

Laboratory efficacy of essential oils against *Pectobacterium carotovorum* subsp. *carotovorum* and *Pectobacterium atrosepticum* causing soft rot of potato tubers

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Abstract

The efficacy of essential oils (EOs) from *Carum carvi*, *Cinnamomum cassia*, *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. Under *in vitro* conditions, the highest efficacy was found for cinnamons EOs against both bacteria. The higher sensitivity of both bacteria was manifested in clove (*Pcc* and *Pa*), mint (*Pcc*), oregano (*Pa*) and thyme (*Pa*) EOs. Rosemary EO was the least effective. The results from the *in vivo* test were not consistent with those of the *in vitro* tests. The most significant antibacterial effect was achieved with mint EO. The bacterial strains *Pcc* and *Pa* were more sensitive to treatment with essential oils of caraway (*Pcc* and *Pa*), cinnamon (*Pcc*), oregano (*Pa*) and thyme (*Pcc* and *Pa*). In particular, based on results of *in vivo* test, mint EO and possibly caraway, oregano and thyme EOs can be recommend for pickling potato tubers against pectinolytic bacteria of the genus *Pectobacterium*.

Introduction

The use of medicinal plants in various industries is well known through history. Nowadays, medicinal plants are increasingly being 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 effects. Essential oils are produced by various differentiated structures, in particular their number and properties are highly variable. Essential oils are localized in the cytoplasm of the secretions of certain plant cells located in one or more organs of the plant. These oils are complex mixtures that can contain more than 300 different compounds (Sell 2006).

In this study, EOs with proven antimicrobial effects were used, namely EOs from *Carum carvi* L., *Cinnamomum cassia* L. (Chahbi et al. 2020), *Cinnamomum zeylanicum* Blume (Bravo Cadena et al. 2018), *Cuminum cyminum* L. (Viuda-Martos et al. 2008), *Eugenia caryophyllus* (Spreng.) Bullock & S.G.Harrison (Lucas et al. 2012), *Foeniculum vulgare* Mill. (Diao et al. 2014), *Melaleuca alternifolia* (Maiden & Betcher) Cheell (Lucas et al. 2012), *Mentha × piperita* L. (Guerra et al. 2014), *Origanum vulgare* L. (Božik, et al. 2017), *Rosmarinus officinalis* L. (Mehrsorosh et al. 2014) and *Thymus vulgaris* L. (Hussain et al. 2011). Other important criteria for the selection of suitable EOs were the possibility of plant cultivation in the conditions of the European continent, as well as the economic aspect. However, any EOs that did not meet some of these criteria were also included in the tests (cinnamon, clove and tea tree) in order to compare a wider range of EOs.

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). One of the host plants of these polyphagous bacteria is *Solanum tuberosum*, in which they cause bacterial blackleg and soft rot of potato. Typical symptoms occur at the base of potato stems, which turn black above the soil surface. The stems are infected from the mother tubers, bacteria from the vascular bundles of the tubers are translocated to the xylem stem (Charkowski et al. 2015; Pérombelon 2002). At the beginning of growth, infected plants tend to be stunted, also have stiff and erect leaves. In wet conditions it is a base stems (up to about 10 cm above the soil surface) black or dark brown, slimy and soft. Blackish streaks of infection are visible on a longitudinal section of the base of the stem, these plants usually die off quickly. In dry conditions, the disease develops less and the basal area the stem is light brown, dry and cracked. Using stolons, bacteria can spread to daughter tubers (Charkowski et al. 2015; Pérombelon and Kelman 1987; Zheng and Joshi 2019). The bacteria are often located in lenticels and tuber injury area (Czajkowski et al. 2009; Pérombelon 2002; Hélias et al. 2000). Tuber rot can occur immediately in the soil, shortly after harvest, or during storage. The subsequent decomposition of tubers is usually accompanied by a strong odor (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 in surface water (Pérombelon and Hyman 1989; Pérombelon and Kelman 1980; Quinn et al. 1980). In particular, potato tubers quality, yield and storage life are significantly reduced, therefore protection is very important to prevent potato tuber infection. Agricultural practices such as the eradication of diseased plants, the destruction of crop residues and the rotation of crops with grasses are recommended. Chemical control is based only on the active substances copper hydroxide and chitosan hydrochloride, which are generally ineffective (Mello et al. 2011). Due to the lack of other registered products, attention is focused on the use of essential oils from different plant species or their individual 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 for testing pickling or control during the storage of potato tubers.

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 Hajian-Maleki et al. (2021) and EUCAST (2003). Bacteria were cultivated on King B medium for 48 h at optimal

temperature 28°C for *Pcc* and 26°C for *Pa*. Bacterial suspensions (1×10^8 CFU/mL) were prepared in Mueller Hinton Broth (MHB) (HiMedia Lab., India). Potato tubers cv. 'Red Anna' were obtained from breeding station VESA Velhartice (Velhartice, CZ). Tubers were immersed in 0.7% sodium hypochlorite for surface disinfection. Then tubers were cut into 1 cm diameter discs, rinsed with tap water and allowed to dry. The discs were placed in plastic boxes on filter paper moistened with sterile distilled water. An impression was made in the center of each disc with 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. The aggressiveness of the bacterial strains was evaluated after 24, 48 and 72 h.

Bacterial strains

Based on the results of the aggressiveness evaluation, strain CPPB 56 *Pcc* and strain CPPB 200 *Pa* were selected for further testing. Prior to each experiment, 48 h cultures of the bacterial strains were used growing on King B medium at optimal temperatures of 28°C for *Pcc* and 26°C for *Pa*. The inoculum was always prepared with standard density (1×10^8 CFU/mL) of 0.5 McFarland.

Essential oils selection

A total of eleven EOs were selected, including seven EOs from plant species belonging to the *Lamiaceae* and *Apiaceae* families (Table 1). For cinnamon, two variants of *Cinnamomum cassia leaf oil* and *Cinnamomum zeylanicum bark oil* were tested in order to compare their efficiency.

Table 1
List of the essential oils and content of selected compounds from GC-MS analysis

Essential oil ^{a)}	INCI name	Family	Content of selected compounds from GC/MS analysis (%) ^{b)}	Test ^{c)}
cinnamon (Ci-EO)	<i>Cinnamomum cassia leaf oil</i>	<i>Lauraceae</i>	cinnamaldehyde (cis + trans) – 90.0 eugenol – 0.5	DDM, MIC, MBC, EoTD
cinnamon 2 (Ci2-EO)	<i>Cinnamomum zeylanicum bark oil</i>	<i>Lauraceae</i>	cinnamaldehyde – 71.0	DDM, MIC, MBC
clove (Cl-EO)	<i>Eugenia caryophyllus leaf oil</i>	<i>Myrtaceae</i>	eugenol – 80.6	DDM, MIC, MBC
tea tree (Tt-EO)	<i>Melaleuca alternifolia leaf oil</i>	<i>Myrtaceae</i>	α -pinen – 2.2 terpinen-4-ol – 39.0	DDM, MIC, MBC
careway (Ca-EO)	<i>Carum carvi oil</i>	<i>Apiaceae</i>	D-carvone – 55.3 limonene – 43.3	DDM, MIC, MBC, EoTD
cumin (Cu-EO)	<i>Cuminum cyminum fruit oil</i>	<i>Apiaceae</i>	cuminaldehyde – 32.5 β -pinen – 12.3	DDM, MIC, MBC
fennel (Fe-EO)	<i>Foeniculum vulgare dulce fruit oil</i>	<i>Apiaceae</i>	trans-Anethole – 84.3 α -pinen – 1.1	DDM, MIC, MBC
mint (Mi-EO)	<i>Mentha piperita oil</i>	<i>Lamiaceae</i>	menthone – 26.1 menthol – 45.2	DDM, MIC, MBC, EoTD
oregano (Or-EO)	<i>Origanum vulgare oil</i>	<i>Lamiaceae</i>	carvacrol – 83.1 thymol – 1.15	DDM, MIC, MBC, EoTD
thyme (Th-EO)	<i>Thymus vulgaris oil</i>	<i>Lamiaceae</i>	thymol – 50.2 carvacrol – 3.0	DDM, MIC, MBC, EoTD
rosemary (Ro-EO)	<i>Rosmarinus officinalis leaf oil</i>	<i>Lamiaceae</i>	α -pinen – 12.5 eucalyptol – 45.3	DDM, MIC, MBC

^{a)} Name of essential oil was followed by the abbreviation of its name used in the text. ^{b)} Content of compounds was taken from the GC/MS analysis protocols from the supplier companies – M + H Mica&Harasta Inc. and Florihana Distillerie. ^{c)} DDM – disc diffusion method, MIC – minimum inhibitory concentration, MBC – minimum bactericidal concentration, EoTD – efficacy of essential oil to reduce soft rot disease on potato tuber discs

All EOs in 100% quality were supplied by M + H Mica&Harasta Inc. (Blansko, CZ) or purchased from Florihana Distillerie (France).

Disc diffusion method (DDM)

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

The EOs (Table 1) were diluted with absolute ethanol to a primary concentration of 100 µg/mL (10% solution). Tween 20 was added improve homogeneity. Additional concentrations (75, 50, 25, 10, and 5 µL/mL) were prepared from the stock dilution by adding sterile distilled water and Tween 20.

The 100 µL of standardized inoculum was pipetted into Petri dishes containing Mueller Hinton agar (MHA) (HiMedia Lab., India) and uniformly spread with a sterile hockey stick. The inoculum was allowed to dry. A sterile filter paper disc (6 mm diameter) was impregnated with 7.5 µL of the EO solution. The EO solution of the given concentration was pipetted onto four discs, which were evenly distributed in a square on the Petri dish. 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 were performed in triplicate, with growth control (discs with sterile distilled water) and ethanol.

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 as insensitive, diameters 9–14 mm as sensitive, diameters 15–19 mm as very sensitive and diameters > 20 mm as extremely sensitive.

Data were processed by analysis of variance (ANOVA) and differences between the means were considered significant at $P \leq 0.05$ using the Tukey-HSD test.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

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

EO solutions (Table 1) were prepared 1.1× more concentrated due to the subsequent addition of bacterial inoculum. EOs were diluted with absolute ethanol with the addition of Tween 20 to prepare a stock concentration of 100 µL/mL. Other concentrations (75, 50, 25, 10, 5, 3, 2.5, 1.5 and 0.5 µL/mL) were prepared from the stock dilution by adding MHB and Tween 20. 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, medium purity control, EO purity control at particular concentration without inoculum.

To the remaining volume of each sample, 15 µL of 0.01% resazurin indicator solution was added. The following methodology was developed by Mann & 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. Mauve and pink colour of the tested sample indicated the presence of live bacteria, while blue colour indicated the presence of dead bacteria. The highest remaining dilution with blue colour indicated the MIC.

Cells from the microtubes showing no growth (10 µL solution) were subcultured onto 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 (EoTD)

The soft rot inoculation assay was conducted to investigate the influence of EOs (Table 1) on reducing the development of tuber tissue maceration. The methodology was adapted from Jiang et al. (2021). Potato tubers cv. 'Red Anna' were surface-sterilized with 0.7% sodium hypochlorite for 10 min, then the tubers were cut into 1 cm diameter discs and rinsed with tap water and air-dried at room temperature. Bacterial suspensions and EO concentration range ($\frac{1}{4}$ MIC, MIC and MBC) were both 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 shaking 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 center 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 growth. The test was performed on six discs of each variant in two replicates. The disease spot area was evaluated after 24, 48 and 72 h.

Results

Aggressiveness evaluation of the strains *Pectobacterium carotovorum* subsp. *carotovorum*

Bacterial strains were evaluated 24, 48, 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.

Aggressiveness evaluation of the strains *Pectobacterium atrosepticum*

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

Disc diffusion method, MIC and MBC for *Pectobacterium carotovorum* subsp. *carotovorum*

The Ci-EO and Ci2-EO were the most effective at concentrations of 100, 75 and 50 µL/mL (Table 2). The 100 µL/mL concentrations of Or-EO, Mi-EO and Cu-EO were comparable to the 75 and 50 µL/mL concentrations of Ci-EO. 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 µL/mL concentrations of Ci-EO, Ci2-EO, Th-EO and Or-EO. In the case of Th-EO, there were no significant differences between individual concentrations, its effectiveness does not decrease with decreasing concentration.

Table 2

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

Essential oil	Average of inhibition zones (mm) ^{a,b)}				MIC ^{c)} (μL/mL)	MBC ^{d)} (μL/mL)
	Concentration (μL/mL)					
	100	75	50	25		
cinnamon	20.96 ^a	17.0 ^c	16.5 ^c	9.29 ^{ijklmno}	< 0.5	2.5
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 ^{mnop}	1.5	2.5
tea tree	12.71 ^{defg}	11.29 ^{fghijkl}	8.54 ^{lmnop}	7.63 ^{nop}	3	5
careway	11.25 ^{fghijkl}	9.96 ^{ghijklmno}	7.5 ^{nop}	7.13 ^{op}	5	10
cumin	13.38 ^{def}	11.46 ^{fghijkl}	7.67 ^{mnop}	6.96 ^{op}	5	5
fennel	10.71 ^{fghijkl}	10.33 ^{fghijklm}	7.38 ^{nop}	7.33 ^{nop}	3	10
mint	14.79 ^{cde}	12.42 ^{efgh}	11.21 ^{fghijkl}	6.0 ^p	2.5	3
oregano	15.0 ^{cde}	12.67 ^{defg}	11.67 ^{fghijk}	8.5 ^{lkmop}	5	5
thyme	12.33 ^{efghi}	11.25 ^{fghijkl}	10.21 ^{ghijklmn}	9.5 ^{hijklmno}	3	10
rosemary	11.96 ^{efghij}	10.25 ^{ghijklmn}	8.65 ^{klmnop}	6.0 ^p	5	50

^{a)} Average of inhibition zones followed by different superscripts alphabets (a–p) showed significant difference ($P \leq 0.05$). ^{b)} Inhibition diameter was given in mm, including diameter of disc 6 mm. ^{c)} MIC – minimum inhibitory concentration. ^{d)} MBC – minimum bactericidal concentration.

The strain 56 of *Pcc* was extremely sensitive to Ci-EO (IZ 20.96 mm) and Ci2-EO (IZ 20.46 mm) at concentration of 100 $\mu\text{L/mL}$, it was still very sensitive at concentrations of 75, 50 $\mu\text{L/mL}$ of these EOs. *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 concentration of 100 and 75 $\mu\text{L/mL}$, as well as in Ci-EO, Ci2-EO and Th-EO at concentration of 25 $\mu\text{L/mL}$, only sensitive *Pcc* reaction was detected.

In the MIC determination, Ci-EO ($< 0.5 \mu\text{L/mL}$) (Online Resource Fig. S1) and Ci2-EO ($< 0.5 \mu\text{L/mL}$) was showed the best antibacterial activity. In the MBC assay, Ci-EO was again the most effective, however, Ci2-EO (5 $\mu\text{L/mL}$) was performed worse than Ci-EO (2.5 $\mu\text{L/mL}$) and Mi-EO (3 $\mu\text{L/mL}$). Pairs of Or-EO with Cu-EO and Th-EO with Fe-EO had identical MIC and MBC results. Additionally, Or-EO and Cu-EO (5 $\mu\text{L/mL}$) had identical MIC and MBC values. The Ro-EO was the least effective (Online Resource Fig. S2).

Disc diffusion method, MIC and MBC for *Pectobacterium atrosepticum*

The Ci-EO and Ci2-EO were the most effective at concentrations of 100, 75 and 50 $\mu\text{L/mL}$, however, but there were significant differences among them (Table 3). 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, Ci-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 Ci-EO was comparable to the efficacy of Th-EO, Ci-EO at 100 $\mu\text{L/mL}$, and Or-EO at 75 $\mu\text{L/mL}$, as well as the 100 $\mu\text{L/mL}$ concentrations of Ro-EO, Mi-EO, Cu-EO, and Tt-EO and Ci2-EO at 25 $\mu\text{L/mL}$.

Table 3

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

Essential oil	Average of inhibition zones (mm) ^{a,b)}				MIC ^{c)} (μ L/mL)	MBC ^{d)} (μ L/mL)
	Concentration (μ L/mL)					
	100	75	50	25		
cinnamon	27.17 ^{ab}	25.27 ^b	22.67 ^c	13.08 ^{efg}	< 0.5	< 0.5
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	10.5 ^{ghijklmn}	9.42 ^{klmnopqr}	7.63 ^{opqrstu}	6.71 ^{stu}	3	5
careway	10.25 ^{hijklmno}	8.71 ^{mnopqrst}	6.79 ^{qrstu}	6.0 ^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 ^{lkmnopqrst}	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 ^{jklmnop}	6.96 ^{qrstu}	5	10
rosemary	12.92 ^{efgh}	11.71 ^{ghijk}	8.0 ^{nopqrstu}	6.42 ^{tu}	5	25
^{a)} Average of inhibition zones followed by different superscripts alphabets (a–u) presented significant difference (P ≤ 0.05). ^{b)} Inhibition diameter was given in mm, including diameter of disc 6 mm. ^{c)} MIC – minimum inhibitory concentration. ^{d)} MBC – minimum bactericidal concentration.						

The strain 81 of *Pa* was extremely sensitive to Ci-EO (IZ 27.17–22.67 mm) and Ci2-EO (IZ 29.58–22.71 mm) at concentrations of 100, 75 and 50 μ L/mL. *Pa* was also very sensitive to Or-EO (IZ 16.58 mm) and Th-EO (15.33 mm) at concentration of 100 μ L/mL. Only sensitive *Pa* reaction was detected with other EOs at concentrations of 100 and 75 μ L/mL, as well as with Ci-EO, Ci2-EO at concentration of 25 μ L/mL. The exception was Ca-EO at 75 μ L/mL, which had an IZ of less than 9 mm.

In MIC and MBC determinations, Ci-EO (MIC and MBC < 0.5 μ L/mL) and Ci2-EO (MIC and MBC < 0.5 μ L/mL) were showed the best antibacterial activity. 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

After 24 h from inoculation (Fig. 3), no disease spot area was recorded for the samples of Ci-EO at MBC, Ca-EO both at MIC and at MBC, Mi-EO at MIC and at MBC. As expected for all EOs, areas of rot were measurable at $\frac{1}{4}$ MIC concentrations, however, these values were lower than those of the positive control – bacterial inoculum. For Or-EO, almost similar values of disease spot area were calculated at MBC (16.5 mm^2) and MIC (17.9 mm^2) concentrations, which was due to the fact that these concentrations were identical ($5 \text{ }\mu\text{L/mL}$). Less effective EOs included Or-EO and Th-EO.

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 $\frac{1}{4}$ MIC, Fig. S7 mint essential oil at MBC, Fig. S8 – mint essential oil at MIC, Fig. S9 – mint essential oil at $\frac{1}{4}$ MIC.

After 48 h from inoculation, the EOs efficiency was worse due to the increase of the rotting area, which was caused by the growth and multiplication of the bacteria. Some values of disease spot area increased by up to 200–300%. However, Ca-EO at MBC and Mi-EO at MBC were still symptom free (Fig. 4). For Ca-EO at MIC and Mi-EO at MIC, lower rotting area values were already measurable. The values for disease spot area were comparable for Ci-EO at MBC and Th-EO at MBC. The value of Or-EO at MBC was unchanged from the previous measurement.

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 $\frac{1}{4}$ MIC, Fig. S7 mint essential oil at MBC, Fig. S8 – mint essential oil at MIC, Fig. S9 – mint essential oil at $\frac{1}{4}$ MIC.

After 72 h from inoculation (Fig. 5), the area of rot remained unchanged from the previous measurement of Mi-EO at MBC (0 mm^2) and Mi-EO at MIC (0.8 mm^2). The Mi-EO at MBC was also the only essential oil that maintained 100% control of *Pcc* even after 72 h of inoculation. Some EOs showed similar efficacy (Ci-EO at MBC $\times$ Th-EO at MBC; Ca-EO at MBC $\times$ Ca-EO at MIC; Or-EO at MBC $\times$ Or-EO at MIC). For some EOs, especially the $\frac{1}{4}$ MIC concentration, the rotting area increased by up to 200%.

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 $\frac{1}{4}$ MIC, Fig. S7 mint essential oil at MBC, Fig. S8 – mint essential oil at MIC, Fig. S9 – mint essential oil at $\frac{1}{4}$ MIC.

Pectobacterium atrosepticum

After 24 h from inoculation, the highest efficacy of EOs on *Pa* (0 mm^2) was found for Ca-EO at both MBC and MIC, the same for Mi-EO at MBC and MIC. The highest efficacy was also found for Th-EO and Or-EO at MBC (Fig. 6). The rotting area values for Ci-EO at MBC and MIC were similar due to the fact that these concentrations were identical ($< 0.5 \text{ }\mu\text{L/mL}$). It was surprising to find that the calculated areas of rot for Th-EO (22.1 mm^2) and Or-EO (39.9 mm^2) at concentration of $\frac{1}{4}$ MIC, as well as Or-EO at MIC (24.9 mm^2), were higher than the values of the positive control – bacterial inoculum (16.6 mm^2).

After 48 h from inoculation (Fig. 7), Mi-EO at MBC ($10 \text{ }\mu\text{L/mL}$) and MIC ($3 \text{ }\mu\text{L/mL}$) and Th-EO at MBC ($10 \text{ }\mu\text{L/mL}$) were still symptom-free. For Ci-EO in both concentrations, a huge increase in rotting area was

noted, almost 2000%. For some EOs, the rotting area was higher than for bacterial inoculum (96.1 mm²), specifically for Ci-EO in both concentrations (MBC – 117.2; MIC 114.7 and ¼ MIC 159.6 mm²) and for Or-EO at MIC (99.2 mm²) and ¼ MIC (104.2 mm²).

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.

The Mi-EO in both MBC and MIC was the only essential oil that maintained 100% control of *Pa* growth and multiplication even after 72 h of inoculation (Fig. 8). For Ca-EO at MBC, the same value of rotting area (0.6 mm²) was found as in the evaluation after 48 h. This essential oil at MBC also had the second highest efficacy on *Pa*. For Ci-EO, Ca-EO and Or-EO at ¼ MIC and for Or-EO at MIC, again the higher rotting area was recorded than for the positive control – bacterial inoculum.

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.

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.

Discussion

Disc diffusion method, MIC and MBC

In vitro tests showed that essential oils from *Cinnamomum cassia* and *Cinnamomum zeylanicum* 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* – 32768 µg/mL, *Tp* – 16384 µg/mL), compared to thyme and oregano essential oils (MIC and MBC > 32768 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 µL/mL).

In the disc diffusion method, it was found that all selected EOs at 100 and 75 µL/mL had an antibacterial effect on *Pcc* and *Pa* strains. However, this claim was not valid for Ca-EO at 75 µL/mL for *Pa* strain. Clerck et al. (2020) observed that *Pcc* seemed to be more sensitive than *Pa*. On the contrary, we found that the sensitivity of the strains of both bacteria was similar to the effect of EOs.

The bacterial strains were also very sensitive to the essential oil from *Origanum vulgare* at 100 µL/mL in DDM but the values of MIC and MBC were shown to be slightly higher (MBC 5 and MIC 2.5–5 µL/mL). Bozin et al. (2006) found that the essential oil of oregano showed the highest antibacterial activity among the tested EOs, even against multiresistant strains of *Pseudomonas aeruginosa* and *Ec*. The MIC of this essential oil reached the lowest values (1–2 µL/mL) of all essential oils tested. Also Viuda-Martos et al. (2008) also listed EO from oregano as the most effective of EOs tested on the growth of some bacteria commonly used in the food industry, followed by EOs from clove and cumin. In our test, MIC (1.5 µL/mL) and MBC (2.5–3 µL/mL) of Cl-EO were found to be one of the lowest for both bacterial strains. Queiroz et al. (2020) found that *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. Lucas et al. (2012) confirmed the antibacterial effect of EOs from thyme, clove, cinnamon and tea tree at 10% concentration on *Xanthomonas vesicatoria* and Alves et al. (2014) observed that EOs from cinnamon at 1% and thyme and clove at 10% effectively inhibited the growth of *Pseudomonas syringae* pv. *tomato*.

Božik et al. (2017) tested EOs against the 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 not effective at the tested concentrations. In our study, we 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 µL/mL were shown by EO from *Rosmarinus officinalis* but Nezhad et al. (2012) even confirmed MBC of 500 µL/mL.

Hussain et al. (2011) found the best antibacterial activity with essential oil of *Thymus vulgaris* with larger inhibition zones 17.1–28.2 mm at 100% and the lowest MIC (70.4–1250.3 µg/mL) against selected strains of pathogenic and food spoilage bacteria. The largest inhibition zone of 16.5 mm at 100% and 10.5 mm at 10% and the lowest MIC and MBC (5 µL/mL) were shown by EO from *Thymus vulgaris* in susceptibility testing of *Pcc* (Alamshahi et al 2010). Mehrsorosh et al. (2014), when testing EOs on *Pectobacterium carotovorum*, found higher values of inhibition zones at 10% concentration of EOs from thyme (17.96 mm) and rosemary (13.7 mm) and lower MIC – for EOs from thyme was 145 µg/mL and from rosemary was MIC ± 200 µg/mL.

In our study by DDM, thyme essential oil showed lower efficacy against *Pcc*, but maintained similar antibacterial activity with decreasing concentration.

In determining the MIC value, we used the dye resazurin to identify live and dead bacterial cells in the samples. 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.

Effectiveness of essential oils to reduce soft rot disease on potato tuber discs

The assumption that *C. cassia* essential oil significantly regulates the rotting area caused by pectinolytic bacteria was not confirmed in our *in vivo* test. 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 to 1% (Alves et al. 2014; 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, 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). The oregano essential oil was phytotoxic to *Echinochloa crus-galli* and *Chenopodium album* (Vasilakoglou et al. 2007). 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, as one possibility of biological control, appears promising for potato tubers. In particular, based on results of *in vivo* test, we can recommend mint EO and possibly caraway, oregano and thyme EOs for pickling potato tubers against pectinolytic bacteria of the genus *Pectobacterium*. For practical use, it is necessary to establish concentrations for individual EOs that suppress bacteria but are not phytotoxic to plants.

Declarations

Competing Interests

The authors have no competing interests to declare that are relevant to the content of this article.

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Ethical Standards

Not applicable.

Author's contribution statements

Barbora Jílková, Jana Víchová, Ludmila Holková and Martin Kmoč designed the study. Material preparation, experiments and data collection were performed by Barbora Jílková, Jana Víchová, Helena Pluháčková and Markéta Michutová. Barbora Jílková performed statistical analyses. Barbora Jílková, Jana Víchová and Helena Pluháčková wrote the article. All authors read and approved the final manuscript.

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Figures

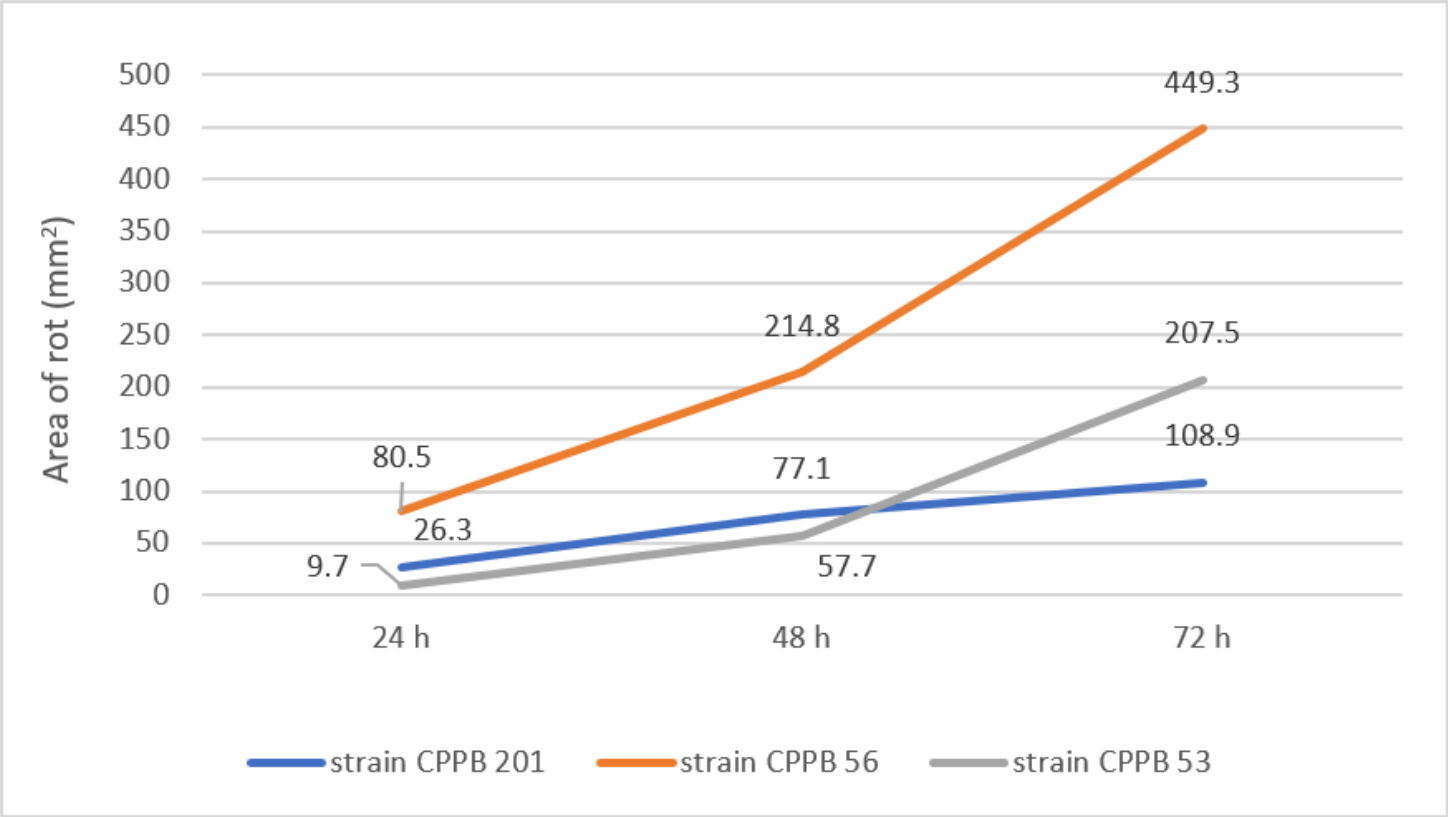


Figure 1

Aggressiveness evaluation of bacterial strains *Pectobacterium carotovorum* subsp. *carotovorum* over time

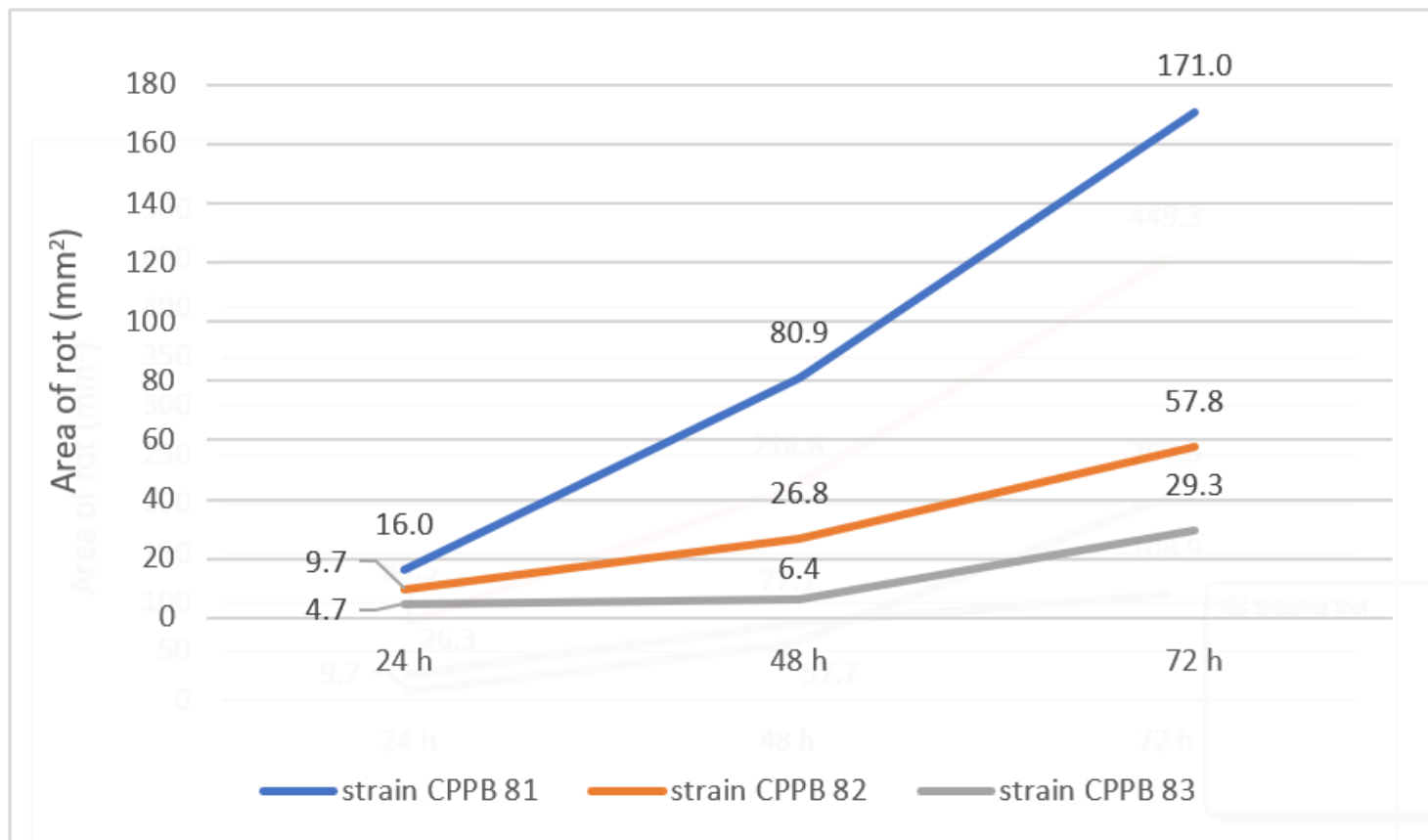


Figure 2

Aggressiveness evaluation of bacterial strains *Pectobacterium atrosepticum* over time

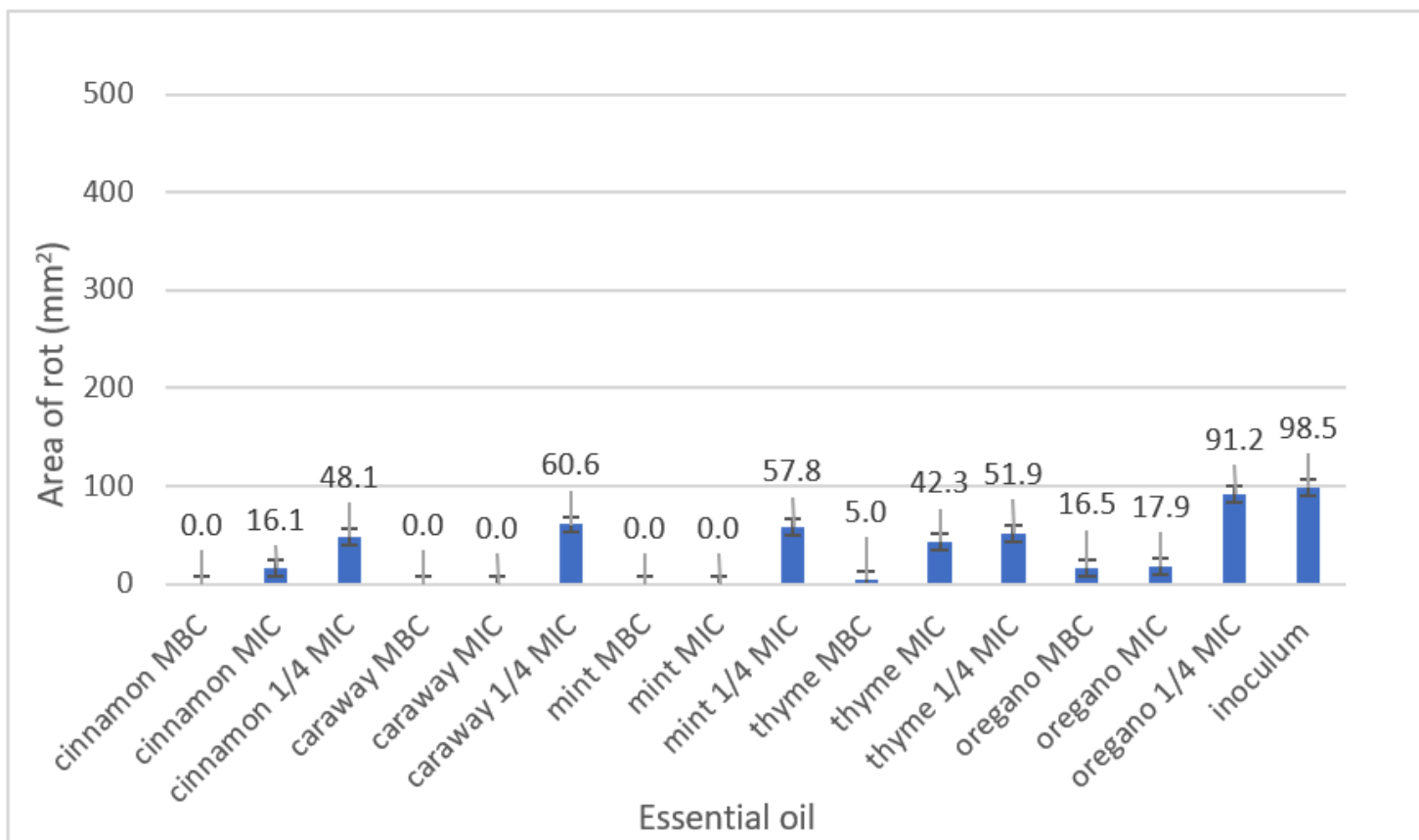


Figure 3

Disease spot area of *Pectobacterium carotovorum* subsp. *carotovorum* in 24 h after treatment with essential oils

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.

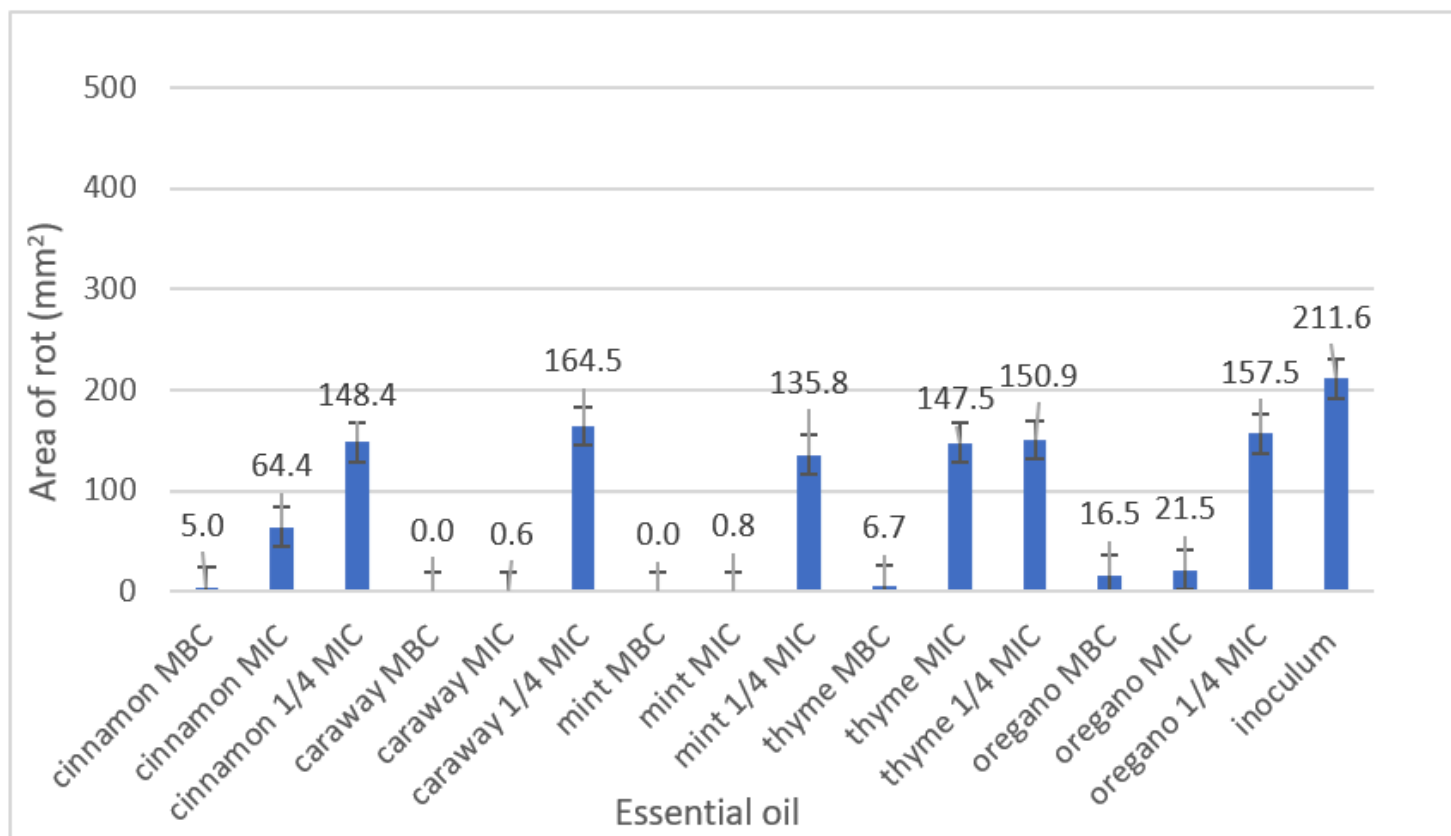


Figure 4

Disease spot area of *Pectobacterium carotovorum* subsp. *carotovorum* in 48 h after treatment with essential oils

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.

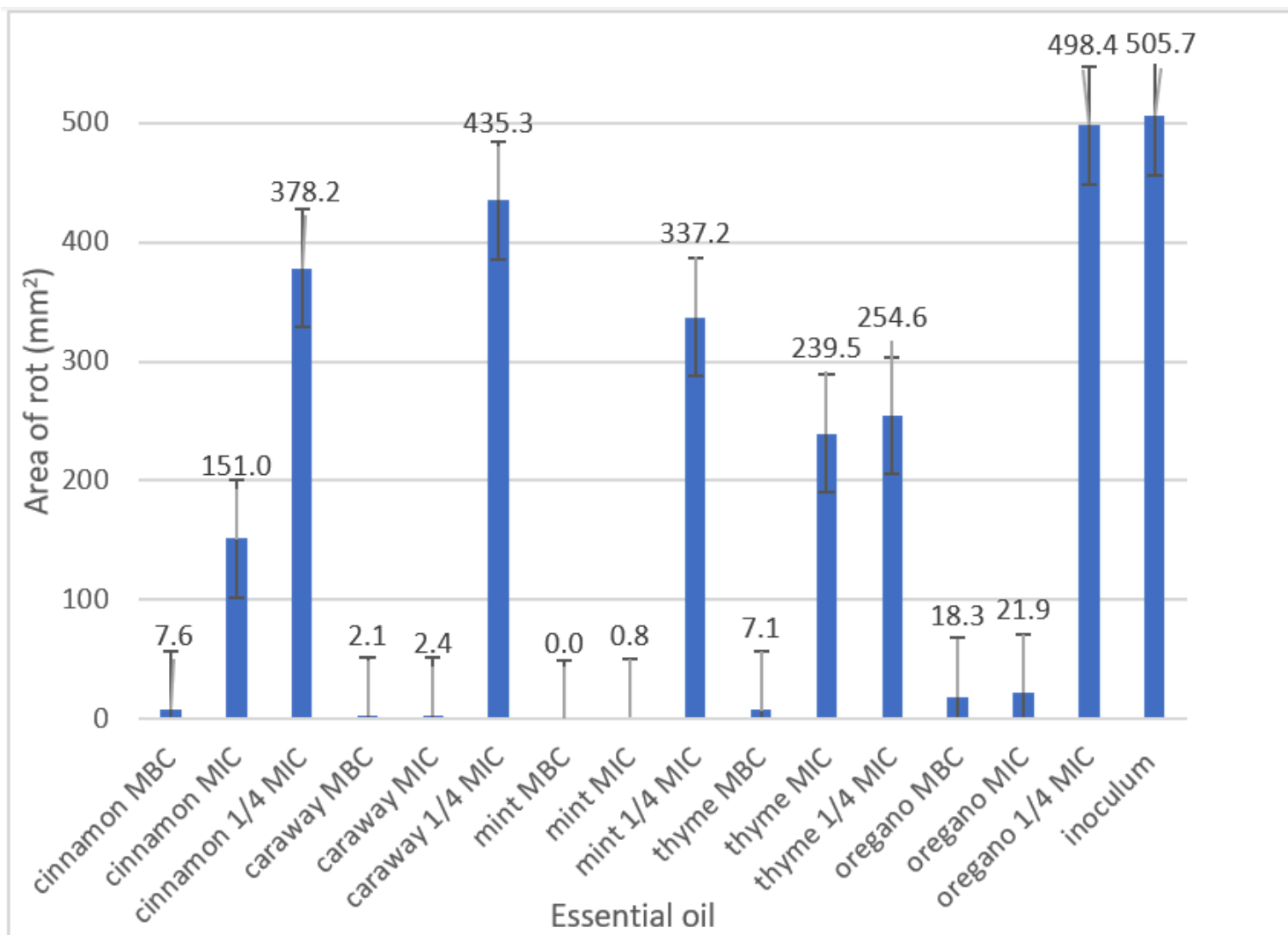


Figure 5

Disease spot area of *Pectobacterium carotovorum* subsp. *carotovorum* in 72 h after treatment with essential oils

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.

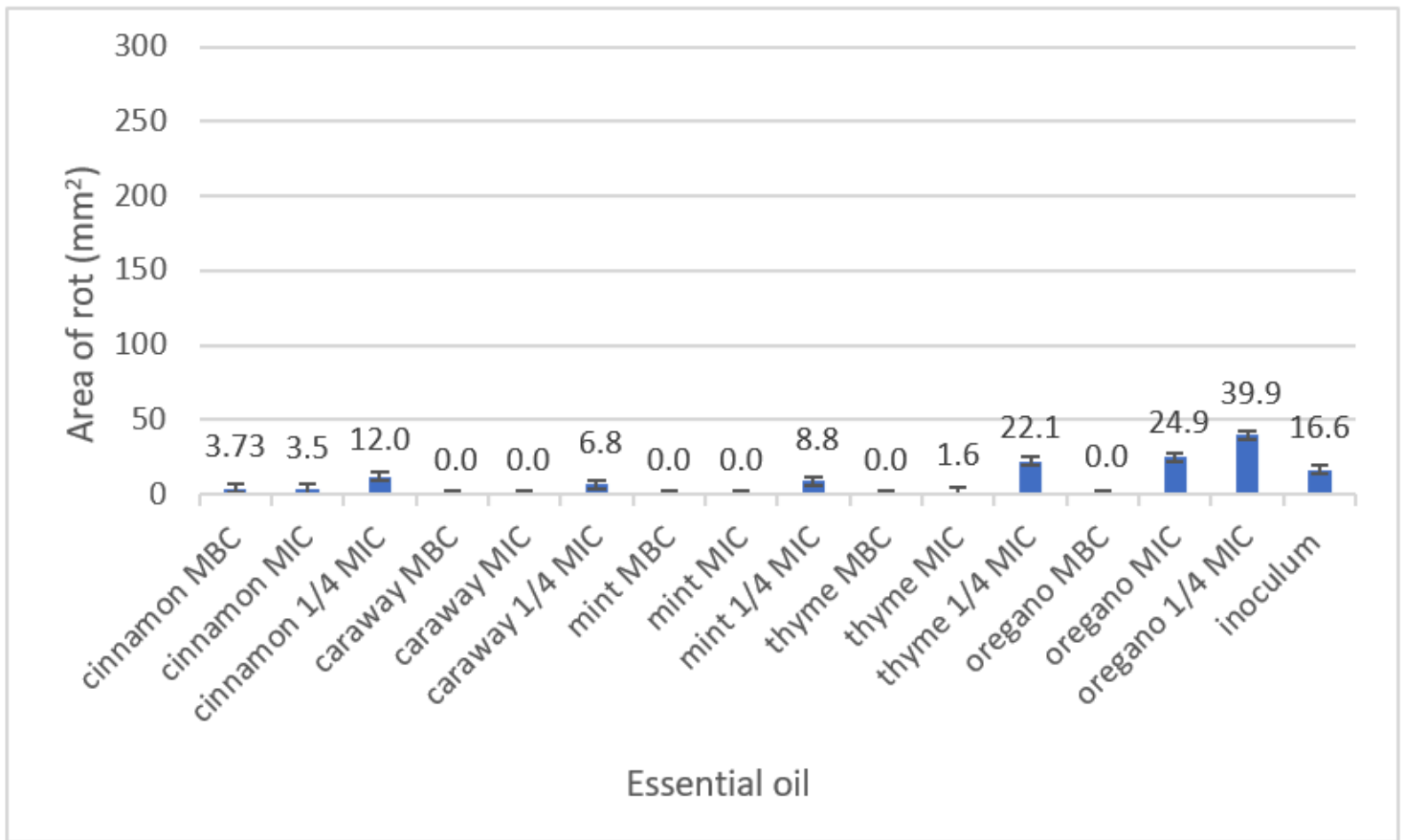


Figure 6

Disease spot area of *Pectobacterium atrosepticum* in 24 h after treatment with essential oils

Online Resource Fig. S10 – inoculum, Fig. S11 – cinnamon essential oil at MBC and MIC, Fig. S12 – cinnamon essential oil at 1/4 MIC, Fig. S13 mint essential oil at MBC, Fig. S14 – mint essential oil at MIC, Fig. S15 – mint essential oil at 1/4 MIC.

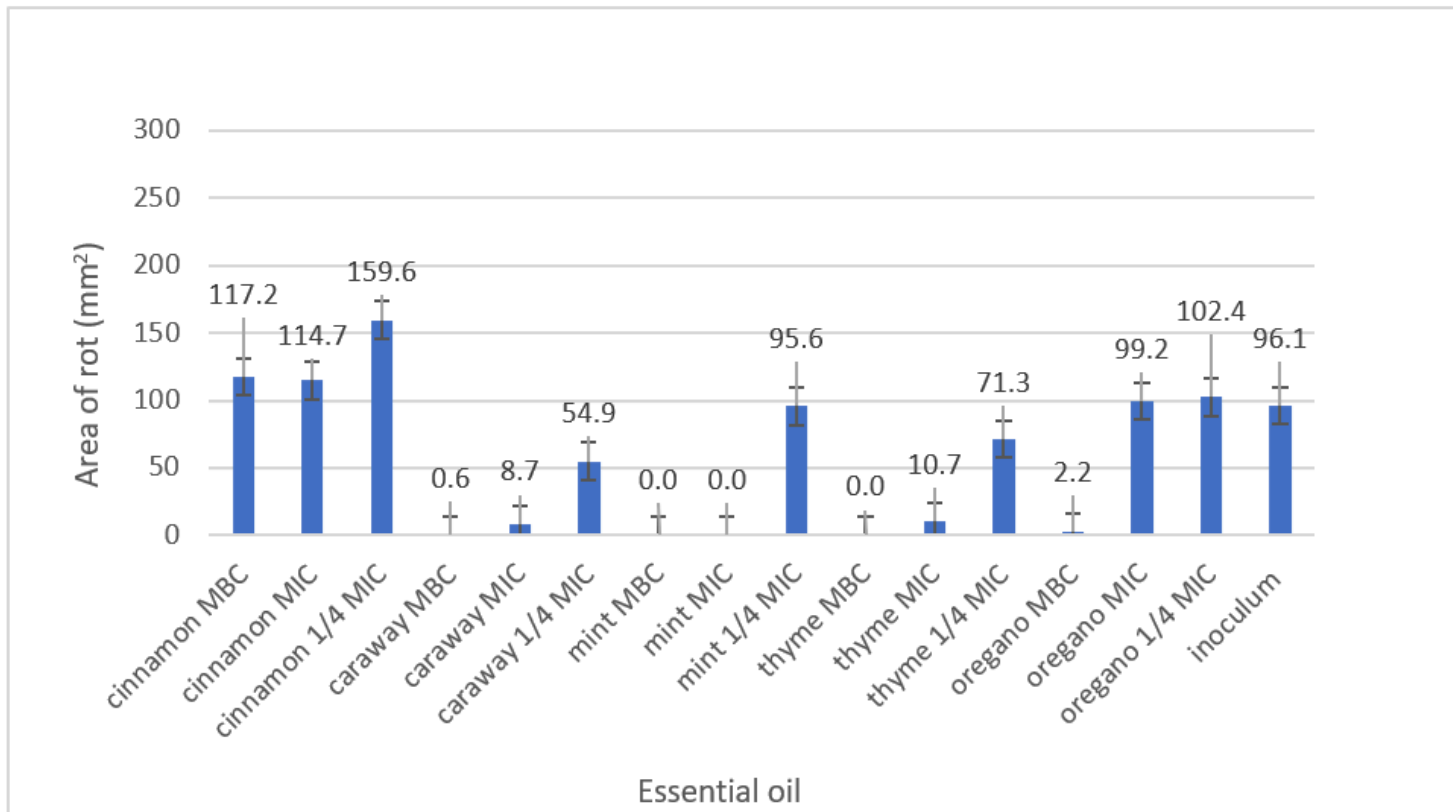


Figure 7

Disease spot area of *Pectobacterium atrosepticum* in 48 h after treatment with essential oils

Online Resource Fig. S10 – inoculum, Fig. S11 – cinnamon essential oil at MBC and MIC, Fig. S12 – cinnamon essential oil at 1/4 MIC, Fig. S13 mint essential oil at MBC, Fig. S14 – mint essential oil at MIC, Fig. S15 – mint essential oil at 1/4 MIC.

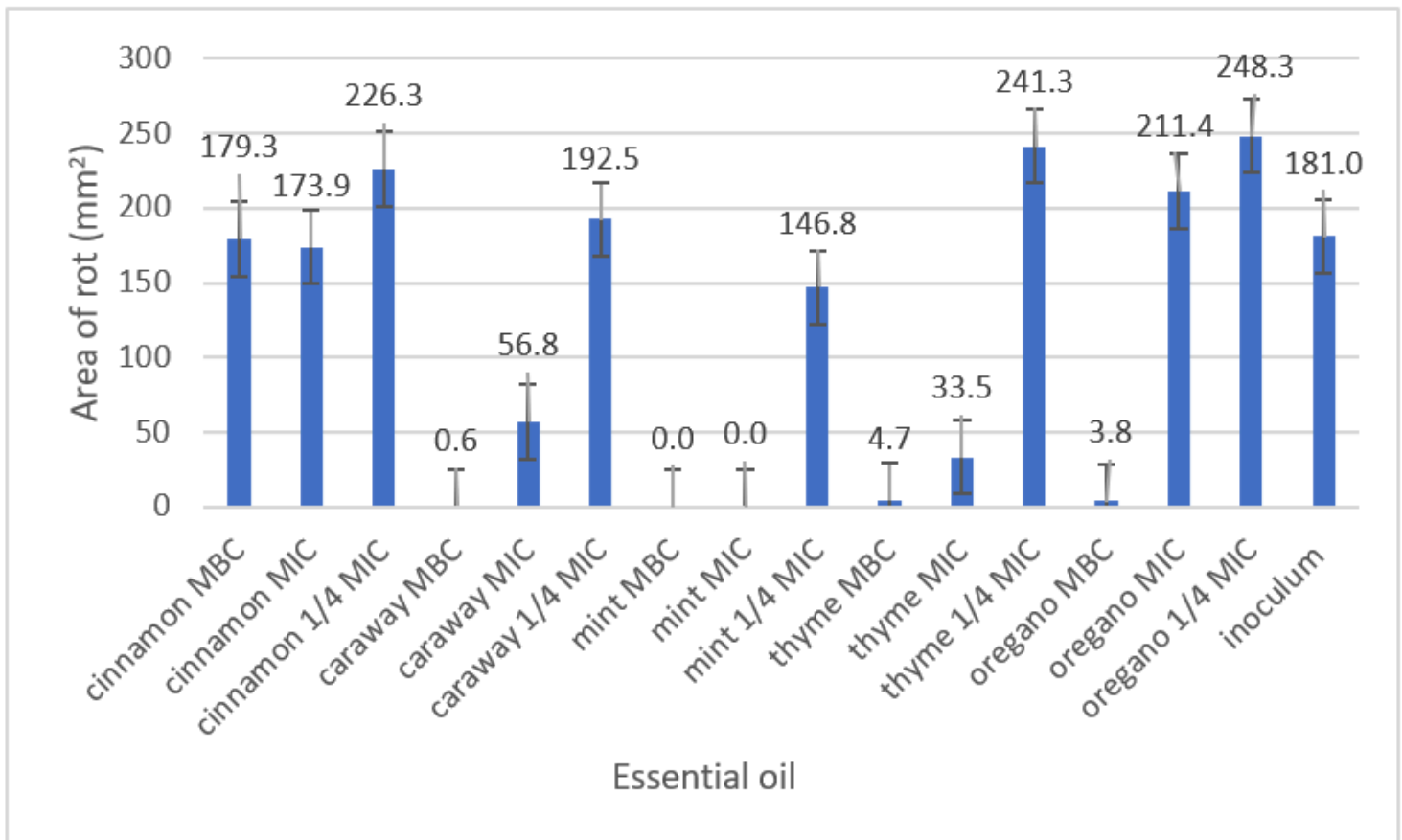


Figure 8

Disease spot area of *Pectobacterium atrosepticum* in 72 h after treatment with essential oils

Online Resource Fig. S10 – inoculum, Fig. S11 – cinnamon essential oil at MBC and MIC, Fig. S12 – cinnamon essential oil at 1/4 MIC, Fig. S13 mint essential oil at MBC, Fig. S14 – mint essential oil at MIC, Fig. S15 – mint essential oil at 1/4 MIC.

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