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Research article

In vitro and *in vivo* antibacterial activity of selected essential oil components against *Pectobacterium carotovorum* subsp. *carotovorum* and *Pectobacterium atrosepticum* causing bacterial soft rot of potato tubers

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ABSTRACT

Pectinolytic bacteria cause bacterial soft rot of potato tubers. The most significant losses occur during storage. The efficacy of essential oil (EO) components carvacrol, cinnamaldehyde, D-carvone, L-menthone, R-(+)-limonene and thymol was tested against *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) and *Pectobacterium atrosepticum* (*Pa*). Disc diffusion, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) tests were performed *in vitro,* as well as potato disc and whole tuber maceration tests *in vivo*.

Under *in vitro* conditions, cinnamaldehyde was the most effective against both bacteria (MIC 0.5 μL/mL, MBC 1.5 μL/mL). Both bacteria were found to be more susceptible to D-carvone (MIC 1.5–2.5 μL/mL, MBC 2.5 μL/mL) and thymol (MIC 2.5–5 μL/mL, MBC 3–5 μL/mL). R-(+)-limonene was the least effective. Results from the potato tuber disc maceration test

confirmed a significant antibacterial effect of cinnamaldehyde at a concentration of 1.5 μL/mL. No rotted area was observed on potato tuber discs after treatment with L-menthone at concentrations of 2.5 μL/mL and 10 μL/mL against *Pcc*. A more pronounced effect was obtained when carvacrol was used at concentrations of 5 μL/mL against *Pcc* and 10 μL/mL against *Pa.* Disease severity tests on potato tubers after soaking for 20 min at MIC concentration of the EO components followed by 7 days of incubation at room temperature and 15 °C confirmed the antibacterial activity of cinnamaldehyde (0.5 μl/ml), L-menthone (2.5 μl/ml) and carvacrol (5–10 μl/ml). Cinnamaldehyde, L-menthone, and carvacrol may be recommended for further testing to treat stored potato tubers.

1. Introduction

Potato (*Solanum tuberosum* L.) is one of the most important vegetable crops for human nutrition worldwide. The quality, yield and storage life of potato tubers can be significantly reduced by pectinolytic bacteria of the genus *Pectobacterium* [1]. *Pectobacterium carotovorum* subsp. *carotovorum* (Jones 1901) Hauben et al., 1999 emend. Gardan et al., 2003 (*Pcc*) and *Pectobacterium atrosepticum*

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(van Hall 1902) Gardan et al., 2003 (*Pa*) are Gram-negative and facultatively anaerobic bacteria previously classified as *Erwinia carotovora* subsp. *carotovora* and *Erwinia carotovora* subsp. *atroseptica* [2]. The virulence of *Pcc* and *Pa* is mainly attributed to several enzymes degrading plant cell walls, such as pectate lyase, polygalacturonase, protease, and cellulase, which are responsible for the maceration of plant tissue [3]. Typical symptoms manifest as a disease called bacterial blackleg and soft rot of potatoes. In growing plants, they occur at the base of potato stems, which become black or dark brown, slimy, and soft in wet conditions. In dry conditions, the disease is less severe, and the base of the stem becomes light brown, dry, and cracked. Bacteria can spread to daughter tubers through the stolons [4–6]. Bacteria are often found in the lenticels and in areas of tuber injury [7–9]. Tuber rot can occur immediately in the soil, shortly after harvest, or during storage. The subsequent decomposition of the tubers is usually accompanied by a strong odour [10]. The bacteria can survive in contaminated and infected tubers, in crop residues in the soil, in the root systems of weeds and crops, and surface water [11,12]. Disease control methods, including crop rotation, soil sanitation, and chemical applications of active substances copper hydroxide and chitosan hydrochloride, are not always as effective as expected [13,14]. Preventive measures must be carefully followed before and during storage, including storing healthy, dry and bacteria-free potato tubers, preventing their mechanical damage and the condensation of free water on the tubers with an appropriate ventilation regime [10].

Plant essential oils (EOs) and plant extracts are gaining popularity as natural antibacterial phytochemicals. Screening of secondary metabolites in plants and herbs can lead to the discovery of effective antimicrobial components [15]. Antimicrobial activities have been reported previously for many EOs [16–18], and some studies have focused on their potential use in bacterial control [18–20]. It is also recognized that the antimicrobial activity of EOs may vary according to their chemical composition, which in turn may vary according to the origin of the plant material collected, the place of cultivation, the environmental conditions and the stage of development [21]. Essential oils are complex mixtures that can contain more than 300 different compounds [22]. These volatile compounds belong to different chemical classes: alcohols, ethers or oxides, aldehydes, ketones, esters, amines, amides, phenols, heterocycles, and especially terpenes.

The aim of our study was to extend the possible range of EO components from plant species with antibacterial activity against *Pcc* and *Pa*. At the same time, we endeavoured to identify EO components with good prospects for further testing for potato tuber pickling or storage control.

2. Materials and methods

2.1. Bacterial strains

In a previous experiment, strain CPPB 56 (CRI Praha-Ruzyně, Czech Republic) *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) and strain CPPB 81 (CRI Praha-Ruzyně, Czech Republic) *Pectobacterium atrosepticum* (*Pa*) were found to be highly aggressive strains on potato tuber discs. Consequently, these strains were selected for further testing. Prior to each experiment, the bacterial strains were cultured for 48 h on King's B medium (HiMedia Laboratories, India) at optimal temperatures of 28 °C for *Pcc* and 26 °C for *Pa*. The inoculum was always prepared at a standard density of 1×10^8 CFU/mL, equivalent to 0.5 McFarland.

2.2. Essential oil components

The most pure EO components were selected for the experiments: carvacrol (Crl) – with a purity of 98 %, cinnamaldehyde (Cin) – ±95 %, D-carvone (Crn) – 100 %, L-menthone (Mnt) – ±96 %, R-(+)limonene (Lim) – 97 %, natural thymol (Tyn) – 100 %, and synthetic thymol (Tys) – 100 %. The components were supplied by M + H Míča&Harašta (Czech Republic) or purchased from Merck KGaA (Germany). These components met our initial requirements: proven antimicrobial activity [23–28] and the most abundant occurrence in European aromatic plants (mint, caraway, oregano, and thyme), which was important from an economic point of view. Cinnamaldehyde was selected as the active ingredient due to the high antibacterial activity demonstrated by cinnamon essential oil, which contains a significant amount of this component [29]. Our laboratory has been conducting tests on this oil for some time.

2.3. Disc diffusion method

A standard agar disc diffusion method (DDM) was used for the antibacterial testing. The methodology was based on EUCAST [30], with cetain modifications.

The EO components (Crl, Cin, Crn, Mnt, Lim, Tyn, and Tys) were diluted with 96 % ethanol to a primary concentration of 100 μL/mL. Subsequently, additional concentrations (75, 50, 25, 10, and 5 μL/mL) were prepared from the stock dilution by the addition of sterile distilled water. To ensure perfect dispersion, a few drops of Tween 20 (0.01 % [w/v]) (Merck KGaA, Germany) were added. One hundred microlitres of the standardised inoculum was pipetted onto Petri dishes containing Mueller Hinton agar (HiMedia Laboratories, India) (MHA) and spread evenly using a sterile hockey stick rod. The inoculum was allowed to dry. A sterile filter paper disc (6 mm diameter) was filled with 7.5 μL of the EO component solution at the specified concentration. The solution of the specified concentration was pipetted onto four discs that were evenly distributed in a square on the Petri dish. The plates were incubated at optimal temperatures of 28 °C for *Pcc* and 26 °C for *Pa*. After 24 h the diameters of the inhibition zones were measured. The tests were performed in triplicate and included a growth control (discs with sterile distilled water) and ethanol.

The sensitivity of bacteria to the components of essential oils was evaluated according to the methodology proposed by Ponce [31]. The diameter of the inhibition zone was used to categorise the sensitivity of the bacteria. Zones with diameters of less than 8 mm were considered insensitive, 9–14 mm sensitive, 15–19 mm very sensitive, and greater than 20 mm extremely sensitive.

2.4. Minimum inhibitory concentration and minimum bactericidal concentration assays

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined according to modified EUCAST [32] and Hajian-Maleki [20] methods.

The component solutions (Crl, Cin, Crn, Mnt, Lim, Tyn, and Tys) were prepared 1.1 × more concentrated due to the subsequent addition of bacterial inoculum. Component solutions were diluted using 96 % ethanol 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 basic dilution by adding Mueller Hinton Broth (HiMedia Laboratories, India) (MHB). Several drops of Tween 20 (0.01 % [w/v]) were added to ensure perfect dispersion in the medium. Due to the volatility of the EO components, 135 μL of the EO component solution at the indicated concentration was added to 0.2 mL microtubes, followed by 15 μL of the inoculum. The microtubes were incubated for 24 h at the optimum temperature for bacterial growth with shaking at 100 rpm. Testing was performed in four replicates with bacterial growth control, medium purity control and EO component purity control at a given concentration without inoculum.

To the remaining volume of each sample, 15 μL of 0.01 % resazurin indicator solution (Merck KGaA, Germany) was added. The methodology developed by Mann and Markham [33] was slightly modified here. The redox dye resazurin sodium salt has been successfully used to evaluate the antibacterial activity of plant extracts and standard pharmaceuticals based on the colour change in a solution as an indicator of live bacterial cells. Subsequently, the microtubes were incubated for a further 2 h with shaking at 120 rpm. The samples were visually inspected for a change in colour. The mauve and pink colours of the tested sample indicated the presence of live bacteria, while the blue colour indicated the presence of dead bacteria. The highest remaining dilution with blue colour indicated the MIC.

Cells from microtubes showing no growth (10 μL of solution) were subcultured onto MHA plates to determine whether the inhibition was reversible or permanent. Subsequently, the incubation period was extended for a further 24 h. The MBC was defined as the highest dilution (lowest concentration) at which no visible growth was observed on the agar plates.

2.5. Potato tuber disc maceration assay

Based on the MIC and MBC test, the components Crl, Cin, Crn, Mnt, Tyn, and Tys were selected. The methodology was adapted from Jiang [34]. Potato tubers of the cultivar 'Red Anna' were obtained from the breeding station of VESA Velhartice a.s. (Czech Republic). The tubers were calibrated to a similar size of approximately 30 × 38 mm. The tubers were surface disinfected with 0.7 % sodium hypochlorite (NaOCl) for 10 min. The tubers were then cut into 1 cm thick slices, rinsed with tap water, and air dried at room temperature. Only discs of similar size were included in the test to ensure comparability of results. Bacterial suspensions and the concentration range of EO components (¼ MIC, MIC, and MBC) were diluted in MHB. Due to the addition of the bacterial suspension, the solutions of EO components were 1.1 × more concentrated. The samples were prepared by mixing 135 μL of EO component 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. A depression was made in the centre of each disc using a sterile metal rod, and 3.5 μL of the mixed sample was pipetted onto the discs. The boxes were lidded and cultured under optimal conditions for bacterial growth. The test was performed on six discs in two replicates of each concentration of the EO component and the positive control – inoculum. At 48 and 72 h after inoculation, the size of the rot patch was measured longitudinally and transversely, and the rotting area was calculated.

2.6. Efficacy of EO components against soft rot on potato tubers

The methodology was partly adapted from Hajian-Maleki [20]. Potato tubers of the cultivar 'Red Anna' were surface disinfected with 0.7 % NaOCl for 10 min, washed with tap water, and left to dry. Subsequently, two points along the longitudinal axis of the tuber were wounded with a sterile borer measuring 2 mm in diameter and 4 mm deep, creating infection sites. The efficacy of the EO components (Crl, Cin, and Mnt) was evaluated individually against *Pcc* and *Pa* at their minimum inhibitory concentrations. The tubers were immersed in the EO component solution for 20 min and then allowed to air dry. Inoculation was performed by adding of 15 μL of a bacterial suspension to each wound. The inoculated tubers were placed in plastic boxes and incubated in a storage room at a temperature of 22 ± 2 °C and a pre-storage temperature of 15 °C for 7 days. Six tubers were used per individual treatment, and the experiment was replicated twice, with a positive control of bacterial inoculum. After seven days, the tubers were cut along the longitudinal axis and across the inoculation site. The tubers were then photographed for subsequent analysis. The disease severity (infection intensity) was quantified using image analysis software for plant disease quantification, namely Assess 2.0 (APS Press, USA). The efficacy of the essential oil component was expressed as a percentage of the rotting area per unit area of healthy tuber.

2.7. Statistical analysis

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

3. Results

3.1. Disc diffusion method, minimum inhibitory concentration, and minimum bactericidal concentration assays

3.1.1. Pectobacterium carotovorum subsp. carotovorum

A comparison of the inhibition zones of the components revealed that the most effective component was Cin at concentrations of 100 and 75 μL/mL (Table 1). The efficacy of Cin at a concentration of 50 μL/mL was comparable to that of Tys, Tyn, and Crl at a concentration of 100 μL/mL. Cinnamaldehyde at a concentration of 25 μL/mL had an antibacterial effect similar to that of Crn, Mnt, and Lim at a concentration of 100 μL/mL. The efficacy of Lim at a concentration of 75 μL/mL was not different from those of Crn, Mnt, Tyn, and Lim at a concentration of 50 μL/mL and from those of Lim, Mnt, Tys, Tyn, and Crl at a concentration of 25 μL/mL. The effect of ethanol, used for the initial dilution of the EO components, was also tested. At the concentration corresponding to a sample of 100 μL/mL, the average IZ for ethanol was measured to be 7.19 mm, and at subsequent concentrations, it was equal to 6 mm.

When evaluating the efficacy of components according to Ponce [31], Bacteria *Pcc* was extremely sensitive to the components Tyn and Tys at a concentration of 100 μL/mL and to Cin down to 50 μL/mL. For other components, except for R-(+)limonene, bacteria were sensitive at concentrations down to 75 μL/mL.

In the MIC and MBC tests, Cin had the strongest effects (Table 1). The second most effective component was Crn. The least effective was Lim.

3.1.2. Pectobacterium atrosepticum

The components Cin (34.42 mm), Tyn (35.0 mm), and Tys (32.67 mm) at a concentration of 100 μL/mL and Cin (30.92 mm) and Tys (29.83 mm) at a concentration of 75 μL/mL showed the greatest efficacy against the *Pa* strain (Table 2). At a concentration of 25 μL/mL, the efficacies of Cin, Tys, and Tyn were comparable to those of Crn, Lim, and Mnt at a concentration of 100 μL/mL. For the components Lim, Mnt, and Crn, no significant difference was found between their concentrations. The results demonstrate that they were less effective EO components. The mean IZ value for ethanol at a concentration corresponding to 100 μL/mL was 7.23 mm, while the mean IZ value for other concentrations was 6 mm.

It was observed that the bacterium *Pa* exhibited extreme sensitivity to the components Cin (100–50 μL/mL), Tyn (100–75 μL/mL), and Tys (100–50 μL/mL). The bacterium demonstrated sensitivity to Crl at a concentration of 100 μL/mL but exhibited an insensitive response at other concentrations. Furthermore, the *Pa* strain demonstrated an insensitive response to Mnt and Lim at all concentrations.

In the MIC and MBC tests (Table 2), the lowest concentration values and, therefore, the greatest efficacy were recorded for Cin. The second most effective component was Crn, which achieved identical MIC and MBC values (2.5 μL/mL). In contrast, R-(+)limonene was the least effective. Synthetic thymol and natural thymol appeared to be moderately effective. The MIC and MBC values for both were identical at 5 μL/mL.

3.2. Potato tuber disc maceration assay

3.2.1. Pectobacterium carotovorum subsp. carotovorum

The most effective components against *Pcc* on potato discs were Cin at MBC (Fig. 1) and Mnt at MBC and MIC. No symptoms of bacterial disease were observed at any point up to 72 h after inoculation. (Table 3). Furthermore, the efficacy of Tyn (MBC) and Crl (MBC and MIC) was observed to be higher at both time points. Forty-eight hours after inoculation, the area of rotting tissue was found to exceed that of the bacterial inoculum (211.6 mm^2) for two components, namely Tys at ¼ MIC and Crl at ¼ MIC. Although Crl (MBC and MIC) was one of the more effective components, Crl at ¼ MIC was, in contrast, the least effective of all the component

Table 1
Disc diffusion method, minimum inhibitory concentration, and minimum bactericidal concentration for Pectobacterium carotovorum subsp. carotovorum.

Essential oil component	Mean inhibition zones (mm) [a,b]				MIC [c] (μL/mL)	MBC [d] (μL/mL)
	Concentration (μL/mL)					
	100	75	50	25		
carvacrol	19.0^{c}	14.25^{de}	11.63^{efgh}	8.42^{ijklm}	5	10
cinnamaldehyde	30.5^{a}	27.08^{b}	21.29^{c}	12.83^{defg}	<0.5	1.5
D-carvone	13.0^{def}	12.21^{efg}	9.04^{hijkl}	8.0^{jklm}	1.5	2.5
L-menthone	12.42^{defg}	11.71^{efg}	8.6^{ijklm}	6.0^{m}	2.5	10
R-(+)limonene	10.42^{fghij}	8.08^{jklm}	6.67^{lm}	6.0^{m}	10	25
natural thymol	20.58^{c}	14.92^{d}	10.29^{ghijk}	7.75^{klm}	3	5
synthetic thymol	20.88^{c}	12.29^{defg}	10.79^{fghi}	6.21^{m}	2.5	3

[a])Means of inhibition zones followed by different superscripts (a–m) indicate significant differences ($P \leq 0.05$) based on Tukey's HSD test.
[b])Inhibition diameter stated in mm, including 6 mm disc diameter. Testing was done in triplicate and included a growth control (discs with sterile distilled water) and ethanol.
[c])MIC – minimum inhibitory concentration.
[d])MBC – minimum bactericidal concentration.

Table 2
Disc diffusion method, minimum inhibitory concentration, and minimum bactericidal concentration for Pectobacterium atrosepticum.

Essential oil component	Mean inhibition zones (mm) [a,b]				MIC [c] (μL/mL)	MBC [d] (μL/mL)
	Concentration (μL/mL)					
	100	75	50	25		
carvacrol	18.96^{def}	7.08^{hi}	6.42^{i}	6.29^{i}	10	25
cinnamaldehyde	34.42^{a}	30.92^{abc}	23.67^{cde}	9.67^{ghi}	<0.5	1.5
D-carvone	9.67^{ghi}	8.96^{hi}	8.29^{hi}	6.83^{hi}	2.5	2.5
L-menthone	7.79^{hi}	7.08^{hi}	6.42^{i}	6.29^{i}	2.5	25
R-(+)limonene	7.88^{hi}	6.79^{hi}	6.20^{i}	6.13^{i}	10	25
natural thymol	35.0^{a}	25.82^{bcd}	16.78^{efg}	14.38^{fgh}	5	5
synthetic thymol	32.67^{ab}	28.83^{abc}	20.58^{def}	9.79^{ghi}	5	5

[a])Means of inhibition zones followed by different superscripts (a–i) indicate significant differences ($P \leq 0.05$) based on Tukey's HSD test.
[b])Inhibition diameter stated in mm, including 6 mm disc diameter. Testing was done in triplicate and included a growth control (discs with sterile distilled water) and ethanol.
[c])MIC – minimum inhibitory concentration.
[d])MBC – minimum bactericidal concentration.

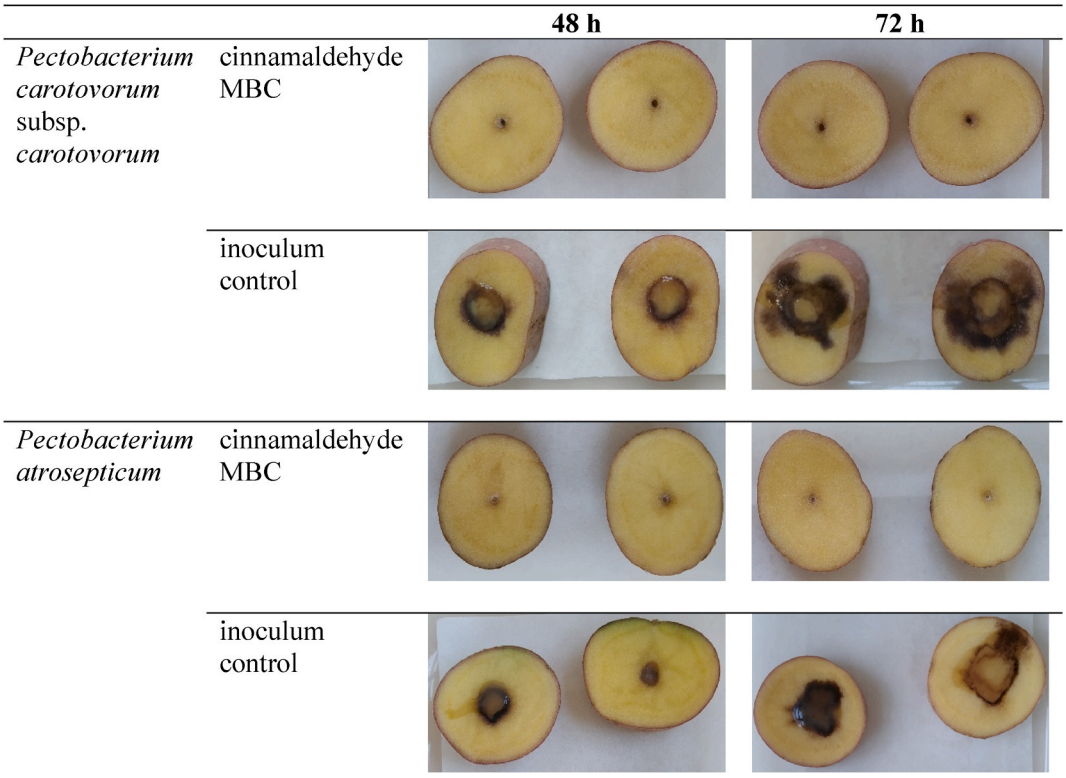

Fig. 1. Potato disc maceration assay for cinnamaldehyde and bacterial inoculum controls. Cinnamaldehyde MBC was at a concentration of 1.5 μL/mL. Bacterial inoculum controls were at a density of 1 × 108 CFU/mL. A depression was made in the centre of each disc using a sterile metal rod, and 3.5 μL of the mixed sample of component and inoculum was pipetted onto the disc. At 48 and 72 h after inoculation, the size of the rot patch was measured longitudinally and transversely, and the rotting area was calculated.

concentrations tested. Seventy-two hours after inoculation, Tys at ¼ MIC exhibited a larger rotting area than the positive control (bacterial inoculum).

3.2.2. Pectobacterium atrosepticum

Cinnamaldehyde at MBC was the only EO component to demonstrate 100 % efficacy against *Pa* even after 72 h (Table 4, Fig. 1). A higher efficacy was observed for Crl (MIC), Crn (MBC and MIC), Mnt (MBC and MIC), Tyn (MBC and MIC), and Tys (MBC and MIC) at both time points. The antibacterial activity of natural and synthetic thymol was comparable, with both displaying the same MBC and MIC value (5 μL/mL). After 48 h, the rotting area observed for Crn at ¼ MIC was greater than that observed for the positive control

Table 3
Potato tuber disc maceration assay for Pectobacterium carotovorum subsp. carotovorum.

Essential oil component	Mean of rotting area (mm^2)[a)]	Mean of rotting area (mm^2)[a)]
	48 h	72 h
carvacrol MBC[b)]	26.69^{ab}	40.62^{ab}
carvacrol MIC[c)]	11.71^{a}	18.58^{a}
carvacrol ¼ MIC[d)]	302.09^{h}	473.75^{ef}
cinnamaldehyde MBC	0.00^{a}	0.00^{a}
cinnamaldehyde MIC	96.49^{bcd}	175.64^{b}
cinnamaldehyde ¼ MIC	152.55^{def}	409.44^{de}
D-carvone MBC	20.28^{ab}	56.78^{b}
D-carvone MIC	112.91^{cde}	324.53^{cd}
D-carvone ¼ MIC	138.36^{cdef}	332.77^{cd}
L-menthone MBC	0.00^{a}	0.00^{a}
L-menthone MIC	0.00^{a}	0.00^{a}
L-menthone ¼ MIC	168.19^{defg}	293.59^{c}
natural thymol MBC	11.12^{a}	12.17^{a}
natural thymol MIC	61.23^{bc}	265.92^{bc}
natural thymol ¼ MIC	182.12^{efg}	329.70^{cd}
synthetic thymol MBC	141.63^{def}	307.72^{cd}
synthetic thymol MIC	205.08^{fg}	453.14^{ef}
synthetic thymol ¼ MIC	233.08^{gh}	538.25^{f}
Pcc inoculum control[e)]	211.56^{fg}	512.28^{ef}

[a])Means of rotting area followed by different superscripts (a–h) for evaluation after 48 h after inoculation and (a–f) for evaluation after 72 h after inoculation indicate significant differences ($P \leq 0.05$) based on Tukey's HSD test.
[b])MBC – minimum bactericidal concentration.
[c])MIC – minimum inhibitory concentration.
[d])¼ MIC – ¼ minimum inhibitory concentration.
[e])*Pcc* – *Pectobacterium carotovorum* subsp. *carotovorum*.

Table 4
Potato tuber disc maceration assay for Pectobacterium atrosepticum.

Essential oil component	Mean of rotting area (mm^2)[a)]	Mean of rotting area (mm^2)[a)]
	48 h	72 h
carvacrol MBC[b)]	12.36^{b}	50.04^{b}
carvacrol MIC[c)]	1.00^{ab}	3.93^{a}
carvacrol ¼ MIC[d)]	67.12^{e}	159.49^{c}
cinnamaldehyde MBC	0.00^{a}	0.00^{a}
cinnamaldehyde MIC	33.04^{c}	184.54^{cd}
cinnamaldehyde ¼ MIC	53.64^{d}	203.38^{cde}
D-carvon MBC	0.15^{a}	2.42^{a}
D-carvon MIC	0.34^{ab}	3.99^{a}
D-carvon ¼ MIC	164.92^{g}	310.60^{f}
L-menthone MBC	1.18^{ab}	7.07^{ab}
L-menthone MIC	5.76^{ab}	28.00^{ab}
L-menthone ¼ MIC	73.33^{e}	223.73^{de}
natural thymol MBC	3.58^{ab}	8.31^{ab}
natural thymol MIC	4.64^{ab}	9.29^{ab}
natural thymol ¼ MIC	69.87^{e}	234.42^{e}
synthetic thymol MBC	3.27^{ab}	7.85^{ab}
synthetic thymol MIC	3.93^{ab}	8.24^{ab}
synthetic thymol ¼ MIC	69.87^{e}	199.65^{cde}
Pa inoculum control[e)]	96.10^{f}	181.01^{cd}

[a])Means of rotting area followed by different superscripts (a–g) for evaluation after 48 h after inoculation and (a–f) for evaluation after 72 h after inoculation indicate significant differences ($P \leq 0.05$) based on Tukey's HSD test.
[b])MBC – minimum bactericidal concentration.
[c])MIC – minimum inhibitory concentration.
[d])¼ MIC – ¼ minimum inhibitory concentration.
[e])*Pa* – *Pectobacterium atrosepticum*.

(bacterial inoculum). After 72 h, the value of the positive control was surpassed by the values of Cin at MIC and all EO components at a concentration of ¼ MIC, except Crl. The least effective concentration against *Pa* in this test was D-carvone at ¼ MIC.

3.3. Efficacy of EO components against soft rot on potato tubers

3.3.1. Pectobacterium carotovorum subsp. carotovorum

The antibacterial activity of the selected EO components was demonstrated at both temperatures, with a significant difference in disease severity between the observed tested components (4.8–31.9 %) and the bacterial inoculum control (56.4–69.3 %) (Table 5). At 15 °C, no significant differences were found between the individual components. At 22 °C, there were significant differences in disease severity between the Cin, Mnt, and Crl components. The smallest area of rot, with a maximum value of 6.7 %, was observed in the case of Cin. L-menthone showed comparable values for the rotting area in both tests (Fig. 2).

3.3.2. Pectobacterium atrosepticum

In the *Pa* bacterial test, there were also significant differences in disease severity between the EO component treatments and the inoculum control (Table 6). Nevertheless, no significant differences were observed between the components at either 15 °C or 22 °C. The smallest area of rot was measured at Mnt (Fig. 2).

4. Discussion

The bioactivity potential of a given essential oil is directly related to the quality and quantity of its chemical components. The contents of individual components in EOs are variable and dependent on a wide range of factors [21]. There is evidence that seemingly minor components play a critical role in biological activities, possibly by producing synergistic effects with other components. Phenols (thymol, carvacrol) play a prominent role. These terpene phenols combine with amine and hydroxylamine groups of proteins in the bacterial membrane, altering the membrane permeability and leading to bacterial death [35,36]. Some activity of an EO could also be directly attributed to one or more specific compounds within the EO [37]. It can be reasonably assumed that the results of tests against pathogens conducted with EO components of defined purity will be of greater explanatory relevance. However, using of purified components would probably be more expensive in practice than using essential oils.

Our results show that DDM was less sensitive than the MIC assay using resazurin sodium salt in a MHB liquid medium for both bacteria. For example, L-menthone and Lim were shown by DDM to be ineffective against *Pa* at a concentration of 100 μL/mL, but Mnt subsequently had MIC of 2.5 μL/mL and Lim MIC of 10 μL/mL. Mann and Markham [33] found that measuring the MIC using the resazurin method gave slightly lower MICs than agar dilution.

Previous studies have mainly concentrated on the efficacy of essential oils against *Pectobacterium* bacteria. However, studies on the individual components of EOs have mostly been carried out on human or foodborne bacterial pathogens.

Our *in vitro* tests identified Cin as the most effective against both bacterial strains of the genus *Pectobacterium*. Its MIC (<0.5 μL/mL) and MBC (1.5 μL/mL) levels were the lowest of all components tested. Abdelrasoul [38] used essential oil components as nanoemulsions to protect against *Pcc* biologically. The antibacterial activity was significantly enhanced by converting pure monoterpenes into nanoemulsions. The cinnamaldehyde nanoemulsion showed the highest inhibition (¼ MIC 60 and 100 mg/L) against *Pcc* significantly. Chahbi [39] reported that the population of *Salmonella enteritidis* was reduced by 0.05 % MIC.

For the components Tyn, Tys, and Crl, larger inhibition zone values were measured at a concentration of 100 μL/mL (19–20.88 mm for *Pcc* and 18.96–35 mm for *Pa*), and for the MIC and MBC assays, concentrations reached MIC 2.5–5 μL/mL and MBC 3–10 μL/mL for *Pcc* and MIC 5–10 μL/mL and MBC 5–25 μL/mL for *Pa*). Eftekhari [40] found that 200 ppm of thymol and carvacrol completely inhibited the growth of 0.1–0.2 optical density *Pcc*. In addition, the maximum inhibition of growth of 2 optical density Pcc by thymol and carvacrol was observed at 300 and 400 ppm, respectively. Burt [13] reported carvacrol activity against *Escherichia* coli in the MIC range of 0.225–5 μL/mL and thymol activity in the MIC range of 0.225–0.45 μL/mL. Cacciatore [41] had found carvacrol to be effective against *E. coli* at MIC of 0.6 mg/mL and MBC of 1.25 mg/mL. Wang [42] determined that thymol (128 μg/mL) and carvacrol (256 μg/mL) had the best inhibitory activities against *Salmonella enteritidis*. Jiang [34] reported a MIC value of 0.1 mg/mL f and a MBC value of 0.2 mg/mL for carvacrol against *Dickeya zeae*. Al-Mariri [43] found the most effective components against the Gram-negative bacteria to be thymol (MIC 0.375–1.5 μL/mL), Crl (MIC 0.375–6.25 μL/mL), and Crn (MIC 3.125–25 μL/mL). Kapp [44] also confirmed a significant effect of carvone against *E. coli* but pointed out that limonene showed no antimicrobial activity. Rasoul [45] found MIC values for thymol at 2000 mg/L, (R)-carvone at 5000 mg/L, and (S)-limonene at 5000 mg/L in their tests with *P. carotovorum*. Eftekhari [40] found that different concentrations of S-carvone did not significantly affect bacterial pathogens compared to the control. According to the results of our study, Crn was one of the less effective components for both bacteria, as indicated by the DDM assay. On the other hand, the MIC value was 1.5 μL/mL, and the MBC value was 2.5 μL/mL for *Pcc*. For *Pa*, MIC and MBC were equal to 2.5 μL/mL. These values ranked Crn among the components with the most significant effect. The component with the lowest antibacterial activity was Lim.

The assumption that the Cin component significantly regulates the rotting area caused by pectinolytic bacteria was confirmed in the maceration test, but only at a concentration of 1.5 μL/mL (MBC). Synthetic thymol was one of the less effective components against *Pcc*. No significant difference was found between cinnamaldehyde at 0.5 μL/mL (MIC) and the *Pa* inoculum control. However, when testing the efficacy of EO components on potato tubers, Cin at MIC was able to regulate both *Pcc* and *Pa* bacterial activity effectively. In some cases, particularly at ¼ MIC concentrations, the rotting area after component treatment was even greater than that of the bacterial inoculum controls. For *Pa*, Crl at MBC produced a significantly greater rotting area than at the lower concentration

Table 5
Efficacy of essential oil components against Pectobacterium carotovorum subsp. carotovorum on potato tubers.

Essential oil component	Disease severity (%)[a)]	Disease severity (%)[a)]
	Room temperature[b)]	15 °C
carvacrol MIC[c)]	31.86[b]	7.96[a]
cinnamaldehyde MIC	6.65[a]	4.83[a]
L-menthone MIC	9.22[a]	9.79[a]
Pcc inoculum control[d)]	69.34[c]	56.40[b]

[a])Disease severity was expressed as a percentage of rotting area to healthy tuber area. Means of disease severity followed by different superscripts (a–c) for room temperature and (a–b) for 15 °C indicate significant differences ($P \leq 0.05$) based on Tukey's HSD test.
[b])Room temperature was 22 ± 2 °C.
[c])MIC – minimum inhibitory concentration.
[d])*Pcc* – *Pectobacterium carotovorum* subsp. *carotovorum*.

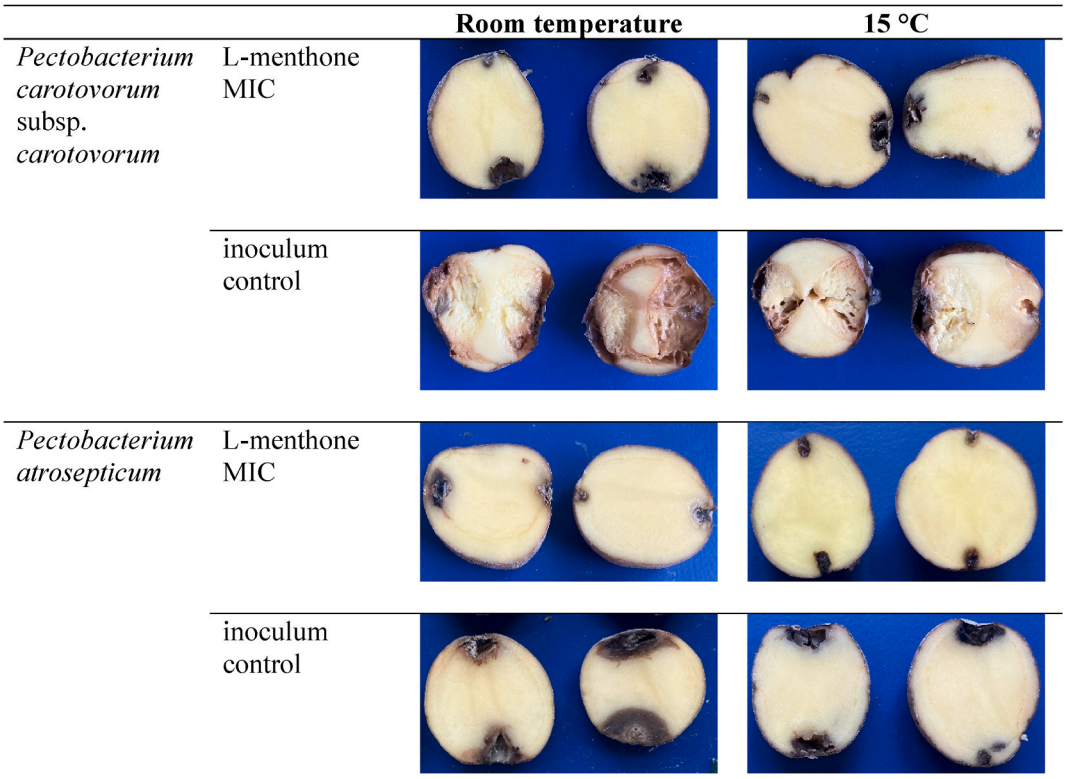

Fig. 2. Efficacy of L-menthone in the control of soft rot on potato tubers. L-menthone MIC was at a concentration of 2.5 μL/mL concentration. Bacterial inoculum controls were at a density of 1 × 108 CFU/mL. The wounded tubers were soaked in EO component solution for 20 min. Inoculation was performed by adding 15 μL of bacterial suspension to each tuber wound. The room temperature was 22 ± 2 °C. Six tubers were used per treatment, and the experiment was replicated twice, with a positive control for bacterial inoculum. After seven days, the tubers were cut along the longitudinal axis and across the inoculation site.

corresponding to the MIC. The possible phytotoxicity of some components on potato tissues or cells could explain this finding. At concentrations of components that are not lethal to the bacteria, the components promote the growth and multiplication of microbes on the damaged potato tuber structures. Queiroz [46] noted that the higher the concentrations of essential oils used to control phytopathogens, the more phytotoxicity they may cause, which may preclude their use as an alternative for plant disease control. Indeed, essential oils or some of their components could be used as active ingredients in producing natural herbicides. Koiou [47] concluded that EO components such as carvacrol, thyme, and carvone have significant phytotoxicity. Pinheiro [48] confirmed that the EO components thymol and carvacrol at a concentration of 0.12 % retarded or inhibited germination and growth in monocot and dicot species and caused changes in the cell cycle of *Lactuca sativa* meristematic cells. Carvacrol has also been shown to be genotoxic at a concentration of 3.0 mmol/L [49]. Araniti [50] postulated that thymol-induced phytotoxicity may be related to a combined osmotic and oxidative stress and increased abscisic acid content, reducing plant development.

Table 6
Efficacy of essential oil components against Pectobacterium atrosepticum on potato tubers.

Essential oil component	Disease severity (%)[a)]	Disease severity (%)[a)]
	Room temperature[b)]	15 °C
carvacrol MIC[c)]	15.96[a]	6.51[a]
cinnamaldehyde MIC	15.73[a]	8.10[a]
L-menthone MIC	8.48[a]	6.16[a]
Pa inoculum control[d)]	36.50[b]	17.58[b]

[a])Disease severity was expressed as a percentage of rotting area to healthy tuber area. Means of disease severity followed by different superscripts (a–b) for room temperature and (a–b) for 15 °C indicate significant differences ($P \leq 0.05$) based on Tukey's HSD test.
[b])Room temperature was 22 ± 2 °C.
[c])MIC – minimum inhibitory concentration.
[d])*Pa* – *Pectobacterium atrosepticum*.

5. Conclusion

All evaluated essential oil components showed antibacterial activity against pectinolytic bacteria causing bacterial blackleg and soft rot under laboratory conditions, both *in vitro* and *in vivo*. However, there were significant differences between the components. Our results show that the MIC assay using resazurin sodium salt in liquid medium MHB was more sensitive than the DDM assay.

Essential oil components are a promising option for biological control on potato tubers. Based on the results of the *in vivo* test, cinnamaldehyde, L-menthone, and carvacrol can be recommended for the preventive treatment of potato tubers against pectinolytic bacteria of the genus *Pectobacterium* under storage conditions. However, before their practical application, it is crucial to determine the concentrations of individual EO components that will effectively suppress bacteria without causing phytotoxicity.

CRediT authorship contribution statement

Jana Víchová: Writing – original draft, Validation, Supervision, Methodology, Investigation, Conceptualization, Data curation, Visualization, Writing – review & editing. **Barbora Jílková:** Writing – original draft, Validation, Supervision, Methodology, Investigation, Formal analysis, Conceptualization, Data curation, Funding acquisition, Project administration, Resources, Visualization, Writing – review & editing. **Markéta Michutová:** Investigation, Writing – review & editing. **Martin Kmoch:** Methodology, Funding acquisition, Conceptualization, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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