Use of cultivar resistance and crop rotation with *Bacillus subtilis* for clubroot control in canola

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Abstract

This study was conducted to assess additional strategies potentially complimentary to cultivar resistance or biocontrol in control of clubroot. New granular *Bacillus subtilis* formulations and a seed dressing method were developed to facilitate biofungicide delivery in field trials. The granular formulations were applied in furrow during seeding at 50 kg/ha to a clubroot resistant (CR) and susceptible (CS) canola cultivar, respectively, in three field trials. The seed dressing applied approximately 1×10^5 to 5×10^6 cfu/seed doses of the biocontrol agent, and was evaluated on the CS cultivar seeded to different crop-rotation scenarios where the plots had a 1-year, 3-year, or 11-year break from last canola crop. Clubroot disease pressure was high at all trial sites with disease severity indexes (DSI) ranging from 69% to 98% on the CS cultivar. None of the granular formulations reduced clubroot substantially, whereas the CR cultivar showed a high effect, reducing DSI to below 15% and doubling the yield over that of CS cultivar. Plots of varying rotation showed a pattern of clubroot pathogen pressure, with those of 1-year break from canola being the highest. The DSI for all rotational scenarios was high, reaching 100% in short-rotation plots. Biofungicide seed dressing did not reduce DSI, but longer crop rotation often reduced gall size slightly, showed much milder above-ground damage, and increased the yield significantly relative to short rotation in two separate trials. Even a 3-year break from canola was highly beneficial, with the yield doubled as opposed to that with only 1-year break from canola.

Introduction

Clubroot, caused by the plasmodiophorid pathogen *Plasmodiophora brassicae* Woronin, is one of the most serious diseases of cruciferous crops worldwide, and an emerging threat to canola (*Brassica napus* L.) production in western Canada (Howard et al. 2009). A total of more than 800 canola fields have been found with clubroot in Alberta thus far and two fields in Saskatchewan were reported with the disease in 2011. Resistant canola cultivars are now available to growers; they are single-gene based and have shown a low level of disease under field conditions (Peng et al. 2011a). Cultivar resistance to clubroot is generally race specific, and historically this type of resistance is not durable because it can be eroded when pathogen race structure changes. Although variety resistance may be a cornerstone for clubroot control on canola, additional measures may help the performance and longevity of resistant cultivars.

The biofungicide Serenade® ASO (AgraQuest, Davis, CA), a liquid formulation of *B. subtilis*, was highly suppressive to clubroot under controlled-environment conditions when applied as a soil drench, especially when pathogen inoculum dose was kept low. It can also boost efficacy of a moderately resistant canola line against a heavy load of clubroot pathogen

inoculum (Peng et al. 2011b). However, a liquid biofungicide will be impractical for application during canola seeding in western Canada due to a general lack of proper equipment. Development of granular or seed-dressing formulations may facilitate the delivery of biofungicide and improve stability in the soil.

There was generally a lack of specific information about the impact of crop rotation on survival of *P. brassicae* in soils, although a long-term rotation was considered beneficial in reducing pathogen inoculum and alleviating clubroot pressure (Wallenhammar 1996). In recent years, however, many growers on the prairies are producing canola in a tightened rotation due to differential commodity prices in marketplaces, and it is not known how much this practice may exacerbate the clubroot problem vs. a 4-year rotation often recommended in the region. Resting spores of *P. brassicae* can survive many years in soil (Dixon 2009) and it would be unrealistic to expect a practical crop rotation removes clubroot pressure entirely. If the 4-year rotation can lower the disease pressure adequately, then an additional treatment such as biofungicide seed dressing may act more effectively in reducing clubroot impact. This study was carried out to assess additional control measures in combination with cultivar resistance to maximize clubroot control, thus reducing inoculum build up when pathogen race structure has changed. This will help extend the longevity of CR cultivars. The objectives were to: 1) Explore interaction of CR cultivars with biofungicides for enahnced clubroot control; 2) Assess the value of longer crop rotation used with a biofungicide seed dressing for control of clubroot in heavily infested fields.

Materials and Methods

Bacillus subtilis formulations

Two granular formulations were produced for *B. subtilis* strain QST713 (AgraQuest Inc. Davis, CA) and tested with CR and CS canola cultivars. Seed dressing was done using the biofungicide Kodiak® FL, a liquid formulation of *B. subtilis* GB03 (Bayer CropScience), and was evaluated in crop-rotation plots.

Cultures of the QST713 strain were started in a liquid medium based on the recipe of Nguyen Thi Minh et al. (2011) for optimal sporulation of *B. subtilis*. The fermentation culture was harvested after 72 h and the sporulation peak was confirmed by plating samples heated at 80°C for 10 min (Nguyen Thi Minh et al. 2011). The average titre for spores in fermentation products was about 2.24E+09 cfu/ml. Two granular formulations were produced for field trials. The formulation A was based on prior experiences on soil-applied biopesticides (Hynes and Boyetchko 2011); it showed a fast disintegration rate in water, which would facilitate rapid release of active ingredients in the soil. The formulation B used corn-cob grits as a low-cost carrier. The formulation composition is as follows:

Formulation A	
Corn starch (Tate and Lyle Ingredients Americas Decatur, IL)	193 g
Peat	32 g
Liquid fermentation product	171 mL
Titre range:	9.26E+08 cfu/g (S.E.±0.54E+08)

Formulation B	
Corn-cub grits (Co-op, Quebec City, QC)	100 g
Liquid fermentation product	60 mL
Titre range:	7.80E+08cfu/g (S.E.±0.36E+08)

Field plots

Plots were established on two commercial farms near Leduc and Edmonton, AB, and also on a AAFC Research Farm in Normandin, QC. All these fields were heavily infested by clubroot, with previous DSI generally greater than 80% on susceptible canola cultivars. Based on the reactions on the Williams' differential set (Williams 1966), the *P. brassicae* populations at the AB sites are predominantly the pathotype 3 while the pathogen in Normandin was pathotype 2. At all locations, each plot consisted of eight 6-m rows with 18cm row spacing (6.5 kg/ha seeding rate). Clubroot severity in each plot was assessed by pulling 25 plants at about the Harper and Berkenkamp (1975) growth stage 4.3 (late flowering) and each plant was assessed for clubroot severity using a 0-3 scale (Strelkov et al. 2006) in which 0 = no galling; 1 = small galls only, on less than 1/3 of roots; 2 = small or medium-sized galls on 1/3 to 2/3 of roots; and 3 = severe galling, medium- to large-sized galls with more than 2/3 of roots affected. A disease severity index (DSI) was calculated over all plants from a plot using the following formula. At crop maturity, each plot was harvested separately, seeds dried to approximately10% moisture, and yields taken.

$$\text{DSI (\%)} = \frac{\sum [(\text{rating class})(\#\text{ plants in the rating class})]}{(\#\text{ plants in the treatment})(3)} \times 100$$

Effect of granular biofungicide formulations on CR and CS canola cultivars

The cultivars 45H-28 (susceptible) and 45H-29 (resistant) were seeded on May 28 (Leduc), June 2 (Edmonton), and June 6 (Normandin), 2011. The two granular formulations of *B. subtilis* strain QST713 were applied in furrow at 50 Kg/ha in mixtures with canola seed. Corn-cub grits coated with the fungicides Allegro® 500F (fluazinam, 725 g/ha) and Ranman® 400SC (cyazofamid, 600 g/ha) were applied similarly for comparisons. The experiment was a split-plot design with cultivar in main plots (4 replicates), and biofungicide/fungicide formulations in sub plots arranged randomly in 4 blocks within each main plot.

Effect of *B. subtilis* GB03 seed dressing in combination with varying crop rotations

The field in Normandin was about 2.8 ha and had been found with clubroot throughout the field since early 1990s. Starting from 1999, this field had been used for a crop rotation study with a total of 112 large plots (8×32 m) maintained as canola-barley-field pea-barley-canola, canola-field pea-barley-barley-canola, and canola-barley-barley-field pea-canola rotational systems, and continuous field pea or barley. The field was chiseled or plowed in the fall annually, and these rotation plots provided choices of varying break duration from a prior canola crop. Plots with 1- and 3-year break were selected to represent 2-year and 4-year crop rotation in canola production, and plots that had been 12 years out canola were used as an extreme scenario for comparisons only. Prior to the trial, 3L soil (top 15 cm) were taken from 5 random sites of each plot and 4 plots from each crop-rotation category (cover both chisel- and mouldboard-ploughed sections) were used to estimate pathogen inoculum levels using a bioassay under controlled-environment conditions.

Four rates of the biofungicide Kodiak (*B. subtilis* strain GB03) was used with the seed-treatment adjuvant L1782 and seeds treated with L1782 alone were used for non-treated controls. Two trials, seeded on June 5 and June 15, 2011 respectively, were set up on different sections of the field in a split-plot design with crop rotation as main plots (4 replicates) and biofungicide seed dressing in sub plots in 4 blocks within each main plot. To assess potential

impact of varying prior crop residuals on canola emergence, plants in two random 1-m rows of a plot were counted at 7, 14, and 21 d after emergence (the same 1-m row section each time) and plant density calculated based on total counts and row spacing.

To obtain an additional estimate of disease pressure in different plots, five plants were pulled randomly from each subplot of control within each main plot, roots washed repeatedly with running tap water to remove debris, soil particles, and pathogen inoculum on the root surface. To determine the amount of *P. brassicae* in roots, pathogen genomic DNA from 0.2g root tissue was extracted with the DNeasy and quantified using quantitative PCR (qPCR) using the procedure described by Sundelin et al. (2010).

In addition to the clubroot severity assessment, the above-ground impact by clubroot was also assessed for each plot visually relative to non-disease plots using a 0-4 scale where: 0 = plants are not affected, having developed normally (**Fig 1A**); 1= plants are slightly affected, with up to 25% of the plants showing the symptom of stunting and yellowing; 2 = about 50% plants showing the symptom; 3 = >75 % plant showing symptoms and the plot is noticeably thinned; and 4 = 100% plants are affected; they are dying or dead, and the plot looks sparse (**Fig 1B**).

Fig 1. Above-ground symptoms as affected by clubroot. A: 0 - a normal crop development and B: 4 –all plants were affected, and were either dying or dead.

Data analysis

DSI data were transformed with arcsine square root prior to analysis of variances (ANOVA) using SAS (v10). The data for above-ground systems used a pre-transformed 0-4 scale (Little and Hills 1978) and were subjected to ANOVA directly. Yield data were analyzed on the per plot basis. PROC UNIVARIATE was used to examine the data normality. Data from repeated trials were pooled when the homogeneity of variance was confirmed using the Bartlett's test or analyzed separately if non-homogeneous. Because no significant interaction was detected in any of the field trials, means were separated using least significant difference (LSD) at $P \leq 0.05$. Untransformed data were reported in results.

Results and Discussion

Effect of biofungicide formulations on CR and CS canola cultivars

The weather condition was generally conducive to *P. brassicae* infection and clubroot development at all sites, with normal spring temperatures and precipitation. There were higher than normal precipitation during the growing season at the Quebec site. The clubroot disease pressure was high at all field sites with DSI ranging from 69% to 98% on the CS cultivar. The CR cultivar 45H29 showed a high level of resistance, lowering DSI to below 15%. None of the biofungicide/fungicide formulations reduced the disease relative to non-treated controls on either CS or CR cultivar. Overall, the yield with the CS cultivar was much lower than that of CR cultivar, with 73% to 81% reduction at the same field site.

Efficacy of *B. subtilis* GB03 seed dressing in combination with varying crop rotations

Plots of varying rotation history showed a pattern of clubroot potential; the plots with only 1-year break from canola had the highest DSI in the bioassay and greatest amount of early pathogen development in canola roots as revealed by qPCR (Table 1).

Table 1. Estimate of *Plasmodiophora brassicae* inoculum pressure based on bioassay of soil samples and early pathogen development in canola roots using qPCR in plots of varying crop-rotation history (2011). [A]

Crop rotation (Break from canola -yrs)	Bioassay (%DSI)	qPCR (ng/g fresh root)	
		Field trial 1	Field trial 2
1	74.8 a [B]	11.6 a	2364 a
3	47.0 b	7.3 b	8.4 b
11	28.3 c	8.7 b	3.2 c

[A] Soil samples were taken prior to the trials and root samples were taken from nontreated control plots 4 weeks after seeding.

[B] The means in a column with the same letter do not differ (LSD, $P = 0.05$).

There was not substantial difference in plant emergence between biofungicide rates: ranging from 37-40 and 39-41 per meter row for trial 1 and 2, respectively. There were no differences in plant counts among different crop rotation scenarios either; the number was 37-40 and 41-42 per meter row, respectively, for trial 1 and 2. This may indicate little allelopathy from varying crop rotation systems used and any variation observed between different

rotational plots will likely be caused by other factors. The DSI in this trial was very high, generally exceeding 90% and reaching 100% in plots of short rotation (**Fig 2**). None of the biofungicide seed dressing rates reduced DSI regardless of crop-rotation scenario. Between the rotational plots, those with a 3-year breaks from canola or longer often showed noticeably smaller galls, but based on the disease severity rating scale used, they still fell into the highest (3) category. Therefore, no substantially difference in DSI was found between different crop rotation scenarios either.

Further assessment of disease impact on crop condition based on above-ground symptoms showed a quite different picture; while there was no effect found with any of the seed-dressing treatments, the crop condition was much better in plots with a longer break from canola (**Fig 3**). The crop in plots with only 1-year break appeared worst, with most plants being either dead or dying (**Fig 1B**).

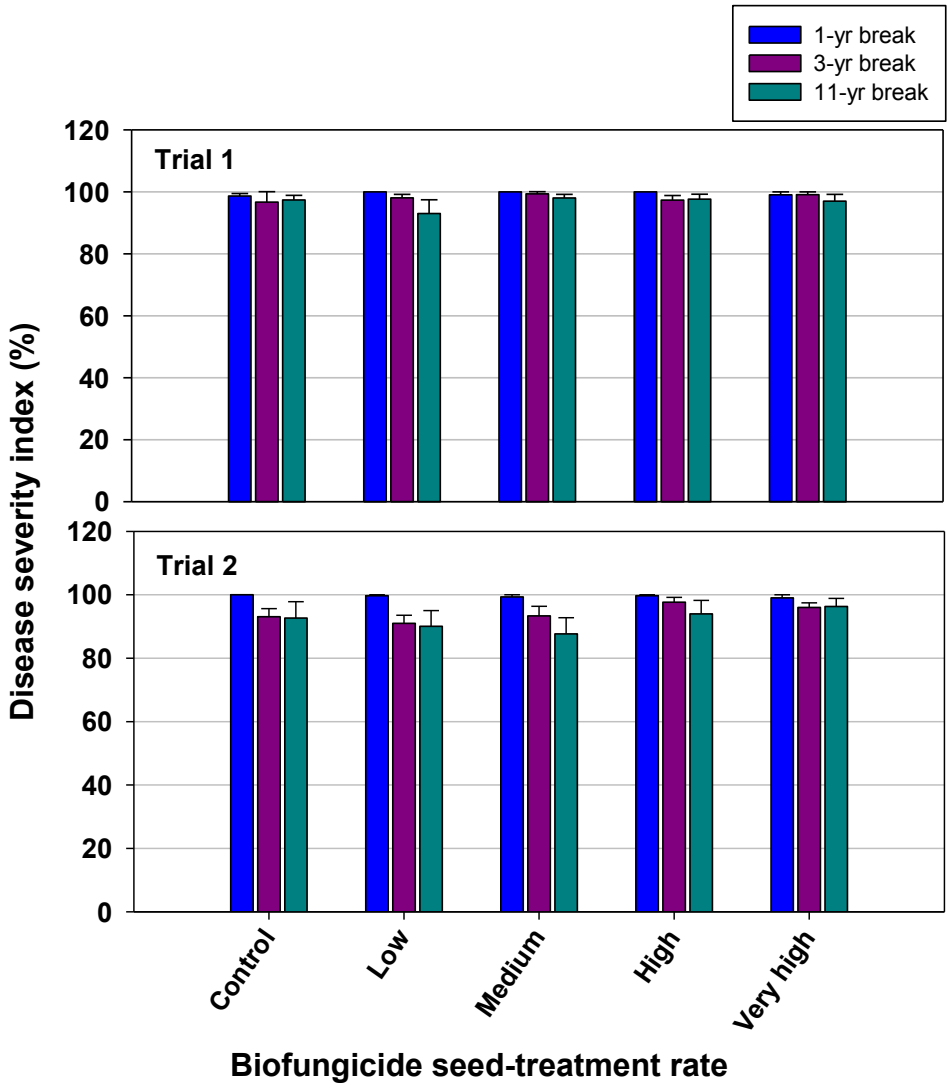

Fig 2. Effect of Kodiak seed treatment (4 rates) on clubroot severity under different crop-rotation scenarios (Normandin, QC, 2011).

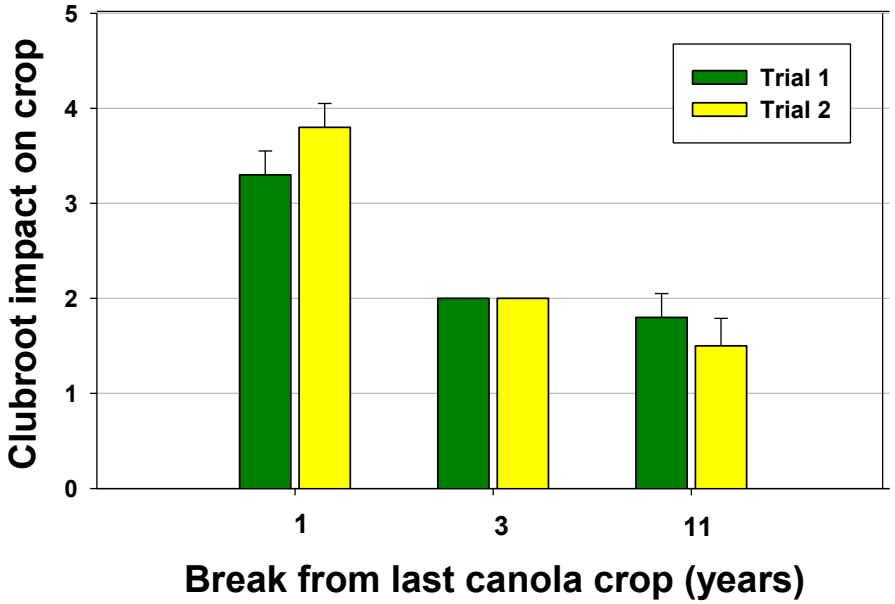

Fig 3. Canola crop conditions at pre ripening stages as affected by clubroot in plots of varying crop-rotation scenarios (Normandin, QC, 2011).

Because the canola cultivar used in this study was a susceptible one, and therefore the yield was much lower than that of a resistant cultivar (45H29) used in the other study at the same location. There was no effect of seed treatment on the yield (**Fig 4**), but a longer break from a canola crop consistently gave higher yields and even a 3-year break was highly beneficial, with the yield doubled as opposed to those with only 1-year break from a canola crop.

This yield benefit was possibly due to the impact of longer crop rotation on clubroot potential reflected in the crop condition assessment (**Fig 3**). Although the rotation is being tightened on many parts of the prairies in favor of canola production, producers need to be aware of the shortfalls for disease management. There are other disease issues with shortened rotation such as blackleg, but if it has to be done, at least a resistance cultivar should be considered to minimize the negative impact by clubroot, especially in fields of heavy infestation.

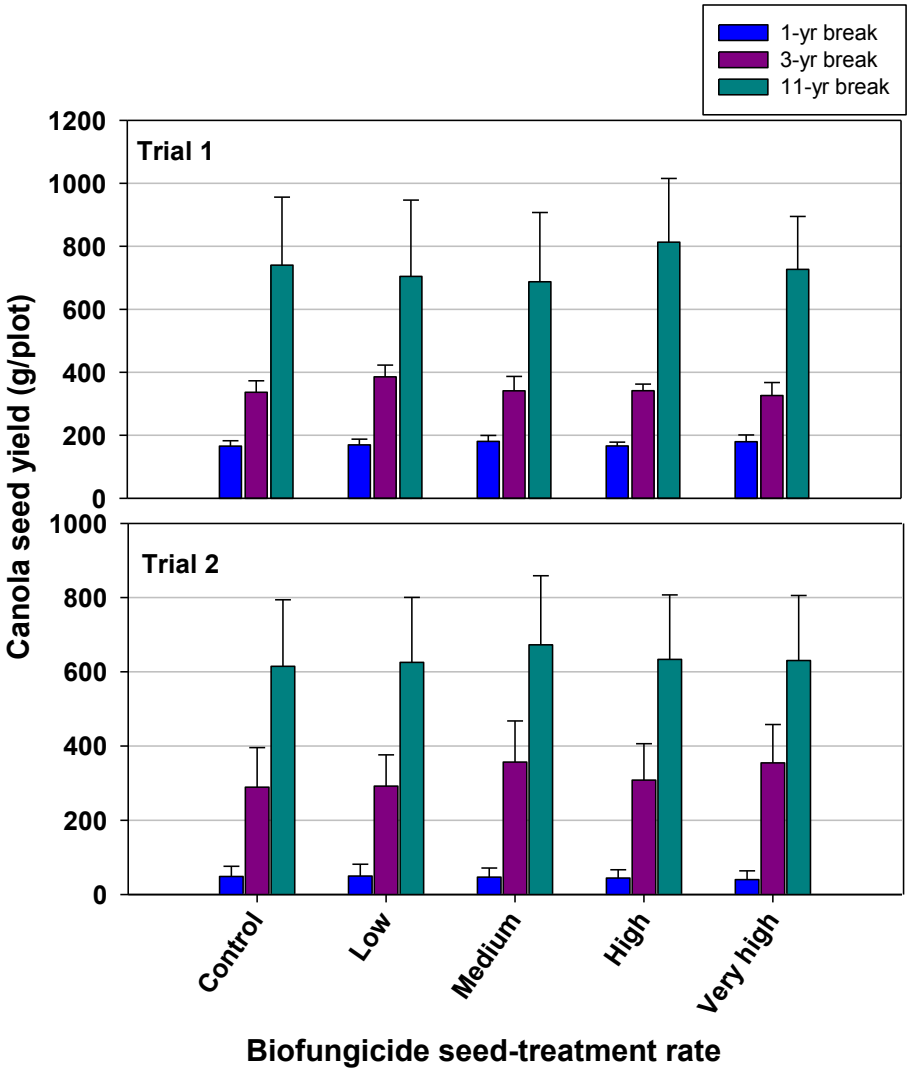

Fig 4. Effect of Kodiak seed treatment and crop rotation on canola yield as affected by clubroot (Normandin, QC, 2011).

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