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Full Length Article

Biological Control Potential of *Trichoderma* Species and Bacterial Antagonists against *Sclerotinia sclerotiorum* on Canola in Western Australia

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Abstract

Fifteen fungal and three bacterial biological control agents (F-BCA and B-BCA, respectively) were isolated from the canola production areas of Western Australia to investigate their potential for controlling sclerotinia stem rot (SSR) caused by *Sclerotinia sclerotiorum* under *in vitro* and field conditions. The capacity of these isolates to inhibit mycelial growth and sclerotia formation by *S. sclerotiorum* was assessed in dual culture tests in Petri dishes. Using Sanger Sequencing of the ITS regions, the F-BCAs were identified as *Trichoderma atroviride* (four isolates), *T. gamsii* (three isolates), *T. koningiopsis* (two isolates), *T. longibrachiatum* (two isolates), *T. paraviridescens* (two isolates), *T. pseudokoningii* (one isolate) and *T. viridescens* (one isolate). Four of the seven *Trichoderma* species (*T. koningiopsis*, *T. gamsii*, *T. atroviride* and *T. viridescens*) are reported for the first time from Western Australia. 16S rRNA sequencing identified B-BCA1 and B-BCA2 as *Serratia proteamaculans* and B-BCA3 as *Ochrobactrum anthropi.* There were significant differences among F-BCAs ($P \leq 0.001$) in their effect on radial mycelial growth (40–60% inhibition) and sclerotia formation (65–100% inhibition). Two isolates of *T. atroviride* (F-BCA12 and F-BCA15) completely blocked sclerotial formation of the pathogen on Potato dextrose agar + 10 ppm/L Aureomycin (PDAA). Incubation of sclerotia in soil inoculated with F-BCA indicated that sclerotia were colonized by the conidia of each F-BCA, and all sclerotia in the presence of F-BCAs failed to germinate on PDAA. The B-BCAs reduced radial mycelial growth by 57–59% and formation of sclerotia by 89–95%. Selected isolates of F-BCAs (*T. koningiopsis* and *T. atroviride*) and B-BCAs (*O. anthropi* and *S. proteamaculans*) significantly reduced disease incidence of *S. sclerotiorum* under glasshouse and field conditions. Field efficacy of tested BCAs was similar or better than the commercial fungicide Prosaro®. © 2022 Friends Science Publishers

Keywords: Inhibition, Mycelial growth, Sclerotia, *Trichoderma* spp., *Serratia proteamaculans*, *Ochrobactrum anthropi*

Introduction

Sclerotinia stem rot (SSR) caused by *Sclerotinia sclerotiorum* (Lib.) de Bary is an important disease of canola causing significant crop losses worldwide (Kamal *et al.* 2016; Smolinska and Kowalska 2018) including West Australian (WA) (Khangura and MacLeod 2012; Khangura *et al.* 2014, Khangura and Van Burgel 2021). Strategies to reduce production losses in canola usually rely on the application of fungicides (Rimmer *et al.* 2007; Khangura and Van Burgel 2021). However, consumer concern on the impact of chemical fungicides has increased the demand for eco-friendly products which are relatively free from chemical residues (Raaijmakers *et al.* 2002).

Biological control is an alternative approach for disease management that is environmentally safe and reduces the amount of human contact with harmful chemicals and their residues. A variety of biocontrol agents, including fungi and bacteria, have been identified but further development and deployment is required (Sharma *et al.* 2017). The limited availability of commercial BCAs has been a major constraint to the development of eco-friendly and sustainable disease management worldwide (Vincent *et al.* 2007). The key factor in developing effective and efficient BCAs is the exploration for potential BCAs across agricultural production regions globally. Therefore, isolation, screening and identification of local BCAs are needed, including in WA which enforces its own biosecurity and quarantine Act.

Several investigations have been conducted to explore potential BCAs from a wide range of niches such as the rhizosphere, phyllosphere, sclerotia and other habitats (Whipps *et al.* 2008). Research has been conducted to test the potential of fungal BCAs against *S. sclerotiorum* such as the use of antagonistic *Coniothyrium minitans* to control SSR disease in some countries (Whipps *et al.* 2008; Yang *et al.* 2011). Some success stories also include the use of antagonistic *Trichoderma* spp. in bean crops against SSR in Brazil (Lopes *et al.* 2012).

There is an opportunity to obtain BCAs from local agricultural regions because potential BCAs have already become established with the pathogen in the ecosystem. However, the concentration and distribution of potential BCAs in ecosystems can be very limited and therefore research to discover new and potential BCAs is invaluable (Lopes *et al.* 2012). In WA, this research is the first to explore the potential of BCAs in managing SSR in canola.

Research on sustainable management of SSR on canola has been conducted including the use of cultural practices (Kharbanda and Tewari 1996) and screening for resistant genotypes (Barbetti *et al.* 2013; Taylor *et al.* 2015). However, due to the quantitative nature of host resistance, it is very difficult to develop completely resistant canola genotypes (Li *et al.* 2006). Currently, no commercial canola variety in Australia is resistant to *S. sclerotiorum*. Therefore, disease control mainly relies on the use of fungicides in combination with cultural practices (Khangura and McLeod 2012). Control of SSR disease by fungicide alone is less effective due to mismatch in spraying time and ascospore release (Bolton *et al.* 2006). Some fungicides gradually lose efficacy as resistant strains of *S. sclerotiorum* emerge (Zhang *et al.* 2003). Decreasing fungicide efficacy over time has reduced the chemical control cost-benefit ratio, at the same period that concern about environmental impacts from chemical fungicides has increased. This has led to research on alternative strategies for controlling *S. sclerotiorum* on canola. Interest in biological control of SSR diseases on canola has increased over recent decades (Saharan and Mehta 2008).

The objectives of this research were: (1) to isolate potential BCAs from WA canola growing areas to control *S. sclerotiorum*; (2) to investigate the efficacy of potential BCAs against *S. sclerotiorum* and their ability to reduce sclerotial formation by the pathogen *in vitro*; (3) to identify the species of potential BCAs; and (4) to investigate the efficacy of newly identified local F-BCAs and B-BCAs in controlling SSR disease on canola under WA field conditions.

Materials and Methods

Isolation and maintenance of potential biological control agents

Potential BCAs were isolated from canola plants, sclerotia of *S. sclerotiorum* and soil. Root, stem and pod samples from approximately 500 healthy and diseased canola plants from the Southern Region of WA were collected and cut into pieces 3 mm in length. Samples were surface sterilised using 1% aqueous $NaHClO_3$ for two minutes and rinsed three times with sterilized distilled water. Samples were dried on tissue paper before being placed into a Petri dish on PDAA medium (Potato dextrose agar + 10 ppm/L Aureomycin) and incubated in a 21ºC growth room with 12 h photoperiod. After 48 h of incubation, potential BCAs that grew from the samples were observed and replated onto new Petri dishes.

Soil samples were collected from canola fields during the WA canola growing season of 2012–2013 in order to isolate the BCAs from soil. The soil samples were placed in small plastic pots (diameter 10 cm) along with ten surface sterilised sclerotia in order to be infected by the potential BCAs. The pots were incubated in a 21ºC growth room for three months. Afterwards, sclerotia were removed and transferred to Petri dishes containing PDAA medium and placed in a growth room. Three days after inoculation, Petri dishes were monitored for potential fungal growth around the sclerotia. Potential F-BCAs, which grew from or around the sclerotia, were isolated onto PDAA medium for further investigation.

Petal samples (approximately 2000 petals) from healthy and diseased canola plants were collected from producers' fields. Petal samples were directly inoculated onto PDA + 10 mL Streptomycin (0.5 mL) and 10 mL Ampicillin (0.5 mL) (PDSA) medium in Petri dishes then incubated in a 21ºC growth room with 12 h photoperiod. After 48 h of incubation, all colonies produced from petal samples were isolated for further investigation.

Approximately 2000 sclerotia were collected from inside the stems of diseased canola plants in 2013. Samples were sterilized using 1% $NaHClO_3$ solution for two minutes and rinsed three times with sterilized distilled water. Sclerotia were directly inoculated on PDAA medium in Petri dishes then incubated in a 21ºC growth room with 12 h photoperiod. After 48 h of incubation, all colonies growing from sclerotia were isolated for further investigation. Potential F-BCAs, which grew from or around the sclerotia, were isolated for further investigation onto PDAA plates and potential B-BCAs were transferred to peptone yeast dextrose agar (PYDA). Potential F-BCAs and B-BCAs were maintained on PDAA and PYDA respectively at 4ºC in a cold room for further investigation.

Identification of potential BCAs

Fifteen potential F-BCAs and three potential B-BCAs were identified to species level at the Australian Genomics Research Facilities (AGRF) laboratories through molecular techniques. For the F-BCAs, the Sanger Sequencing method was used to sequence the purified PCR products. The samples were prepared based on DNA sample preparation instructions by the AGRF (www.agrf.org.au). Each reaction mixture contained 6–12 ng of PCR product and 0.8 pmol/μL

of the specific primer in 12 μL with H_2O. Purified PCR products were Sanger-sequenced with Big-Dye 3.1 (PerkinElmer, Waltham, MA), using PCR primers ITS1-f forward and reverse (Gardes and Bruns 1993; de la Cerda *et al.* 2007) and ITS4 (White *et al.* 1990) and analysed using an ABI3730xl analyzer (Thermofisher).

For the B-BCAs, the Australian Genomics Research Facilities 16S sequencing process employed universal primers to interrogate an approximate 800 bp region of the 16S ribosomal RNA (www.agrf.org.au). Bacterial samples were subjected to an initial amplification using the universal 16S primers (www.agrf.org.au). The process included PCR amplification, QC gel assessment, PCR purification, sequencing and purification, capillary separation and analysis. The amplified product was visualized on a 2% agarose gel to confirm successful amplification. The amplified product was then subjected to a manual purification employing magnetic beads; the purified product was resuspended in HPLC grade water, then a Big Dye Terminator sequencing reaction for both the forward and reverse directions was performed using the previously mentioned universal primers. The sequencing products were then subjected to an automated purification employing magnetic beads; the purified product was re-suspended in EDTA. Samples were separated by capillary electrophoresis (96 capillary) using an Applied Biosystems 3730xl Genetic Analyzer (Thermofisher). All samples were then blasted against the AGRF in-house 16S database; this database was derived from publicly available 16S databases (green genes). A positive control and a negative control were processed in triplicate using 16S sequencing batch. Positive control samples consistently generated the same BLAST result (Altschul *et al.* 1990; Altschul *et al.* 1997).

Mycelial and colony growth of potential BCAs

F-BCAs: A 3-day-old on PDAA grown, 5 mm of mycelial disc from each F-BCA was placed in the centre of each of 3 Petri dishes and then incubated in growth room at 21ºC with 12 h photoperiod. Radial mycelial growth was recorded at 24 and 48 h after incubation. All potential F-BCAs were grown up to 7 days to determine colony colour and photographs were then taken.

B-BCAs: A 1-day-old, PYDA grown bacterial BCA was streaked onto new PYDA in Petri dishes and incubated in a growth room at 21ºC with 12 h photoperiod. Three replicates were prepared for each isolate. Growth and colony colour were observed 24 h after incubation and photographs were taken. The relative extent of colonization of the Petri dish was used to rank the colony growth rate.

***In vitro* biological control of *S. sclerotiorum* by potential BCAs**

Dual culture test of BCAs: A 3-day-old mycelial plug (5 mm diameter) of each F-BCA isolate was incubated on PDAA. A mycelial plug of an aggressive isolate (SS12) of *S. sclerotiorum* was removed from the colony margin of a 3-day-old culture grown on PDAA and placed 7 cm distance from the plug of the F-BCA isolate in the same Petri dish. Three replicates were prepared. Petri dishes inoculated similarly, but with F-BCAs or *S. sclerotiorum* alone, were used as controls. Plates were incubated in a 21ºC growth room and observed after 72 h for inhibition zones between F-BCAs and the *S. sclerotiorum* isolate. After incubation period, radial mycelial growth was recorded. Reduction in the radial mycelial growth was calculated as follows:

$$\text{Inhibition percentage of radial mycelial growth} = \left[\frac{(C-T)}{C}\right] \times 100$$

Where C is the pathogen radial mycelial growth measurement in control plates, and T is the pathogen radial mycelial growth in presence of F-BCAs (Simonetti *et al.* 2012). After incubated for two weeks, the number of sclerotia formed by *S. sclerotiorum* in each Petri dish was recorded and the percentage inhibition of sclerotia formation was calculated with the same formula above with adjustment for sclerotia formation.

Three isolates of B-BCAs were tested for their antagonistic effect in dual culture tests. For dual culture tests, two inoculation methods were used for the pathogen, either mycelial plugs or sclerotia. In the mycelial plug method, a 3-day-old mycelial plug with 5 mm diameter of *S. sclerotiorum* isolate SS12 was placed on PYDA about 1 cm away from the edge of each Petri dish. In the sclerotia method, a sclerotium produced by *S. sclerotiorum* isolate SS12 (after 2 weeks of incubation in Petri dishes) was incubated on PYDA about 1 cm away from the edge of each Petri dish. A 3-day-old culture of B-BCAs grown on PYDA was streaked 7 cm away from the plug/sclerotia of the pathogen isolate in the same petri dish. Petri dishes inoculated similarly with each B-BCA or *S. sclerotiorum* isolate SS12 alone were used as controls. There were three Petri dishes replication for each treatment. Plates were incubated in a 21ºC growth room and were observed after 48 h for calculation of inhibition zones between B-BCAs and *S. sclerotiorum*. Radial growth was calculated after incubation period. The numbers of sclerotia formed by the pathogen were recorded two weeks after incubation. Inhibition in radial growth and sclerotial production by each B-BCA was calculated as described for the F-BCAs.

Hyper-parasitisation of *S. sclerotiorum* sclerotia by BCAs under artificial inoculation in pots

Ten inoculum discs of each of the 15 F-BCAs were mixed in separate 100 mm diameter plastic pots (Burnell Agencies Pty Ltd) which were filled with a commercial bulk potting mix produced by Baileys (http://www.baileysfertiliser.com.au/). Each F-BCA was replicated in three plastic pots. The plastic pots were placed in a growth room at 21ºC with a 12 h photoperiod for about one week. Ten sclerotia of *S. sclerotiorum* isolate SS12 were placed at a depth of 5 cm in

each plastic pot. The plastic pots were watered by hand every morning. After a week, two sclerotia from each pot were plated onto PDAA and grown for seven days to test the viability of sclerotia.

In planta testing the efficacy of BCA's

Glasshouse experiment: Canola plants were grown in 30 cm diameter plastic pots that were arranged in a Randomized Complete Block Design (RCBD). Each pot had one canola plant and there were four replicate pots per treatment. A potting mix (http://www.baileysfertiliser.com.au/) was used as the growth medium in the glasshouse; each pot was mixed with 10 g of NPK (19:19:19) fertilizer before sowing. The glasshouse trial was undertaken at the same time as the 2015 field experiments. The B-BCA1 (*S. proteamaculans*), B-BCA2 (*O. anthropi*), F-BCA1 (*T. koningiopsis*) and F-BCA2 (*T. atroviride*) were sprayed at the green bud stage. The *S. sclerotiorum* isolate SS12 was sprayed at 10, 30 or 50% flowering stages. The aim of the glasshouse experiment was to evaluate the effectiveness of the BCAs in suppressing SSR for comparison with the field experiments.

Field experiments: Field experiments were conducted in 2014 and 2015 to evaluate the effectiveness of selected BCAs under field conditions at the Department of Primary Industries and Regional Development field experimental area in South Perth. The aim of the 2014 field experiments was to investigate the efficacy of newly identified local F-BCAs and B-BCAs in controlling SSR disease on canola by co-inoculating at 50% flowering. The 2014 field experiment was established as a RCBD comprising seven treatments and three replicate plots. Individual plot size was 2 x 2 m^2. Seed (cultivar Crusher) was sown by hand to a depth of 1 cm on the 29 May 2014. Fertilizer was applied based on common practice for canola in Australia: 110 kg N/ha, 15 kg P/ha, 12 kg K/ha and 20 kg S/ha (Norton *et al.* 2011). The BCA and pathogen treatments were applied at 50% flowering (Table 1).

To prepare the pathogen inoculum, ten agar plugs (5 mm diameter) were cut from the margin of actively growing 3-day-old colonies and transferred to a 250 mL conical flask contained sterile liquid medium (24 g/L potato dextrose broth with 10 g/L peptone) and shaken at 250 rpm. The inoculated medium was incubated for 3 days at 21°C. The *S. sclerotiorum* colonies were harvested and rinsed three times with sterile deionized water. Before inoculation on plants, the harvested mycelial mats were transferred into 150 mL of liquid medium and homogenized at medium speed in a blender for 1 min. The macerated mycelia were filtered through three layers of cheesecloth and suspended in the same liquid medium. Then, the concentration of mycelia was counted using a haemocytometer and adjusted to the required concentration for the experiment. Similar procedures were applied to produce inocula of the B-BCAs. Suspensions (100 mL) containing either mycelia of F-BCAs or colonies of B-BCAs were sprayed at concentrations of ~10^6 fragment mL^{-1} and ~10^{10} CFU mL^{-1}, respectively, in each treatment plot. Mycelia of the pathogen were also sprayed at 100 mL per plot at a concentration of ~10^6 fragment mL^{-1}. The fungicide Prosaro® was sprayed based on the recommended dose of 450 mL ha^{-1} (equal to 0.2 mL $plot^{-1}$) to compare the efficacy of potential BCAs with a fungicide recommended for Sclerotinia control in canola in Australia. The control treatment plots were sprayed with water only. The number of infected plants in every plot was counted and disease incidence was calculated for each treatment two weeks after inoculation. Plots were harvested at maturity and seed yield was obtained for each plot.

In 2015, two field experiments were carried out to optimise the timing of application of BCAs against *S. sclerotiorum*. In the first experiment, the BCAs were sprayed at the green bud stage, while in the second experiment; the BCAs were sprayed one week before the pathogen and at the same time as the pathogen at 30% bloom. In experiment 1, a Randomized Complete Factorial Design (RCFD) was used consisting of sixteen treatments with pseudo-replication inside the treatment due to limited space (Table 2). Rows were 7 m long. There were 3 buffer rows on each side of the treatment rows to prevent inoculum drift. Cultivar Hyola 404 was hand sown @ 0.8 g (approximately 150 seeds) per row on 1st June 2015. In this experiment, the BCAs (*S. proteamaculans, O. anthropi, T. koningiopsis*, and *T. atroviride*) were applied as foliar sprays at the green bud stage and at the same concentrations and water volume as in 2014. Mycelia of the pathogen isolate SS12 were sprayed at 100 mL per plot at a concentration of ~10^{12} fragment mL^{-1} at 10% and 50% bloom stages, respectively.

The design of field experiment 2 was the same as experiment 1 except there were fifteen treatments (Table 3). Row length, row spacing, cultivar, time of seeding, fertilization, treatment application (method and rate) were as in experiment 1. In this experiment, each BCA (*S. proteamaculans, T. atroviride*) was applied one week prior to pathogen inoculation at 10 and 50% bloom stages or both the BCA and the pathogen were applied at the same time at 10 and 50% bloom stages. Spray application of the fungicide Prosaro® at the same rate as the previous field experiment was included as a positive control and applied at the same timings as the two BCAs.

For field experiments 1 and 2, the number of infected plants in each treatment row was counted 2 weeks after inoculation and disease incidence (DI) and disease control efficiency for each treatment were calculated as:

Disease control efficiency = (Mean of DI in pathogen treated plots-Mean of DI in treatment plots)*100/Mean of DI in pathogen treated plots.

Seed was harvested from each treatment row and middle buffer row at maturity.

Statistical analysis

Analysis of variance (ANOVA) of radial mycelial growth and inhibition by F-BCAs at 24 and 48 h after incubation,

Table 1: Details of treatments for field experiment in 2014

No	Treatment	Code
1	Pathogen only (mycelia of *S. sclerotiorum*)	PO
2	Pathogen + Fungicide (Prosaro®)	P-Fc
3	Pathogen + F-BCA1	P-FBCA1
4	Pathogen + F-BCA2	P-FBCA2
5	Pathogen + B-BCA1	P-BBCA1
6	Pathogen + B-BCA2	P-BBCA2
7	Untreated Control	Control

Table 2: Details of treatments of field experiment 1 in 2015

No	Treatment
1	F-BCA1 at green bud followed by pathogen at 10% flowering
2	F-BCA1 at green bud followed by pathogen at 30% flowering
3	F-BCA1 at green bud followed by pathogen at 50% flowering
4	F-BCA2 at green bud followed by pathogen at 10% flowering
5	F-BCA2 at green bud followed by pathogen at 30% flowering
6	F-BCA2 at green bud followed by pathogen at 50% flowering
7	B-BCA1 at green bud followed by pathogen at 10% flowering
8	B-BCA1 at green bud followed by pathogen at 30% flowering
9	B-BCA1 at green bud followed by pathogen at 50% flowering
10	B-BCA2 at green bud followed by pathogen at 10% flowering
11	B-BCA2 at green bud followed by pathogen at 30% flowering
12	B-BCA2 at green bud followed by pathogen at 50% flowering
13	Pathogen at 10% flowering
14	Pathogen at 30% flowering
15	Pathogen at 50% flowering
16	Un-inoculated control

Table 3: Details of treatment of field experiment 2 in 2015

No	Treatment
1	F-BCA at 1 week before pathogen 10% flowering
2	F-BCA + pathogen together at 10% flowering
3	B-BCA at 1 week before pathogen 10% flowering
4	B-BCA + pathogen together at10% flowering
5	F-BCA at 1 week before pathogen at 30% flowering
6	F-BCA + pathogen together at 30% flowering
7	B-BCA at 1 week before pathogen at 30% flowering
8	B-BCA + pathogen together at 30% flowering
9	Pathogen a week before Prosaro® at 10% flowering
10	Pathogen a week before Prosaro® at 30% flowering
11	Pathogen + Prosaro® at 10% flowering
12	Pathogen + Prosaro® at 30% flowering
13	Pathogen only at 10% flowering
14	Pathogen only at 30% flowering
15	Un-inoculated control

sclerotial formation by F-BCAs and data of infected plants were performed using GenStat 16® software (Release 16, Lawes Agricultural Trust – Rothamsted Experimental Station) followed by mean separation by LSD ($P \leq 0.05$). Percentage of disease incidence data for the glasshouse experiment were analysis. Data of field experiments with pseudo-replication were predicted using Restricted Maximum Likelihood (REML) analysis.

Results

Isolation and mycelial growth of potential F-BCAs and B-BCAs

In this study we excluded fungal isolates that were not of interest (genera other than *Trichoderma*). The percentage of *Trichoderma* spp. isolated by all methods was 5–10%. In total, fifteen potential *Trichoderma* species were isolated. Mycelial colour of the *Trichoderma* species showed wide variation, being dark green, light green, green, whitish green, brownish green, yellowish white, and white (Fig. 1).

There were significant differences ($P \leq 0.001$) in growth rate among isolates at 24 and 48 h of incubation. At 24 h, Isolate F-BCA9 had the highest radial mycelial growth with diameters of 3.17 cm (24 h) and 8.5 cm (48 h), followed by isolates F-BCA11 (2.83 cm at 24 h, 7.2 cm at 48 h) and F-BCA14 (2.63 cm at 24 h, 7.53 cm at 48 h). Isolate F-BCA12 had the smallest radial mycelial growth at 24 h (2.03 cm) but had accelerated growth at 48 h (6.53 cm) (Fig. 2).

Three isolates of potential B-BCAs were obtained. Colony colour of isolates of B-BCA1, B-BCA2 and B-BCA3 were light yellow, yellow, and whitish yellow, respectively. Isolate B-BCA3 had the fastest colony growth rate and morphologically had the softest and more watery colony; whereas isolate B-BCA2 had a much drier colony compared with isolates B-BCA1 and B-BCA3 (Fig. 3).

Molecular identification of F-BCAs and B-BCAs

F-BCAs: The *Trichoderma* isolates were identified as *Trichoderma atroviride* (four isolates), *T. gamsii* (three isolates), *T. koningiopsis* (two isolates), *T. longibrachiatum* (two isolates), *T. paraviridescens* (two isolates), *T. pseudokoningii* (one isolate) and *T. viridescens* (one isolate). *Trichoderma atroviride*, *T. gamsii*, *T. koningiopsis*, and *T. viridescens* are reported for the first time from Western Australia. Accession numbers are provided in Table 6.

B-BCAs: Isolates B-BCA1 and B-BCA2 were identified as *Serratia proteamaculans* and isolate B-BCA3 as *Ochrobactrum anthropi*. The highest level of identity for *S. proteamaculans* and *O. anthropi* were 99.58 and 100%, respectively. Accession numbers are given in Table 7.

In vitro biological control of *S. sclerotiorum* by potential BCAs (inhibition of mycelial growth and hyper-parasitisation of *S. sclerotiorum* sclerotia by F- BCAs)

F-BCAs: All potential F-BCA isolates showed some capacity to reduce mycelial growth and the number of sclerotia of *S. sclerotiorum* SS12 in dual culture tests in Petri dishes. The morphology of the dual cultures of the potential F-BCAs with SS12 is shown in Fig. 4. There were significant differences ($P \leq 0.001$) in inhibition of both radial mycelial growth and sclerotia formation by the pathogen among the 15 F-BCAs. Mycelial growth of SS12 was inhibited by 46–60%. The highest inhibition was caused by F-BCA9 (60%). The presence of F-BCAs decreased sclerotia formation by 65–100%. Isolates F-BCA12 and F-BCA15 completely inhibited the formation of sclerotia by the pathogen, while isolates F-BCA13 and F-BCA10 had the least potential to inhibit sclerotia formation, reducing the number of sclerotia by 65 and 70%, respectively. There was an antagonistic effect of F-BCA against sclerotia in soil in pots. After a week on PDAA, no new sclerotia were formed (Fig. 7).

Fig. 1: Mycelial growths of fifteen isolates of potential F-BCAs from WA on PDAA media 7 days after incubation. From left to right: top row F-BCA1, F-BCA2, F-BCA3, F-BCA4, F-BCA5; second from top row F-BCA6, F-BCA7, F-BCA8, F-BCA9, F-BCA10; and bottom row F-BCA11, F-BCA12, F-BCA13, F-BCA14, F-BCA15

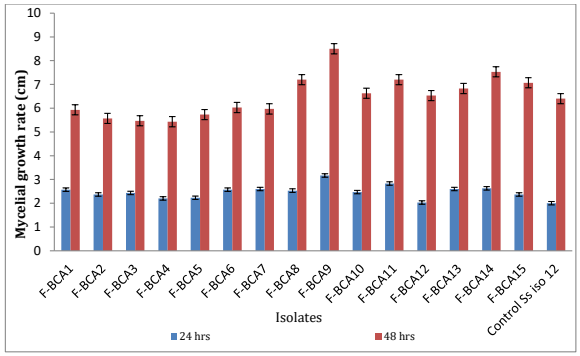

Fig. 2: Radian mycelial growth of fifteen potential F-BCAs on PDAA media at 24 and 48 hours after incubation. Least significant difference of means (LSD) at 5% level at 24 hours after incubation was 0.1909 and at 48 hours was 0.3014. Columns having different letters at each incubation time are highly significant different. Values are means (n=3)

Fig. 3: Bacterial colonies from three isolates of potential B-BCAs from WA on PYDA media after 24 hours incubation. From left to right: B-BCA1, B-BCA2 and B-BCA3

B-BCAs: Using the mycelial plug method, the three potential B-BCAs inhibited *in vitro* radial mycelial growth of SS12 by 57–79% and sclerotia formation by 89–95% (Fig. 5), but there was no significant difference among isolates in inhibition of mycelial growth (P=0.934) or sclerotia (P=0.78). The three isolates also inhibited mycelial growth and sclerotia formation using the sclerotium inoculation method (Fig. 6). There were significant differences

Fig. 4: Dual cultures of fifteen potential F-BCAs against *S. sclerotiorum* (isolate 12) on PDAA media. Each F-BCA is on the left side and the pathogen is in the right side of the Petri dish. From left to right: top row: F-BCA1, F-BCA2, F-BCA3, F-BCA4, F-BCA5; second from top row: F-BCA6, F-BCA7, F-BCA8, F-BCA9, F-BCA10; and bottom row: F-BCA11, F-BCA12, F-BCA13, F-BCA14, F-BCA15

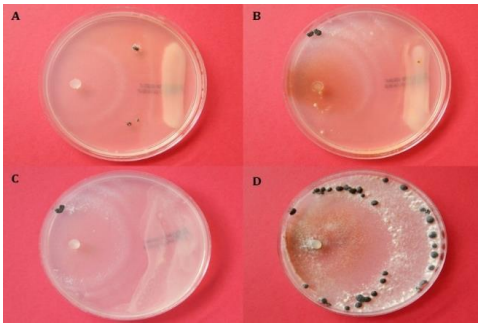

Fig. 5: Dual culture tests of three potential B-BCAs against *S. sclerotiorum* isolate 12 on PYDA media: (A) B-BCA1, (B) B-BCA2, (C) B-BCA3 and (D) Control pathogen only. Inoculum source of pathogen was from a mycelial agar plug placed in left side of each Petri dish

(P=0.029) in inhibition of radial mycelial growth among the three B-BCAs but there were no differences (P=0.072) among the B-BCAs in the inhibition of sclerotia.

In-planta testing of BCAs against *S. sclerotiorum*

Glasshouse experiment: In the glasshouse experiment, all BCA's were applied at the green bud growth stage of canola and the pathogen was inoculated at 10, 30 or 50% bloom stages. No disease developed in the un-inoculated control. Significantly higher levels (100%) of disease developed when the pathogen was applied at 10% bloom compared with at 30 and 50% bloom. F-BCA1 (*T. koningiopsis*) was very effective against the pathogen at 10% bloom. Likewise, incidence of the disease was reduced significantly with *O. anthropi* when the pathogen was inoculated at 10% bloom. The fungal F-BCA1 (*T. koningiopsis*) and F-BCA2 (*T. atroviride*) were significantly more effective in reducing incidence of the disease when the pathogen was applied at 30% bloom compared with the B-BCAs. Due to very low disease incidence with pathogen application at 50% bloom,

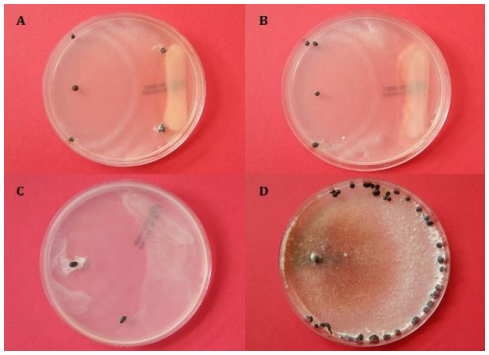

Fig. 6: Dual culture tests of three potential B-BCAs against *S. sclerotiorum* isolate 12 on PYDA media: (A) B-BCA1, (B) B-BCA2, (C) B-BCA3, (D) Control sclerotium only. Inoculum source of pathogen was sclerotium placed in left side of each Petri dish

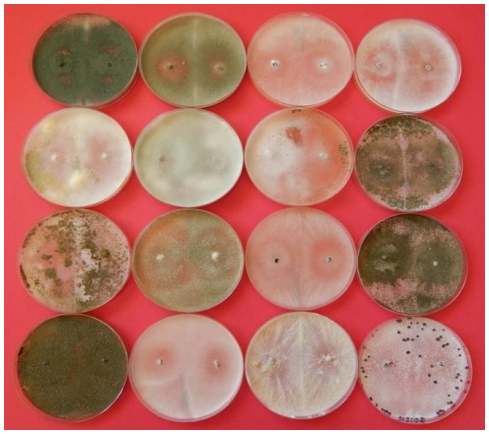

Fig. 7: Plating of *S. sclerotiorum* sclerotia after being colonized by each isolate of F-BCAs in soil for a week. From left to right: top row (sclerotia in the presence of F-BCA1, F-BCA2, F-BCA3, F-BCA4); second from top row (F-BCA5, F-BCA6, F-BCA7, F-BCA8); third from top row (F-BCA9, F-BCA10, F-BCA11, F-BCA12); bottom row (F-BCA13, F-BCA14, F-BCA15, control sclerotia only)

only *T. atroviride* and *O. anthropi* provided complete suppression of disease incidence (Fig. 8).

Field experiments

2014 field experiment: Very low levels of disease (<5% disease incidence in *S. sclerotiorum* inoculated plots) developed in the 2014 field experiment (data not shown). However, there was a clear difference in the appearance of each treatment plot. Plots sprayed with fungicide Prosaro® and *O. anthropi* were much greener with denser foliage and greater leaf area compared to other treatment plots. There were no significant yield differences, but the yield of Fungicide Prosaro®+Pathogen and *O. anthropi*+Pathogen treatments trended higher compared to pathogen only and other treatments where yield was increased by 19 and 18% with *O. anthropi* and Prosaro®, respectively, compared with the pathogen only treatment (Fig. 9).

2015 field experiments: Effect of each treatment and their interaction in field experiment 1 were predicted using Restricted Maximum Likelihood (REML) analysis (Table 4). There were significant differences ($P<0.001$) among the BCAs in controlling SSR disease. There were significant differences ($P<0.001$) in disease incidence of SSR with application of the pathogen at different flowering stages. In addition, there were significant ($P<0.001$) interactions between BCAs and pathogen application at various flowering stages. There was significantly less disease when the pathogen was applied at 30 and 50% compared with 10% flowering. Both bacterial B-BCA1 (*S. proteamaculans*) and B-BCA2 (*O. anthropi*) were significantly more effective than the fungal F-BCA1 (*T. koningiopsis*) and F-BCA2 (*T. atroviride*) in reducing the disease incidence when the pathogen was applied at 10% flowering. The maximum disease control efficiency (89%) was achieved with *O. anthropi* (Fig. 10). However, when the pathogen was applied at 50% flowering stage, both the fungal BCAs were significantly more effective than the bacterial BCAs in reducing the disease incidence.

For experiment 2, predicted treatment and interaction effects from REML analysis are given in Table 5. There was a significant difference ($P<0.001$) among the BCAs. Time of application of *S. sclerotiorum* was highly significant ($P<0.001$) but application time of fungicide was not significant ($P=0.901>0.001$). There were also highly significant differences between time of application of BCAs ($P<0.001$), but no significant difference ($P=0.382>0.001$) between interaction of BCAs and time of spraying the pathogen. In addition, there was a significant ($P=0.002<0.005$) interaction of BCAs and timing of application. High level of disease developed when the *S. sclerotiorum* was applied at 10% flowering. Disease development was negligible when the *S. sclerotiorum* was applied at 30% flowering. There was a significant reduction in disease incidence when *T. atroviride* was applied either one week before the pathogen or at the same time as the pathogen at 10% flowering, with the disease control efficiency being 86 and 98%, respectively. However, *S. proteamaculans* and fungicide Prosaro® were more effective when applied at the same time as the pathogen at 10% flowering resulting in disease control efficiencies of 75 and 100%, respectively (Fig. 11).

There was a significant ($P=0.12$) interaction of BCA and time of application of the pathogen on yield. Significant yield responses were achieved when *T. atroviride* and *S. proteamaculans* were applied one week before *S. sclerotiorum* and when *S. proteamaculans* and *S. sclerotiorum* were applied together at 10% flowering. Despite negligible levels of disease with *S. sclerotiorum* inoculation at 30% flowering, there was a significant yield response (19.6 and 19.8% enhancement respectively) to the fungal BCA (*T. atroviride*) when applied a week before or at the same time as the pathogen (Fig. 12).

Table 4: Effect of applying BCAs and the pathogen *S. sclerotiorum* during the different flowering stages and their interaction from field experiment 1 in growing season 2015 based on REML analysis

Change	d.f	deviance	Mean deviance	Deviance ratio	Approx. chi pr
BCAs	4	44.80	11.20	11.20	*<0.001*
PATH_Flowering	2	283.85	141.92	141.92	*<0.001*
Residual	8	101.19	12.65		
BCAs.PATH_Flowering	8	101.19	12.65	12.65	*<0.001*
Total	14	429.84	30.70		

Table 5: Effect of each treatment and their interaction from field experiment 2 in growing season 2015 based on REML analysis

Change	d.f	deviance	Mean deviance	deviance ratio	approx chi pr
BCAs	3	59.186	19.729	19.73	*<0.001*
PATH_Flowering	1	129.170	129.170	129.17	*<0.001*
Timing_Fungicide	1	0.016	0.016	0.02	*0.901*
Timing_BCAs	2	71.811	35.905	35.91	*<0.001*
BCAs.PATH_Flowering	1	0.764	0.764	0.76	*0.382*
BCAs.Timing_Fungicide	0	0.000	*		
Residual	3	15.211	5.070		
BCAs.Timing_BCAs	3	15.211	5.070	5.07	*0.002*
PATH_Flowering.Timing_Fungicide	0	0.000	*		
PATH_Flowering.Timing_BCAs	0	0.000	*		
BCAs.PATH_Flowering.Timing_Fungicide	0	0.000	*		
BCAs.PATH_Flowering.Timing_BCAs	0	0.000	*		
Total	11	276.157	25.105		

Table 6: Accession numbers of F-BCAs used in the investigations

FASTA ID	Accession #	Gene description
F_BCA1_F_A01	MW268857	Trichoderma ovalisporum isolate MI98 internal transcribed spacer 1
F_BCA1_R_D01	MT529291	Trichoderma sulphureum clone SF_15 small subunit ribosomal RNA gene
F_BCA2_F_A02	MT137373	Trichoderma sp. strain 21F13C_AC small subunit ribosomal RNA gene
F_BCA2_R_D02	MT529291	Same as F_BCA1_R_D01
F_BCA3_F_A03	MT529638	Trichoderma sulphureum clone SF_362 small subunit ribosomal RNA gene
F_BCA3_R_D03	MT732907	Trichoderma sp. PB-2018 strain 56E small subunit ribosomal RNA gene
F_BCA4_F_A04	MW269180	Trichoderma neokoningii isolate MI479 small subunit ribosomal RNA gene
F_BCA4_R_E12	MK808808	Trichoderma sp. isolate DS554 small subunit ribosomal RNA gene
F_BCA5_F_A05	MK862245	Trichoderma erinaceum isolate SWFU000006 internal transcribed spacer 1
F_BCA5_R_D05	MK808808	Same as F_BCA4_R_E12
F_BCA6_F_F01	MK862247	Trichoderma samuelsii isolate SWFU000004 internal transcribed spacer 1
F_BCA6_R_D06	MK460812	Trichoderma atroviride strain CSK3_13 small subunit ribosomal RNA gene
F_BCA7_F_A07	MK910067	Trichoderma longibrachiatum isolate BM12 small subunit ribosomal RNA gene
F_BCA7_R_D07	MF076623	Trichoderma reesei isolate S254 small subunit ribosomal RNA gene
F_BCA8_F_A08	MK871246	Trichoderma sp. isolate SDAS203393 small subunit ribosomal RNA gene
F_BCA8_R_D08	MF076590	Trichoderma koningii isolate S54 small subunit ribosomal RNA gene
F_BCA9_F_A09	MN795754	Trichoderma atroviride strain p18 small subunit ribosomal RNA gene
F_BCA9_R_D09	MF076590	Same as F_BCA8_R_D08
F_BCA10_F_A10	MK910067	Trichoderma longibrachiatum isolate BM12 small subunit ribosomal RNA gene
F_BCA10_R_D10	MF076623	Same as F_BCA7_R_D07
F_BCA11_F_A11	MK407088	Uncultured Trichoderma clone D1314ITS internal transcribed spacer 1
F_BCA11_R_D11	MK460812	Same as F_BCA6_R_D06
F_BCA12_F_A12	MN795754	Same as F_BCA9_F_A09
F_BCA12_R_D12	MF076590	Same as F_BCA8_R_D08
F_BCA13_F_B01	MK333266	Trichoderma citrinoviride isolate MTAT17 small subunit ribosomal RNA gene
F_BCA13_R_E01	EU280097	Trichoderma pseudokoningii strain DAOM 167678 18S ribosomal RNA gene
F_BCA14_F_B02	MW269180	Same as F_BCA4_F_A04
F_BCA14_R_E02	MK808808	Same as F_BCA4_R_E12
F_BCA15_F_B03	MN795754	Same as F_BCA9_F_A09
F_BCA15_R_E03	MF076590	Same as F_BCA8_R_D08

Table 7: Accession numbers of B-BCAs used in the investigations

FASTA ID	Accession #	Gene description
16SQ11665-1_B_BCA1	MT561436	*Serratia* sp. strain CT197 16S ribosomal RNA gene
16SQ11665-2_B_BCA2_R_E03	MK530301	*Serratia proteamaculans* strain Sample_92 16S ribosomal RNA gene
16SQ11665-3_B_BCA3	MG430400	*Ochrobactrum pituitosum* strain AA2 16S ribosomal RNA gene

Discussion

The application of biological control agents is a promising tool to manage the damage caused by plant pathogens. Biological control treatments for soil-borne plant pathogens must provide enhanced levels of disease suppression and consistency of control over diverse soils before their wide-scale application on a commercial scale (Hu *et al.* 2019).

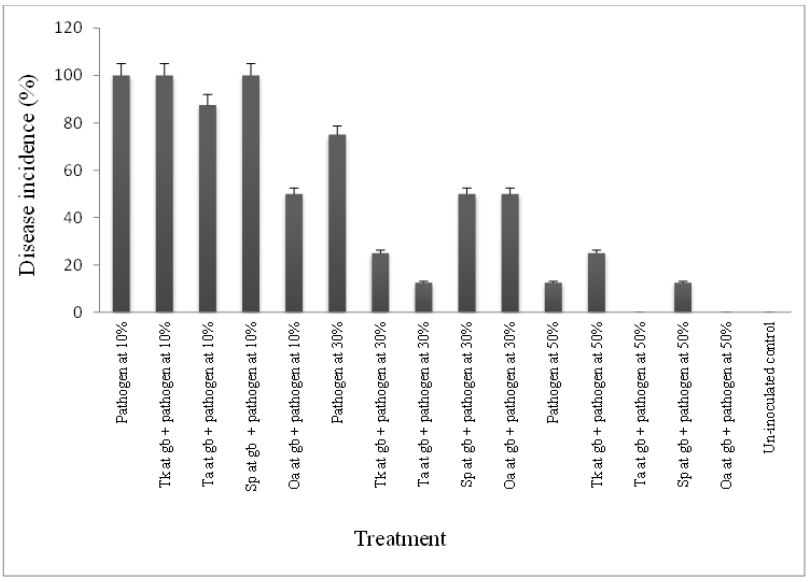

Fig. 8: Effect of various BCA's on the disease incidence of *S. sclerotiorum* in canola under glasshouse conditions. Tk = *T. koningiopsis*, Ta = *T. atroviride*, Sp = *S. proteamaculans*, Oa = *O. anthropi*, gb = green bud. Values are means (n=4). Error bars represent Standard Error (SE)

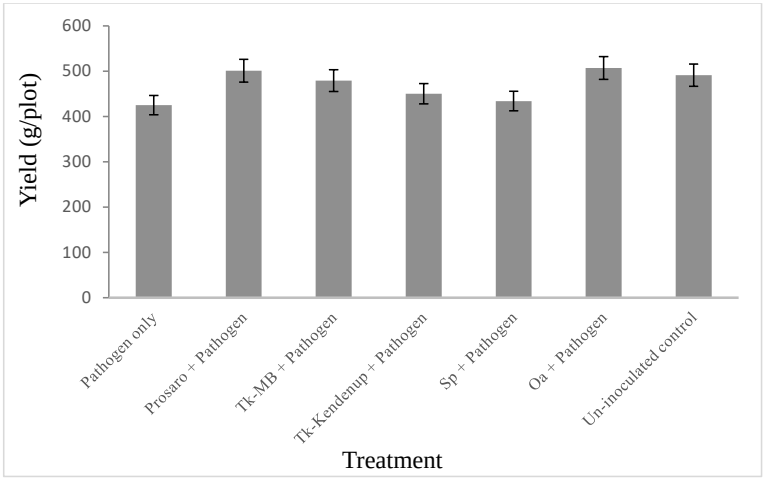

Fig. 9: Effect of various BCAs on yield of canola under field conditions during 2014. Tk-MB = *T. koningiopsis* isolate Mount Baker, Tk-Kendenup = *T. koningiopsis* isolate Kendenup, Sp = *S. proteamaculans*, Oa = *O. anthropi*. The pathogen is *S. sclerotiorum*. Values are means (n = 3). Error bars represent LSD

We exploited a range of techniques to isolate naturally occurring fungal and bacterial BCAs with view to their future use to manage *S. sclerotiorum* in canola. We isolated 18 taxa that are known to have biological control potential in a number of host-pathogen systems (Ghazanfar *et al.* 2018; Kshetri *et al.* 2019).

The fifteen isolates of *Trichoderma* had moderate to fast radial mycelial growth rates on PDAA medium. Rapid growth is one of the important competitive advantages antagonistic fungi have over plant pathogenic fungi. It enables them to compete for space and nutrients. Furthermore, some *Trichoderma* species can induce host resistance responses against pathogens (Harman *et al.* 2004). Several studies have shown the biocontrol potential of *Trichoderma* species in controlling pathogens in *in vitro* and *in vivo* conditions (Ojaghian 2011; Saxena *et al.* 2015). For example, an *in vivo* seed coating test using thiophanate-methyl or *Trichoderma* spp. substantially improved soybean germination and suppressed growth of *S. sclerotiorum* (Macena *et al.* 2020). Our results are consistent with previous research where colonies of *T. longibrachiatum*, *T. atroviride* and *T. harzianum* grew faster than *S. sclerotiorum* in both single or mixed cultures (Matroudi and Zamani 2009). Our studies also showed that isolates of *T. atroviride* were highly effective in reducing mycelial growth and completely inhibiting sclerotia production by the pathogen as also reported by Gupta *et al.* (2014). Furthermore, *T. atroviride* has been shown to reduce colony growth by 93 and 85% in two isolates of *S.*

sclerotiorum from canola (Matroudi and Zamani 2009).

Knowledge on the effectiveness of the new isolates of BCAs on controlling the critical stages of the life cycle of a particular pathogen is very important to determine the most effective isolates for commercialisation. The survival of *S. sclerotiorum* depends on the production and viability of sclerotia that can remain viable in soil for more than 7 years (Kora *et al.* 2008; Smolińska *et al.* 2018). Therefore, to control this pathogen, the key is to reduce production and viability of sclerotia. Since the sclerotia reside in soil, using chemical sprays to reduce the inoculum load in broad-acre crops is not feasible. Thus, effective biological products may be more feasible to reduce the density of sclerotia in soil. A commercially available product Contan®WG (a formulation of *C. minitans*) is reported to control sclerotial populations in soil in canola and other hosts including carrots and soybean (Fernando *et al.* 2004; McQuilken and Chalton 2008; Zeng *et al.* 2012). This product was not tested in the current studies as, due to strict quarantine regulations in WA, it was not possible to import commercial *C. minitans*. Our *in vitro* experiments revealed significant inhibition of sclerotia formation by *Trichoderma* species both in dual plate and soil inoculation treatments. The nearly complete inhibition of sclerotial formation could possibly be due to reduced viability of mycelia. It could also be attributed to competition for space and nutrients or mycoparasitism reducing growth and consequently inhibiting the sclerotia formation ability of the pathogen. Our results corroborate those of Abdullah *et al.* (2008) who reported that *T. harzianum* had an ability to control both mycelial growth and sclerotial production by *S. sclerotiorum* when tested on the same plate.

Molecular identification indicated that bacterial isolates B-BCA1 and B-BCA2 are species of *S. proteamaculans*. *Serratia* is a diverse and widely dispersed group of gamma proteobacteria (Grimont and Grimont 2006). Some species of *Serratia* have beneficial effects on economically and ecologically important crops (Kalbe *et al.* 1996; Kurze *et al.* 2001) and others are indicated as opportunistic pathogens for humans and other organisms (Grimont and Grimont 2006). *Serratia* associated with plants has considerable interest in agriculture and some strains have been investigated as BCAs in field crops (Kalbe *et al.* 1996; Kurze *et al.* 2001) and as plant growth promoting rhizobacteria (PGPR) (Bababola 2010). Furthermore, some isolates of *S. proteamaculans* can stimulate plant growth and suppress growth of some important soil-borne fungal pathogens (Neupane *et al*. 2013). B-BCA3 was identified as *Ochrobactrum anthropi*, a species known for its potential as a BCA and PGPR (Chakraborty *et al*. 2009; Bababola 2010). This species is a gram-negative bacterium that has a structure membrane composed of an outer membrane, periplasmic space, and inner membrane (Bababola 2010). *Ochrobactrum anthropi* isolated from the rhizosphere of *Camellia* produces IAA and siderophores *in vitro*, and have potential for biological control (Chakraborty *et al*. 2009).

Our *in vitro* experiments showed that the B-BCAs inhibited radial mycelial growth of the pathogen and sclerotia production by 60 to 95%. Ability of the BCAs to reduce sclerotial production *in planta* or their viability in soil can substantially curtail the primary inoculum source of *S. sclerotiorum* for susceptible crops. A possible mechanism of supressiveness by B-BCAs *in vitro* is the production of antibiotics (Abdullah *et al.* 2008). Raaijmakers *et al.* (2002) argued that antibiotics produced by antagonistic microorganism are evidence they can play an important role in the suppression of some soil-borne pathogens. Further investigation is needed to determine if antibiotics were produced by the WA B-BCAs in our study.

Application of BCAs in the field can sometimes give unexpected results due to factors that can be attributed to the behaviour of BCAs and environmental conditions (Saharan and Mehta 2008). However, application methods that are consistent with the cropping system may enhance biological control of *S. sclerotiorum* (Li *et al.* 2020). In our studies, selected *Trichoderma* isolates including *T. koningiopsis* and *T. atroviride* and an isolate each of the bacterial BCA (*O. anthropi* and *S. proteamaculans*) significantly reduced disease incidence of *S. sclerotiorum* under glasshouse and field conditions. However, the effectiveness of the BCAs varied with the growth stage of canola and in particular the timing of application of the antagonist and the pathogen. Under glasshouse conditions, the fungal BCAs were more effective than the bacterial BCAs when applied at the green bud stage followed by inoculation of *S. sclerotiorum* at 30% flowering. However, under field conditions, bacterial BCAs were significantly more effective than the fungal BCA's when applied at the green bud stage followed by inoculation of *S. sclerotiorum* at 10% flowering. In field experiment 2, the fungal BCA *T. atroviride* was substantially superior in reducing disease incidence when applied either a week before the pathogen or co-inoculated with the pathogen at 10% flowering compared with the bacterial BCA treatments at the same growth stage. These results indicate that the disease suppression window of bacterial BCA's is comparatively shorter than that of the fungal BCA's.

To investigate the beneficial effect of disease reduction by the BCA's on canola yield, the field experiments in 2014 and 2015 were hand harvested and seed yield for each treatment was measured. Dry seasonal conditions in 2014 resulted in very low disease incidence in pathogen only inoculated plots, consequently, yield responses to BCA application were not expected. However, the greening effect with the bacterial BCA- *O. anthropi* and fungicide Prosaro® was evident by eye and seed yield in both these treatments was marginally (19 and 18%, respectively) higher than the *S. sclerotiorum* only treatment. This improvement in yield in the absence of disease is possibly due to the growth promoting effect of *O. anthropi*. Likewise, in field experiment 2 in 2015, a significant increase (21%) in yield with *T. atroviride* over the pathogen only treatment at 30% flowering was observed. These results are encouraging in the context that these BCA's can improve canola yield in both the presence or absence of

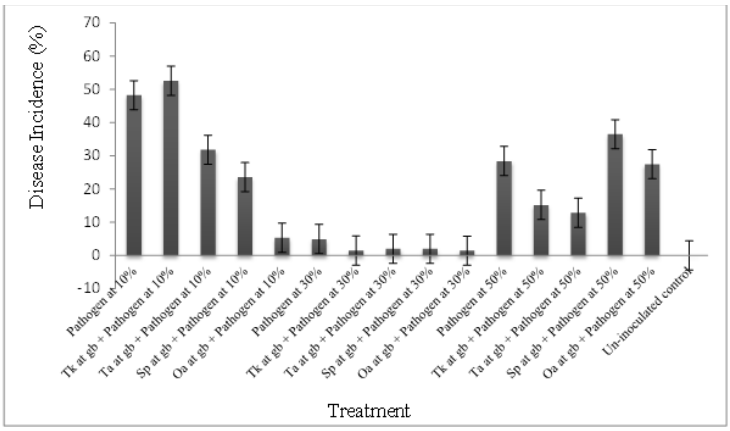

Fig. 10: Effect of various BCA's on disease incidence of *S. sclerotiorum* in canola in field experiment 1 during 2015. Tk = *T. koningiopsis*, Ta = *T. atroviride*, Sp = *S. proteamaculans*, Oa = *O. anthropi*, gb = green bud. Error bars represent LSD

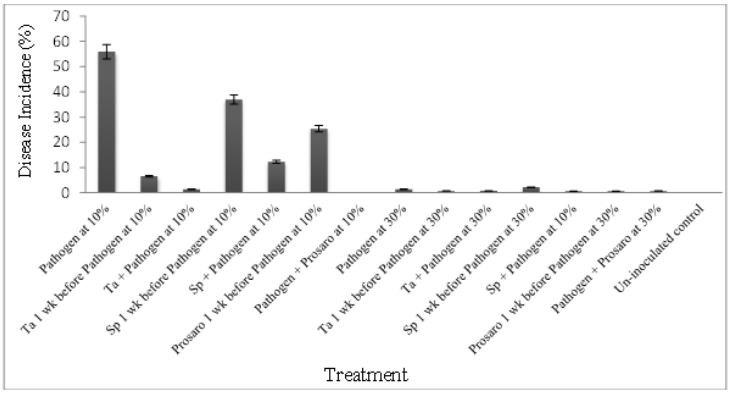

Fig. 11: Effect of various BCA's on disease incidence of *S. sclerotiorum* in canola in Field experiment 2 during 2015. Ta = *T. atroviride*, Sp = *S. proteamaculans*. Error bars represent Standard Error (SE)

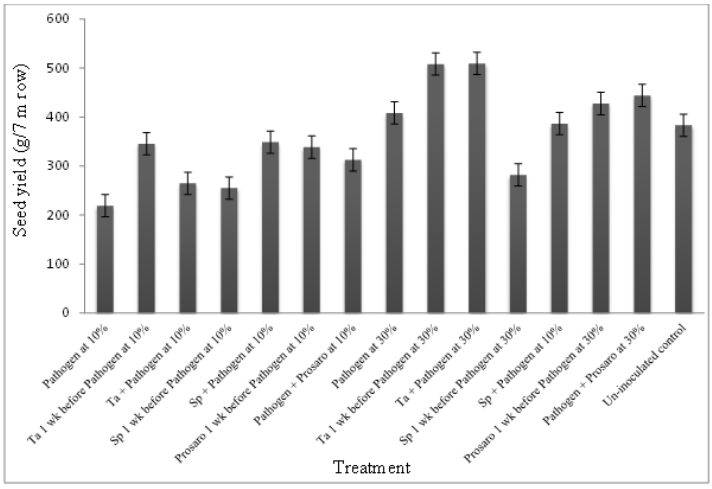

Fig. 12: Effect of various BCA's and *S. sclerotiorum* on seed yield (g/7m row) of canola in field experiment 2 during 2015. Ta = *T. atroviride*, Sp = *S. Proteamaculans.* Error bars represent LSD

disease thus making it a useful additional tool for alleviating both biotic and abiotic stresses in canola. Moreover, it is noteworthy that the efficacy of the tested BCA's in the current studies was similar to that of a commercial product Prosaro® implying that BCAs can potentially be used as an alternative to fungicides or as an additional tool in the integrated management of SSR in canola. Growth promoting and pathogen suppression ability of *T. atroviride*, *T. koningiopsis*, *S. proteamaculans* and *O. anthropi* is well documented in other host pathogen systems (Chakraborty *et al.* 2009).

However, further field studies with large field plots at multiple locations in a range of environments are required to validate these preliminary findings and develop commercial formulations and spray regimes. Furthermore, combinations of some BCAs may increase the possibility of synergetic action in suppressing the pathogen (Jain *et al.* 2011). For example, a triple-compatible microbial consortium increased enzyme activities and phenol accumulation 1.4 to 4.6 times compared with individual and dual consortia (Jain *et al.* 2011). Future research to screen multiple combinations of these in field trials may further enhance the disease control potential of these beneficial micro-organisms.

Biological control products should be extensively evaluated with robust testing under local conditions before deployment. Not only must they be effective, BCAs should also be easy to use, non-toxic, economical, environmentally safe, meet biosecurity concerns, and be acceptable to growers, consumers, and regulatory agencies. In this regard, Western Australia has its own biosecurity and plant quarantine regulations and commercial BCAs from outside of the state would need to be rigorously evaluated before release to primary producers. Hence, the identification of effective local BCAs is a priority for research.

Conclusion

For the first time we identified potential fungal and bacterial BCAs from Western Australia that suppressed both growth and sclerotial formation of *S. sclerotiorum in vitro* and reduced disease incidence when applied as foliar applications under glasshouse and field conditions. Mycelial and sclerotial inhibition ranged from 40–60% and 65–100% for the F-BCAs and 57–59% and 89–95% for the B-BCAs, respectively. Selected isolates of F-BCAs (*T. koningiopsis* and *T. atroviride*) and of B-BCAs (*O. anthropi* and *S. proteamaculans*) significantly reduced disease incidence of *S. sclerotiorum* under glasshouse and field conditions. Under field conditions, *O. anthropi* provided the maximum disease control efficiency (89%) when the pathogen was applied at 10% flowering. Field efficacy of tested BCAs was similar or better than the commercial fungicide Prosaro®. Further studies are required to understand their mechanism of suppression against *S. sclerotiorum* and their ability to persist and to provide protection under field conditions. In addition, the life-cycle of the beneficial organisms needs to be understood in an environment where the climate has increasing variability due to climate change.

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Author Contributions

BN Hidayah (main contributor): planned work, conducted the experiments, and wrote the manuscript; R Khangura and B Dell (supporting contributors): supervised work and proof read the manuscript.

Conflict of Interest

The authors declare that they have no known conflict of interest.

Data Availability

Data presented in this study are available on fair request to the corresponding author.

Ethic Approval

Not applicable

References

Abdullah MT, NY Ali, P Suleman (2008). Biological control of *Sclerotinia sclerotiorum* (Lib.) de Bary with *Trichoderma harzianum* and *Bacillus amyloquefaciens*. *Crop Prot* 27:1354–1359

Altschul SF, W Gish, W Miller, EW Myers, DJ Lipman (1990). Basic Local Alignment Search Tool. *J Mol Biol.* 215:403–410

Altschul SF, TL Madden, AA Schaffer, J Zhang, Z Zhang, W Miller, DJ Lipman (1997). Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res* 25:3389–3402

Bababola OO (2010). Beneficial bacterial of agricultural importance. *Biotechnol Lett* 32:1559–1570

Barbetti MJ, SK Banga, TD Fu, YC Li, D Singh, SY Liu, XT Ge, SS Banga (2013). Comparative genotype reactions to *Sclerotinia sclerotiorum* within breeding populations of *Brassica napus* and *B. juncea* from India and China. *Euphytica* 197:27–59

Bolton MD, BPHJ Thomma, BD Nelson (2006). *Sclerotinia sclerotiorum* (Lib.) de Bary: Biology and molecular traits of a cosmopolitan pathogen. *Mol Plant Pathol* 7:1–16

Chakraborty U, BN Chaktaborty, M Basnet, AP Chaktaborty (2009). Evaluation of *Ochrobactrum anthropi* TRS-2 and its talc based formulation for enhancement of growth of tea plants and management of brown root rot disease. *J Appl Microbiol* 107:625–634

de la Cerda KA, GW Douhan, FP Wong (2007). Discovery and characterization of *Waitea circinata* var. *circinata* affecting annual bluegrass from the western United States. *Plant Dis* 91:791–797

Fernando WGD, S Nakkeeran, Y Zhang (2004). Ecofriendly methods in combating *Sclerotinia sclerotiorum* (Lib.) de Bary. *Recent Res Dev Environ Biol* 1:329–347

Gardes M, TD Bruns (1993). ITS primers with enhanced specificity for Basidiomycetes - Application to the identification of mycorrhizae and rusts. *Mol Ecol* 2:113–118

Gupta KV, M Schmoll, A Herrera-Estrella, RS Upadhyay, I Druzhinina, MG Tuohy (2014). *Biotechnology and Biology of Trichoderma.* Elsevier. 255 Wyman Street, Waltham, Massachusetts, USA

Ghazanfar MU, M Raza, W Raza, MI Qamar (2018). *Trichoderma* as potential biocontrol agents, its exploitation in agriculture: A review. *Plant Prot* 2:109–135

Grimont F, PAD Grimont (2006). The genus *Serratia*. *In*: *The Prokaryotes Volume 6: Proteobacteria: Gamma Subclass*, pp: 219–244, 3rd edn. Dworkin M, S Falkow, E Rosenberg, KH Schleifer, E Stackebrandt (Eds.). Springer, New York USA

Harman GE, CR Howell, A Viterbo, I Chet, M Lorito (2004). *Trichoderma* species – Opportunistic, avirulent plant symbionts. *Nat Rev Microbiol* 2:43–56

Hu X, DP Roberts, L Xie, L Qin, Y Li, X Liao, P Han, C Yu, X Liao (2019). Seed treatment containing *Bacillus subtilis* BY-2 in combination with other *Bacillus* isolates for control of *Sclerotinia sclerotiorum* on oilseed rape. *Biol Cont* 133:50–57

Jain A, S Singh, BK Sarma, HB Singh (2011). Microbial consortium-mediated reprogramming of defence network in pea to enhance tolerance against *Sclerotinia sclerotiorum*. *J Appl Microbiol* 112:537–550

Kalbe C, P Marten, G Berg (1996). Strains of the genus *Serratia* as beneficial rhizobacteria of oilseed rape with. *Microbiol Res* 151:433–439

Kamal MM, KD Lindbeck, S Savocchia, GJ Ash (2016). Biology and biocontrol of *Sclerotinia sclerotiorum* (Lib.) de Bary in oilseed Brassicas. *Aust Plant Pathol* 45:1–14

Khangura R, WJ MacLeod (2012). *Managing the risk of Sclerotinia stem rot in canola*. *Farm note* 546. Department of Agriculture and Food, Western Australia

Khangura R, AJ Van Burgel (2021). Foliar fungicides and their optimum timing reduce sclerotinia stem rot incidence, improve yield and profitability in canola (*Brassica napus* L.). *Ind Phytopathol* 11:1170

Khangura R, A van Burgel, M Salam, M Aberra, WJ MacLeod (2014). Why Sclerotinia was so bad in 2013? Understanding the disease and management options. *In Proceedings of the 2014 Crop Updates* 24–25 Feb 2014, Perth, WA Australia

Kharbanda PD, JP Tewari (1996). Integrated management of canola diseases using cultural methods. *Can J Plant Pathol* 18:168–175

Kora C, MR McDonald, GJ Boland (2008). New progress in the integrated managment of sclerotinia rot. *In: Integrated Management of Plants Pests and Diseases: Integrated Managment of Diseases caused by Fungi, Phytoplasms and Bacteria*, pp:243–270. Ciancioa A, KG Mukerhi (eds.). Springer, Dordrecht, The Netherlands

Kshetri L, F Naseem, P Pandey (2019). Role of *Serratia* sp. as biocontrol agent and plant growth stimulator, with prospects of biotic stress management in plant. *In: Plant Growth Promoting Rhizobacteria for Sustainable Stress Management*. *Microorganisms for Sustainability* Vol 13, Sayyed R (ed.). Springer, Singapore

Kurze S, H Bahl, R Dahl, G Berg (2001). Biological control of fungal strawberry diseases by *Serratia plymuthica* HRO-C48. *Plant Dis* 85:529–534

Li CX, H Li, K Sivasithamparam, TD Fu, YC Li, SY Liu, MJ Barbetti (2006). Expression of field resistance under Western Australian conditions to *Sclerotinia sclerotiorum* in Chinese and Australian *Brassica napus* and *Brassica juncea* germplasm and its relation with stem diameter. *Aust J Agric Res* 57:1131–1135

Li Y, L Qin, DP Roberts, X Hu, L Xie, C Gu, X Liao, P Han, X Liao (2020). Biological fertilizer containing *Bacillus subtilis* BY-2 for control of *Sclerotinia sclerotiorum* on oilseed rape. *Crop Prot* 138:105340

Lopes FAC, AS Steindorff, AM Geraldine, RS Brandao, VN Monteiro, M Lobo Junior, ASG Coelho, CJ Ulhoa, RN Silva (2012). Biochemical and metabolic profiles of *Trichoderma* strains isolated from common bean crops in the Brazilian Cerrado, and potential antagonism against *Sclerotinia sclerotiorum*. *Fungal Biol* 116:815–824

Macena AMF, NN Kobori, GM Mascarin, JB Vida, GL Hartman (2020). Antagonism of *Trichoderma*-based biofungicides against Brazilian and North American isolates of *Sclerotinia sclerotiorum* and growth promotion of soybean. *BioControl* 65:235–246

Matroudi S, M Zamani (2009). Antagonistic effects of three species of *Trichoderma* sp. on *Sclerotinia sclerotiorum*, the causal agent of canola stem rot. *Egypt J Biol* 11:37–44

McQuilken MP, C Chalton (2009). Potential for biocontrol of sclerotinia rot of carrot with foliar sprays of Contans® WG (*Coniothyrium minitans*). *Biocontr Sci Technol* 19:229–235

Neupane S, LA Goodwin, N Högberg (2013). Non-contiguous finished genome sequence of plant-growth promoting *Serratia proteamaculans* S4. *Stand Genome Sci* 8:441–449

Norton R, T Jensen, V Nosov (2011). *Balanced nutrition in Brassica napus production with emphasis on S fertilizer requirements*. 17th Australian Research Assembly on Brassicas (ARAB). Wagga Wagga, Australia

Ojaghian MR (2011). Potential of *Trichoderma* spp. and *Talaromyces flavus* for biological control of potato stem rot caused by *Sclerotinia sclerotiorum*. *Phytoparasitica* 39:185–193

Raaijmakers JM, M Vlami, JT De Souza (2002). Antibiotic production by bacterial biocontrol agents. *Antonie van Leeuwenhoek* 81:537–547

Rimmer SR, VI Shattuck, L Buchwaldt (2007). Compendium of brassica diseases. *Amer Phytopathol Soc* (APS Press), St. Paul, Minnesota, USA

Saharan GS, N Mehta (2008). *Sclerotinia Diseases of Crop Plants: Biology, Ecology, and Disease Management*. Springer Science + Business Media B.V., Berlin, Germany

Saxena A, R Raghuwanshi, HB Singh (2015). *Trichoderma* species mediated differential tolerance against biotic stress of phytopathogens in *Cicer arietinum* L. *J Basic Microbiol* 55:195–2016

Sharma M, A Tarafdar, R Ghosh R, S Gopalakrishanan (2017). Biological control as a tool for eco-friendly management of plant pathogens. Biological control as a tool for eco-friendly management of plant pathogens. *In: Advances in Soil Microbiology: Recent Trends and Future Prospects. Microorganisms for Sustainability*, Vol 4. Adhya T, B Mishra, K Annapurna, D Verma, U Kumar (eds.). Springer, Singapore

Simonetti E, AI Hernandez, NL Kerber, NL Pucheu, MA Carmona, AF Garcia (2012). Protection of canola (*Brassica napus*) against fungal pathogens by strains of biocontrol rhizobacteria. *Biocontr Sci Technol* 22:111–115

Smolinska U, B Kowalska (2018). Biological control of the soil-borne fungal pathogen *Sclerotinia sclerotiorum*—a review. *J Plant Pathol* 100:1–12

Taylor A, E Coventry, JE Jones, JP Clarkson (2015). Resistance to a highly aggressive isolate of *Sclerotinia sclerotiorum* in a *Brassica napus* diversity set. *Plant Pathol* 64:932–940

Vincent C, MS Goettel, G Lazarovits (2007). *Biological control: a global perspective*. CABI, Wallingford, England

Whipps JM, S Sreenivasaprasad, S Muthumeenakshi, CW Rogers, MP Challen (2008). Use of *Coniothyrium minitans* as a biocontrol agent and some molecular aspects of sclerotial mycoparasitism. *Eur J Plant Pathol* 121:323–330

White TJ, T Bruns, S Lee, J Taylor (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In: PCR Protocols : A guide to Methods and Applications*, pp: 315–322. Innis MA, DH Gelfand, JJ Sninsky, TJ White (eds.). Academic Press, San Diego, California, USA

Yang L, G Li, J Zhang, D Jiang, W Chen (2011). Compatibility of *Coniothyrium minitans* with compound fertilizer in suppression of *Sclerotinia sclerotiorum*. *Biol Cont* 59:221–227

Zeng W, W Kirk, J Hao (2012). Field management of Sclerotinia stem rot of soybean using biological control agents. *Biol Contr* 60: 141–147

Zhang X, X Sun, G Zhang (2003). Preliminary report on the monitoring of the resistance of *Sclerotinia libertinia* to carbendazim and its internal management. *J Pesticide Sci Admin* 24:18–22