



Laboratory Efficacy of Essential Oils Against *Pectobacterium carotovorum* Subsp. *carotovorum* and *Pectobacterium atrosepticum* Causing Soft Rot of Potato Tubers

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Received: 31 July 2023 / Accepted: 28 May 2024 / Published online: 2 July 2024
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Abstract

The antibacterial activity of essential oils (EOs) from *Carum carvi*, *Cinnamomum zeylanicum*, *Cuminum cyminum*, *Eugenia caryophyllus*, *Foeniculum vulgare*, *Melaleuca alternifolia*, *Mentha × piperita*, *Origanum vulgare*, *Rosmarinus officinalis* and *Thymus vulgaris* was tested against *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) and *Pectobacterium atrosepticum* (*Pa*), which cause soft rot of potato tubers. In disc diffusion, minimum inhibitory concentration and minimum bactericidal concentration (MBC) tests, cinnamon EO was found to be most effective against both bacteria. The inhibition zones ranged from 20.46 to 29.58 mm for a concentration of 100 µL/mL. The minimum inhibitory concentration was 0.5 µL/mL, and MBC was between 0.5 and 5 µL/mL. The higher sensitivity of bacteria was manifested in clove (*Pcc* and *Pa*), mint (*Pcc*), oregano (*Pa*) and thyme (*Pa*) EOs. Rosemary EO was the least effective. The results of the *in vivo* test were not entirely consistent with those of the *in vitro* tests. The most significant antibacterial effect was achieved with mint EO. The treatment of potato tuber discs with mint EO at a concentration of 3 µL/mL for *Pcc* and 3–10 µL/mL for *Pa* was 100% effective. The efficacy of the essential oils of caraway (5–10 µL/mL), thyme (10 µL/mL) and oregano (5 µL/mL) also ranged from 95.7 to 99.7%. Based on the results of the *in vivo* test, it may be recommended that mint EO and potentially caraway, oregano and thyme EOs be further tested for pickling potato tubers against bacteria of the genus *Pectobacterium*.

Keywords Biological control · Disc diffusion method · Minimum inhibitory concentration · Minimum bactericidal concentration · *Solanum tuberosum*

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Introduction

The use of medicinal plants in various industries is well-known throughout history. Medicinal plants are increasingly used in integrated pest management, especially their secondary metabolites. Secondary metabolites include essential oils (EOs) (Hussain et al. 2008). Essential oils are extracted from the plant material, most commonly by steam distillation or mechanical processes from the pericarp (Dewick 2002). Essential oils are chemical signals that allow a plant to control or regulate its environment (ecological role): attracting pollinating insects, repelling predators, inhibiting seed germination or communication between plants. In addition, EOs also have an antimicrobial or insecticidal and repellent effect. Various differentiated structures produce essential oils; their number and properties are highly variable. Essential oils are found in the cytoplasm of specific plant cells located in one or more plant organs. These oils are complex mixtures that can contain more than 300 different compounds. Some compounds have significant antimicrobial effects (Sell 2006).

Pectobacterium carotovorum subsp. *carotovorum* (Jones 1901) Hauben et al. 1999 emend. Gardan et al. 2003 (*Pcc*) and *Pectobacterium atrosepticum* (van Hall 1902) Gardan et al. 2003 (*Pa*) are pectinolytic bacteria that are generally known as tissue maceration agents (Charkowski et al. 2015). These bacteria are Gram-negative and facultatively anaerobic and were previously classified as *Erwinia carotovora* subsp. *carotovora* and *Erwinia carotovora* subsp. *atroseptica* (Gardan et al. 2003). *Solanum tuberosum* L. is one of the host plants affected by these polyphagous bacteria, which cause bacterial blackleg and soft rot in potatoes. Symptoms typically manifest at the base of the potato stems, which turn black above the soil surface. Longitudinal sections of the stem base reveal blackish streaks of infection and affected plants usually die rapidly. In dry conditions, the disease develops less severely, and the basal part of the stem becomes light brown, dry and cracked. Bacteria can infect stems through vascular bundles from mother tubers. They can also spread to daughter tubers using stolons (Charkowski 2015; Pérombelon and Kelman 1987; Pérombelon 2002; Zheng and Joshi 2019). Bacteria are commonly found in lenticels and in the area of tuber wounds (Czajkowski et al. 2009; Hélias et al. 2000), which can cause tuber rot immediately in the soil, shortly after harvest, or during storage. The subsequent decomposition of tubers is usually accompanied by a strong odour (Hausvater and Doležal 2014). The bacteria can survive in contaminated and infected tubers, in crop residues in the soil, in the root system of weeds and field crops and surface water (Pérombelon and Hyman 1989; Pérombelon and Kelman 1980; Quinn et al. 1980). In particular, potato tuber quality, yield and storage life are significantly reduced; therefore, protection is very important to prevent potato tuber infection. Agricultural practices such as eradicating diseased plants, destroying crop residues and rotating crops with grasses are recommended. Chemical control is limited to using copper hydroxide and chitosan hydrochloride, which are generally less effective. (Mello et al. 2011). Due to the lack of other registered products, attention is focused on using essential oils from different plant species or their purified components (Hajian-Maleki et al. 2021).

The aim of our study was to expand the possible spectrum of essential oils from plant species that would show antibacterial effects on *Pcc* and *Pa*. At the same time, they would be promising to test pickling or control during the storage of potato tubers. The main criteria for selecting essential oils were proven antimicrobial properties, essential oils from plants grown in Europe for economic reasons or essential oils from commercially interesting plants.

Materials and Methods

Aggressiveness Evaluation of Bacterial Strains

The aggressiveness of three strains of *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) (CPPB 56, CPPB 53 and CPPB 201 from CRI Prague-Ruzyne, CZ) and three strains of *Pectobacterium atrosepticum* (*Pa*) (CPPB 81, CPPB 82 and CPPB 83 from CRI Prague-Ruzyne, CZ) was tested in order to select them for the further experiments. The experiment was inspired by the methods of Jiang et al. (2021). Bacteria were cultivated on King's B medium (HiMedia Laboratories, India) for 48 h at optimal temperatures of 28 °C for *Pcc* and 26 °C for *Pa*. Bacterial suspensions (1×10^8 CFU/mL) were prepared in Mueller Hinton Broth (MHB) (HiMedia Laboratories, India). Potato tubers of the cultivar 'Red Anna' were obtained from the breeding station VESA Velhartice a.s. (Czech Republic). Tubers were surface-disinfected with 0.7% sodium hypochlorite (NaOCl) for 10 min. Then, tubers were cut into 1-cm-diameter discs, rinsed with tap water and then air-dried at room temperature. Only discs of similar size were included in the test for comparison. The discs were placed in plastic boxes on filter paper moistened with sterile distilled water. An impression was made in the centre of each disc using a sterile metal rod, and 3.5 µL of bacterial inoculum was pipetted onto it. The boxes were lidded and incubated under optimal conditions for each bacteria growth. The test was performed on six discs from each strain in two replicates and the control of MHB. The aggressiveness of the bacterial strains was evaluated after 24, 48 and 72 h after inoculation.

Bacterial Strains

Based on the results of the aggressiveness evaluation, strain CPPB 56 *Pcc* and strain CPPB 81 *Pa* were selected for further testing. Before each experiment, 48-h cultures of the bacterial strains were used, growing on King's B medium at optimal temperatures of 28 °C for *Pcc* and 26 °C for *Pa*. The inoculum was prepared for all tests with a standard density corresponding to 0.5 McFarland (1×10^8 CFU/mL).

Essential Oils

A total of eleven EOs were selected (Table 1). Mint (Mi-EO), oregano (Or-EO), thyme (Th-EO) and rosemary (Ro-EO) belong to the *Lamiaceae* family. Caraway (Ca-EO), cumin (Cu-EO) and fennel (Fe-EO) belong to the *Apiaceae* family. Clove

Table 1 List of the essential oils and content of selected components from GC–MS analysis

Essential oil ^a	INCI name	Content of selected components from GC/MS analysis (%) ^b
Cinnamon (Ci-EO)	<i>Cinnamomum cassia</i> leaf oil	Cinnamaldehyde (cis + trans) — 90.0 Eugenol — 0.5
Cinnamon 2 (Ci2-EO)	<i>Cinnamomum zeylanicum</i> bark oil	Cinnamaldehyde — 71.0
Clove (Cl-EO)	<i>Eugenia caryophyllus</i> leaf oil	Eugenol — 80.6
Tea tree (Tt-EO)	<i>Melaleuca alternifolia</i> leaf oil	α -pinen — 2.2 Terpinen-4-ol — 39.0
Caraway (Ca-EO)	<i>Carum carvi</i> oil	D-carvone — 55.3 Limonene — 43.3
Cumin (Cu-EO)	<i>Cuminum cyminum</i> fruit oil	Cuminaldehyde — 32.5 β -pinen — 12.3
Fennel (Fe-EO)	<i>Foeniculum vulgare</i> dulce fruit oil	Trans-anethole — 84.3 α -pinen — 1.1
Mint (Mi-EO)	<i>Mentha piperita</i> oil	Menthone — 26.1 Menthol — 45.2
Oregano (Or-EO)	<i>Origanum vulgare</i> oil	Carvacrol — 83.1 Thymol — 1.15
Thyme (Th-EO)	<i>Thymus vulgaris</i> oil	Thymol — 50.2 Carvacrol — 3.0
Rosemary (Ro-EO)	<i>Rosmarinus officinalis</i> leaf oil	α -pinen — 12.5 Eucalyptol — 45.3

Many essential oil components showed to have antibacterial effects, e.g. carvacrol (Eftekhari et al. 2016), cinnamaldehyde (Chahbi et al. 2020), D-carvone (Chan et al. 2016), L-menthone (Zaia et al. 2016) and thymol (Eftekhari et al. 2016)

^aThe name of the essential oil was followed by the abbreviation of its name used in the text

^bThe content of components was taken from the GC/MS analysis protocols from the supplier companies — M + H Míča&Harašta s.r.o. and Florihana Distillerie

(Cl-EO) and tea tree (Tt-EO) belong to the *Myrtaceae* family. For cinnamon, two variants of *Cinnamomum cassia* leaf oil (Ci-EO) and *Cinnamomum zeylanicum* bark oil (Ci2-EO) were tested in order to compare their efficiency. The essential oil from *Cinnamomum cassia* is used in our laboratory as a positive control.

All EOs in 100% quality were supplied by M + H Míča&Harašta s.r.o. (Czech Republic) or purchased from Florihana Distillerie (France).

Analysis of Essential Oils by GC–MS

The GC/MS analyses were carried out using a Thermo Trace GC Ultra equipped with a TriPlus autosampler, coupled with an ion-trap Polaris Q mass spectrometer (EI mode at 70 eV). The chromatographic separation was performed on the DB-5 capillary column (30 m×0.25 mm i.d., film thickness 0.25 μ m); the following temperature program was used: 45 °C held for 2 min, then increased to 250 °C at a rate of 10 °C/min, then increased to 300 °C at a rate of 30 °C/min and finally held at 300 °C for 2 min. The carrier gas was helium with a flow rate of 0.8 mL/min.

The sample (1 μL) dissolved in n-hexane was injected according to a splitless mode. The mass scan range (m/z) was 50–650 amu, data acquired at full scan mode with solvent delay for 4.7 min. Both the injector and the transfer line temperatures were set at 225 °C and ion source at 220 °C. The data were analyzed using Xcalibur 2.2, with identification of the individual components performed by comparison with co-injected pure standards and by matching the MS fragmentation patterns and retention indices with the built-in libraries or literature data (Adams) or commercial mass spectral libraries (NIST MS Search 2.0 library).

Disc Diffusion Method

The standard agar disc diffusion method (DDM) was used for the antibacterial assay. The methodology was based on EUCAST (2021) and partially modified.

The EOs (Ca-EO, Ci2-EO, Cl-EO, Cu-EO, Fe-EO, Mi-EO, Or-EO, Ro-EO, Th-EO and Tt-EO) were diluted with 96% ethanol to a primary concentration of 100 $\mu\text{g/mL}$ (10% solution). Additional concentrations (75, 50, 25, 10 and 5 $\mu\text{L/mL}$) were prepared from the stock dilution by adding sterile distilled water. Several drops of Tween 20 (0.01% [v/v]) (Merck KGaA, Germany) were added to improve homogeneity.

One hundred microliters of standardized inoculum was pipetted onto Petri dishes containing Mueller Hinton agar (MHA) (HiMedia Lab., India) and uniformly spread with a sterile hockey stick rod. The inoculum was allowed to dry. Four sterile filter paper discs (6 mm diameter), which were evenly spaced in a square on the Petri dish, were each filled with 7.5 μL of EO solution of the given concentration. The plates were incubated in a thermostat at the optimal temperature for bacterial growth, and after 24 h, the diameters of the inhibition zone (IZ) were measured. Test was done in triplicate, with bacterial growth control (negative control), ethanol and the Ci-EO (positive control).

Bacterial sensitivity to the different essential oils was evaluated according to Ponce et al. (2003). The diameter of the inhibition zone < 8 mm was considered insensitive, diameters 9–14 mm as sensitive, diameters 15–19 mm as very sensitive and diameters > 20 mm as extremely sensitive.

Determination of Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) were determined according to the modified procedure of Hajian-Maleki et al. (2021) and EUCAST (2003).

The EO solutions (Ca-EO, Ci2-EO, Cl-EO, Cu-EO, Fe-EO, Mi-EO, Or-EO, Ro-EO, Th-EO and Tt-EO) were prepared $1.1 \times$ more concentrated due to the subsequent addition of bacterial inoculum. EOs were diluted with 96% ethanol to prepare a 100- $\mu\text{L/mL}$ stock concentration. Other concentrations (75, 50, 25, 10, 5, 3, 2.5, 1.5 and 0.5 $\mu\text{L/mL}$) were prepared from the stock dilution by adding MHB. Several

drops of Tween 20 (0.01% [v/v]) (Merck KGaA, Germany) were added to improve homogeneity.

Due to the volatility of the EO components, 135 μL of EO solution indicated concentrations was added to 0.2 mL microtubes, followed by 15 μL of the inoculum. The microtubes were incubated for 24 h at the optimal temperature for bacterial growth with shaking at 100 rpm. Testing was performed in four replicates, with bacterial growth control (negative control), the Ci-EO solutions (positive controls), medium purity control and EO purity control at a particular concentration without inoculum.

To the remaining volume of each sample, 15 μL of 0.01% resazurin (Merck KGaA, Germany) indicator solution was added. The following methodology was developed by Mann and Markham (1998) and slightly modified. The redox dye resazurin sodium salt has been successfully used to evaluate the antibacterial activity of plant extracts and standard drugs based on the colour change in the solution as an indicator of living bacterial cells. Microtubes were incubated for another 2 h with shaking at 120 rpm. The samples were visually inspected for colour change. The mauve and pink colour of the tested sample indicated the presence of live bacteria, while the blue colour indicated the presence of dead bacteria. The highest remaining dilution in blue indicated the MIC.

Cells from the microtubes showing no growth (10 μL of solution) were subcultured on MHA plates to determine whether the inhibition was reversible or permanent. Incubation continued for an additional 24 h. The MBC was determined as the highest dilution (the lowest concentration), at which no growth occurred on the plates.

Efficacy of Essential Oils in Reducing Soft Rot on Potato Tuber Discs

The soft rot inoculation assay was conducted to investigate the influence of EOs on reducing the development of tuber tissue maceration. Based on the MIC and MBC assay, the Ca-EO, Mi-EO, Or-EO and Th-EO were selected. The methodology was adapted from Jiang et al. (2021). Potato tubers of the cultivar ‘Red Anna’ were calibrated to a similar size of about 30×38 mm. Tubers and discs were prepared for testing in the same way as for the *Aggressiveness evaluation of bacterial strains*. Only discs of similar size were included in the test for comparison. Bacterial suspensions and EO concentration range ($\frac{1}{4}$ MIC, MIC and MBC) were diluted in MHB. Due to the addition of the bacterial suspension, the EO solutions were 1.1× more concentrated. Samples were prepared by mixing 135 μL of EO solution and 15 μL of bacterial inoculum in a microtube and then shaken at 150 rpm for 1 h and at the optimal temperature for bacterial growth. The discs were placed in plastic boxes on filter paper moistened with sterile distilled water. An impression was made in the centre of each disc with a sterile metal rod, and 3.5 μL of the mixed sample was pipetted onto it. The boxes were lidded and cultivated under optimal conditions for each bacteria’s growth. The test was performed on six discs of each variant in two replicates. The positive control was Ci-EO, and the negative control was bacterial inoculum. At 24, 48 and 72 h after inoculation, the size of the rot patch was measured longitudinally and transversely. The rotting area

was calculated. Disease level over time was assessed using the area under the disease progression curve (*AUDPC*) according to the following formula, as described by Maden et al. (2007).

$$AUDPC = \sum_{i=1} \left(\frac{y_i + y_{i+1}}{2} \right) \times (t_{i+1} - t_i)$$

in which $y_i + y_{i+1}$ — values of the detected infestation in the given term and $t_{i+1} - t_i$ — t_i is the time interval between two assessments in days.

The efficacy of EO on the bacterial strains was evaluated as the percentage difference between the average rotting area of the negative control and the average rotting area of the essential oil treatment.

Statistical Analysis

Statistical analyses were performed using TIBCO Statistica® version 14.0.0.15 (USA). Data from the disc diffusion assay were analysed using factorial analysis of variance (ANOVA) and the other tests using one-way ANOVA. The significance between the mean values was determined at $P \leq 0.05$ using Tukey's HSD test.

Results

Aggressiveness Evaluation of Bacterial Strains

Pectobacterium carotovorum subsp. *carotovorum*

Bacterial strains were evaluated 24, 48 and 72 h after inoculation. Lesion diameters were measured, and values were converted to disease spot area. Symptoms caused by all strains were measurable as early as 24 h after inoculation (Fig. 1). The largest disease spot areas were measured in strain CPPB 56 at all time intervals. This strain was selected for subsequent in vitro and in vivo evaluation of the EO efficacy.

Pectobacterium atrosepticum

Symptoms caused by the strains CPPB 81, CPPB 82 and CPPB 83 were measurable already after 24 h (Fig. 2). In strain CPPB 83, the rate of rotting was low and gradual compared to other strains. The most significant disease spot area was measured in strain CPPB 81 in all time intervals. This strain was selected for subsequent in vitro and in vivo evaluations.

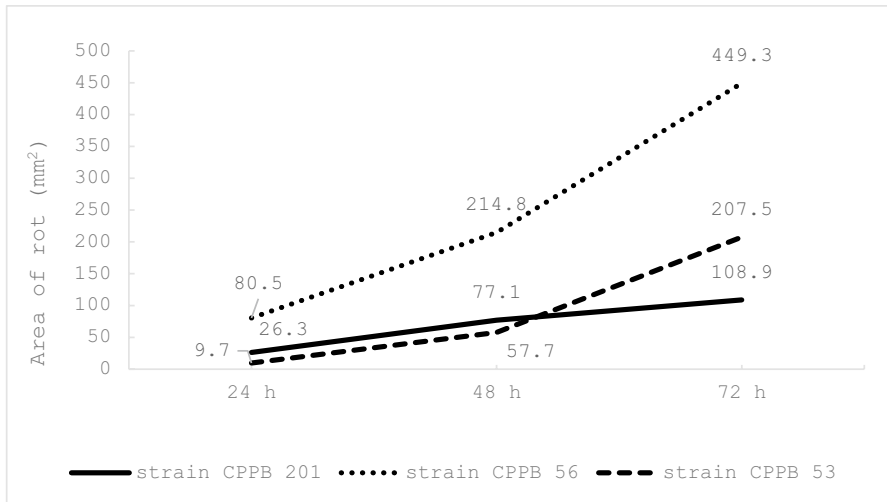


Fig. 1 Aggressiveness evaluation of bacterial strains *Pectobacterium carotovorum* subsp. *carotovorum* over time. Evaluation was carried out 24, 48 and 72 h after inoculation

Disc Diffusion Method, Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

Pectobacterium carotovorum subsp. *carotovorum*

The Ci2-EO was the most effective at concentrations of 100, 75 and 50 $\mu\text{L/mL}$ (Table 2). The values of IZ diameters were similar to the positive control Ci-EO. The 100- $\mu\text{L/mL}$ concentrations of Or-EO, Mi-EO and Cu-EO were comparable to the 75- and 50- $\mu\text{L/mL}$ concentrations of Ci-EO (positive control). The Th-EO, Ro-EO, Ca-EO and Fe-EO were least effective at the highest concentration. At this concentration, Ca-EO and Fe-EO were comparable to the 25 $\mu\text{L/mL}$ concentrations of Ci2-EO, Th-EO, Or-EO and Ci-EO (positive control). In the case of Th-EO, there were no significant differences between individual concentrations; its effectiveness did not decrease with decreasing concentration. The effect of ethanol, which was used for the initial dilution of the essential oil, was also tested. At the concentration corresponding to a 100- $\mu\text{L/mL}$ sample, the average IZ for ethanol was measured to be 7.19 mm, and at subsequent concentrations, it was equal to 6 mm.

The strain CPPB 56 of *Pcc* was extremely sensitive to Ci2-EO (IZ 20.46 mm) at a concentration of 100 $\mu\text{L/mL}$. It was still very sensitive at concentrations of 75 and 50 $\mu\text{L/mL}$ of this EO. *Pcc* was also very sensitive to Or-EO (IZ 15.0 mm) at a concentration of 100 $\mu\text{L/mL}$. In other EOs at concentrations of 100 and 75 $\mu\text{L/mL}$,

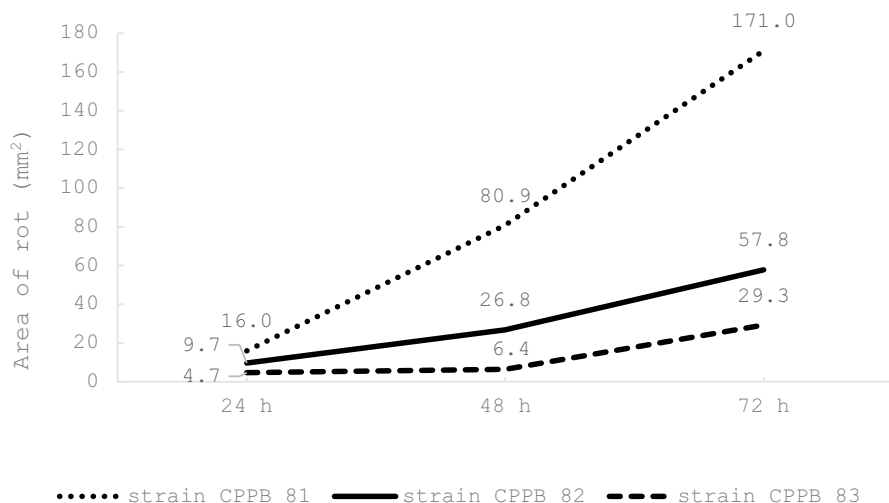


Fig. 2 Aggressiveness evaluation of bacterial strains *Pectobacterium atrosepticum* over time. Evaluation was carried out 24, 48 and 72 h after inoculation

Table 2 Disc diffusion method, minimum inhibitory concentration and minimum bactericidal concentration for *Pectobacterium carotovorum* subsp. *carotovorum*

Essential oil	Average of inhibition zones (mm) ^{1,2}				MIC (μL/mL)	MBC (μL/mL)
	Concentration (μL/mL)					
	100	75	50	25		
Cinnamon 2	20.46 ^{ab}	17.54 ^{bc}	15.58 ^{cd}	9.54 ^{hijklmno}	<0.5	5
Clove	12.83 ^{defg}	12.08 ^{efghij}	9.08 ^{ijklmno}	7.88 ^{mno}	1.5	2.5
Tea tree	12.71 ^{defg}	11.29 ^{fghijkl}	8.54 ^{lmnop}	7.63 ^{no}	3	5
Caraway	11.25 ^{fghijkl}	9.96 ^{ghijklmno}	7.50 ^{no}	7.13 ^{op}	5	10
Cumin	13.38 ^{def}	11.46 ^{fghijkl}	7.67 ^{mno}	6.96 ^{op}	5	5
Fennel	10.71 ^{fghijkl}	10.33 ^{fghijklm}	7.38 ^{no}	7.33 ^{no}	3	10
Mint	14.79 ^{cde}	12.42 ^{efgh}	11.21 ^{fghijkl}	6.00 ^p	2.5	3
Oregano	15.00 ^{cde}	12.67 ^{defg}	11.67 ^{fghijk}	8.50 ^{klmno}	5	5
Thyme	12.33 ^{efghi}	11.25 ^{fghijkl}	10.21 ^{ghijklmn}	9.50 ^{hijklmno}	3	10
Rosemary	11.96 ^{efghij}	10.25 ^{ghijklmn}	8.65 ^{klmnop}	6.00 ^p	5	50
Cinnamon ³	20.96 ^a	17.00 ^c	16.50 ^c	9.29 ^{ijklmno}	<0.5	2.5

MIC minimum inhibitory concentration, MBC minimum bactericidal concentration

¹The average of inhibition zones followed by different superscripts alphabets (a–p) showed a significant difference ($P \leq 0.05$) based on Tukey's HSD test. Testing was done in triplicate and included a bacterial growth control (discs with sterile distilled water — negative control), ethanol and positive control of cinnamon essential oil

²Inhibition diameter was given in millimeters, including the diameter of disc of 6 mm

³Cinnamon essential oil was taken as a positive control

as well as in Ci2-EO and Th-EO at concentrations of 25 $\mu\text{L/mL}$, only sensitive *Pcc* reaction was detected.

During the MIC determination, it was found that Ci2-EO ($<0.5 \mu\text{L/mL}$) exhibited the best antibacterial activity, with a MIC value identical to that of the positive control Ci-EO. However, in a surprising turn of events, the MBC assay revealed that Ci2-EO (5 $\mu\text{L/mL}$) performed less effectively than Cl-EO (2.5 $\mu\text{L/mL}$) and Mi-EO (3 $\mu\text{L/mL}$). The Or-EO and Cu-EO (5 $\mu\text{L/mL}$) had identical MIC and MBC values, indicating their comparable performance. The Ro-EO was the least effective (Online Resource Fig. S2).

Pectobacterium atrosepticum

The Ci2-EO was the most effective at concentrations of 100, 75 and 50 $\mu\text{L/mL}$; however, there were significant differences among them (Table 3). Inhibition zone values were similar to those of the positive control Ci-EO. A larger inhibition zone was also found with Or-EO at a concentration of 100 $\mu\text{L/mL}$ (16.58 mm), but when compared with Th-EO, Cl-EO at 100 $\mu\text{L/mL}$ and Or-EO at 75 $\mu\text{L/mL}$, no significant differences were found among them. The 25- $\mu\text{L/mL}$ concentration of Ci2-EO was comparable to the efficacy of Cl-EO, Cu-EO, Mi-EO and Ro-EO at 100 $\mu\text{L/mL}$ and Or-EO at 75 $\mu\text{L/mL}$. An average IZ value of 7.23 mm was measured for

Table 3 Disc diffusion method, minimum inhibitory concentration and minimum bactericidal concentration for *Pectobacterium atrosepticum*

Essential oil	Average of inhibition zones (mm) ^{1,2}				MIC (μL/mL)	MBC (μL/mL)
	Concentration (μL/mL)					
	100	75	50	25		
Cinnamon 2	29.58 ^a	26.08 ^b	22.71 ^c	12.42 ^{fghij}	<0.5	<0.5
Clove	14.79 ^{def}	12.75 ^{efghi}	10.13 ^{ijklmno}	7.54 ^{pqrstu}	1.5	3
Tea tree	10.50 ^{ghijklmn}	9.42 ^{klmnopqr}	7.63 ^{opqrstu}	6.71 ^{stu}	3	5
Caraway	10.25 ^{hijklmno}	8.71 ^{mnopqrst}	6.79 ^{qrstu}	6.00 ^u	2.5	10
Cumin	10.83 ^{ghijklm}	9.46 ^{klmnopq}	8.83 ^{mnopqrst}	7.71 ^{opqrstu}	2.5	10
Fennel	10.08 ^{ijklmnop}	9.29 ^{klmnopqrs}	8.92 ^{klmnopqrst}	6.75 ^{rstu}	3	10
Mint	11.67 ^{ghijk}	11.54 ^{ghijkl}	8.63 ^{mnopqrstu}	8.13 ^{nopqrstu}	3	10
Oregano	16.58 ^d	14.75 ^{def}	11.04 ^{ghijklm}	8.48 ^{mnopqrstu}	2.5	5
Thyme	15.33 ^{de}	11.67 ^{ghijk}	9.75 ^{ijklmnop}	6.96 ^{qrstu}	5	10
Rosemary	12.92 ^{efgh}	11.71 ^{ghijk}	8.00 ^{nopqrstu}	6.42 ^{tu}	5	25
Cinnamon ³	27.17 ^{ab}	25.27 ^b	22.67 ^c	13.08 ^{efg}	<0.5	<0.5

MIC minimum inhibitory concentration, MBC minimum bactericidal concentration

¹The average of inhibition zones followed by different superscripts alphabets (a–u) presented a significant difference ($P \leq 0.05$) based on Tukey's HSD test. Testing was done in triplicate and included a bacterial growth control (discs with sterile distilled water — negative control), ethanol and positive control of cinnamon essential oil

²Inhibition diameter was given in mm, including diameter of disc of 6 mm

³Cinnamon essential oil was taken as a positive control

ethanol at a concentration corresponding to 100 $\mu\text{L/mL}$ sample and 6 mm for other concentrations.

The strain CPPB 81 of *Pa* was extremely sensitive to Ci2-EO (IZ 29.58–22.71 mm) at 100, 75 and 50 $\mu\text{L/mL}$ concentrations. *Pa* was also very sensitive to Or-EO (IZ 16.58 mm) and Th-EO (15.33 mm) at a concentration of 100 $\mu\text{L/mL}$. Only sensitive *Pa* reaction was detected with other EOs at concentrations of 100 and 75 $\mu\text{L/mL}$, as well as with Ci2-EO at a concentration of 25 $\mu\text{L/mL}$. The exception was Ca-EO at 75 $\mu\text{L/mL}$ with an IZ of less than 9 mm.

In MIC and MBC determinations, Ci2-EO (MIC and MBC < 0.5 $\mu\text{L/mL}$) showed the best antibacterial activity. These values were the same as the positive control Ci-EO. The Cl-EO belonged to more significantly effective EOs. Pairs of Ca-EO with Cu-EO and Mi-EO with Fe-EO had identical MIC and MBC results. The Ro-EO was the least effective.

Efficacy of Essential Oils in Reducing Soft Rot on Potato Tuber Discs

Pectobacterium carotovorum subsp. *carotovorum*

The AUDPC parameter was used to compare changes in efficacy over time (Fig. 3). The AUDPC values were significantly higher for the $\frac{1}{4}$ MIC of essential oils and also for the treatment with thyme essential oil at MIC.

After 24 h from inoculation (Table 4), no disease spot area was recorded for the samples of Ca-EO both at MIC and at MBC and Mi-EO at MIC and at MBC. The

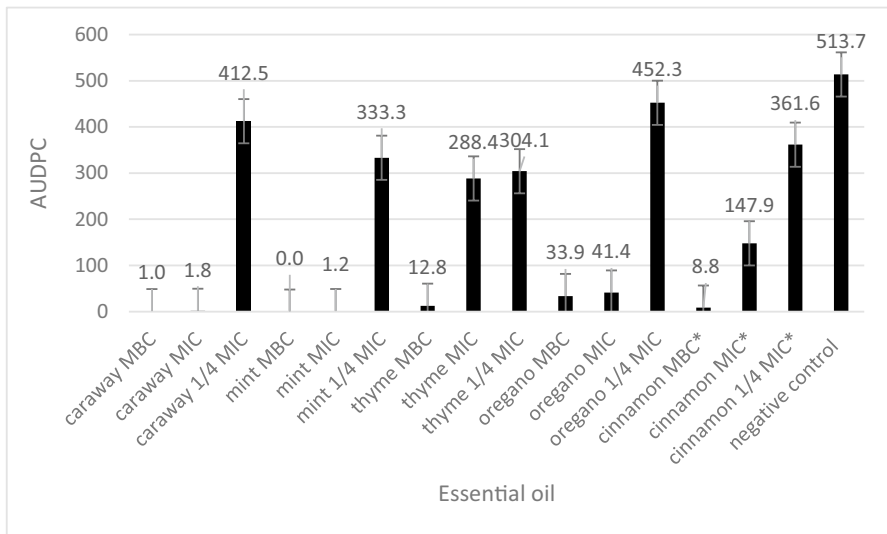


Fig. 3 Area under disease progress area (AUDPC) of mean rotting area caused by *Pectobacterium carotovorum* subsp. *carotovorum* after the treatment with essential oils. The evaluations were carried out 1, 2 and 3 days after inoculation. *Positive control. Negative control was *Pectobacterium carotovorum* subsp. *carotovorum*

Table 4 Potato tuber discs maceration assay for *Pectobacterium carotovorum* subsp. *carotovorum*

Essential oil	Mean of rotting area (mm ²) ¹		
	24 h	48 h	72 h
Caraway MBC	0.0 ^a	0.0 ^a	2.1 ^a
Caraway MIC	0.0 ^a	0.6 ^a	2.4 ^a
Caraway ¼ MIC	60.6 ^b	164.5 ^{cd}	435.3 ^{ef}
Mint MBC	0.0 ^a	0.0 ^a	0.0 ^a
Mint MIC	0.0 ^a	0.8 ^a	0.8 ^a
Mint ¼ MIC	57.8 ^b	135.8 ^c	337.2 ^{de}
Thyme MBC	5.0 ^a	6.7 ^a	7.1 ^a
Thyme MIC	42.3 ^b	147.5 ^{cd}	239.5 ^{bc}
Thyme ¼ MIC	51.9 ^b	150.9 ^{cd}	254.6 ^{cd}
Oregano MBC	16.5 ^a	16.5 ^a	18.3 ^a
Oregano MIC	17.9 ^a	21.5 ^a	21.9 ^a
Oregano ¼ MIC	91.2 ^c	157.5 ^{cd}	498.4 ^f
Cinnamon MBC ²	0.0 ^a	5.0 ^a	7.6 ^a
Cinnamon MIC ²	16.1 ^a	64.4 ^b	151.0 ^{bc}
Cinnamon ¼ MIC ²	48.1 ^b	148.4 ^{cd}	378.2 ^{def}
Negative control ³	98.5 ^c	211.6 ^d	505.7 ^f

Online Resource Fig. S3 — inoculum; Fig. S4 — cinnamon essential oil at MBC; Fig. S5 — cinnamon essential oil at MIC; Fig. S6 — cinnamon essential oil at ¼ MIC; Fig. S7 mint essential oil at MBC; Fig. S8 — mint essential oil at MIC; Fig. S9 — mint essential oil at ¼ MIC

MBC minimum bactericidal concentration, MIC minimum inhibitory concentration, ¼ MIC ¼ minimum inhibitory concentration

¹Means of rotting area followed by different superscripts (a–c) for evaluation after 24 h, (a–d) for evaluation after 48 h and (a–f) for evaluation after 72 h after inoculation indicate significant differences ($P \leq 0.05$) based on Tukey's HSD test. The superscripts apply only for a given column, i.e. the assessment after a given time point

²Positive control

³*Pectobacterium carotovorum* subsp. *carotovorum*

results were comparable to or better than the Ci-EO positive control. For Or-EO, almost similar values of disease spot area were calculated at MBC (16.5 mm²) and MIC (17.9 mm²) concentrations, which was because that these concentrations were identical (5 µL/mL). All treatments had a lower rot area value than the negative control.

After 48 h of inoculation, a significant increase in the rotting area was observed, which was due to the growth and multiplication of bacteria (Table 4). However, Ca-EO at MBC and Mi-EO at MBC were still symptom-free. Lower rotting area values were already measurable for Ca-EO at MIC and Mi-EO at MIC. The value of Or-EO at MBC was unchanged from the previous measurement. Thyme at the MIC

was found to have the highest rotting area of all the MIC concentrations of EOs, and this value was comparable to that of the negative control.

After 72 h from inoculation (Table 4), Mi-EO at MBC was also the only essential oil that maintained 100% control of *Pcc* even after 72 h of inoculation. Of all the MICs, the largest rotting areas were found in the thyme and cinnamon treatments.

The efficacy of essential oil treatment after 72 h after inoculation ranged from 52.6 to 100% at MBC and MIC (Table 6). Mint had 100% efficacy at MBC, while Ci-EO (positive control) had 70.1% at MIC and Th-EO only 52.6% at MIC.

Pectobacterium atrosepticum

The AUDPC parameter showed that the negative control's disease area was exceeded by some variants treated with essential oils, specifically oregano at MIC and thyme and oregano at 1/4 MIC. At all concentrations tested, the positive control of cinnamon had a higher rotting area value than the negative control (Fig. 4).

After 24 h after inoculation, the variants treated with Ca-EO and Mi-EO at both MBC and MIC and Th-EO and Or-EO at MBC were asymptomatic (Table 5). The rotting area values for Ci-EO at MBC and MIC were similar because that these concentrations were identical ($<0.5 \mu\text{L/mL}$). The largest rotting area of all MIC was found in the oregano treatment at MIC. This value was even higher than the negative control value.

After 48 h from inoculation (Table 5), Mi-EO at MBC (10 $\mu\text{L/mL}$) and MIC (3 $\mu\text{L/mL}$) and Th-EO at MBC (10 $\mu\text{L/mL}$) were still symptom-free. A huge increase in rotting area was noted for Ci-EO in all concentrations. Disease spot area values

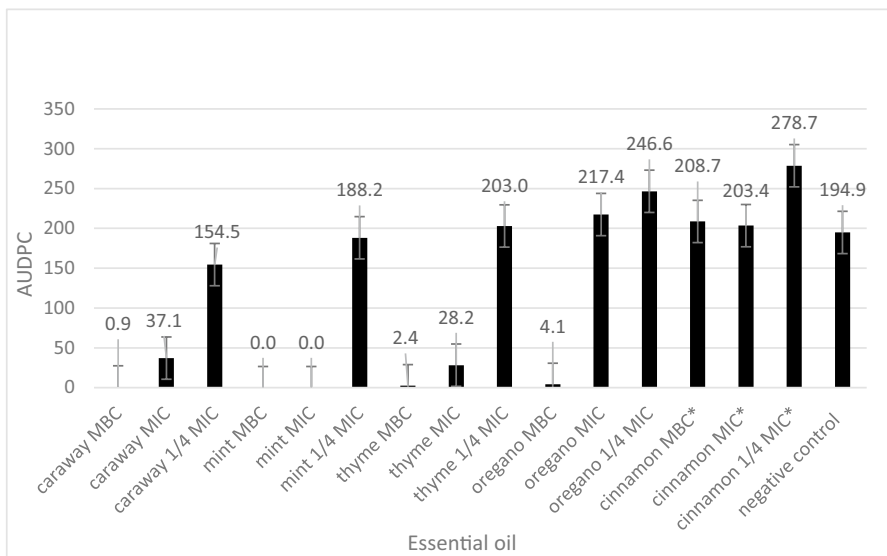


Fig. 4 Area under disease progress area (AUDPC) of mean rotting area caused by *Pectobacterium atrosepticum* after the treatment with essential oils. The evaluations were carried out 1, 2 and 3 days after inoculation. *Positive control. Negative control was *Pectobacterium atrosepticum*

Table 5 Potato tuber discs maceration assay for *Pectobacterium atrosepticum*

Essential oil	Mean of rotting area (mm ²) ¹		
	24 h	48 h	72 h
Caraway MBC	0.0 ^a	0.6 ^a	0.6 ^a
Caraway MIC	0.0 ^a	8.7 ^a	56.8 ^b
Caraway ¼ MIC	6.8 ^{ab}	54.9 ^b	192.5 ^d
Mint MBC	0.0 ^a	0.0 ^a	0.0 ^a
Mint MIC	0.0 ^a	0.0 ^a	0.0 ^a
Mint ¼ MIC	8.8 ^{ab}	95.6 ^{cd}	146.8 ^c
Thyme MBC	0.0 ^a	0.0 ^a	4.7 ^a
Thyme MIC	1.6 ^a	10.7 ^a	33.5 ^{ab}
Thyme ¼ MIC	22.1 ^{bc}	71.3 ^{bc}	241.3 ^d
Oregano MBC	0.0 ^a	2.2 ^a	3.8 ^a
Oregano MIC	24.9 ^{bc}	99.2 ^{cd}	211.4 ^d
Oregano ¼ MIC	39.9 ^c	102.4 ^{cd}	248.3 ^d
Cinnamon MBC ²	3.73 ^{ab}	117.2 ^d	179.3 ^{cd}
Cinnamon MIC ²	3.5 ^{ab}	114.7 ^d	173.9 ^c
Cinnamon ¼ MIC ²	12.0 ^b	159.6 ^e	226.3 ^d
Negative control ³	16.6 ^b	96.1 ^{cd}	181.0 ^d

Online Resource Fig. S10 — inoculum, Fig. S11 — cinnamon essential oil at MBC and MIC, Fig. S12 — cinnamon essential oil at ¼ MIC, Fig. S13 mint essential oil at MBC, Fig. S14 — mint essential oil at MIC, Fig. S15 — mint essential oil at ¼ MIC

MBC minimum bactericidal concentration, *MIC* minimum inhibitory concentration, ¼ *MIC* ¼ minimum inhibitory concentration

¹Means of rotting area followed by different superscripts (a–c) for evaluation after 24 h, (a–d) for evaluation after 48 h and (a–f) for evaluation after 72 h after inoculation indicate significant differences ($P \leq 0.05$) based on Tukey's HSD test. The superscripts apply only for a given column, i.e. the assessment after a given time point

²Positive control

³*Pectobacterium atrosepticum*

were higher than the negative control value for Ci-EO at all concentrations and Or-EO at MIC and ¼ MIC; significant difference was found only with Ci-EO at ¼ MIC.

The Mi-EO in MBC and MIC was the only essential oil that maintained 100% control of *Pa* growth and multiplication even after 72 h of inoculation (Table 5). For Ca-EO at MBC, the same value of rotting area (0.6 mm²) was found as in the evaluation after 48 h. For Ci-EO, Ca-EO, Or-EO and Th-EO at ¼ MIC and Or-EO at MIC, again, the higher rotting area was recorded than for the negative control, but there were no statistical differences between them.

The efficacy of essential oil treatments was varied. Mint at MBC and MIC had 100% efficacy. On the other hand, zero efficacy was found for Or-EO at MIC and ¼ MIC, Ca-EO, Th-EO and Ci-EO at ¼ MIC (Table 6). High efficacy was also found

Table 6 Efficacy of essential oils against *Pectobacterium carotovorum* subsp. *carotovorum* and *Pectobacterium atrosepticum* after 72 h after treatment

Essential oil	Efficacy (%) ¹	
	<i>Pcc</i>	<i>Pa</i>
Caraway MBC	99.6 ^d	99.7 ^d
Caraway MIC	99.5 ^d	68.6 ^c
Caraway ¼ MIC	13.9 ^{ab}	0.0 ^a
Mint MBC	100.0 ^d	100.0 ^d
Mint MIC	99.8 ^d	100.0 ^d
Mint ¼ MIC	33.3 ^b	18.9 ^b
Thyme MBC	98.6 ^d	97.4 ^d
Thyme MIC	52.6 ^{bc}	81.5 ^{cd}
Thyme ¼ MIC	49.7 ^{bc}	0.0 ^a
Oregano MBC	96.4 ^d	97.9 ^d
Oregano MIC	95.7 ^d	0.0 ^a
Oregano ¼ MIC	1.4 ^a	0.0 ^a
Cinnamon MBC ²	98.5 ^d	0.9 ^a
Cinnamon MIC ²	70.1 ^c	3.9 ^{ab}
Cinnamon ¼ MIC ²	25.2 ^b	0.0 ^a

MBC minimum bactericidal concentration, MIC minimum inhibitory concentration, ¼ MIC ¼ minimum inhibitory concentration

¹Efficacy followed by different superscripts (a–d) for *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) and (a–d) for *Pectobacterium atrosepticum* (*Pa*) indicate significant differences ($P \leq 0.05$) based on Tukey’s HSD test. The superscripts apply only for a given column, i.e. the assessment for each bacterium separately

²Positive control

for Ca-EO (at MBC), Th-EO (at MBC and MIC) and Or-EO (at MBC). Surprisingly, the positive control Ci-EO (at MBC) had the lowest efficacy of all the essential oils.

Discussion

In vitro tests showed that essential oils from *Cinnamomum zeylanicum* and *Cinnamomum cassia* (positive control) were the most effective against both bacterial strains of the genus *Pectobacterium*. Paiano et al. (2023) reported that in an experiment with *Escherichia coli* (*Ec*) and *Trueperella pyogenes* (*Tp*), cinnamon essential oil had the lowest MIC values (*Ec* — 2048 µg/mL, *Tp* — 512 µg/mL and MBC (*Ec* — 32,768 µg/mL, *Tp* — 16,384 µg/mL), compared to thyme and oregano essential oils (MIC and MBC > 32,768 µg/mL). Chahbi et al. (2020) confirmed the low MIC values of *C. cassia* EO (MIC 0.05%) and high values of inhibitory zones 16.3–27.7 mm at 100% EO concentration in the study of the sensitivity of *Salmonella* strains. In our study, only 10% EO solution (100 µL/mL) was determined as the highest concentration for testing. The IZs of Ci-EO and

Ci2-EO at this concentration were measured to be 20.46–29.58 mm, and MIC for these EOs was less than 0.05% ($< 0.5 \mu\text{L/mL}$).

In the disc diffusion method, it was found that all selected EOs at 100 and 75 $\mu\text{L/mL}$ had an antibacterial effect on *Pcc* and *Pa* strains. However, this claim was not valid for caraway essential oil at 75 $\mu\text{L/mL}$ for the *Pa* strain.

The second lowest MIC and MBC values were for clove essential oil. The bacterial strains were also sensitive to the essential oil from *Origanum vulgare* at 100 $\mu\text{L/mL}$ in DDM, but the values of MIC and MBC were shown to be slightly higher (MBC 5 and MIC 2.5–5 $\mu\text{L/mL}$). Zhang et al. (2023) found that oregano and cloved oil treatment could significantly reduce *Pcc* cell viability. The minimum inhibitory and bactericidal concentrations were 200 $\mu\text{L/L}$ for oregano and 400–500 $\mu\text{L/L}$ for clove. Queiroz et al. (2020) found the in vitro sensitivity of *Pectobacterium carotovorum* subsp. *brasiliensis* to EOs from rosemary 1%, tea tree 0.75% and clove 0.25% completely inhibited pathogen growth after 48 h of incubation. The oregano and tea tree EOs at 0.5–1 mg/mL concentrations exerted a significant and similar antibacterial effect against *Ec* (Blejan et al. 2021).

Božik et al. (2017) tested EOs against bacterial postharvest pathogens. The most effective against *Pcc* were the EOs from cinnamon (MIC 128 mg/L) and oregano (MIC 256 mg/L). The rosemary and tea tree (MIC > 1024 mg/L) were ineffective at the tested concentrations. Our study also obtained results classifying Ro-EO as an EO with lower effects on both bacterial strains. Alamshahi et al. (2010) reported that on Petri dishes with *Pcc*, the IZ 8.1 mm by 100%, 6 mm by 10% and MIC and MBC 250 $\mu\text{L/mL}$ were shown by EO from *Rosmarinus officinalis*, but Nezhad et al. (2012) even confirmed MBC of 500 $\mu\text{L/mL}$.

For the foodborne bacteria, thyme EO showed an inhibitory effect at concentrations ranging from 1.48 to 7.01 mg/mL and a bactericidal effect at concentrations between 2.61 and 15.07 mg/mL (de Almeida et al. 2023). The largest inhibition zone of 16.5 mm at 100% and 10.5 mm at 10% and the lowest MIC and MBC (5 $\mu\text{L/mL}$) were shown by EO from *Thymus vulgaris* in susceptibility testing of *Pcc* (Alamshahi et al. 2010). Mehrsoroosh et al. (2014), when testing EOs on *Pectobacterium carotovorum*, found higher values of inhibition zones at a 10% concentration of EOs from thyme (17.96 mm) and rosemary (13.7 mm) and lower MIC — for EOs from thyme was 145 $\mu\text{g/mL}$ and from rosemary was MIC $\pm 200 \mu\text{g/mL}$. In our study by DDM, thyme essential oil showed lower efficacy against *Pcc* but maintained similar antibacterial activity with decreasing concentration.

We used the dye resazurin to identify live and dead bacterial cells in the samples and determine the MIC value. Hussain et al. (2011) also concluded that the modified resazurin assay could be effectively used for reliable evaluation of the antibacterial activity of the tested essential oils against several Gram-positive and negative bacterial taxa. Reduction results in an easily detectable colour change, which occurred at cell densities meaningful for MIC testing. Mann and Markham (1998) found that the MIC results obtained by the resazurin method were slightly lower than those obtained by agar dilution. The treatment of potato tuber discs with mint essential oil at a concentration of 3 $\mu\text{L/mL}$ for *Pcc* and 3–10 $\mu\text{L/mL}$ for *Pa* was 100% effective. The high efficacy of caraway (5–10 $\mu\text{L/mL}$), thyme (10 $\mu\text{L/mL}$) and oregano (5 $\mu\text{L/mL}$) EOs was clearly demonstrated in the study. Zhang et al. (2023) reported that

in the groups treated with oregano EO, the severity of soft rot could be suppressed with an inhibition rate of 62.32 to 100%. The assumption that the essential oil of *C. cassia*, chosen as a positive control, would significantly affect the rot caused by pectinolytic bacteria was only confirmed in our in vivo test at a concentration of 2.5 $\mu\text{L/mL}$ for *Pcc*. On the contrary, in some cases, the rotting area after the treatment with Ci-EO was even higher than the control of *Pa* inoculum. The same case was also observed with Or-EO and Th-EO, especially at lower concentrations ($\leq 2.5 \mu\text{L/mL}$). This fact could be explained by the possible phytotoxicity of these EOs on potato tissue or cells. The principle would be that at non-lethal concentrations of EO, the bacteria would be supported in growth and reproduction on the damaged structures of the potato tuber. The essential oils employed in phytopathogen control are generally used at concentrations between 0.1 and 1% (Huang and Lakshman 2010; Guerra et al. 2014). Queiroz et al. (2020) stated that the higher these concentrations, the more phytotoxicity they could cause, which eliminates their use in the alternative control of plant diseases. Essential oils from aromatic plants are a promising source of bioherbicides. Abd-ElGawad et al. (2021) summarized in their review that EO chemical components α -pinen, carvacrol, 1,8 cineole and many others had significant phytotoxicity. The valuable component of *Origanum vulgare* EO is carvacrol, in *Thymus vulgaris* EO thymol. Pinheiro et al. (2015) confirmed that the EO components thymol and carvacrol at 0.12% retarded or inhibited germination and growth in monocot and dicot species and caused changes in the cell cycle of meristematic cells *Lactuca sativa*. Carvacrol also showed a genotoxic effect at the concentration of 3.0 mmol/L (Alves et al. 2018). Araniti et al. (2020) postulated that thymol-induced phytotoxicity could be related to a combined osmotic and oxidative stress and an increase in ABA content, resulting in reduced plant development. Werrie et al. (2022) highlighted the modification of oxidative stress-related metabolites (mainly glutathione and malondialdehyde) following the application of *C. cassia* essential oil on *Malus domestica* tree in a dose–response relationship. Increases in the expression levels of specific genes belonging to PR-proteins, hormonal signalling and oxidative stress pathways were observed following EO cinnamon application.

In conclusion, all essential oils used showed an antibacterial effect against pectinolytic bacteria, causing potato soft rot in laboratory conditions in vitro and in vivo. The use of essential oils from different plant species appears promising for potato tubers as one possibility of biological control. According to the results of in vivo tests, further testing of essential oils of mint and potentially caraway, oregano and thyme may be recommended for pickling potato tubers against *Pectobacterium* pectinolytic bacteria. It is necessary to establish concentrations for practical use of individual EOs that suppress bacteria but are not phytotoxic to plants.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s11540-024-09743-y>.

Acknowledgements We are grateful to M+H Míča&Harašta s.r.o., for providing essential oils for testing.

Author Contribution Statement Barbora Jílková, Jana Víchová, Ludmila Holková and Martin Knoch designed the study. Material preparation, experiments and data collection were performed by Barbora

Jílková, Jana Víchová and Markéta Michutová. Barbora Jílková performed statistical analyses. Barbora Jílková, Jana Víchová and Helena Pluháčková wrote the original draft. Barbora Jílková and Jana Víchová wrote the revised and edited draft. All authors read and approved the final manuscript.

Funding Open access publishing supported by the National Technical Library in Prague. The study was financially supported by the National Agency for Agricultural Research, Ministry of Agriculture of the Czech Republic, project No. QK21010083 — Ecological protection of ware potatoes as a healthy vegetable against selected soilborne and seedborne pathogens.

Data Availability Data will be made available on request.

Declarations

Ethical Standards Not applicable.

Competing Interests The authors declare no competing interests.

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