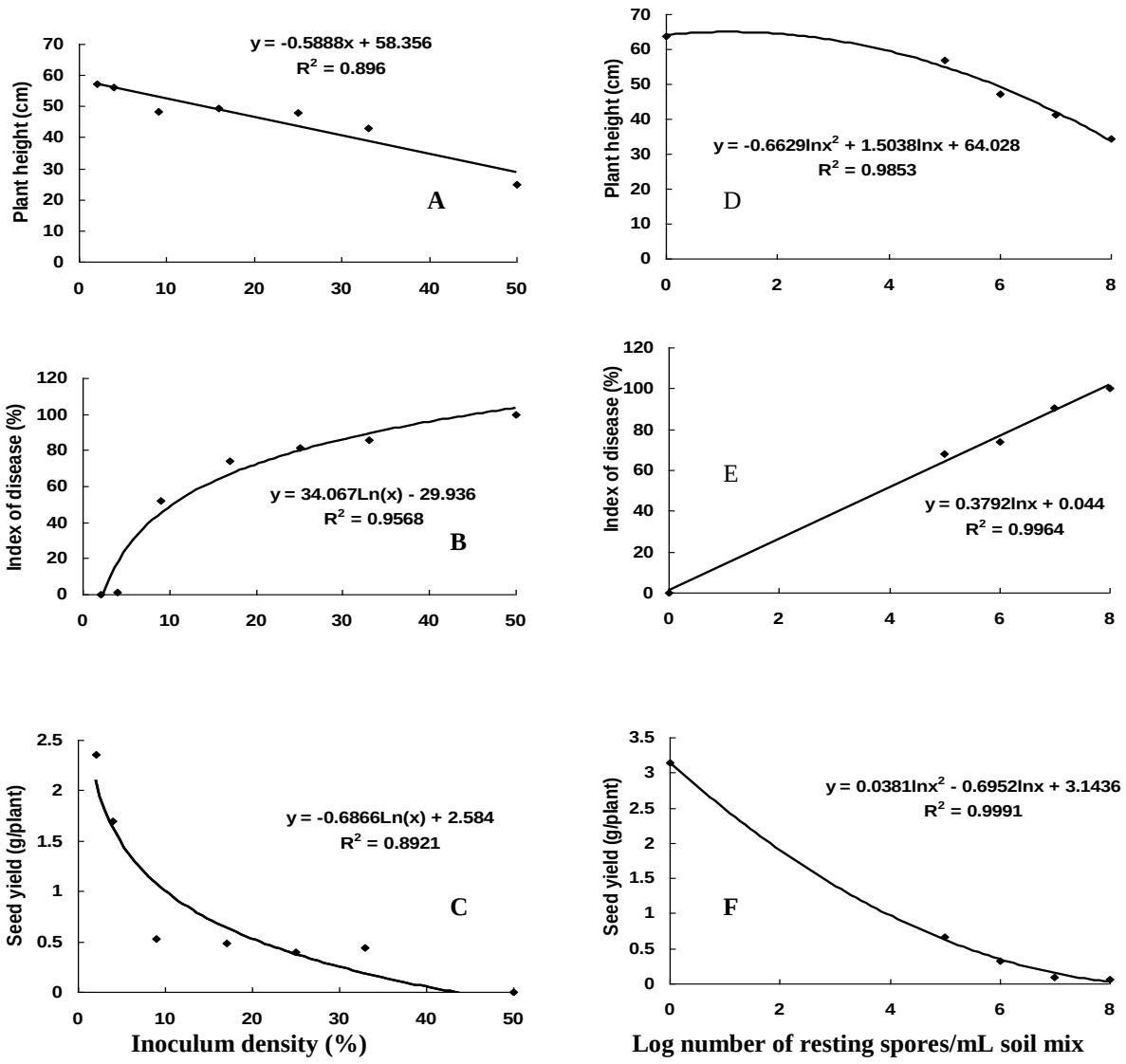

Figure 8

Effect of inoculum density on plant height, clubroot severity (index of disease), and seed yield of canola (A, B, C: dilution of naturally infested soil; D, E, F: soil inoculated with known concentrations of resting spores extracted from root galls).

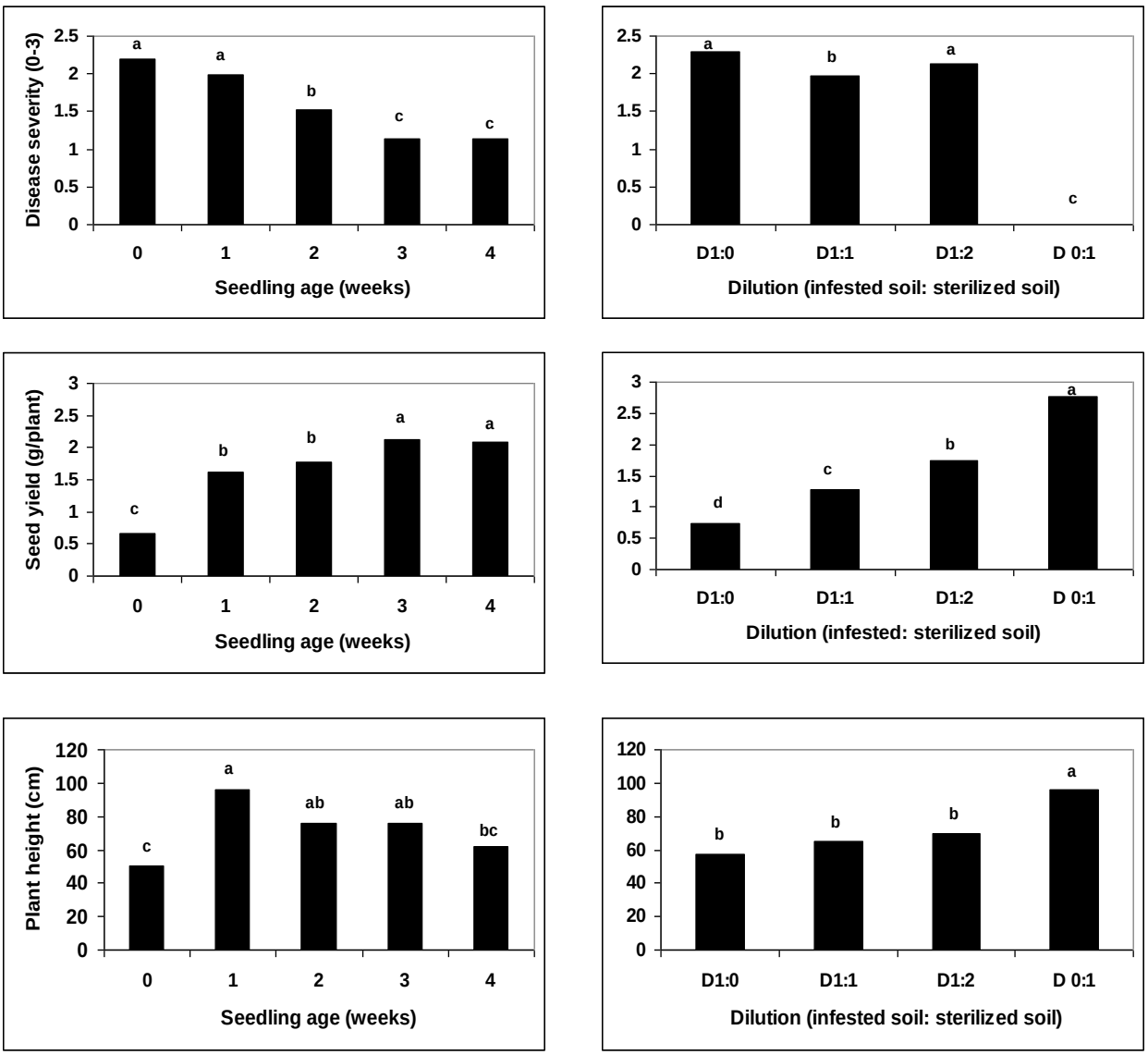

Figure 9

Effect of seedling age at first exposure to infection and pathogen inoculum density (infested soil: sterilized soil (v/v)) on clubroot severity, plant height and yield of canola under greenhouse conditions. No clubroot developed in the sterilized soil control, so these data were dropped from the analysis. Bars with the same letter within a graph do not differ according to Fisher's protected least significant difference test at $P \leq 0.05$.

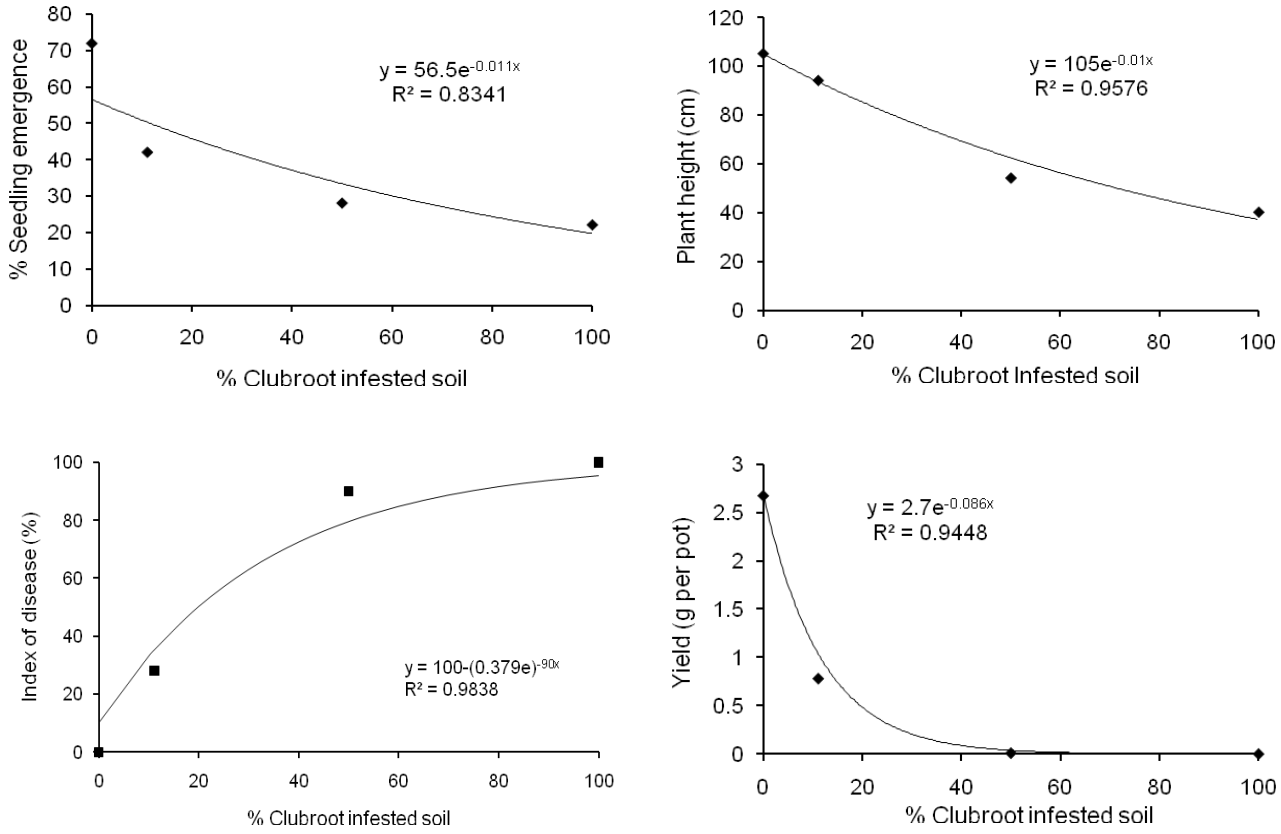

Figure 10
Relationship of *Plasmodiophora brassicae* inoculum density (% clubroot infested soil) with seedling emergence, plant height, index of disease, and yield (g per pot) in a clubroot-susceptible cultivar ('45H26') of canola. Each point represents the mean of five replicates.

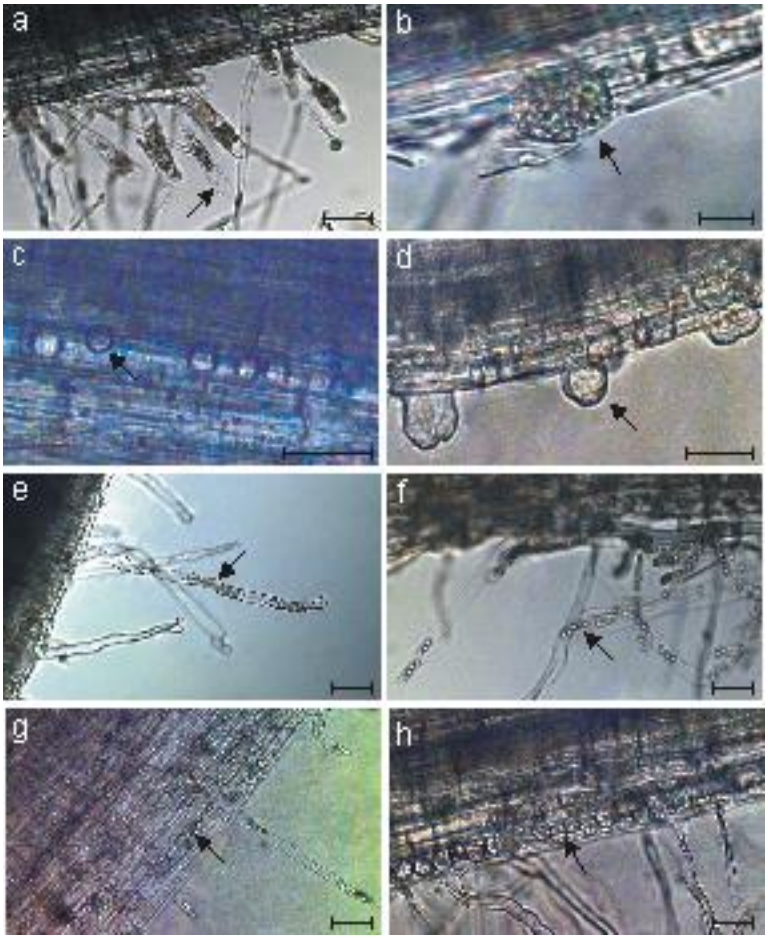

Figure 11
Root hair colonization by *Plasmodiophora brassicae* in susceptible ('45H26') (a-d) and resistant ('45H29') (e-h) canola cultivars. (a) Primary plasmodium in root hair of susceptible cultivar prior to secondary zoospore formation; (b) plasmodium-like structure just below the root epidermis; (c) infection and formation of secondary plasmodia in the inner cortex of the root; (d) very young galls emerging through root epidermis; (e) young primary plasmodium in the root hair; (f) cleavage of the primary plasmodium and formation of secondary zoospores; (g)

Figure 12
Effect of resting spore density on colonization of clubroot resistant ('45H29') and susceptible ('45H26') canola seedlings by *Plasmodiophora brassicae*. Seedlings were grown in a soil-less potting mixture inoculated with different densities of resting spore inoculum. Columns in each concentration with the same letter do not differ at $P \leq 0.05$.

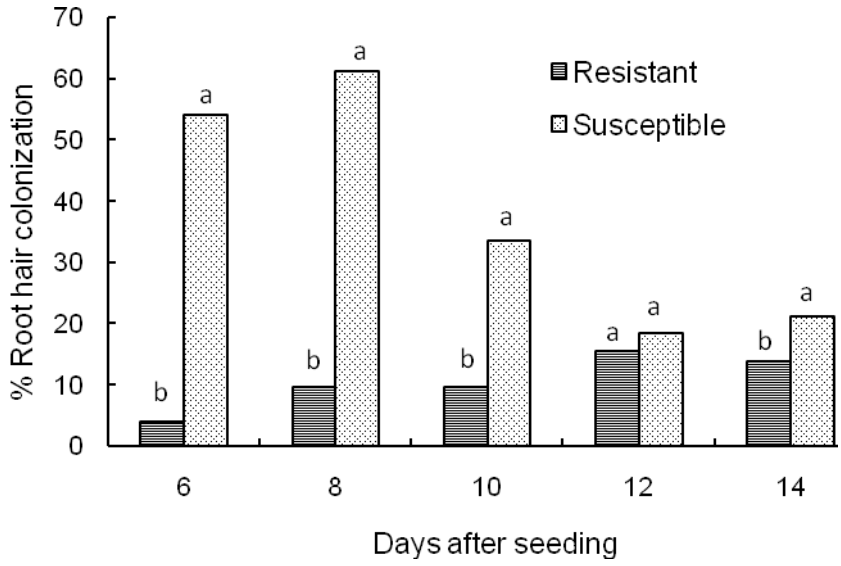

Figure 13
Colonization of resistant ('45H29') and susceptible ('45H26') canola seedlings by *Plasmodiophora brassicae* at 6-12 days after sowing in a soil-less potting mixture inoculated with resting spores of the pathogen (1×10^6 resting spores/g soil). Root hairs were microscopically examined for the presence of *P. brassicae* plasmodia at each time-point. Columns in each sampling date with the same letter do not differ at $P \leq 0.05$.

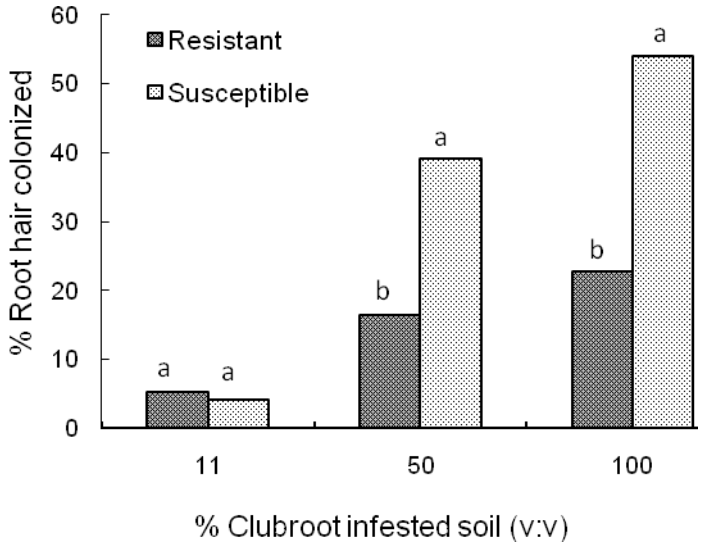

Figure 14
Effect of inoculum density on colonization of root hairs of resistant and susceptible canola cultivars by *Plasmodiophora brassicae* in infested field soil diluted with soil-less mix. Columns in each soil dilution with the same letter do not differ at $P \leq 0.05$.

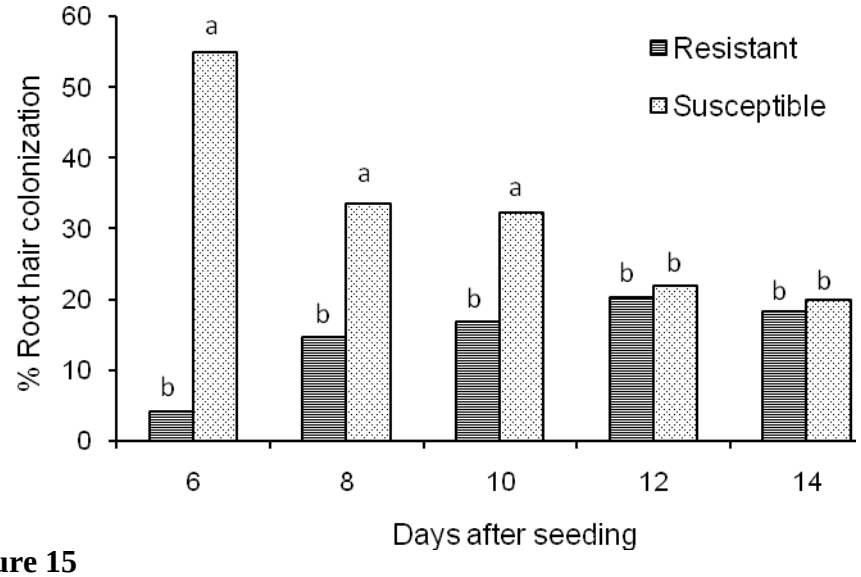

Figure 15
Colonization of resistant ('45H29') and susceptible ('45H26') canola seedlings by *Plasmodiophora brassicae* at 6-12 days after sowing (0:1, 1:8, 1:1, and 1:0 naturally infested soil: soil-less mix). Root hairs were microscopically examined for the presence of *P. brassicae* plasmodia at each time-point. Columns in each sampling date with the same letter do not differ at $P \leq 0.05$.

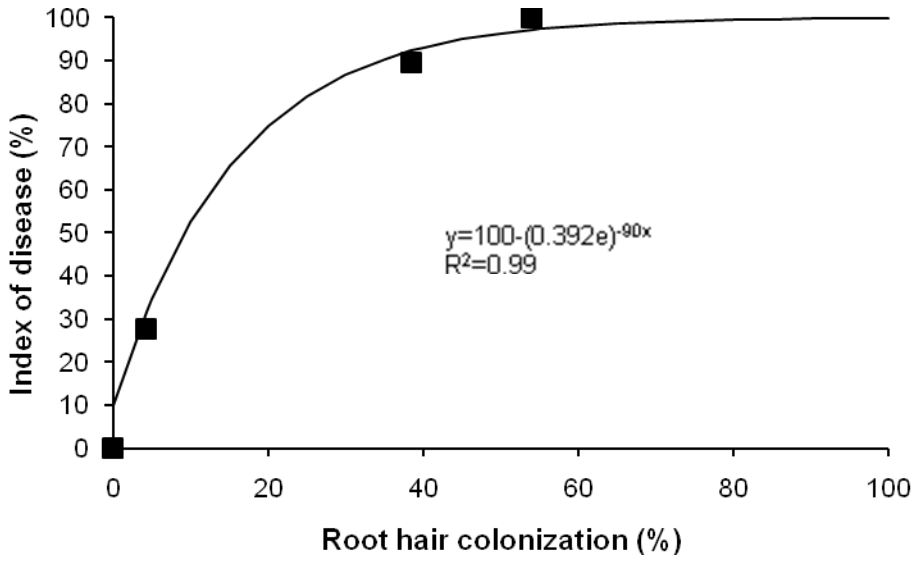

Figure 16
Relationship of root hair colonization and clubroot index of disease in a susceptible cultivar of canola ('45H26') grown on *Plasmodiophora brassicae*-infested soil (0:1, 1:8, 1:1, and 1:0 infested soil: soil-less mix) under greenhouse conditions. Each point represents the mean of 100 root hairs on the x-axis and the mean of five replicates on the y-axis.