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Abstract

The seed-borne bacterial disease black rot is caused by *Xanthomonas campestris* pv. *campestris* (*Xcc*) is one of the economically important diseases of cruciferous plants worldwide. Seed treatments are commonly referred to as control seed-borne diseases. This study objected to identifying black rot disease based on biochemical and MALDI-TOF MS and management using several seed treatments on cabbage. Seed treatments including plant essential oils (*Thymus vulgaris*, *Syzygium aromaticum*, and *Citrus sinensis*) and experimental biocontrol agents such as fluorescence *Pseudomonas* sp., *Pseudomonas chloropsis*, *Bacillus* sp., *Bacillus subtilis* selected from an in vitro assay, and hot water treatment were evaluated in separate growth chamber assays. Non-treated, *Xcc* inoculated seeds and non-treated, non-inoculated cabbage seeds (*Brassica oleracea*) served as controls. According

to *in-vitro* assay results, essential oils at several rates, and biocontrol agents were screened. Also, seeds were exposed to hot water treatment at different temperatures and durations. The result showed that seed treatment at 50 °C for 30 min significantly reduced black rot incidence (80.67%) on cabbage cotyledon leaves compared to the non-treated, inoculated control. Seed treatments with *Bacillus subtilis* and, with *Thymus vulgaris* at 62.50 µl/ml essential oil significantly decreased the number of disease seedlings (94.68% and 85.34%, respectively). The present study has shown that all applications were effective as a seed treatment protecting the seedlings from black rot disease.

Keywords: black rot, cabbage seed, hot water, antagonist, plant essential oils

Introduction

Brassicas are generally cultivated as oilseeds and vegetables take a place in an important group of vegetable crops. The annual production of about 97 million tons of Brassica vegetables worldwide describes its position and importance in food and the economy. In Turkey, production of Brassica vegetables was estimated to be 1.2 million tons (FAO, 2021). Black rot is caused by *Xanthomonas campestris* pv. *campestris* (*Xcc*) is reported as a major disease of brassicas in Asia, Europe, and North America, which significantly reduces the quality and quantity of crops in the World (Fargier et al. 2011; Lange et al. 2016; Singh et al. 2016). Last decades, bacterial disease has led to yield losses of around 23% to 60% in Canada, India, and Russia. Also, the disease incidence was reported as 10-80% on the Worldwide (CABI 2022). The pathogen bacteria that is rod-shaped, non-sporulating, and Gram-negative can penetrate the plant through the roots, wounds, leaf stomata, and hydathodes and spread into the leaf through the vascular system (Vicente and Holub 2013). The main symptoms are V-shape chlorotic, yellow lesions at the margins of the leaves with darkening of veins (Schaad and Alvarez 1993). For the characterization of the bacteria, classical methods and advanced molecular techniques are commonly referred (Schaad et al. 2001; Ruella et al. 2004).

Xcc is a seed-borne and vascular disease, transmitted by contaminated seeds (Cook et al. 1952) and also wind, insects, irrigation water, farm equipment, and workers are other important factors in pathogen dispersal (Vicente and Holub 2013). Moreover, the bacteria were reported and caused losses on certificated and hybrid Brassica seeds (van Zijl de Jong et al. 2010; Little 2021). The pathogens can survive in cruciferous weed hosts, seeds, and infected crop residues in the soil which are created main source of inoculum (Lopez et al. 1999; Gazdik et al. 2021). Therefore, using healthy propagating material such as *Xcc*-free seed can help to prevent or suppress the spreading of the pathogen and decrease yield losses.

Management of black rot is very challenging like many seed-borne diseases because it can colonize in the seed embryo or coat (Schaad and Alvarez 1993; Rodriguez et al. 2018). The pathogen has different races that have a specific reaction to Brassica genotypes. Hence, it might be difficult to develop breeding-resistant cultivars (Vicente et al. 2001; Vicente and Holub, 2013; Cruz et al., 2017). The pathogen can survive on infected plant debris for 24 months. So, crop rotation can be suggested for 3-4 years to remove the bacterial population (Köhl et al. 2011). Also, there are suggested copper-based compounds known as effective in controlling most of the plant pathogens. However, using copper-based compounds can cause several problems such as damaging soil structure and microbiome, and creating resistant strains to copper. So, it might be a side effect on the sustainability of agriculture in the future (Aktar et al. 2009; Lamichane et al. 2018).

Seed treatments are a highly common and recommended application against plant pathogens (Afza et al. 2020). General seed treatments can be listed as hot water treatments, heat application, using chemical compounds, nanoparticles, and disinfectants like hydrogen peroxide, sodium hypochlorite, biological control agents, bacteriophages and plant extracts could provide alternative methods to control black rot disease (Carisse et al. 2000; Duval and Nesmith 2000; Balogh et al. 2008; van der Wolf et al. 2008; Mishra and Arora 2012; Ghazalibiglar 2014; Mandiriza et al. 2018; Sanna et al. 2022)

It is known that biological control of using antagonistic bacteria is a promising strategy to control plant pathogens (Kloepe et al., 1999). Previous studies showed that some *Bacillus* spp., *Paenibacillus* spp., and *Pseudomonas* spp. with good antagonistic potential against black rot (Chen et al. 1995; Mguni 1996; Assis et al. 1998; Luna et al. 2002; Massomo et al. 2004; Mishra and Arora 2011; Ghazalibiglar 2014). Moreover, some non-pathogenic *Xanthomonas* sp. suggested that effective disease control was achieved (Azegami et al. 2006; Nagai et al. 2014). Any other alternative to control black rot includes the use of plant extract and hot water treatments. Some researchers reported that the extract of some plants reduced the severity of black rot (van Der Wolf et al. 2008; Szopinska et al. 2010; Pawar 2001; Mandiriza et al. 2018). Application of plant extracts for controlling plant disease in non-pollutive, cost-effective, non-hazardous, and harmonious ecological balance (Thoudam and Dutta 2014). Similarly, the effectiveness of hot water treatment in reducing bacterial disease incidence, severity, and seed-borne inoculum was demonstrated (Forsberg et al. 2002; Nega et al. 2003; Mandiriza et al. 2018; Kreitlow et al. 2022). When using hot water seed treatments, their temperatures and durations will vary depending on the particular crop. It provides an advantage to hot water seed treatment that works better (Toporek and Hudelson 2017).

The aims of presented study were aimed to achieve mainly two objectives: a) to identify the *Xcc* strains caused by black rot disease on cabbage, and b) to investigate the antagonistic effect of bacterial strains, antibacterial activity, and potential of some plant extracts, and hot water seed treatments against black rot disease on artificially inoculated cabbage seeds under *in vitro* and plant assay.

Materials and methods

Isolation and identification of pathogen and candidate biological control agents

Pathogen: The pathogens were isolated from diseased cabbage plants (*Brassica oleracea*). Twelve cabbage fields were sampled in Adana during January and March 2018. The leaf samples that typical black rot V-shaped lesions symptoms were collected. From each leaf sample, 1 to 2 cm² of leaf tissue was excised with a sterile scalpel from the margin of a lesion and placed in a drop of sterile nutrient broth (NB) for 15 min. A loopful of NB from a point with bacterial ooze was streaked on nutrient agar (NA) and incubated at 28°C for 48 h and observed for typical *Xcc* colonies. A typical colony from each isolation plate was transferred to NA. Then isolates were tested for pathogenicity on 3 to 4-week plant of cabbage cv. Yalova sarmalık. Each isolate was tested three times in the same inoculation date. Bacteria were grown on NA at 28°C for 48 h, and inoculum was prepared by suspending a small amount of growth in NB. The suspensions were adjusted to approximately 10⁸ CFU ml⁻¹. Each pot with three plants was sprayed with 10 ml of bacterial suspension. After inoculation, plants were kept in a transparent polyethylene tent for 24 h to increase the humidity. The temperature was arranged between 28°C and 30°C and a

day length of approximately 16 h. The plants were assessed for symptoms 4 weeks after inoculation (Vicente et al. 2006). According to pathogenicity test results, re-isolates were obtained.

All re-isolates of *Xcc* were characterized by the methods of Dye (1962) and Lelliot and Stead (1987). Isolates were tested for Gram reaction, catalase reaction, oxidase reaction, starch and gelatin hydrolysis, and hypersensitive reaction of tobacco leaves (HR). After the biochemical test results, all were identified with MALDI-TOF MS which is the ability to identify each microbe by its own unique protein fingerprint by matching the abundance of the biomarkers with the proteome database (Ruella et al. 2004; Soylu et al. 2020). Then, one isolate (*Xcc* 776) among all was randomly selected to be maintained for the next experiments.

Candidate biological control agents: The candidate biological control agents were collected from healthy cabbage leaves and soil in Tekirdağ. Soil and leaf samples were processed according to protocols described by Cazorla et al. (1998) and Romero et al. (2004). Then all candidate antagonist isolates were tested on tobacco leaves to observe HR. After HR test, the isolates were selected as candidate biological control agents. The effective candidate biological control agents were identified by MALDI-TOF MS.

***In vitro* screening of antibacterial activity**

Assessment of antagonistic activity

Dual-culture assay: The dual-culture assay was performed in order to screen the antagonist activity of the candidate biological control agent against *Xcc*. The candidate biological control agents were inoculated to described by three inoculated equidistant points of the NA petri plate (90-mm-diameter). After 24 h, the *Xcc* suspension was adjusted to $0.2 (10^8 \text{ CFU ml}^{-1})$ using a spectrophotometer and sprayed on the same plates. Following the inoculation, plates were incubated for 48 h 28°C . Inoculated plates with *Xcc* were used as a control. The antagonistic activity was expressed as an inhibition zone. The inhibition zones were measured by Vernier caliper and recorded (Ullah et al. 2017; Bozkurt and Soylu. 2019). The experiments were conducted twice and three replicates were prepared for each antagonist

Assessment of plant essential oils

The commercial plant essential oils used in this study were *Tymus vulgaris*, *Syzygium aromaticum*, *Citrus sinensis*, *Juniper communis*, *Salvia officinalis*, *Lavandula officinalis*, and *Allium sativum*.

Disk diffusion assay: The paper disk diffusion assay according to Mangama and Sreeramulu (1981) and Mirik and Aysan (2006) was performed to screen the antibacterial activity of plant essential oils. The *Xcc* suspension was prepared in sterile nutrient broth (NB) and adjusted to 10^8 CFU ml^{-1} ($\text{OD}_{600}:0.2$). Then 100 μl of *Xcc* suspension was streaked on NA plates. After that sterile paper disks (5-mm-diameter) were placed on the NA surface and 15 μl of essential oil was dropped on the paper disk. After 48h of incubation, the inhibition zone was measured by Vernier caliper, recorded, and considered as an indication of antibacterial activity. The experiment was repeated twice and included three replications.

Determination of minimum inhibitory concentrations (MIC's) of the plant's essential oils: MIC is defined as the lowest concentration of the antimicrobial agent that inhibits microbial growth after 24 h of incubation (Mostafa et al. 2018) This method according to Eloff (1998), Masangwa et al. (2013) and Mandiriza et al. (2018) with few

modifications was used for the antibacterial assay to determine the MIC's for each of the plant essential oils tested against *Xcc*. NA medium was autoclaved and cooled until 50°C. Different rates (5, 7.81, 15.62, 31.25, 62.50, 125, 250, 500, and 1000 µl ml⁻¹) of plant essential oils were added into NA medium and transferred on petri plates. The *Xcc* isolate was grown on NA media at 28 °C for 48 h and single colonies were transferred to sterile NB. The density of the bacterial suspension was adjusted to 0.2 (10⁸CFU ml⁻¹, OD₆₀₀:0.2) using a spectrophotometer, and 100 µl of *Xcc* suspension was streaked on plates including NA amended different rates of plant essential oil. As a control, the *Xcc* suspension was streaked on NA medium plates. After incubation at 48 h 28 °C, the bacterial colonies were counted and recorded to compare with control. Three plates were used for each rate of plant essential oil. The experiment was performed twice.

Artificial inoculation

Seeds of cabbage (cultivar Yalova sarmalık) were surface sterilized and artificially inoculated with *Xcc* suspension (10⁸ CFU ml⁻¹, OD₆₀₀:0.2) using a vacuum for 2 h. After inoculation, seeds were kept in the laminar flow cabinet overnight air drying to use seed treatments.

Seed treatments

Seed treatment with plant essential oils: Plant essential oils used for seed treatments were selected based on the results of thorough disk diffusion and MIC's assays. The cabbage seeds, which were artificially inoculated with *Xcc*, were immersed in the essential oils for 2 h with an orbital shaker (150 rpm). Inoculated, untreated seeds were presented as a positive control, and non-inoculated seeds were immersed in sterile distilled water as a negative control. The experiment was conducted with 50 seeds per treatment in three replicates and two independent trials. Then, the seeds were placed into the tray (5x5x12 cm) and kept in the climatic room at 24 °C (photoperiod of 16 h dark and 8 h light) to monitor *Xcc* presence in germinated plants.

Seed treatments with candidate biological control agents: Candidate biological agents were selected based on dual culture assay. The suspension of effective biological control agents (10⁸ CFU ml⁻¹ · OD₆₀₀:0.2) was applied by shaking (150 rpm) for 2 h with cabbage seeds. Inoculated, untreated seeds were presented as a positive control, and non-inoculated seeds were immersed in sterile distilled water as a negative control. The experiment was conducted with 50 seeds per treatment in three replicates and two independent trials. The seeds were planted in the tray (5x5x12 cm) and kept in the climatic room at 24 °C (photoperiod of 16 h dark and 8 h light) to monitor *Xcc* presence in germinated plants.

Seed treatment with hot water: Artificially infected seeds were applied hot water treatments using a thermostatically controlled in bain-marie according to Mandiriza et al (2018). The treatments were: 45 °C for 25 min, and 30 min, 50 °C for 10 min, 20 min and 30 min, 53 °C for 10 min, 20 min, and 25 min. The controls included *Xcc* inoculated seeds soaked in SDW for 30 min at room temperature, untreated inoculated seeds, and healthy seeds (each replicate of 50 seeds). After treatment, the seeds were laminar flow cabinet for 24 h. All plants are kept in the climatic room at 24 °C (photoperiod of 16 h dark and 8 h light)

Data analysis

All the results were statistically analyzed by SPSS statistical software with Duncan multiple range. Differences in mean values were considered significant when $P < 0.05$.

Results

Isolation and identification of pathogens and candidate biological control agents

The isolates produced yellow mucoid growth on NA and 19 isolates produced V-shaped lesions on the leaves of *B. oleracea*. According to biochemical test results, all 19 re-isolates were Gram (-), they were positive for catalase and oxidase reaction, starch, and gelatin hydrolysis, and HR on tobacco leaves. They also were identified as *Xcc* by MALDI-TOF MS. So, they were considered to be *Xcc*. Isolate *Xcc* 776 was randomly selected among them, and seed treatments were conducted with *Xcc* 776.

For candidate biological control agents, 57 isolates were given negative results HR test on tobacco leaves. They were considered to be non-pathogen. All isolates used dual culture tests to determine their antagonistic ability against *Xcc*.

In vitro screening of antibacterial activity

The results of the disk diffusion assay indicated that *J. communis*, *S. officinalis*, *L. officinalis*, and *A. sativum* did not suppress pathogen growth. Therefore, these plant essential oils did not have any impact on *Xcc*. Moreover, *T. vulgaris*, *S. aromaticum*, and *C. sinensis* essential oils produced an inhibition zone range of 10.42 ± 2.52 , 4.33 ± 0.79 , and 3.31 ± 0.72 mm respectively. These essentials were considered to continue in the following studies.

The MIC studies were conducted by using various rates of *T. vulgaris*, *S. aromaticum*, and *C. sinensis* like 5, 7.81, 15.62, 31.25, 62.50, 125, 250, 500 ve 1000 μ l/ml. It was shown that the growth inhibition was observed to significantly rate 62.50 μ l/ml of *T. vulgaris* essential oil. The rate of *T. vulgaris* essential oil was inhibited *Xcc*. However, the following rates 125 μ l/ml and 500 μ l/ml of *S. aromaticum* and *C. sinensis* were inhibited *Xcc in vitro* conditions. So these effective doses were applied to seed treatments with essential oils.

Among the 57 bacterial isolates that were tested for dual culture plate assay, 5 isolates were found to be antagonistic to *Xcc* on NA (Table 1). Those 5 isolates called MM64, MM100, MM103, MM104, and MM112 were identified *Pseudomonas chloropsis*, *Bacillus* sp., *Bacillus subtilis* (both MM103 and MM104), and *Pseudomonas* sp. respectively using MALDI-TOF MS. According to the dual culture assay, the isolate MM103 (*B. subtilis*) showed maximum suppression on *Xcc*. Its antagonistic effect was measured as 166 mm (Table 1). The isolates MM104 (also *B. subtilis*), MM64 (*P. chloropsis*), MM112 (*Pseudomonas* sp.), and MM100 (*Bacillus* sp.) have followed with the antagonistic effect of 132 mm, 84 mm, 41 mm, and 30 mm respectively.

Table 1. Determination of antagonistic activity on *Xcc*

Isolates	Growth of antibacterial strains (mm)	Inhibition zone (mm)	Antagonistic effect (mm)
MM64 (<i>Pseudomonas chloropsis</i>)	86	73	0.84
MM100 (<i>Bacillus</i> sp.)	100	30	0.30
MM103 (<i>Bacillus subtilis</i>)	50	83	1.66
MM104 (<i>Bacillus subtilis</i>)	83	110	1.32
MM112 (<i>Pseudomonas</i> sp.)	120	50	0.41

217

218 **Seed treatments**

219 **Seed treatment with plant essential oil:** According to MIC results, the effective plant essential oils on *Xcc* were
 220 determined as *C. sinensis* (250, 500, and 1000 µl/ml), *S. aromaticum* (62.50, 125, and 250 µl/ml), and *T. vulgaris*
 221 (31.25, 62.50, and 125 µl/ml). The plant essential oils significantly reduce the percentage of diseased seedlings.
 222 However, the percentage of germinating seed increased when compared to the untreated inoculated control (Table
 223 2). At 250 µl/ml of *S. aromaticum* and 31.25 µl/ml of *T. vulgaris* recorded the highest germination percentage
 224 (80.66%). The 125 µl/ml of *T. vulgaris* caused a decrease in the percentage of diseased seedlings (10.66 %). On
 225 the other hand, the percentage of germinating seeds reduced when compared to all treatments. When evaluated
 226 both percentages of germinating seed (77.33%) and diseased seedlings (14.66 %) 62.50 µl/ml of *T. vulgaris* were
 227 found effective treatment against *Xcc*. Also, seed treatment with *T. vulgaris* at 62.50 µl/ml plant essential oil
 228 decreased black rot incidence (85.34%) on cabbage cotyledon leaves compared to non-treated inoculated control
 229 (Table 2).

230 **Table 2.** Effect of seed treatments with plant essential oils on cabbage seed germination and percentage of
 231 seedlings infected by *Xcc*

Treatments	Germination (%)	Diseased seedlings (%)
<i>Citrus sinensis</i> (250 µl/ml)	73.00 abc*	39.33 b
<i>Citrus sinensis</i> (500 µl/ml)	73.00 abc	26.66 d
<i>Citrus sinensis</i> (1000 µl/ml)	67.33 c	24.00 d
<i>Syzygium aromaticum</i> (62.50 µl/ml)	73.00 abc	34.66 bc
<i>Syzygium aromaticum</i> (125 µl/ml)	74.00 abc	28.00 cd
<i>Syzygium aromaticum</i> (250 µl/ml)	80.66 ab	26.66 d
<i>Thymus vulgaris</i> (31.25 µl/ml)	80.66 ab	22.66 d
<i>Thymus vulgaris</i> (62.50 µl/ml)	77.33 abc	14.66 e
<i>Thymus vulgaris</i> (125 µl/ml)	71.33 bc	10.66 e
Untreated inoculated control	84.66 a	61.33 a
Untreated control	84.66 a	3.33 f
<i>P</i>	<0.05	<0.05

232 *Values within columns followed by the same letter are not significantly different from each other according to
 233 SPSS statistical software with Duncan's multiple range.

234 **Seed treatments with candidate biological control agents:** Seed treatments with the antagonists, *P. chloropsis*
 235 (MM64), *Bacillus* sp. (MM100), *Bacillus subtilis* (MM103-MM104), and *Pseudomonas* sp. (MM112) enhanced
 236 germination of seeds (Table 3). The most efficient antagonist in promoting germination was *Pseudomonas* sp at
 237 86.64%. The untreated control seeds recorded a higher percentage of germinated seeds at 86.64% such as
 238 *Pseudomonas* sp. when compared to all treatments. However, treating the seed with *B. subtilis* was obtained
 239 reducing the percentage of diseased seedlings (5.32%). So *B. subtilis* has been the most efficient antagonist
 240 compared to all treatments and also untreated inoculated control. In other words, *B. subtilis* significantly decreased

the number of disease seedlings (94.68%). Besides, all antagonist treatments were found effective on both germinations of seeds and diseased seedlings (Table 3).

Table 3. Effect of seed treatments with antagonist bacteria on cabbage seed germination and percentage of seedlings infected by *Xcc*

Treatments	Germination (%)	Diseased seedlings (%)
MM64 (<i>Pseudomonas chloropsis</i>)	69.32 b*	14.64 ab
MM100 (<i>Bacillus</i> sp.)	80.00 b	6.63 bc
MM103 (<i>Bacillus subtilis</i>)	73.32 b	5.32 bc
MM104 (<i>Bacillus subtilis</i>)	81.32 b	5.32 bc
MM112 (<i>Pseudomonas</i> sp.)	86.64 a	10.64 abc
Untreated inoculated control	54.64 b	17.32 a
Untreated control	86.64 a	2.64 c
<i>P</i>	<0.05	<0.05

*Values within columns followed by the same letter are not significantly different from each other according to SPSS statistical software with Duncan multiple range.

Seed treatment with hot water: All the hot water treatments reduced seed germination when compared to the untreated inoculated control and untreated control (Table 4). Seed immersed at 45 °C for 20 min, the percentage of seed germination was obtained as 78.66%. On the other hand, the 53 °C for 25 min, the percentage of seed germination decreased (38.66%). The treatment of 50 °C for 30 min high effective in reducing the percentage of diseased seedlings (19.33%). Following 53 °C for 25 min treatment reduced diseased seedlings (18.66%). According to the results, all treatments showed the effect of decreasing the percentage of diseased seedlings to compare the untreated inoculated control (Table 4). When to evaluated together with the germination of seeds and diseased seedlings, the most effective hot water treatment at 50 °C for 30 min significantly reduced black rot incidence (80.67%) on cabbage cotyledon leaves compared to the non-treated inoculated control.

Table 4. Effect of hot water treatment on cabbage seed germination and seedlings infected with *Xcc*

Treatments	Germination (%)	Diseased seedlings (%)
45 °C 20 min	78.66 a*	43.33 b
45 °C 30 min	70.66 b	42.00 b
50 °C 10 min	68.00 bc	30.66 c
50 °C 20 min	65.33 bc	27.33 cd
50 °C 30 min	61.33 c	19.33 de
53 °C 10 min	51.33 d	24.00 cde
53 °C 20 min	42.00 e	24.66 cde
53 °C 25 min	38.66 e	18.66 e
Untreated inoculated control	84.66 a	61.33 a
Untreated control	84.66 a	3.33 f
<i>P</i>	<0.05	<0.05

*Values within columns followed by the same letter are not significantly different from each other according to SPSS statistical software with Duncan multiple range.

Discussion

Brassicas are economically important vegetable crops based on their richness in nutritional composition and oil production in the world. Black rot caused by the plant-pathogenic bacterium *Xcc* is one of the most important bacterial diseases that cause yield and economic losses of crucifers because of its epidemiology (Popovic et al. 2013). Since young plants are raised from infected seeds, cotyledons show dark discoloration in the margins which later turn black, shrivel, and drop off (Maroni and Germani 2014). In the leaves, blackened vascular tissues, and V-shaped chlorotic to necrotic lesions along the leaf margins are characteristic symptoms (Alvarez 2000). In the presented study, the cabbage plants that showed the symptoms were collected and isolated. The isolates that were used in this study were characterized by the methods of Dye (1962) and Lelliot and Stead (1987). The isolates were grown small and yellow on NA. The tests were done to identify such as pathogenicity, Gram-stained, oxidase and catalase reaction, and starch hydrolysis. Also, all isolates were Gram-negative, catalase-positive, oxidase-negative, and hydrolyzed starch. Our results showed similarity with the literature description for *Xcc* given by Ozturk et al. (2019). Conventional methods are still common to identify bacteria. However, recent new tools such as MALDI-TOF MS technique have an advantage for identification based on the short duration time of identification (Weis et al. 2020). In the presented study, the bacterial isolates were analyzed related to fingerprinting analyses of ribosomal proteins using MALDI-TOF MS. According to the results, the isolates from cabbage were identified as *Xcc* by pathogenicity, biochemical tests, and also MALDI-TOF MS analysis.

The evaluation of different seed treatments was performed such as hot water, plant essential oils, and biocontrol agents under controlled conditions to observe whether the seed treatments suppress disease symptoms from plants while raising from artificially inoculated seed. Generally, essential oils are known for their microbial capability and have the potential to control plant disease caused by bacteria (Guenther 1948; Mourey and Carillac 2002; van der Wolf et al. 2008). The applications of essential oils inhibited the growth of infected seedlings. The most promising results were obtained for *T. vulgaris* plant essential oil treatment. It has provided a decrease in infected seedlings by around 89.44%. In particular, thyme essential oils have more ability to suppress *Xanthomonas* species (Cyorgyi et al. 2004). Previous studies indicated that thymol, which is one of the major components of essential oils of *T. vulgaris* showed bactericidal effects on *Xcc* (Amini et al. 2018; da Silva et al. 2019; Hakalova et al. 2022). Also, Nguetack et al. (2005) obtained, *T. vulgaris* essential oil applied significantly reduced the seed infection by 32% on *Xanthomonas* sp. Like previous findings, the presented study observed that the inoculated seed treated with essential oils might help suppress black rot disease in cabbage.

Biological control of plant pathogens using antagonistic bacteria is another approach to sustainable disease management (Nelson 2004). Biological control agents and their products have been shown to be efficient in the control of plant bacterial diseases caused by *Xanthomonas* species (Mirik and Aysan 2005; Lanna Filho et al. 2010; Murate et al. 2015). In this present study, five out of 57 bacterial isolates were selected as they exhibited antagonistic activity against *Xcc* *in vitro* assay which are generally used for pre-selection of candidate antagonist bacteria. *Pseudomonas* sp. and *Bacillus* sp. have been selected thanks to their biocontrol activity against *Xcc*. Several studies have also demonstrated the antagonistic activity of *Bacillus* and *Pseudomonas* species against several bacterial diseases (Mirik et al. 2008; Mishra and Arora 2012; Fira et al. 2018). The plant assay pointed out

the potential biocontrol ability of antagonistic bacteria selected in the *in vitro* study. The biocontrol activity of *P. chloropsis* (MM64), *Bacillus* sp (MM100), *B. subtilis* (MM103 and MM104), and *Pseudomonas* sp (MM112) was highlighted in tomato plants. These antagonist bacteria reduced pathogen infection in the plants. In the previous report, *B. subtilis* and *P. fluorescens* displayed a suppressive effect against *Xcc* (Sing et al. 2010). Also, *P. aeruginosa* and *B. thuringiensis* provided effective control of black rot disease caused by *Xcc* (Mishra and Arora 2012). Our result has demonstrated the high antagonistic activity of *B. subtilis* (MM103 and MM104 isolate) against disease seedlings showed around 5.32%. It makes possible significant reductions in the disease severity. Similarly, treatment of rapeseeds with *Bacillus* sp. significantly reduced the *Xcc* infection seeds, decreased the number of diseased seedlings, and increased seed germination Mandiriza et al (2018). Antagonist bacteria have shown promising ability to control several plant diseases. Many known *Bacillus* and *Pseudomonas* species are the most common antagonist bacteria that exhibit remarkable effects against plant bacterial diseases (Guldogan et al. 2022; Tümen et al. 2022). Our study revealed the significant antibacterial activity of *Pseudomonas* sp. and *Bacillus* sp. against *Xcc* on cabbage and showed that they suppressed diseased seedlings and provided seed germination. Many known hot water is one of the common physical seed treatments. Previous studies have reported that hot water treatments are of increasing importance for the control of seed-borne bacterial diseases (Koch et al. 2000; Linders 2000; Permana et al. 2017). However, one of the possible hurdles of the hot water treatment is to find a reliable heating temperature and time that are effective on seed germination to achieve control of seed-borne pathogens (Ivey and Miller 2005). The potential of hot water seed treatment is referred to at 50 °C or 53 °C for 10-30 min had provided effective phytosanitary control of seed-borne diseases. Previous studies pointed out that hot water treatment has emerged as an important method to control *Xanthomonas* species. This treatment on cabbage and cauliflower seeds demonstrated a reduction in *Xcc* infections between 25 and 90% (Babadost et al. 1996). Moreover, recent studies reported that this hot water treatment was able to reduce bacterial diseases such as *Xcc* (Nega et al. 2003; Mandiriza et al. 2018). In the case of hot water treatment, another important point is without reducing seed germination (Nega et al. 2003). Hot water seed treatment can have the beneficial effect of faster seed germination as it might have cracked the hard seed coat (Baskin 2003). However, the treatments also might decrease the germination rates. The current study is indeed this point showing that the high temperature of the treatment decreased seed germination by around 71% at 53 °C 25 min. So, our study observed that hot water treatment at 50 °C for 30 min was effective in controlling the black rot of cabbage with a minimum percentage of infected seedlings.

In conclusion, control of plant bacterial diseases remains difficult due to the limited availability of effective chemicals that are not eco-friendly and the ability of many bacteria to spread. So, some alternative methods including seed treatments were focused on controlling *Xcc* in cabbage. The present study has shown that all those were effective as a seed treatment protecting the seedlings from black rot diseases. However, the biocontrol agent *B. subtilis* applied as seed treatments has the highest potential among plant essential oils and hot water treatments to control *Xcc* in cabbage. Together, these results demonstrate the potential achievement of seed-based applications suppressing or reducing pathogen infections in cabbage. Furthermore, these promising treatments mark a significant advancement in the exploration of their combinatory potential against *Xcc* in future studies.

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Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Cansu Oksel, Benian Pinar Aktepe, Mustafa Mirik and Yesim Aysan. The first draft of the manuscript was written by Cansu Oksel and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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