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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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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 min 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 min. In the greenhouse trials, acetone extracts of *A. caulescens* (15 mg/ml), *Paenibacillus* sp., and hot water at 50 °C for 30 min 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.

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

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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 h. 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 h 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 h 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 min 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 h 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 h 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*