The evaluation of plant extracts, biocontrol agents and hot water as seed treatments to control black rot of rape in South Africa

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Highlights

- Three seed treatments were shown to be effective in controlling black rot of rape.
- *Paenibacillus* sp. reduced *Xanthomonas campestris* on rape seeds and reduced black rot.
- *Agapanthus caulescens* (indigenous to South Africa) at 15 mg/ml reduced black rot.
- Hot water seed soak at 50 °C for 30 min effectively controlled black rot.

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ABSTRACT

Black rot disease, which is caused by the pathogen *Xanthomonas campestris* pv. *campestris* (*Xcc*), is a major challenge to brassica vegetable production by smallholder farmers. The pathogen is seed-borne making it difficult to control the disease. In this study various plant extracts, commercial biocontrol agents (BCAs) and hot water treatments were evaluated for their antibacterial activity, and as seed treatments of rape (*Brassica napus* L.) against *Xcc in vitro* and under greenhouse conditions. The microtitre double-dilution assay showed that acetone extracts of *Cymbopogon citratus* had strong antimicrobial activity with the lowest minimum inhibitory concentration (MIC) of 0.19 mg/ml, which was comparable to the antibiotic neomycin (0.2 mg/ml). Using the agar well diffusion method the BCA *Paenibacillus* sp. (3×10^9 cfu/ml) recorded the highest antibacterial activity with a maximum zone of inhibition of 17 mm. Seed treatment with hot water at 50°C for 30 minutes reduced the bacterial population to 3.1 cfu/ml compared to the untreated inoculated control (6.0 cfu/ml). Significantly higher germination percentage (84%) was recorded after seed treatments with acetone extracts of *Agapanthus caulescens* (15 mg/ml) and hot water at 50°C for 30 minutes. In the greenhouse trials, acetone extracts of *A. caulescens* (15 mg/ml), *Paenibacillus* sp., and hot water at 50°C for 30 minutes significantly increased seedling emergence and reduced black rot incidence and severity on rape leaves. The present study showed that plant extracts, commercial BCAs and hot water have potential as seed treatments for the control of *Xcc* and black rot disease.

Keywords: *Brassica*; biocontrol; hot water; plant extracts; seed treatment; *Xanthomonas campestris* pv. *campestris*; black rot

1. Introduction

Brassicas are an important group of vegetable crops grown by smallholder farmers in Africa (Massomo et al., 2003; Bila et al., 2009). In South Africa, production of cabbage (*Brassica oleracea* L.) and other brassicas in 2013 was estimated to be 132 600.00 tonnes (FAOSTATS, 2016). A survey conducted by Mandiriza-Mukwirimba et al. (2016) showed that rape (*Brassica napus* L.), a leafy vegetable, was the second (to cabbage) most popular brassica vegetable grown by the smallholder farmers in the Gauteng Province and Waterberg District in the Limpopo Province. Rape is nutritious, rich in nutrients such as vitamins A, B and C, calcium, magnesium, phosphorus and iron (Toxopeus and Mvere, 2004). Smallholder farmers produce brassica crops for home consumption and for sale (Mandiriza-Mukwirimba et al., 2016). However, brassica cultivation by the farmers is severely hampered by black rot disease (Massomo et al., 2003; Mandiriza-Mukwirimba et al., 2016) caused by *Xanthomonas campestris* pv. *campestris* (Pammel) Dowson (*Xcc*) (Neergaard, 1977). The disease is seed-borne and infected seeds are a primary source of inoculum (Schaad and Alvarez, 1993), resulting in poor germination and seedling emergence. Black rot is very destructive, particularly when warm and humid conditions exist (Meenu et al., 2013). Severe yield losses including whole fields have been reported (Massomo, 2002; Massomo et al., 2004). Moreover, lesions on affected crops reduce market value and shorten the shelf life of produce.

Current management of black rot involves the use of resistant varieties; pathogen free seed; crop rotation with non-brassicas; removal of crop residues; and, chemical control (Trench et al., 1992; Mishra and Arora 2012; Vicente and Holub, 2013). Currently, no bactericides are registered for use as seed treatments for the control of bacterial pathogens such as *Xcc*. Copper based fungicides are sometimes used to control such pathogens (Pacific Northwest Extension, 2015) but at times they are relatively ineffective (Lenka and Ram,

1997; Mikicinski et al., 2012). However, the use of chemicals is becoming restricted due to the perceived toxic effects on humans and the environment (Aktar et al., 2009). Furthermore, most smallholder farmers lack the knowledge on pesticide use and safety (Dinham, 2003), which exacerbates the risk of human exposure and environmental toxicity.

Seed treatments with alternative methods such as plant extracts and microbial biological control agents (BCAs), could potentially provide control of *Xcc* and black rot disease (van der Wolf et al., 2008; Mishra and Arora, 2012; Ghazalibiglar, 2014). Hence there is need for research on such alternative methods to control the pathogen including the use of hot water treatment, which is effective in reducing seed-borne inoculum (Forsberg et al., 2002) and controlling bacterial pathogens (Nega et al., 2003). Some plant extracts have been reported to be effective chemotherapeutants (Pawar, 2011) and they have the advantage of being biodegradable (van der Wolf et al., 2008; Szopinska et al., 2010). In addition, the use of antagonistic microorganisms such as *Bacillus* spp. and *Paenibacillus* spp. applied as seed treatments on brassicas against the black rot pathogen have been reported to be effective (Massomo et al., 2004; Ghazalibiglar, 2014).

The current study investigated the antibacterial activity and potential of various plant extracts and commercial BCAs as seed treatments on artificially *Xcc* inoculated rape seeds *in vitro*. The effect of the seed treatments on rape seed germination *in vitro* was determined. Furthermore, the efficacy of plant extracts, biocontrol agents and hot water seed treatments against black rot disease on artificially inoculated rape seeds was evaluated under greenhouse conditions.

2. Materials and methods

2.1. Plant material, antagonists and pathogen

The plant material used in the present study was *Lantana camara* L. (leaves and flowers, family Verbanaceae), *Agapanthus caulescens* Spreng (leaves, family Agapanthaceae), *Lavandula angustifolia* Mill (leaves, flowers and stem, family *Lamiaceae*), *Chlorophytum comosum* (Thunb) Jacq (whole plant, family Anthericaceae), *Tagetes minuta* L. (leaves, flowers and stem, family Asteraceae) and *Cymbopogon citratus* Stapf (leaves and stem, family Poaceae). Specimens of each of the selected plants were deposited in the H.G.W.J. Schweickerdt Herbarium, University of Pretoria, Pretoria, Republic of South Africa (RSA) and voucher numbers were assigned. *Chlorophytum comosum* (PRU119732) and *A. caulescens* (PRU119729) were collected from the Manie van der Schijff Botanical Garden at the University of Pretoria, Pretoria, RSA. *Lantana camara* (PRU119730) was collected from Louis Trichardt, Limpopo Province, RSA whilst *Tagetes minuta* (PRU119727), *L. angustifolia* (PRU119728) and *C. citratus* (PRU119731) were obtained from home gardens in the eastern suburbs of Pretoria, RSA. Plant selection was based mostly on reports that they have antimicrobial activity against plant pathogens (Muyima *et al.*, 2004; Somda *et al.*, 2007; Shah *et al.*, 2011; Masangwa *et al.*, 2013). In addition, plants of the genus *Agapanthus* are native to South Africa and are found in many homestead gardens making them readily available (Pienaar, 2001; Pretorius *et al.*, 2002).

The formulated commercial biocontrol products, which were obtained from companies in South Africa, were *Paenibacillus* sp. (3×10^9 cfu/ml), *Bacillus* sp. (2×10^{10} cfu/ml) and *Bacillus subtilis* (5×10^7 cfu/g). For the *B. subtilis* powder formulation, 200 g was dissolved in 800 ml sterile distilled water (SDW) to make a suspension (manufacturer's recommendation).

The *Xcc* pathogen (PPRI BD 1476, GenBank accession number: KT964517) used for inoculation in the current study was isolated from diseased rape leaves showing typical black rot symptoms in a smallholder farmer field in the Gauteng Province. It was selected based on pathogenicity tests on rape plants and results indicated that it was the most virulent isolate (data not shown).

2.2. Preparation of crude plant extracts

The collected plant materials were all air dried on a laboratory bench at room temperature and ground to a fine powder using a Macsalab mill (Model 200 LAB Eriez®, Erie, USA). For each of the ground plant material (500 g or 1 kg), sequential extractions were conducted with 1 l (for 500 g material) or 2 l (for 1 kg material) of acetone followed by 1 or 2 l of sterile distilled water (SDW). The soaked extracts were placed on a laboratory shaker at 100 rpm for 48 hours. After filtration, the acetone was removed by evaporation using a Büchi Rotavapor (Model R-200, Flawil, Switzerland) at a temperature of ±50°C. Aqueous extracts were concentrated to a powder by freeze drying at -80°C (Edwards High Vacuum International, Sussex, England). Final crude plant extracts harvested were weighed, recorded and stored in glass vials at 4°C until further use.

2.3. Microtitre double-dilution assay

The microtitre double-dilution assay according to Eloff (1998) and Masangwa et al. (2013) with few modifications was used for the antibacterial assay to determine the minimum inhibitory concentrations (MICs) for each of the plant extracts tested against *Xcc*. The pathogen was cultured in sterile nutrient broth and incubated for 48 hours at 30°C. The optical density of the bacterial suspension (in broth) was adjusted to 0.5 McFarland standard (10^8 cfu/ml) using a spectrophotometer.

A stock solution of 50 mg/ml was prepared for each plant extract by dissolving the extracts in 10% dimethyl sulphoxide (DMSO). The controls included sterile nutrient broth (sterile control), the antibiotic neomycin (0.2 mg/ml) and 10% DMSO. After a series of dilutions of plant extracts, antibiotic neomycin and 10% DSMO, 100 µl of the bacterial suspension was added to all wells except column 10, which represented the sterile control. The microtitre plates were incubated at 30°C for 24 hours and after incubation, 40 µl iodonitrotetrazolium chloride (INT) (0.2 mg/ml) was added to all the wells excluding columns 3, 6 and 9 that were colour controls. After further incubation for 30 minutes MIC values for the plant extracts were recorded as the lowest concentration value of extract that completely inhibited bacterial growth (Eloff, 1998). The experiment was performed twice.

2.4. Agar well diffusion assay

The antibacterial activity of the commercial BCAs was determined by using the agar well diffusion assay as described by Mishra and Arora (2012) with some modifications. Besides testing the formulated products produced by the manufacturer, each of the biocontrol products was further diluted up to 1:1000 to test its bio-efficacy. *Xanthomonas campestris* pv. *campestris* suspension was prepared in sterile saline (0.85% sodium chloride (NaCl) solution) and was adjusted to 10^8 cfu/ml. The bacterial suspension was spread evenly on solidified Luria Bertani (LB) agar medium. Using a sterile cork borer, three wells of 9 mm diameter that were equidistant from each other were punched into the LB agar. Fifty µl of each of the biocontrol suspensions were added into the wells. The control plates had the antibiotic neomycin (0.2 mg/ml) and SDW added into the wells and each treatment consisted of three replicates. Petri dishes were incubated at 30°C for 48 hours and thereafter the zone of inhibition was measured (in mm) from the edge of the well (Mishra and Arora, 2012). The experiment was done twice.

2.5. Artificial inoculation

Seeds of rape (cultivar English Giant), obtained from a seed company in South Africa, were artificially inoculated by soaking in bacterial suspension of *Xcc,* adjusted to 10^8 cfu/ml, for one hour with occasional hand shaking. After inoculation, the bacterial suspension was drained and seeds were left to dry for 48 hours in a laminar flow cabinet.

2.6. Seed treatments

2.6.1. Seed treatment with plant extracts

Plant extracts used for seed treatments were selected based on the results of the microtitre double-dilution assay. The selected plant extracts were acetone extracts of *A. caulescens, T. minuta,* and *C. citratus* and water extracts of *C. citratus.* For the *in vitro* investigation, water extracts were dissolved directly in SDW and acetone extracts were dissolved in 1% or 10% DMSO to yield final concentrations of 10, 15 and 20 mg/ml. Evaluation of the plant extracts against black rot disease in the greenhouse was performed using acetone extracts of *A. caulescens* (15 mg/ml) and *C. citratus* (10 mg/ml), which showed the best activity *in vitro* as seed treatments. Artificially *Xcc* inoculated rape seeds were soaked in the respective extracts for 3 hours at 25°C in the dark with occasional hand shaking. The controls included *Xcc* inoculated seeds soaked in SDW, 1% or 10% DMSO, untreated *Xcc* inoculated seeds and healthy seeds. After soaking the extracts were drained and seeds were allowed to air dry in a laminar flow cabinet for 48 hours.

2.6.2. Seed treatments with commercial biological control agents

The liquid formulations of *Paenibacillus* sp. and *Bacillus* sp. were applied at recommended rates of 40 ml/kg seed and at 1.6 ml/kg seed, respectively. The rate used for the powder formulation of *Bacillus subtilis* was 200 g/12.5 kg seed and a few drops of the

supplied sticker were added to allow even mixing. The BCAs were applied as slurries for 2 hours and seeds were then left to dry overnight in Petri dishes inside a laminar flow cabinet *(in vitro* tests) or sown immediately (greenhouse tests). Controls included *Xcc* inoculated seeds soaked in SDW, untreated *Xcc* inoculated seeds and healthy disease free seeds.

2.6.3. Seed treatment with hot water

Artificially infected seeds were subjected to different hot water treatments using a thermostatically controlled water bath (Labotec model 132 A, Johannesburg, South Africa) as described by Nega *et al.* (2003) with slight modifications. The treatment parameters were: 45°C for 25 minutes, 45°C for 30 minutes, 50°C for 10 minutes, 50°C for 20 minutes, 50°C for 30 minutes, 53°C for 10 minutes, 53°C for 20 minutes and 53°C for 25 minutes. The controls included *Xcc* inoculated seeds soaked in SDW for 30 minutes at room temperature, untreated inoculated seeds and healthy seeds. After treatment, seeds were dried in a laminar flow cabinet for 48 hours.

2.6.4. Detection of *X. campestris* pv. *campestris* post seed treatments

Detection of *Xcc* after various seed treatments was done using the International Seed Testing Association (ISTA, 2017) validated method on detection of *Xcc* on *Brassica* spp. seed using grinding and with some modifications. The extraction procedure was performed on 300 seeds for each treatment from plant extracts, BCAs and hot water treatments. A dilution series was conducted from the undiluted (stock) up to 10^{-5} for each treatment and 100 µl was pipetted from each dilution including the undiluted (stock) solution onto Petri dishes containing solidified mCS20ABN media (selective for *Xcc*) (ISTA 2017) and each dilution had three replicates. Petri dishes were incubated at 30°C for four days. After incubation, the

numbers of *Xcc* colonies were recorded and cfu/ml was determined. The experiments for the different seed treatments were conducted twice.

2.6.5. Germination tests

Germination tests were performed using the blotter paper method according to ISTA rules (2017) with slight modifications. Two hundred artificially inoculated seeds (four replicates of 50 seeds) were used for each of the respective seed treatments. Three filter papers were placed in sterile glass Petri dishes, wetted with SDW and excess water was drained. Seeds were placed on top of the moistened filter paper in each Petri dish and incubated at ±22°C. Germination was evaluated two times at day 5 and day 7. The percentage of germinated seeds and diseased seedlings were determined at the end of incubation. Germination tests were done twice.

2.7. Greenhouse trials

Greenhouse trials were carried out at the Plant Pathology Greenhouses on the Hatfield Experimental Farm of the University of Pretoria, South Africa (25° 45' S, 28°15’ E, 1 380 m a.s.l.). Following seed treatments, rape seeds were sown in seedling trays (128 cells, dimensions of 675 mm (L) x 345 mm (W) x 60 mm (H); Isowall, Pretoria, South Africa) filled with an organic germination mix (Varing Kwekery, Pretoria, South Africa). In each cell a single seed was sown and covered with a thin layer of vermiculite. The trays were then arranged in a randomised complete block design (RCBD) in the greenhouse, each treatment with four replicates of 25 seeds. The arranged trays were kept on benches at 25°C for four weeks and were watered daily using a fine spray. To evaluate the effectiveness of the treatments, the number of emerged seedlings and healthy seedlings were recorded two weeks after sowing (WAS). Disease severity for black rot was recorded at 4WAS using a scale of 0-

9 adapted from Massomo et al. (2004). The scores were then used to calculate the external black rot index (EBRI) on the leaves as ∑ (number of plants in class X severity class)/total number of plants (Alvarez et al., 1994; Massomo et al., 2004). Shoot length (mm), dry root mass and dry shoot mass (g) were measured at harvest (4WAS). The trial was conducted twice.

2.8. Data analysis

All the results were statistically subjected to analysis of variance (ANOVA) using SAS version 9.3 statistical software (SAS Institute, 2010).Data for experiments one and two were combined and then subjected to analysis of variance and the means of the two experiments are presented. Percentage emergence and healthy plants data was not combined and was arcsine transformed (although values are presented as untransformed data). To separate means, the data was subjected to Fischer's least significant difference (LSD) test. All experiments and trials were done twice.

3. Results

3.1. Antimicrobial activity of plant extracts and commercial biocontrol agents

The results of the microtitre double-dilution assay indicated that the best activity was shown by acetone extracts of *C. citratus* with an MIC of 0.19 mg/ml which closely matched the activity of neomycin (0.2 mg/ml). *Agapanthus* acetone extracts and *C. citratus* water extracts were moderately active, both with MICs of 0.39 mg/ml. Acetone and water extracts of *L. angustifolia* showed low activity (both with MICs of 1.56 mg/ml) against *Xcc*. None of the extracts of *L. camara*, *C. comosum* and DMSO were active against *Xcc*.

Paenibacillus sp. at its maximum population (undiluted, 3 x 10^9 cfu/ml) inhibited the growth of *Xcc* forming an inhibition zone of 17 mm, which was significantly (P<0.001) larger than that of neomycin (0.2 mg/ml). *Paenibacillus* sp. at 1:10 dilution and *B. subtilis* undiluted (5 x 10^7 cfu/ml) both formed an inhibition zone of 12 mm whilst *Bacillus* sp. undiluted (2 x 10^{10} cfu/ml) formed an inhibition zone of 11 mm and did not differ significantly (P<0.001) from neomycin. However, as the dilution of the BCAs increased from 100 fold to 1000 fold, the radius of the inhibition zone significantly decreased (P<0.001).

3.2. Effect of seed treatments on X. campestris pv. campestris population on rape seeds

All the plant extract seed treatments at 10, 15 and 20 mg/ml concentrations significantly (P<0.001) reduced *Xcc* bacterial population on the seed when compared to the untreated inoculated control (Table 1). Acetone extracts of *C. citratus* were the most effective at 20 mg/ml reducing the *Xcc* population to 3.8 cfu/ml.

Table 1 Effect of plant extract treatments at concentrations of 10, 15 and 20 mg/ml on the population of *X. campestris* pv. *campestris* on rape seeds.

	*Concentration (Average log cfu/ml)		
Treatments	10 mg/ml	15 mg/ml	20 mg/ml
Tagetes minuta (A) - 1% DMSO	5.2 b	5.1 d	4.3 e
Agapanthus caulescens (A) - 1% DMSO	4.4 c	4.3 e	4.2 f
Cymbopogon citratus (A) - 1% DMSO	4.2 d	4.2 f	3.8 g
Cymbopogon citratus (A) - 10% DMSO	N/A	4.1 g	3.8 g
Cymbopogon citratus (W)	5.2 b	5.2 c	5.2 bc
SDW inoculated control	5.2 b	5.3 b	5.2 bc
1% DMSO inoculated control	5.2 b	5.2 c	5.1 c
10% DMSO inoculated control	N/A	5.1 d	5.0 d
Untreated inoculated control	6.0 a	6.0 a	5.9 a
Healthy control	0.0 e	0.0 h	0.0 h
P	<0.001	<0.001	<0.001

* Values within columns followed by the same letter are not significantly different from each other according to Fisher's LSD test (P = 0.05). Values are the means of two experiments, each treatment with three replicates. A, acetone extracts; W, water extracts; SDW, sterile distilled water; 1% DMSO, dissolved in 1% dimethyl sulphoxide; 10% DMSO, dissolved in 10% dimethyl sulphoxide; N/A, not tested.

Compared to the untreated inoculated control, seed treatments with *Paenibacillus* sp., *Bacillus* sp. and *B. subtilis* (B-RUS) significantly (P<0.001) reduced the *Xcc* population on rape seed. Treatment with *Paenibacillus* sp. showed the best activity by significantly reducing (P<0.001) the bacterial count by approximately 50% (3.2 cfu/ml) when compared to the untreated inoculated seeds (5.6 cfu/ml) (Fig. 1.).

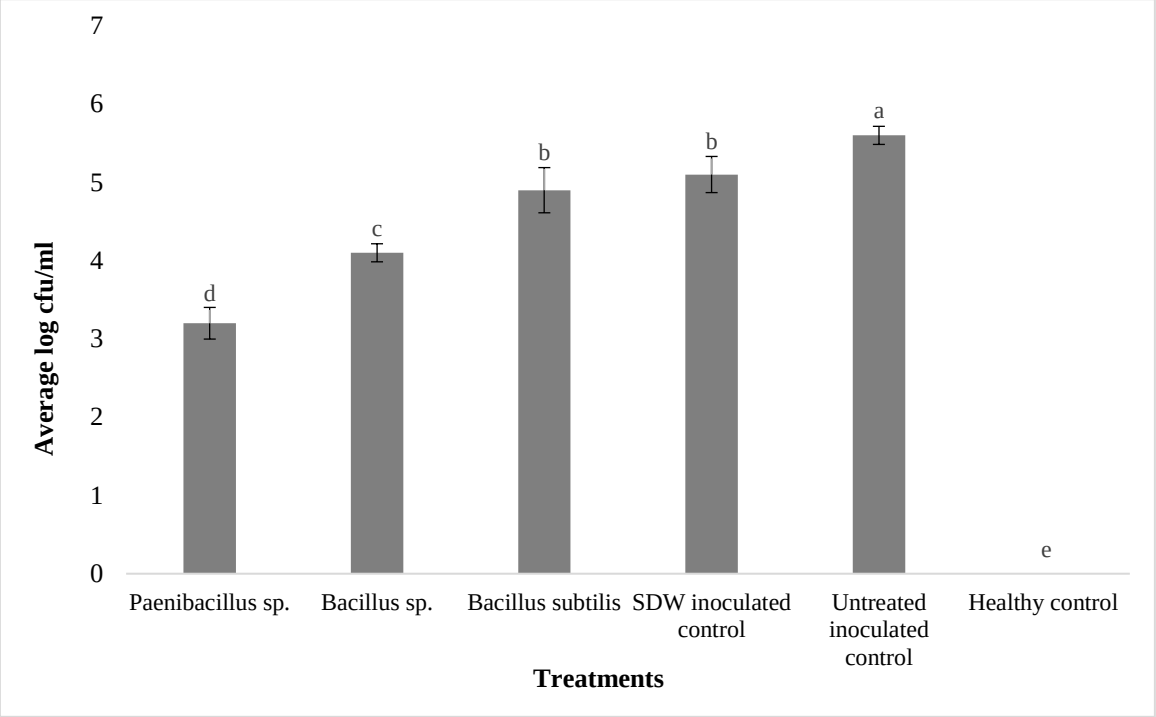

Fig. 1. Effect of seed treatments with commercial biocontrol agents on *X. campestris* pv. *campestris* population on rape seeds.

Values of bars followed by the same letter are not significantly different based on Fisher's LSD test (P = 0.05). Values are the means of two experiments, each treatment with three replicates.

All the hot water treatments reduced the *Xcc* population significantly (P<0.001) when compared to the untreated inoculated control that had a count of 6.0 cfu/ml. Seeds immersed at 50°C for 30 minutes and 50°C for 25 minutes reduced the population of *Xcc* to 3.1 cfu/ml

and 3.6 cfu/ml, respectively. Submerging seeds at 45°C for 25 minutes and at 50°C for 10 minutes provided the least control of *Xcc* (5.1 cfu/ml) (Fig. 2.)

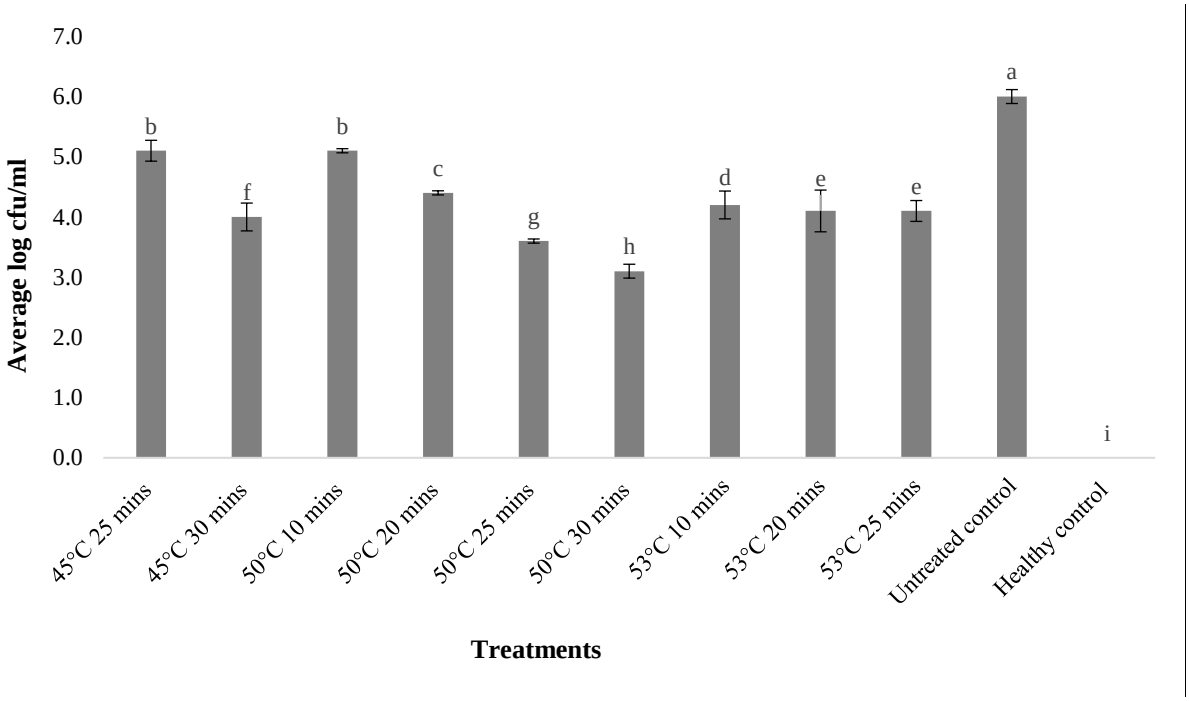

Fig. 2. Effect of hot water seed treatments on *X. campestris* pv. *campestris* population on rape seeds.
Values of bars followed by the same letter are not significantly different based on Fisher's LSD test (P = 0.05). Values are the mean of two experiments, each treatment with three replicates. mins = minutes, untreated control = untreated inoculated control.

3.3. Effect of alternative seed treatments on rape seed germination

Treatment of rape seeds with plant extracts at 10 mg/ml significantly (P<0.001) increased the percentage of germinating seed when compared to the untreated inoculated control (Table 2). At 15 mg/ml acetone extracts of *A. caulescens* recorded the highest germination percentage (84%) amongst the *Xcc* inoculated and plant extract treated seeds. Generally percentage seed germination was reduced as the concentration of plant extracts increased to 20 mg/ml. Treatments with all the plant extracts at 15 and 20 mg/ ml caused a significant (P<0.001) decrease in the percentage of diseased seedlings when compared to the untreated inoculated control (Table 2). The healthy seeds had 0% diseased seedlings.

Table 2 Effect of seed treatments with plant extracts at 10, 15 and 20 mg/ml concentrations on rape seed germination.

Treatments	*Germination (%)			*Diseased seedlings (%)		
	10 mg/ml	15 mg/ml	20 mg/ml	10 mg/ml	15 mg/ml	20 mg/ml
Tagetes minuta (A)- 1% DMSO	78.7 b	69.0 d	61.0 b	23.3 a	15.0 b	9.5 c
Agapathus caulescens (A) - 1% DMSO	76.0 bc	84.0 b	63.0 b	15.0 b	12.5 b	8.0 cde
Cymbopogon citratus (A)-1% DMSO	76.0 bc	73.5 cd	40.0 c	12.5 b	7.5 c	4.5 e
Cymbopogon citratus (A)-10% DMSO	NA	38.0 f	41.0 c	NA	6.5 c	5.5 de
Cymbopogon citratus (W)	73.5 c	75.3 c	60.5 b	22.5 a	14.0 b	9.0 cd
SDW inoculated control	60.5 d	60.5 e	60.5 b	23.5 a	22.5 a	24.5 a
1% DMSO inoculated control	59.0 d	59.0 e	59.0 b	25.5 a	25.5 a	25.5 a
10% DMSO inoculated control	NA	41.5 f	41.5 c	NA	15.0 b	15.0 b
Untreated inoculated control	54.5 e	60.0 e	58.5 b	26.5 a	23.5 a	26.0 a
Healthy control	89.0 a	93.0 a	94.0 a	0.00 c	0.00 d	0.0 f
P	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

* Values within columns followed by the same letter are not significantly different from each other according to Fisher's LSD test (P = 0.05). Values are the mean of two experiments, each with four replicates of 50 seeds. A, acetone extracts; W, water extracts; SDW, sterile distilled water; 1% DMSO, dissolved in 1% dimethyl sulphoxide; 10% DMSO, dissolved in 10% dimethyl sulphoxide; N/A, not tested.

Seed treatments with the antagonists, *B. subtilis*, *Paenibacillus* sp. and *Bacillus* sp. enhanced germination of seeds (Table 3). The most efficient BCA in promoting germination was *B. subtilis* at 83.5%. The healthy control seeds recorded a higher percentage of germinated seedlings when compared to all treatments. Treating the seeds with the BCAs decreased the percentage of diseased seedlings when compared to the untreated inoculated control.

With the exception of the 53°C for 20 minutes and 53°C for 30 minutes treatments, hot water treatments significantly (P<0.0001) increased seed germination when compared to the untreated inoculated control (Table 4). The most effective hot water treatment at increasing percentage germination was 50°C for 30 minutes. All hot water treatments decreased the

percentage of diseased seedlings with 53°C for 30 minutes (2.0%) being most effective, not differing significantly (P<0.0001) from the healthy control at 0% (Table 4).

Table 3 Effect of seed treatments with commercial biocontrol agents on rape seed germination.

Treatments	*Germination (%)	*Diseased seedlings (%)
Paenibacillus sp.	76.0 c	4.0 c
Bacillus sp.	71.5 d	5.5 c
Bacillus subtilis	83.5 b	10.0 b
SDW inoculated control	52.0 f	20.0 a
Untreated inoculated control	56.0 e	22.0 a
Healthy control	89.5 a	0.0 d
P	<0.0001	<0.0001

* Values within columns followed by the same letter are not significantly different from each other according to Fisher's LSD test (P = 0.05). Values in columns are the means of two experiments with four replicates of 50 seeds for each treatment. SDW, sterile distilled water.

Table 4 Effect of hot water seed treatment on rape seed germination

Treatments	*Germination (%)	*Diseased seedlings (%)
45°C 25 minutes	72.5 de	20.0 bc
45°C 30 minutes	71.0 e	18.5 c
50°C 10 minutes	72.0 e	22.0 b
50°C 20 minutes	74.5 cde	13.5 d
50°C 25 minutes	78.5 c	10.5 e
50°C 30 minutes	84.0 b	7.5 f
53°C 10 minutes	74.7 cde	6.0 fg
53°C 20 minutes	62.5 f	4.5 gh
53°C 30 minutes	60.5 f	2.0 hi
Untreated inoculated control	61.0 f	26.5 a
Healthy control	90.0 a	0.0 i
P	*P*<0.0001	*P*<0.0001

*Values in columns followed with the same letter are not significantly different according to Fisher's LSD (P = 0.05). Values in columns represent the actual untransformed data but ANOVA was applied to arcsine transformed data. Values are the means of two experiments each with four replicates of 50 seeds per treatment.

3.4. Effect of alternative seed treatments against black rot disease under greenhouse conditions

In both trials the tested alternative seed treatments significantly ($P<0.0001$) increased emergence and number of healthy plants when compared with the untreated inoculated control, with the hot water treatment at 50°C for 30 minutes and *Paenibacillus* sp. not differing significantly ($P<0.0001$) from the healthy control (Table 5).

Seed treatments with hot water, the BCA and plant extracts under greenhouse conditions significantly ($P<0.001$) increased the seedling length when compared to the untreated inoculated control. Plants from seeds treated with *Paenibacillus* sp. had the greatest shoot length at 110.3 mm (Table 5). In both trials, all four alternative treatments *Paenibacillus* sp., 50°C for 30 minutes, acetone extracts of *C. citratus* (10 mg/ml) and *A. caulescens* (15 mg/ml) significantly increased ($P<0.001$) total dry mass when compared with the untreated inoculated control (Table 5) and they did not differ significantly from the healthy control except for *C. citratus* (10 mg/ml) in trial two.

Table 5 Effect of alternative seed treatments against *X. campestris* pv. *campestris* on rape seedling emergence and health.

Treatments	*Emergence (%)		*Healthy plants (%)		*Total dry mass (g)		*Shoot length (mm)
	I	II	I	II	I	II	
Paenibacillus sp.	94.0 a	93.0 ab	91.0 ab	92.0 ab	0.81 a	0.69 a	110.3 a
50°C for 30 minutes	95.0 a	96.0 a	92.0 ab	95.0 a	0.80 a	0.69 a	97.4 b
Cymbopogon citratus (A) 10 mg/ml	82.0 bc	80.0 c	70.0 c	73.0 cd	0.75 a	0.53 b	73.7 d
Agapanthus caulescens (A) 15 mg/ml	92.0 a	82.0 bc	87.0 b	80.0 bc	0.81 a	0.69 a	84.3 c
1% DMSO inoculated	84.0 b	79.0 c	66.0 c	66.0 d	0.57 b	0.47 bc	58.8 e
SDW (30 minutes) inoculated	77.0 c	75.0 cd	55.0 d	61.0 de	0.52 bc	0.41 c	50.4 f
Untreated inoculated control	69.0 d	68.0 d	49.0 e	50.0 e	0.47 c	0.39 c	47.1 f
Healthy control	97.0 a	98.0 a	97.0 a	98.0 a	0.83 a	0.67 a	88.2 c
P	<0.0001	<0.0001	<0.0001	<0.0001	<0.001	<0.001	<0.001

*Values within columns followed by the same letter are not significantly different from each other according to Fisher's LSD test (P = 0.05). Shoot length values are the mean of two trials each with eight treatments and four replicates of 25 seeds. I, Trial one; II, Trial two; SDW, sterile distilled water; A, acetone extract; 1% DMSO, dissolved in 1% dimethyl sulphoxide.

At 4WAS the tested alternative seed treatments significantly (P<0.001) reduced the incidence of black rot disease on rape plants when compared to the untreated inoculated control. Treatments with hot water treatment at 50°C for 30 minutes, *Paenibacillus* sp. and acetone extracts of *A. caulescens* (15 mg/ml) did not differ significantly (P<0.001) from the healthy control (Table 6).

Table 6 Effect of alternative seed treatments on incidence and severity of black rot disease on rape seedlings under greenhouse conditions.

Treatments	*Disease incidence (%)	* External black rot index
Paenibacillus sp.	2.5 c	0.7 de
50°C for 30 minutes	2.0 c	0.5 de
Cymbopogon citratus (A) 10 mg/ml	10.0 b	2.2 c
Agapanthus caulescens (A) 15 mg/ml	3.5 c	0.8 d
1% DMSO inoculated control	15.5 a	3.2 b
SDW (30 min) inoculated control	17.0 a	3.3 b
Untreated inoculated control	19.0 a	4.1 a
Healthy control	0.0 c	0.0 e
P	<0.001	<0.001

* Values in columns with different letters indicate significant differences at P = 0.05 according to Fisher's LSD. Values in columns are the means of two greenhouse experimental trials each with four replicates of 25 seeds. SDW, sterile distilled water; A, acetone extract; 1% DMSO, dissolved in 1% dimethyl sulphoxide.

The black rot severity was moderate to low within the greenhouse trials. Seed treatment with all the alternative methods significantly (P<0.001) reduced the external black rot index (EBRI) with plants from the hot water treatment at 50°C for 30 minutes, and *Paenibacillus* sp. not differing statistically from the healthy control plants (Table 6).

4. Discussion

The present study tested plant extracts, commercial BCAs and hot water as seed treatments for the control of *Xcc* and black rot disease. The results showed that *C. citratus*

(lemongrass) extracts were active against *Xcc* and the acetone extracts gave the highest MIC value. Ferdinand et al. (2009) reported good antibacterial activity by organic extracts of lemongrass. In the current study, seed treatments with acetone extracts of *C. citratus* at low and high concentrations decreased the bacterial population and at 10 mg/ml the extracts enhanced seed germination. Not much literature is available on lemongrass plant extracts as seed disinfectants against bacterial seed-borne pathogens. However, Nguefack et al. (2008) found that seed treatment of rice (*Oryza sativa* L.) with essential oils of lemongrass significantly reduced seed infection by *Bipolaris oryzae* (Breda de Haan) Shoemaker and *Fusarium moniliforme* J. Sheld. (now *F. verticillioides*) and increased the germination of rice cultivars when compared to the untreated controls. In the greenhouse study the acetone extract of *C. citratus* (10 mg/ml) provided moderate control of black rot disease denoting the potential antibacterial activity of lemongrass against *Xcc*. Lucas et al. (2012) found that lemongrass essential oils reduced the severity of bacterial spot disease (*Xanthomonas vesicatoria* Vauterin et al.) on tomato (*Solanum lycopersicum* L.) plants under greenhouse conditions. The inhibitory effect of lemongrass has been attributed to bioactive compounds such as alkaloids, phenols, citral, saponins, geraniol, flavonoids, ketones etc. (Santin et al., 2009; Hindumathy, 2011; Shah et al., 2011). These compounds can either cause growth inhibition of a pathogen, inactivation of enzyme activities or rupturing of the cytoplasmic membrane (Jafari et al., 2012).

Plants of the genus *Agapanthus* are associated with the production of saponins and these are biologically active with antimicrobial properties (Pretorius et al., 2002; Singh et al., 2008). Although there are a few known studies on the antifungal properties of *Agapanthus* against plant pathogens (Pretorius et al., 2002; Tegegne et al., 2008; Masangwa et al., 2013), not much is known of the antibacterial activity and use as a seed treatment. Acetone extracts of *Agapanthus* exhibited antibacterial properties against *Xcc*; the extracts significantly

inhibited growth of the pathogen in the microtitre double-dilution assay. This is in contrast with the findings of Pretorius (2012) where methanol crude extracts of *Agapanthus africanus* (L.) Hoffmanns did not inhibit growth of plant pathogenic bacteria such as *Agrobacterium tumefaciens* Smith and Townsend, *Clavibacter michiganensis* subsp. *michiganensis* Davis et al. 1984 and *Xanthomonas campestris* pv. *phaseoli* (Smith) Dye *in vitro.* The differences in activity of the plant extracts could be due to differences in *Agapanthus* species, the pathogens tested or the type of solvents used during extraction of the plant products. Seed treatments with *Agapanthus* acetone extracts at 10 mg/ml showed good antibacterial effects on *Xcc.* As the concentration was increased to 15 mg/ml significantly higher antibacterial properties were demonstrated with a significant increase in seed germination, reduction in the number of diseased seedlings and the pathogen population on rape seed. In the greenhouse trial, acetone extracts of *A. caulescens* effectively controlled black rot disease and seedling emergence was promoted.

In the present study, the commercial biocontrol agents demonstrated good antibacterial activity against the black rot pathogen *in vitro*. Strong antagonism was shown by *Paenibacillus* sp. against growth of *Xcc* in the agar well diffusion assay. Previous studies reported that *Paenibacillus polymyxa* HKA-15 demonstrated antibacterial activity against *X. campestris* pv. *phaseoli in vitro* and resulted in increased seed germination of French bean (*Phaseolus vulgaris* L.) (Mageshwaran et al., 2011, 2012). Seed treatments with *Paenibacillus* sp. significantly reduced the population of *Xcc*, reduced the percentage of diseased seedlings and increased seed germination. In the greenhouse trials, rape seed treatment with *Paenibacillus* sp. reduced black rot severity and incidence, seedling emergence was increased and rape plant growth was enhanced resulting in the highest shoot length and total dry mass, comparable to the healthy control. Similarly, Ghazalibiglar (2014) found that treatment of cabbage seeds (*Brassica oleracea* L.) with a *Paenibacillus* isolate

(P16) reduced black rot incidence on cabbage true leaves and cotyledons from the onset of symptoms up to the end of experiment and increased plant growth. Several reports have linked the antagonistic activity of *Paenibacillus* spp. to the production of peptide antibiotics, extracellular hydrolytic enzymes and induction of systemic resistance (Beatty and Jensen 2002; Benitez et al., 2004; Mageshwaran et al., 2012).

Although *Bacillus* spp. were not evaluated as seed treatments in the greenhouse trials, the tested isolates i.e., *B. subtilis* and *Bacillus* sp. effectively inhibited growth of *Xcc in vitro*. Treatment of rape seeds with the *Bacillus* sp. significantly reduced the *Xcc* infection on seeds, decreased the number of diseased seedlings and increased seed germination. However, only *Paenibacillus* sp. was tested further in greenhouses as it provided the best results in the *in vitro* test.

Several studies have reported on the effectiveness of hot water treatments against bacterial diseases (Linders, 2000; Nega et al., 2003; Schmitt et al., 2006). Hot water treatments for control of *Xcc* have been reported at 50°C for 10-60 minutes (Shekawhat et al., 1982; Sha et al., 1985; Babadoost et al., 1996; Nega et al., 2003). Generally physical seed treatment with hot water demonstrated good antibacterial effect against *Xcc* on rape seeds in the current study and treatment at 50°C for 30 minutes controlled *Xcc* on seeds and increased germination. Under greenhouse conditions the same hot water treatment increased rape seedling emergence, effectively reduced black rot incidence and was comparable to the healthy control. However, as the treatment temperature increased to 53°C and with an increase in treatment time to 20 minutes or 30 minutes, *Xcc* populations were significantly reduced but rape seed germination was adversely affected. Ivey and Miller (2005) reported that even though hot water treatment is effective in decreasing pathogen levels on vegetables, sometimes reduction in seed germination rates has been recorded, and Nega et al. (2003)

recommended that for high treatment temperatures a shorter duration is needed especially with sensitive crops such as brassicas.

In conclusion, the present study has shown that plant extracts and commercial BCAs applied as seed treatments have potential to control *X. campestris* pv. *campestris* in brassica vegetables. Hot water treatment at 50°C for 30 minutes and the commercial BCA *Paenibacillus* were effective as a seed treatment protecting the seedlings from black rot disease. Furthermore, the plant extracts of *A. caulescens* (acetone, 15 mg/ml) provided control of black rot disease (*Xcc*) under greenhouse conditions. However, there is a need to further investigate the tested alternative seed treatments against *Xcc* under field conditions and to identify the phytochemicals responsible for the plant extract's antibacterial activity.

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