

# Effect of timing of application and population dynamics on the degree of biological control of *Sclerotinia sclerotiorum* by bacterial antagonists

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## Abstract

Antagonistic *Pseudomonas* spp. (DF-41 and PA-23) were evaluated for inhibition of germination of ascospores, and for the effect of timing of application and its effect on biological control of *Sclerotinia sclerotiorum* (Lib.) de Bary, causal agent of stem rot of canola. Population dynamics were also assessed. In all studies, a petal inoculation technique was used. Significant inhibition ( $P < 0.05$ ) of germination of ascospores was observed at both log 4 and log 8 cfu (colony forming units) ml<sup>-1</sup> of bacterial populations. In the population study, the pathogen had no significant effect ( $P < 0.05$ ) on bacterial populations; however, a significant ( $P < 0.05$ ) increase in bacterial populations was observed after 24 h and a decrease occurred between 96 and 120 h. Significant differences in disease severity ( $P < 0.05$ ) were found with respect to timing of ascospore applications in the control treatments (ascospores only). One isolate completely suppressed disease when co-applied with ascospores, while only minor suppression occurred when applied 24 or 48 h after. Results from all studies indicate PA-23 and DF-41 to be effective biocontrol agents against *S. sclerotiorum* of canola and to have practical implications for biological control of this disease by bacteria in the field.

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## 1. Introduction

Understanding population dynamics of potential biological control organisms (or antagonists) is fundamental to the implementation of these organisms into a disease management strategy and has been recognized in several reviews and studies [1–3]. The rapidly changing environment of the phylloplane often presents a challenge to successful colonization by antagonists, given that they can be affected by temperature, leaf wetness, competition from other microbes, pesticide applications, insects, relative humidity, pH levels, as well as the host plant itself [4]. Bacterial populations are known to

fluctuate rapidly as a result of these factors [4], and can rapidly decline to levels ineffective for antagonism [5]. Also, certain population levels must be reached to induce production of antibiotics and the formation of biofilms [6,7], both of which have been shown to have a significant impact in terms of competition and survival on the phyllosphere [8–10].

*Sclerotinia sclerotiorum* (Lib.) deBary is one of the most important diseases of canola in Western Canada [11] and is found world-wide, causing infection on more than 400 species of plants [12]. Research on biocontrol of *S. sclerotiorum* continues to be focused primarily on control of carpogenic germination or apothecia production [13,14], and on hyperparasitism by fungal antagonists to reduce sclerotial load in the soil [15]. However, a reduction in the number of sclerotia in a field does not necessarily eliminate the risk of significant yield losses. There exists, then, a need for research into

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biocontrol of *S. sclerotiorum* on canola through application of antagonists to the site of entry for this pathogen, the petal. Previous studies have demonstrated, firstly, that this fungus can be effectively controlled by application of bacterial antagonists to the petal [3,16], and, secondly, that the degree of control may be strongly affected by population levels and time of application of such antagonists [17].

Preliminary screenings for natural antagonists of *S. sclerotiorum* were carried out in a previous study. Several promising antagonists were identified, two of which have been chosen for use in this study. Isolates DF-41 and PA-23 both demonstrated significant inhibition *in vitro*, in the greenhouse, and in the field and were initially isolated from canola (cv Cresor) root tips (1998) and soybean root tips (1995), respectively. They were both found to be pseudomonads; PA-23 was identified as *Pseudomonas chlororaphis* (fluorescent biotype D), and DF-41 was identified to the genus level only.

The objectives of this study were: (1) to determine the degree of variability of populations of two potential antagonists on the phylloplane of canola petals, and the effect of *S. sclerotiorum* on this variability; (2) to determine how the population variability was related to the degree of inhibition given different competition levels of the pathogen (by use of different inoculation regimes) and (3) to determine the degree of antagonism occurring at the microscopic level given different initial concentrations of the antagonist.

## 2. Materials and methods

### 2.1. Antagonists

Bacterial antagonists used in this study were: *P. chlororaphis* (fluor. biotype D) strain PA-23 and *Pseudomonas* spp., strain DF-41.

### 2.2. *Sclerotinia sclerotiorum* inoculum production

Ascospores used in this study were produced using modifications of the protocol described by Lefol [18]. Sclerotia that had been grown on agar plates containing potato dextrose as a nutrient source (PDA plates) at 15 °C were incubated at 4 °C for two weeks. They were then surface sterilized with a 10% (v/v) NaOCl in distilled water (store brand bleach having an initial concentration of 4.0%) solution prior to placement on the vermiculite. Stipes appeared after 2–4 months, at which time sclerotia were transferred to 10-mm diameter Petri dishes containing 1.0% water agar. Ascospores were harvested from apothecia after approximately 2–5 weeks, using vacuum filtration onto a Millipore® membrane filter (type GS, 47 µm) placed in a 150-ml bottle top filter (0.22 µm CN) with 45 mm neck (Corn-

ing®, Corning, NY). They were stored in a desiccator at 4 °C until ready for use. Excellent spore viability has been reported after 24 mo using this protocol [19].

Spores were recovered using the methods of Hunter et al. [19]. Tween 20 (polyoxyethylene (20) sorbitan monolaurate, Mallinckrodt OR®, Paris, KY) was added as a surfactant to the spore suspension at a rate of 10 µl ml<sup>-1</sup>. The mixture was vigorously vortexed for 1 min to break up clumps of spores prior to enumeration using a hemacytometer. For all experiments, the spore density used was 1.0 × 10<sup>5</sup> ascospores ml<sup>-1</sup>.

### 2.3. Canola growth parameters

Plants were seeded in peat moss (Metro Mix® consisting of peat, vermiculite and perlite) and transplanted at the 3-leaf stage into a standard 2:1:1 soil mix: 2 parts soil, 1 part sand, 1 part peat. For all experiments, inoculations were carried out at the 30–50% bloom stage (the window of infection for the pathogen in the field [16]) and plants were incubated in a humidity chamber for 24 h following inoculation. They were then placed in the greenhouse and grown at 18 °C with a 16-h photoperiod for the duration of the experiments.

### 2.4. Effect of timing of application of BCA experiment

Disease suppression by strain 41 was investigated using a petal inoculation technique. Petals that had been randomly collected from plants grown in the greenhouse were dipped into either strain 41 or ascospore suspension and placed onto potato dextrose agar (PDA, Difco Laboratories, Detroit, MI) plates for 24–72 h before being used to inoculate the plants. Eight different inoculation regimes were analyzed for efficacy using differential timing of application of both organisms in a completely randomized design (CRD) (Table 1). Incubation temperature was maintained at 22 °C and petals were placed onto new PDA plates after each spore or bacterial treatment. Ten plants were used for each treatment, each plant having two leaves inoculated, with one petal placed into the axil of each leaf. Plants were incubated in a humidity chamber for 24 h following inoculation and then placed in the greenhouse and grown at 18 °C with a 16-h photoperiod for the duration of the experiment. Disease severity ratings, taken on days 1, 3, 6, 9, 12, and 14, were based on the following scale: 0: no visible stem or leaf infection, 1: leaf infection with no visible stem infection, 2–10: leaf infection present in all cases and disease severity based on size of stem lesion (mm), 2: 1–20, 3: 21–40, 4: 41–60, 5: 61–80, 6: 81–100, 7: 101–130, 8: 131–160, 9: 161–190, 10: >190 mm, or plant death. The experiment was repeated once.

Results from both studies were analyzed using Analysis of Variance (SAS Institute), looking at differences between each treatment on each day that disease

Table 1  
Timing of inoculation of canola petals (*Brassica napus*) with *S. sclerotiorum* and/or bacterial isolate 41

Treatment	Day			
	1	2	3	4
A	Ascospores			↑
B	Ascospores		Bacteria	
C	Bacteria	Ascospores		
D		Ascospores	Bacteria	Plant inoculations
E		Ascospores		
F		Bacteria	Ascospores	
G			Ascospores	
H			Bacteria and ascospores	↓

severity ratings were carried out [20]. When applicable (based on Tukey–Kramer Honestly Significant Differences (HSD)), data from different treatments were grouped for further analysis.

### 2.5. Strain development for bacterial population studies

To study the fluctuations in bacterial populations on canola petals in the greenhouse, a destructive sampling technique was used in combination with plating on selective LBA media containing 150 ppm rifampicin (95% a.i. 3-[4-methylpiperazinyliminomethyl], SIGMA®, St. Louis, MI) (LBA-R). Spontaneous rifampicin-resistant mutants of strain DF-41 were generated on LBA-R. The plating efficiency on LBA-R was determined to be equivalent to that on non-amended media, by growth curve comparison, which was repeated three times for accuracy.

### 2.6. Monitoring population changes in the absence of pathogen

In the first trial (I), canola petals were dipped into a log 8 suspension of rifampicin-resistant (RR) bacterial strain DF-41 (DF-RR41) and placed onto PDA plates for 3 days before being used in plant inoculations. A control treatment was also carried out for this trial, in order to verify the validity of the populations obtained for bacterial treatments. Petals were dipped into a solution of Tween 20 and water, and populations were monitored on 10 petals on days 1, 3, 5, 6, and 7 in the manner described below for the bacterial treatments. There were 10 plants used for each treatment, each having two leaves inoculated, with one petal placed into the axil of each leaf. Plants were incubated in a humidity chamber for 24 h following inoculation and then placed into the greenhouse and grown at 18 °C with a 16-h photoperiod for the duration of the experiment.

### 2.7. Monitoring population changes in the presence of pathogen

The second trial (II) included a treatment with bacteria, and another treatment having bacteria and as-

cospores present. Petals were dipped into an ascospore suspension 24 h after bacterial inoculation and 48 h before plant inoculation. Petal sampling for population levels was carried out right after bacterial inoculation (at 0 h) (Day –3), at 24 h (Day –2), at 48 h (Day –1), on the day of placement onto the leaves (Day 0), and on days 1, 3, 5, 7, and 10 (from plants that were in the greenhouse). Six petals were sampled (prior to plant inoculation), from which bacteria were quantified by sonicating the petals in a flask of sterile, distilled water, one flask per six petals. From this, six separate serial dilutions were carried out using half strength nutrient agar (NA, Difco Laboratories, Detroit, MI) amended with rifampicin and nystatin (mycostatin, 4460 USP U mg<sup>-1</sup>, SIGMA, St. Louise, MI). For the remaining days, destructive sampling was carried out on the plants in the greenhouse. Three leaves per plant were used (one petal per leaf) with a total of 10 plants being sampled for each treatment each day (30 petals were bulked together and assessed for population levels for each treatment). Leaves from each plant were bulked-assessed for populations as previously described for days –3 to –1.

Population means were derived from the log<sub>10</sub>-transformed populations of 6 or 30 replicate blossoms (6 for days –3, –2, and –1). Similar results were found for each trial; therefore, the data from each trial were tested for homogeneity of variance [20] within treatments and the trials combined for statistical analysis. Statistically significant differences were determined using the Student's *t* test.

### 2.8. Ascospore germination experiment

The effect of bacterial strains 41 and PA-23 on inhibition of ascospore germination and germtube elongation was investigated using microscopic techniques. Petals were dipped into an ascospore suspension 24 h after bacterial inoculation and 48 h before plant inoculation. In each of the three trials carried out (I–III), the control treatments were petals inoculated with ascospores only. Treatments in the trials were as follows: I (1) strain 41, (2) strain PA-23 (both at log 8 cfu ml<sup>-1</sup>), and (3) control; II (1) strain 41 at log 8 cfu ml<sup>-1</sup>, (2) strain 41

at log 4 cfu ml<sup>-1</sup>, and (3) control; III (1) strain 41 at log 4 cfu ml<sup>-1</sup>, (2) control. At 0, 6, 14, and 24 h, 25 petals were randomly sampled from the PDA plates and treated and stained as described in Fernando and Linderman [21]. Briefly, petals were transferred from the PDA plates onto filter papers in glass Petri plates that had been placed on absorbent cotton wads previously soaked in a 50:50 mixture (v/v) of 95% ethanol and glacial acetic acid (Fisher Scientific, Nepean, ON). Plates were sealed with parafilm and incubated at room temperature for approximately three days, or until all of the pigment had left the petals. Cleared petals were then placed onto 25 × 75 mm microscope slides and stained with a drop of 0.01% cotton blue in lactoglycerine (1:2:1 water:glycerol:lactic acid). Ascospores were considered to have germinated when the germtube was longer than the length of the ascospore itself.

### 2.9. Germtube elongation experiment

One hundred ascospores were randomly selected per treatment per sampling time for all trials from ascospore germination experiment. Germtube lengths were measured using a compound light microscope (Wild M20 Heerbrugg (Made in Switzerland), Microtech scientific sales, 67 Rockcliffe Rd., Winnipeg, MB) and classified into four categories as follows: 0–40, 41–90, 91–180, and >180 µm.

The Pearson  $\chi^2$  [20] test statistic was used to test for differences in germtube initiation and growth between treatments at each sampling time.

## 3. Results

### 3.1. Timing of BCA application

Statistical evaluation of results from both greenhouse trials revealed that they were not statistically different, and as such, the results from only the first trial are reported here.

Analysis of variance of the results found there to be a significant difference ( $P < 0.05$ ) between the treatments on all of the days. Disease progressed at the highest rate when the ascospores were present on the petals before the bacteria (B, D, Fig. 1), or when there were no bacteria present at all (E, A, G, Fig. 1). When bacteria were inoculated prior to or at the same time as *S. sclerotiorum* (co-inoculation), there was complete inhibition of disease (DSR = 0 at day 14).

Treatments E, B, and D (in which petals were inoculated with ascospores 24 h before plant inoculation (BPI), ascospores 72 h BPI and bacteria 48 h after ascospores, and ascospores 48 h BPI and bacteria 24 h after ascospores) had the highest Disease Severity Ratings (DSR) overall and showed a similar pattern of

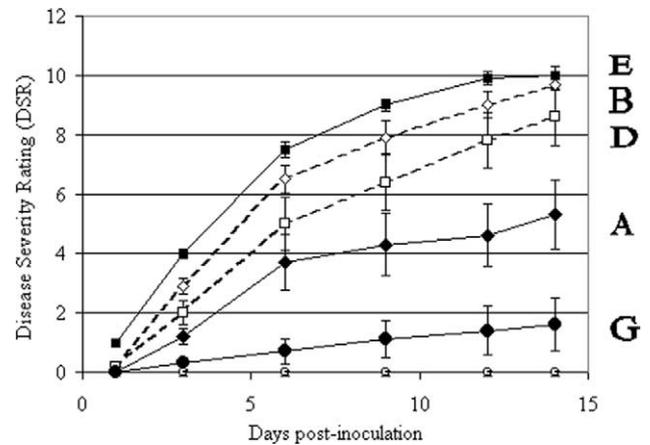


Fig. 1. Effect of inoculation time on *S. sclerotiorum* disease development. (A, E, G) had only ascospore inoculum (applied 72, 48, and 24 h before petals were placed onto leaves, respectively), (B, D) had ascospore inoculation 48 and 24 h prior to the addition of bacterial isolate 41, respectively. In treatments (F) and (C) bacterial addition preceded ascospores by 24 h, with ascospore inoculations being carried out 48 and 24 h prior to petal placement on leaves, respectively. (H) was a co-inoculation treatment with both bacteria and ascospores. All plants were inoculated on the same day. Points represent the mean DSR of 10 plants (representative data from one experimental trial). Error bars represent SE at the 95% CI (for  $n = 10$ ).

disease progression over the 14-day period (Fig. 1). Sclerotinia stem rot symptoms were visible as water-soaked lesions on the leaf axils of these plants (at the point of petal placement) immediately upon removal from the humidity chamber for all three of the treatments. Lesions had progressed into the stem by day 3 (DSR  $\geq 2$ ), and DSR ratings were either 9 or 10 by day 14. Treatment A, in which the ascospores were applied 72 h before plant inoculation, exhibited intermediate levels of disease, with stem infection visible by day 6 and reaching a maximum DSR of 5, while G (with ascospores applied 24 h BPI) had stem infection present on only one of the 10 replicate plants (hence the average DSR never reached 2). Treatments A, E, and G were significantly different ( $P < 0.05$ ) from each other at all of the time points tested according to Tukey–Kramer HSD, as were treatments A and B. Treatments D and E were significantly different ( $P < 0.05$ ) at all time points except day 14. Treatments B and D were compared to C, F, and H on days 1, 3, 9, and 14 (as the groups of treatments were found to be statistically comparable based on Tukey–Kramer HSD and on homogeneity of variance), and there was found to be a significant ( $P < 0.05$ ) difference between the groups of treatment for all of the aforementioned days.

### 3.2. Monitoring bacterial antagonist population changes

Bacteria that could be tentatively characterized as isolate RR41, based on morphological characteristics on the rifampicin plates, were significantly greater on

inoculated petals than on the control petals on days 1 and 3, but were almost identical by day 5 (192 h after bacterial inoculation) (Fig. 2(I)). Therefore, it can be concluded that RR41 bacteria was present during the first three days following petal placement onto the plants.

After inoculation, bacterial populations decreased, with an initial bacterial suspension of  $\log 8.0 \text{ cfu ml}^{-1}$  resulting in a per petal amount of  $\log 4.8$  and  $\log 3.8 \text{ cfu petal}^{-1}$  at time 0 (Fig. 2(I) and (II)). Populations increased to  $\log 7.5$  and  $7.7$  after 24 h for trials I and II, respectively, with a second increase to  $\log 9.8 \text{ cfu petal}^{-1}$  by 96 h for trial I. In trial II, populations of isolate 41 inoculated alone remained relatively stable for 96 h before they rapidly declined. In the first trial, populations steadily decreased after 96 h and were at  $\log 2.8 \text{ cfu petal}^{-1}$  by 192 h (5 days). In the second trial (II), populations decreased to  $\log 0.9 \text{ cfu petal}^{-1}$  by 144 h (3 days) and then increased to  $\log 1.6 \text{ cfu petal}^{-1}$  by 192 h. Populations of putative isolate RR41 on petals, where *S. sclerotiorum* ascospores were present (Fig. 2(II)), appeared larger than those with the bacteria in isolation,

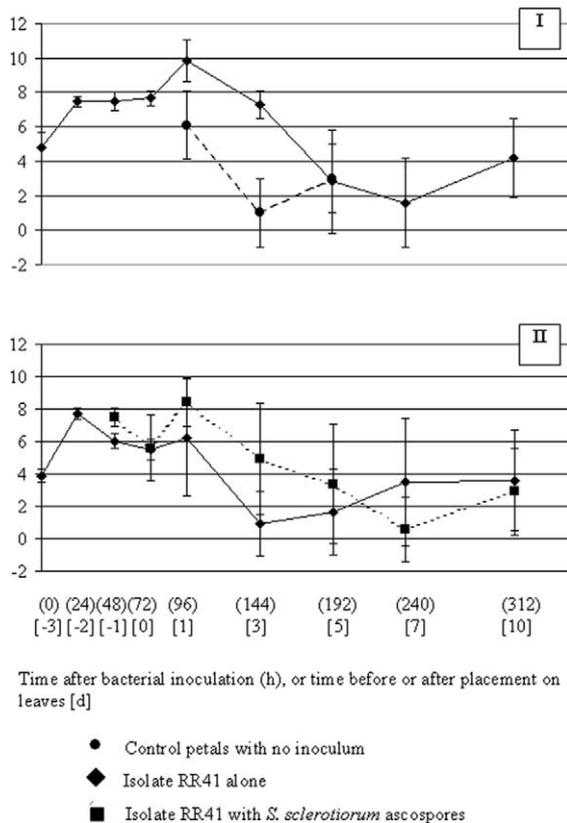


Fig. 2. Bacterial populations on canola petals placed onto PDA media and then onto leaf axils of *B. napus* (cv Westar) at 72 h. (I) isolate RR41 inoculated alone (◆), control petals having no bacterial inoculation (●), (II) isolate RR41 alone (◆), and with ascospores of *S. sclerotiorum* applied 48 h after bacterial inoculation (■). Bars represent the mean population of 6 (time 0, 24, and 48 h) and 10 (72–144 h) petals ( $n = 6$  and  $n = 10$ , respectively). Representative data from one experimental trial is shown. Error bars represent SD.

following the same patterns of growth throughout the five sampling periods, but the differences were not significant at the 5% level.

In examining population variations within each treatment over the sampling period (Fig. 2), it can be seen that there was a significant ( $P < 0.05$ ) increase in the bacteria-only treatments from 0 to 24 h and a significant ( $P < 0.05$ ) decrease from 96 to 120 h for all three treatments. Populations were relatively stable for all treatments between 48 and 96 h with the exception being trial 1 (isolate RR41 only), where there was a significant ( $P < 0.05$ ) increase between 72 and 96 h. Placement of the petals on the leaves did not seem to have an immediate impact on the bacterial populations. In addition, putative RR41 were apparently present on the petals during the critical stages of ascospore infection (prior to spore addition and during the first three days after petals were placed on the leaves).

### 3.3. Ascospore germination and germtube growth

Ascospores on petals treated with bacteria exhibited significantly less germination than those growing in isolation on the petals (Fig. 3). As well, there was less total growth and the germtubes were shorter.

Germination of ascospores on petals incubated on PDA plates was significantly inhibited in the presence of isolate RR41 at a concentration of  $\log 8 \text{ cfu ml}^{-1}$ , but not at  $\log 4 \text{ cfu ml}^{-1}$  (Fig. 3). Near complete inhibition was observed for the higher concentration in both trials I and II, at over 80% after 24 h, while only 63% and 5% was reported with the lower concentration at the same time point (Fig. 3). Spore germination followed a linear growth pattern over the 24-h sampling period for petals treated with PA-23, reaching 69% growth by 24 h, while growth rate was significantly ( $P < 0.005$ ) less and only reached 18% and 8% in trials I and II, respectively, for petals treated with isolate 41 (Fig. 3). Growth of the spores followed an exponential rate during the sampling period, reaching 75% in the first trial and 100% in the second two trials. Control of germination using isolate 41 at  $\log 4 \text{ cfu ml}^{-1}$  was more variable, demonstrating a pattern similar to that shown by isolate PA-23 in the second trial, and exhibiting almost no inhibition at all in the third trial.

Using the Pearson  $\chi^2$ -test statistic, it was determined that there was a significant treatment effect at each sampling time ( $P < 0.005$  in all cases except at 6 h in trial I,  $P < 0.02$ ).

The degree of inhibition by isolate 41 at  $\log 8 \text{ cfu ml}^{-1}$  can clearly be seen in Fig. 4, in which the images on the right-hand side, depicting the isolate 41 treatment, can be compared with those on the left, depicting the control treatment. It can clearly be seen that there is almost complete inhibition by isolate RR41 (A–D), while there is considerable branching of germtubes by 14 h (G), and

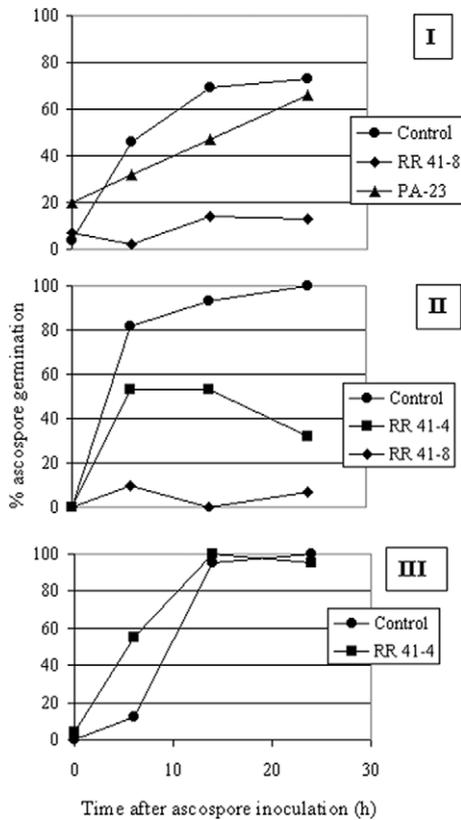


Fig. 3. Inhibition of ascospore germination and growth by bacterial isolates 41 and PA-23. Bacteria were inoculated 24 h prior to ascospores ( $\log 5$  ascospores  $\text{ml}^{-1}$ ). Representative data from one experimental trial is shown. SE was found to be  $<1\%$  for all trials.

petal colonization by 24 h (H), in control treatments. Bacteria can be seen clustering around the ascospores after 24 h in image (D). Also in this figure, pollen grains can be seen (in C, F–H) as spherical structures with a radius of about 10–15  $\mu\text{m}$ .

#### 4. Discussion

Successful colonization by *Pseudomonas* sp. isolate 41 and effective competition with *S. sclerotiorum* (at  $\log 4$  and  $8 \text{ cfu ml}^{-1}$ ) was observed in this investigation, both macroscopically and microscopically.

In the greenhouse study, there were variations in degree of colonization on the petals by both antagonist and pathogen from the various inoculation regimes. The greatest amount of mycelial growth could be seen in the treatment in which the ascospores were applied 72 h before plant inoculation, followed by a treatment in which ascospores were applied 72 h before plant inoculation and bacteria were applied 72 h after the ascospore, and a treatment in which ascospore treatment was applied at 48 h before plant inoculation. The treatment consisting of concurrent application of bacteria and ascospores, conversely, showed visible signs of

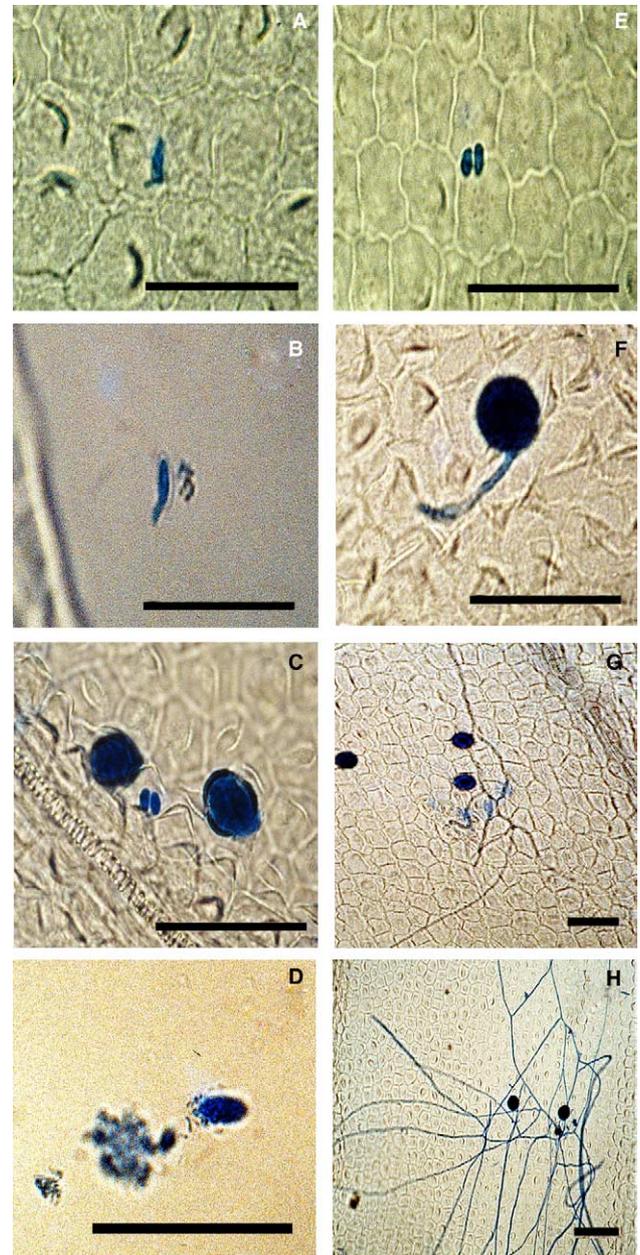


Fig. 4. Microscopic examination of ascospore germination and growth on canola petals in the presence and absence of antagonist. (A–D) Isolate RR41 inoculated at  $\log 8 \text{ cfu ml}^{-1}$  24 h prior to inoculation with ascospores, (E–H) *S. sclerotiorum* ascospores inoculated without antagonist present. Top to bottom: after 0, 6, 14, and 24 h of incubation on PDA plates, respectively. (G) and (H) are at  $160\times$  magnification, (D), (C), and (F) are at  $400\times$ , and the rest are at  $630\times$  magnification (bar =  $20 \mu\text{m}$ ).

bacterial infestation. Furthermore, the treatment in which bacteria were applied 24 h after ascospores also showed bacterial colonization. These latter plates had displayed mycelial growth at the time of bacterial application. This indicates that bacteria can effectively compete for nutrients, even after growth and establishment of the pathogen. However, it should be noted that this part of the experiment was conducted on Petri

plates containing a nutrient source other than just the petals, which would be the case in the field. Studies by Yuen et al. [17] found that populations of bacteria applied directly to bean blossoms decreased by 2.5 log units in the first 24 h after application and that bacterial populations in the field were consistently lower than those recorded in the greenhouse. The treatment in which ascospores were applied 24 h before plant inoculation showed no visible signs of mycelia, while the petals that had the bacterial treatment prior to ascospore inoculation were heavily infested with bacteria, again, likely attributable in large part to the nutrient-rich environment of the Petri plates.

Disease progressed at a similar rate within each treatment, with the difference being in the overall severity rating at each time point between treatments (Fig. 1). In terms of the treatments in which only ascospores were applied to the petals (A, E, G), the highest rate of disease progression was observed in E, where they were applied 48 h pre-plant inoculation (PPI), followed by A at 72 h pre-plant inoculation, and G at 24 h before plant inoculation. This variation could be explained as a response to nutrient depletion in the 72-h treatment, and by insufficient time for complete petal colonization by the ascospores in treatment G. Abawi et al. [22] found that appressoria start to form 10–12 h after placement on bean plant tissues. Branching began after 24 h, and by 48 h many branched appressoria had been formed by the majority of the fungal colonies. There might have been too few appressoria formed by 24 h in treatment G to prevent spores from washing off in the humidity chamber treatment. This supposition is substantiated by Boosalis et al. [23], who report on improved “stickiness” of ascospores as a result of a new method for storage and recovery that limits the disruption of the gelatinous coating on the spores. This removal, they stated, was likely caused by storage on and removal of ascospores from filter papers, a technique that was used in their earlier studies [19] and was the same one used in this study.

Analysis of the effect of adding bacteria after ascospores indicates that treatments in which bacteria were added to the petals 48 and 24 h after ascospores had higher rates of disease progression than those with ascospore inoculation only at 72, 48, and 24 h pre-plant inoculation. Bacteria applied 48 h after ascospore inoculation could have acted as a nutrient source for the pathogen, thus breaking the dormancy assumed to be occurring in treatment (A). Evidence for this was found in the microscopic experiments carried out in this study and will be described in more detail when these results are discussed.

The superior competitive ability of isolate 41 was demonstrated by its complete suppression of disease development when applied as a co-inoculation treatment or prior to ascospore inoculation. This treatment, when

grouped together with (F) and (H) was found to be statistically different from treatments (B) and (D) (also compared as a group) on days 1, 3, 9, and 14 ( $P < 0.05$ ). The difference between these two groups was that in the first, bacteria were applied after the ascospores, while in the second group they were either applied before or at the same time. It would appear, then, that bacteria are able to significantly inhibit disease when applied before, or even at the same time as ascospores. In a practical sense, this could mean that a field application of antagonist could be concurrent with infection by the pathogen.

In comparing the treatment in which the bacteria were applied 24 h after the pathogen and the treatment that had the same overall time scale (48 h from pathogen inoculation to plant inoculation) but no bacterial application, there was significantly more disease in the latter treatment (Fig. 1). This suggests the ability of the bacterial isolate to compete with ascospores already established on the petals, and has significant implications in the field, in that it can be difficult to assess the exact timing required for sprays in order to achieve adequate disease control in the field. An antagonist with this kind of curative potential could therefore be an invaluable resource.

Bacterial populations were not significantly affected by the presence of the pathogen 72 h after bacterial inoculation and onward. This can be seen in Fig. 2, in which the population levels of the treatment with ascospores and bacteria present (trial II) were not significantly different from the bacteria-only treatment. If nutrient competition is a significant factor in disease suppression by isolate 41, the populations for the treatment with pathogen and bacteria present would be expected to be lower throughout the duration of the experiment due to niche overlap [24].

An alternate explanation might be that isolate RR41 is hyperparasitic (or an organism parasitising a primary parasite) towards the pathogen, as populations were significantly greater in the treatment with *S. sclerotiorum* than in that having bacteria only (Fig. 2(I), 48 h after bacterial inoculation). Further evidence of the possible role of hyperparasitism is that bacteria can be seen “eating” the ascospores in Fig. 4(D). As well, there were considerably fewer ascospores overall on the petals at 14 and 24 h, and at 6, 14, and 24 h for the log 4 and log 8  $\text{cfu ml}^{-1}$  treatments, respectively. If hyperparasitism is indeed occurring, increased disease pressure in the form of more ascospores landing on the petals could, in fact, increase the longevity of the bacteria on the phylloplane by acting as a nutrient source. This phenomenon was observed in a three-year field trial by McLaren et al. [15], in which hyperparasitic fungi reduced the inoculum load of the pathogen.

Rapid colonization of the petals was seen in the increase of bacteria from approximately log 4 to

log 8 cfu ml<sup>-1</sup> between 0 and 24 h (Trials I and II, Fig. 2). An increase from log 8 to log 10 cfu ml<sup>-1</sup> was observed by Kloepper [25] in his study using *Pseudomonas fluorescens* (strain Pf-5), but this was on hypocotyls, which are known to be less nutrient-rich than petals [26]. Results from this study suggest that the carrying capacity of the petals may be as high as log 10 cfu ml<sup>-1</sup>. There is a marked contrast between the controlled environmental conditions present in the greenhouse, and the rapidly changing field environment, in which it has been found that populations of bacteria and levels of protection against *S. sclerotiorum* may not be as high as those seen in the greenhouse [17]. Ascospores are known to remain viable for approximately 12 days in the field [27]; hence bacterial numbers would have to remain high for weeks in order to provide adequate control. Fig. 2 shows that populations decreased significantly within 7 days after inoculation of petals, or 3 days from petal emplacement, for all treatments. However, the fact that RR41 displayed a fungicidal effect on ascospores in the germination experiment and disease was completely suppressed for 2 weeks after the co-application treatment of bacteria and pathogen suggests that the bacterial longevity might not be an issue in providing adequate disease control. In fact, further results from this lab have strongly suggested that induced systemic resistance (or ISR, in which a colonization by one type of pathogen or micro organism elicits a resistance response by the plant protecting it from subsequent infection [28]) might play a role in the suppression of disease by this isolate (*Yilan Zhang, personal communication*).

Spread of bacteria from treated to control plants in the humidity chamber was assumed to be the cause of the relatively large bacterial populations (log 6.1 cfu petal<sup>-1</sup>) isolated from the control petals on day 1 (96 h). These numbers were, nonetheless, considerably lower than those retrieved from the treated petals (approximately log 9.7 cfu petal<sup>-1</sup>). This could be attributed to cross-contamination in the humidity chamber, since there is a significant amount of moisture and air circulation in this environment, both of which could have acted to spread bacteria from the treated to the control plants. This could have a positive impact in the field, where plant contact would be occurring on a regular basis.

Control of ascospore germination and growth was observed when isolate 41 was applied at log 8 cfu ml<sup>-1</sup>, less at log 4, and intermediate inhibition for isolate PA-23 (log 8 cfu ml<sup>-1</sup>) (*unpublished data*). The latter is an important finding in that it verifies that the significant inhibition of disease observed in the greenhouse studies for isolate 41 at the higher concentration was not simply a physical “artifact” of the ascospores not being able to penetrate through a bacterial coating. Yuen et al. [3] found less inhibition of ascospore germination in their study using *Erwinia herbicola* and *Bacillus polymyxa* to control *S. sclerotiorum* on detached bean blossoms. At

concentrations of log 6.3 and 8.4, a 64% and 49% degree of inhibition was observed for each treatment, respectively, after 4 h of incubation. In the present study, however, it was found that RR41 can inhibit at log 8 and log 4 cfu ml<sup>-1</sup>, indicating superior control of this pathogen. It is interesting to note the increase in inhibition at log 4 cfu ml<sup>-1</sup> seen at 24 h in trial II. This is likely not a result of decreased germination of the spores, as the control demonstrated increased overall growth in this trial as compared to trial I. It is known that the pathogens are very vulnerable at the germination stage of infection [29].

It has been suggested that diseases caused by *Sclerotinia* spp. may be spread through infection and subsequent transport of infected pollen grains by wind or insects [13]. Infection of pollen grains could clearly be seen on numerous petals in all of the treatments in this study (Fig. 4(F)). Ascospores of *S. sclerotiorum* were identified using the description and images in the study by Lefol and Morrall [30], and are seen as the ovoid structures approximately 8 µm in length in this figure. Pollen did not inhibit *S. sclerotiorum* infection. Complete petal colonization was observed by 24 h (Fig. 4(H)), in agreement with findings with this pathogen on bean flowers [22].

Isolate 41 fits with criteria that have been mentioned in previous studies involving biological control of *S. sclerotiorum*. Yuen et al. [3] reported that epiphytic bacteria that are able to colonize the blossoms quickly would be the ideal candidates for biological control. Isolate 41 demonstrated this ability given the conditions used in this study, and a field study conducted in 2003 substantiated these results by showing significant disease suppression by this isolate [31].

Long-term control of disease outbreaks would be necessary since sclerotia have been known to remain viable up to 10 years in the field [32]. This would take the form of annual bacterial applications, targeting the flowering period of the crop (the window of infection for *S. sclerotiorum*). However, it has recently been shown that sclerotial degradation took place within a year when sclerotia were placed at 5 or 10 cm depths in the soil [33] and as such, annual carry-over inoculum might be reduced. Reduction of disease incidence and severity of *S. sclerotiorum* by *Bacillus* bacteria in the field has been demonstrated in a field study by Tu [34] in which the effectiveness of biocontrol was cumulative over the 2 year period.

Mercier and Reeleder [35] suggested that rapid nutrient acquisition would limit pathogen penetration and infection. Previous studies using this isolate (*unpublished data*, *S. Savchuk*) have found that log 4 cfu ml<sup>-1</sup> was effective in controlling the pathogen, and this was seen in the significant inhibition of ascospore germination that occurred at this concentration in one repetition of this experiment (see Fig. 3(II)).

In summary, isolate 41 is able to effectively colonize canola petals for several days and effectively control disease development, even when applied to canola petals at the same time as the pathogen (in the greenhouse). This isolate thus warrants further investigation in a long-term study as a potential foliar biocontrol agent of *S. sclerotiorum*, as it has demonstrated adaptability and longevity in greenhouse trials, and (in previous studies in this lab, S. Savchuk) the ability to decrease infection in the field.

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