Control of clubroot on canola using the biofungicide Serenade plus cultivar resistance

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Abstract
In a trial under controlled-environment conditions, the biofungicide Serenade® (active agent, Bacillus subtilis) was evaluated on canola cultivars that differed in resistance to clubroot. The biofungicide was applied as a soil drench to clubroot susceptible (CS), moderately resistant (MS), and resistant (CR) cultivars, and efficacy was higher on the MS than on CS cultivar. In 2010, the biofungicides Serenade and Prestop, and fungicides fluazinam and cyazofamid were applied in-furrow (500 L water ha^{-1}) at seeding to a CS and CR cultivar in two field trials. Under high disease pressure, the CR cultivar reduced clubroot severity by 85-92% and increasing canola seed yield by over 40% relative to the CS cultivar. None of the products (fungicides or biofungicides) provided effective control of clubroot. To better understand how Serenade works, two of the product components, i.e., B. subtilis and its metabolites (product filtrate) were assessed for their relative contribution in clubroot control under controlled conditions. Each component alone was only partially effective, while Serenade reduced clubroot severity by over 90%. This pattern of response was confirmed using quantitative PCR assessment of P. brassicae DNA in treated roots at 2 and 3 wks after treatment. We conclude that an effective formulation will be required to support biofungicide performance under field conditions.

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) where 5 million ha of spring-canola crops are grown yearly. Several CR cultivars became available in last two years; they are single-gene based and have shown a low level of clubroot severity under field conditions (Peng et al. 2010). Cultivar resistance is generally race specific and long-term use of this option alone is insufficient because the resistance can be eroded when the pathogen race structure changes. An integrated approach may be required for sustainable management of clubroot.

The commercial biofungicide Serenade® (Bacillus subtilis QST 713) showed substantial efficacy against clubroot when applied as a soil drench under controlled-environment conditions (Peng et al. 2009). In field trials, however, its efficacy was inconsistent (Peng et al. 2010). The objectives of this study were to: 1) evaluate potential interaction of this biofungicide with cultivar resistance for clubroot control, and 2) investigate the mechanism of Serenade in mediating clubroot control. The information may be used to guide the development of new biofungicide formulations for optimal product efficacy and delivery.

Materials and Methods

I. Interaction of cultivar resistance with biofungicides against clubroot

Under controlled conditions: Plastic root trainers (conetainer, 3.5-cm in diam, 20-cm tall) were filled with Sunshine #3 soil-less planting mix (pH 5.8–6.2) amended with 1% (v/w) of 16-8-12 (N:P:K) control-released fertilizer. Clubroot galls (pathotype 3) were homogenized in a blender for 1 min, filtered through 3 layers of nylon cloth (0.3-mm mesh), and adjusted to desired concentration using a haemocytometer. A spore suspension was added to each conetainer 2 days prior to seeding.

Highly (45H29) and moderately (P2009-9) resistant, and susceptible (45H26) canola cultivars were seeded in the P. brassicae infested growth medium (2×10^7 spores g^{-1}) in conetainers. The biofungicide Serenade ASO (5%, v/v) was applied as a soil drench to all cultivars at 25 mL per conetainer (about 1.3×10^9 cfu of B. subtilis) and water was used as controls. Seven plants were used in each treatment, and the treated plants were kept at 23º/18º C in a growth cabinet. A randomized block design (RBD) was used and the trial repeated at two different times (blocks). Clubroot severity

was assessed 4 wks after treatment (WAT) using a 0 to 3 scale described by Strelkov et al. (2006) and a disease severity index (DSI) calculated over each replicate using the following formula:

$$DSI = \frac{\sum (\text{scale class})(\text{no. of plants in the class})}{(\text{total no. plants in the rep}) \times 3} \times 100\%$$

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Field trials: The Pioneer canola cultivars 45H26 (CS) and 45H29 (CR) were seeded at 6.5 kg/ha between mid and late May in two field plots near Leduc and Edmonton, Alberta in 2010. The plots, each consisting of four 6-m rows (21-cm row spacing) were laid out as a RBD with 4 replicates. A liquid preparation of Serenade was applied (13L ha^{-1}) in furrow at 500 L/ha of carrier volume. The biofungicide Prestop® (1.4 kg ha^{-1}), fungicides fluazinam (2.9L Allegro 500F ha^{-1}) and cyazofamid (0.54L Ranman ha^{-1}) were applied similarly as additional treatments. Clubroot severity was assessed on up to 25 plants in each plot at late flowering using the 0-3 scale. Plots were harvested and seed yields taken at the end of season.

II. The mechanism of Serenade against clubroot

Effect of Serenade components: Four treatments were applied as a soil drench (25 mL per conetainer, equivalent to the field-application rate) immediately after seeding: i) Serenade product (5%, v/v), ii) a cultural suspension of the B. subtilis strain QST713, iii) Serenade product filtrate (5%, v/v), and iv) water control. Treated plants were kept at 23º/18ºC for 4 wks and assessed for clubroot severity using the 0-3 scale. A RBD was used and the trial was replicated at three different times (blocks) with 7 plants per treatment in a block.

Impact on infection: A quantitative PCR (q-PCR) assay was used to determine the amount of P. brassicae DNA in canola roots. About 0.2 g of roots were sampled from each treatment at 2 and 3 WAT, washed repeatedly, and frozen in liquid nitrogen. Roots from control plants (with and without pathogen inoculation) were used as controls. P. brassicae genomic DNA in the root samples was extracted, and the primers Pb4-1 (TACCATACCCAGGGCGATT) and PbITS6 (CAACGAGTCAGCTTGAATGC) used to amplify a 139-bp product (Sundelin et al. 2010). The amplification was done in triplicates using a StepOne real-time thermocycler, and a standard curve between the threshold cycle and logarithm of template concentrations was included with each run. After amplification, melting-curve and electrophoresis analyses were performed to ensure that only the target PCR product had been amplified.

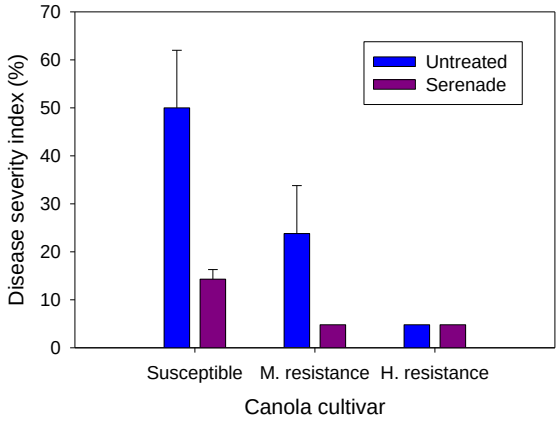
III. Data analysis

DSI data were transformed with arcsine square root, and analyzed for variances. Means were separated with Protected LSD ($P \leq 0.05$) for multiple comparisons.

Results and Discussion

Effect of biofungicide and cultivar resistance on clubroot

Under controlled conditions: There was a slight interaction between the cultivar resistance and the biofungicide treatment; on the CS and MS cultivars, Serenade substantially reduced the clubroot severity relative to the untreated controls, whereas on the highly-resistant cultivar which showed a very low level of clubroot in the untreated control, the biofungicide did not reduce the disease further. It seems that, on certain CR cultivars, the Serenade treatment is beneficial in further reducing the clubroot impact. CR canola cultivars can have 10-20% disease under field conditions and a low-cost treatment may help reduce pathogen inoculum load in the soil and re occur. This may be of value to resistance stewardship.



Under field conditions: In Leduc and Edmonton, AB, the DSI on the CS cultivar was 61% and 68%, respectively. The CR cultivar was highly effective; with 85 and 92% lower DSI and more than 40% canola yield increase, relative to the CS cultivar. None of the treatments was substantially effective. On the CR cultivar which showed 10-15% of DSI in untreated control plots, the treatments failed to

reduce the disease further. Several of these products showed significant efficacy against clubroot under controlled conditions (Peng et al. 2009), but it appears that an in-furrow application has little value in reducing clubroot on either CS or CR canola cultivar. The poor efficacy may be related to fluctuating field conditions (Peng et al. 2010) but until a formulation is developed to address the environmental stress, efficacy of these products will likely continue to be inconsistent under field conditions.

Mechanisms of Serenade against clubroot

Effect of Serenade components: On average, the Serenade product formulation reduced clubroot by more than 90% relative to the pathogen control (Table 1), whereas the product filtrate or B. subtilis suspension had only about 60% efficacy. At 4-6 WAT, root enlargement was substantial in controls, noticeable in bacterial or product-filtrate treatment, but generally absent in the Serenade treatment. Throughout the trial, the Serenade product was consistently more effective than B. subtilis or the product filtrate alone. Possibly both components contribute to the efficacy of this biofungicide. It is known that B. subtilis QST713 produces lipopeptides (Kinsella et al. 2009), which may facilitate rhizosphere colonization by the bacterium via minimizing the competition from other soil microbes.

Table 1: Effect of Serenade or its components on clubroot of canola (n = 3).

Treatment	Incidence (%)	DSI (%)
Control	100 b	100 c
B. subtilis	81 b	40 b
Product filtrate	71 b	39 b
Serenade	19 a	6 a

Means with the same letter do not differ (LSD, $P = 0.05$).

Effect on infection: Pathogen DNA samples showed a single PCR amplicon of about 139 bp (data not shown) and the q-PCR assay detected the amplicon in canola roots inoculated with P. brassicae, but not in non-inoculated root samples. This confirmed the pathogen presence within the roots and/or root hairs at 2 or 3 WAT. A melt-curve analysis showed only one peak in the q-PCR results, indicating the specificity of the primers used. The amount of pathogen DNA detected in P. brassicae-inoculated roots treated with Serenade or the product components was substantially lower ($P < 0.0001$) than that in roots inoculated only with the pathogen (Table 2). The DNA amount increased substantially in roots of the pathogen control between 2 and 3 WAT, only slightly with the B. subtilis treatment, but not at all in roots treated with Serenade or product filtrate.

Table 2: The amount of *Plasmodiophora brassicae* DNA detected in canola roots (n = 3).

Treatment	*P. brassicae* DNA (ng/g of root)	
	2 WAT	3 WAT
Control	0.52 a	13.35 a
B. subtilis	0.18 b	1.16 b
Product filtrate	0.04 c	0.03 c
Serenade	0.02 c	0.01 c

Means with the same letters do not differ (LSD, $P = 0.05$).

The significant lower amount of pathogen DNA detected at 2 and 3 WAT in the root samples treated with Serenade or its components, may indicate that these treatments possibly suppress root-hair and cortical infection, because this biofungicide has little effect on the germination of resting spores (Lahlali et al. 2010). Root-hair infection tends to peak at 2 WAT (Sharma et al. 2010). In the current trial, the q-PCR data were correlated with the frequency of root-hair infection ($r = 0.86$) assessed microscopically at 2 WAT (data not shown).

In controlled-environment conditions, the biofungicide Serenade helped reduce clubroot severity on the moderately-resistant cultivar but this synergistic interaction was not observed in field trials due to general ineffectiveness of the biofungicides. Over several field trials in 2 years, biofungicides and synthetic fungicides applied in furrow as a liquid generally showed inconsistent and unsatisfactory efficacy. This insufficient performance under field conditions may have resulted from poor distribution (only 500 L carrier volume per ha) and short life span of these products in the soil (Peng et al. 2010). Some of the challenges may be tackled with more durable product formulations such as seed coating and granules. In light of the peak for production of secondary zoospores (Sharma et al. 2010) and cortical infection, it is likely that the products need to be effective in the soil for at least 2-3 WAT. These aspects are being investigated for selected biofungicides.

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