Species-specific antimicrobial activity of essential oils and enhancement by encapsulation in mesoporous silica nanoparticles.

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Abstract

Essential oils are volatile plant compounds that are biologically active and play an important role in natural plant protection. There are currently ~3000 essential oils known, of which over 300 are commercially important for a variety of industries. In fact, the essential oil global market has been estimated to reach 13.94 billion USD with a demand of over 370,000 tons by 2024. These compounds have a wide variety of applications in agriculture, food and beverage, cosmetics, medicine, amongst other industries. A promising application of essential oils is as antimicrobials to target the vast number of diseases affecting crops. However, their volatile nature limits their effective use as free agents. To overcome this, we have investigated the use of mesoporous silica nanoparticles to protect essential oils from evaporation and degradation and to enhance their antimicrobial activity against bacterial phytopathogens. Silica nanoparticles were used due to their potential to be produced at an industrial scale and their biocompatibility. As a proof of concept, we evaluated 41 essential oils against bacterial phytopathogen *Pseudomonas syringae* pv. *pisi,* causative agent of pea bacterial blight. Additionally, we compared the effect of such essential oils against *Pectobacterium carotovorum* subsp. *carotovorum* and *Pseudomonas fluorescens.* Two of the most effective antimicrobials, cinnamon (*Cinnamomum zeylanicum*) and mustard (*Brassica nigra*) oils, were able to inhibit bacterial growth after 24h at a concentration as low as 0.016% (v/v). Besides efficacy, the species-specificity of essential oils was demonstrated with >67% of oils tested displaying specificity towards pathogenic *P. syringae* pv. *pisi* over non-pathogenic *Pseudomonas fluorescens.* Furthermore, the encapsulation of essential oils into

mesoporous silica nanoparticles (MSNPs) as a means of extending and improving their antimicrobial effect was found to enable a 10-fold increase in potency compared to the free essential oil. Cinnamaldehyde immobilised onto MSNPs proved to be the most effective antimicrobial, eliminating >99.8%, >99.9%, and >95% bacterial growth of *P. fluorescens, P. syringae* pv. *pisi* and *P. carotovorum* subsp. *carotovorum,* respectively. This system has the potential to be used to treat and prevent bacterial infections in crops and to enable a more controlled and effective exploitation of volatile compounds as antimicrobials.

Keywords

Essential oils, crop protection, bacterial phytopathogens, antimicrobials, mesoporous silica nanoparticles

1. Introduction

Essential oils (EOs) are naturally occurring odorous, volatile, oily liquids that are biologically active and produced by secondary metabolism in aromatic plants. They comprise complex combinations of different volatile compounds (Bajpai et al., 2011; Yap et al., 2014) and are widely used in industries such as foods, perfumes and pharmaceuticals; many being classified as GRAS (Generally Regarded as Safe) and appearing in the EAFUS list (Everything Added to Food in the US). These compounds can be used to substitute chemicals or antibiotics since they are safe to use and have low toxicity, fewer effects on the environment, and most importantly, known antimicrobial properties (Bajpai et al., 2011). Currently, the treatment of bacterial diseases in crops is

mainly restricted to two antibiotics; streptomycin and oxytetracycline, or copper products. However, their use is tightly regulated due to the emergence of bacterial resistance together with negative environmental and health impacts. Essential oils are, therefore, promising candidates for alternative agents.

A wide range of essential oils have been reported to possess antimicrobial activity against a number of bacterial species, including a range of *Pseudomonas* (Amlai Todi Poswal and Witbooi, 1997; Curtis et al., 2004; de Sousa et al., 2013; Iacobellis et al., 2005; Kavanaugh and Ribbeck, 2012; Kokoskova et al., 2011; Lo Cantore et al., 2004; Tyagi and Malik, 2010; Verma and Agrawal, 2015) and *Pectobacterium* species (Al-Ani et al., 2012; Badawy and Abdelgaleil, 2014; Curtis et al., 2004; Iacobellis et al., 2005; Lo Cantore et al., 2004; Ngadze et al., 2012). Herein we aimed to directly compare the inhibitory effects of 41 essential oils against the agriculturally important pathogens *Pseudomonas syringae* pv. *pisi* and *Pectobacterium carotovorum* subsp. *carotovorum* (previously *Erwinia carotovora* pv. *carotovora*), and also against the ubiquitous organism *Pseudomonas fluorescens.*

P. syringae pv. *pisi* is a seed-borne and seed-transmitted bacterial pathogen that causes pea bacterial blight. It was first recorded in the United States in 1915 (Sackett, 1916) and has since been reported to occur in most pea growing areas worldwide, causing devastating effects that reduce yield and seed quality. *P. carotovorum* subsp. *carotovorum* causes soft rot of tubers and storage organs of a variety of plants such as potato, tobacco, cabbage, and peppers, amongst others, by secreting enzymes responsible for plant cell wall degradation (Lim et al., 2013). *P. fluorescens* is generally non-pathogenic and a ubiquitous organism that can thrive on soil, plants and aqueous surfaces and is used in

84 industrial and commercial sectors. The strain used in this study (biovar I) is not toxic to any
85 aquatic or terrestrial organisms and has low environmental and human health hazards
86 (Government of Canada, 2015).

87 Despite the potential antimicrobial activity of essential oils and their use as free agents in
88 agriculture and other areas, application can be hindered by their inherent characteristics,
89 in particular their high volatility, susceptibility to degradation in aqueous conditions and
90 hydrophobicity. To overcome these drawbacks, we examined the effectiveness of a novel
91 approach whereby the essential oils were encapsulated in nanoparticles, so providing
92 protection and preventing volatilisation of the oils, whilst improving their stability, long-
93 term effects, and immiscibility in aqueous solutions. EOs have been previously
94 encapsulated into different materials including polymeric particles, liposomes and solid
95 lipid NPs, for a detailed review about EO encapsulation see (Asbahani et al., 2015).

96 Mesoporous silica nanoparticles (MSNPs) are potential candidates for essential oil
97 encapsulation due to their porous structure, chemical stability, biocompatibility (Slowing
98 et al., 2008), tuneable pore size and porosity (Trewyn et al., 2007), simple and low-cost
99 synthesis (Kwon et al., 2013), and potential scale-up for industrial use. Additionally, silica
100 is biologically inert and has the ability to decompose into relatively harmless silicic acid by-
101 products (Diaconu et al., 2010), making it a useful material for biocide delivery
102 applications in agriculture.

103 An additional advantage of MSNPs is that they can be capped to further protect the
104 encapsulated EOs against degradation or evaporation so enabling a controlled and

prolonged release of the antimicrobial. Carbohydrates are promising candidates for nanoparticle functionalisation since they have a chemically well-defined structure, are biocompatible and biodegradable, are available on a large scale, are protein-repellent and highly water soluble, do not aggregate and are natural targeting agents (Biao Kang et al., 2015). We therefore evaluated a lactose derivative (a sugar molecule attached to 3-aminopropyltriethoxysilane, APTES) as a capping agent to gate the MSNPs pores thus preventing the premature release of the EOs whilst enhancing their antimicrobial activity. Lactose was selected since it can be assimilated by a broad range of bacteria. Also, its chemical properties make it amenable to the capping technology employed in this study.

The objectives of this study therefore were (i) to determine the specificity and effectivity of 41 essential oils (EOs) against the seed-borne pathogen *P. syringae* pv *pisi* in comparison to *Pectobacterium carotovorum* and *Pseudomonas fluorescens*; (ii) to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for the most effective oils; (iii) to load synthesised MSNPs with the selected EOs, and (iv) to assess the antimicrobial efficiency of EO-loaded lactose-capped and uncapped MSNPs *in vitro* against *P. syringae* pv. *pisi*, *P. carotovorum* and *P. fluorescens*.

2. Materials and Methods

2.1 Test Microorganisms and Growth Conditions

Pseudomonas fluorescens NCPPB 1964 and *Pectobacterium carotovorum* subsp. *carotovorum* NCPPB 1274 were obtained from the National Collection of Plant Pathogenic

Bacteria (NCPPB), UK. *Pseudomonas syringae* pv. *pisi* race 2 strain 203 (NCPPB 2585) was obtained from the Department of Plant Sciences, University of Oxford, UK. Bacterial stocks were prepared with 80% glycerol to a final glycerol concentration of 32% and maintained at -80°C in 2 ml Cryotubes. Test microorganism cultures were prepared from glycerol stocks, streaking onto Mueller-Hinton Agar (MHA; Sigma Aldrich, UK) plates for antimicrobial tests and onto Luria-Bertani Agar (LBA; Sigma Aldrich, UK) plates for all other tests. Plates were incubated overnight at 28°C before inoculating 25 ml of Mueller-Hinton Broth (MHB; Oxoid Ltd, UK) for antimicrobial tests or Luria-Bertani Broth (LB; Sigma Aldrich, UK) for all other tests with one colony and incubating at 28°C and 220 rpm overnight. Overnight cultures were used to inoculate 25 ml of broth in 50 ml Falcon tubes, which were incubated until mid-exponential phase was achieved. Bacterial cells were harvested by centrifugation at 4000 rpm and 10°C for 15 minutes and washed twice with phosphate-buffered saline (PBS; Sigma-Aldrich, UK). Turbidity was adjusted to 0.5 McFarland Standard (OD_{600}=0.132) to achieve a bacterial density of ~10^8 CFU/ml.

Escherichia coli NCIMB 8879 and *Pseudomonas aeruginosa* NCIMB 950 were used during the *in vitro* tests of loaded nanoparticles to compare results with the previous three bacterial species and were cultured as described with incubation parameters of 37°C and 150 rpm.

2.2 Natural Biocides – Essential Oils

All essential oils (100%) used are commercially available and were used as purchased. Caraway and garlic essential oils were obtained from G. Baldwin & Co (UK); mustard,

147 turmeric and ajwain essential oils were obtained from Herbalveda (UK). The remaining 29
148 essential oils were obtained from Oils4Life (UK).

149 **2.3 Antimicrobial Susceptibility Tests (ASTs) - Disk Diffusion Assay**

150 The disk diffusion assay was performed as described by the European Committee on
151 Antimicrobial Susceptibility Testing (EUCAST, 2017) with a few modifications, to evaluate
152 the antimicrobial activity of 41 essential oils against *P. syringae* pv. *pisi*, *P. fluorescens* and
153 *P. carotovorum*. Bacterial suspensions (OD_{600}=0.132) were homogenously streaked onto
154 MHA plates using sterile cotton swabs. Whatman antibiotic assay discs (6mm diameter;
155 GE Healthcare, UK) were saturated with 10 µl of each essential oil (100%) and placed on
156 the agar surface using sterile tweezers. Plates were left at room temperature for 15
157 minutes before incubating at 30°C (28°C for *P. syringae* pv. *pisi*) overnight. Streptomycin
158 sulphate salt (Sigma-Aldrich, UK) was used as a positive control at concentrations of 1
159 mg/ml and 10 mg/ml (a calibration curve on the effect of streptomycin at increasing
160 concentrations on *P. syringae* pv. *pisi* was obtained using the disk diffusion assay) and
161 sterile water and empty disks were used as negative controls. Inhibition zones were
162 measured to the nearest millimetre after 24h of incubation. Essential oils with the largest
163 inhibition zones as well as oils with specificity for pathogenic strains were selected for
164 further testing. All tests were performed in triplicate.

165 **2.4 Antimicrobial Susceptibility Tests (ASTs) - Broth Microdilution Test**

166 The broth microdilution test was carried out to study the effect of varying concentrations
167 of twelve selected essential oils on phytopathogenic bacteria *P. syringae* pv. *pisi*. A two-

168 fold dilution series of essential oils ranging from 1% to 0.016% was prepared by dissolving
169 the essential oils in ethanol to increase solubility at a ratio of 1:3 (for the highest essential
170 oil concentration, 1%, the concentration of ethanol was 3%) and then further dissolving
171 with MHB containing 0.05% (w/v) 2,3,5-Triphenyltetrazolium chloride (TTC; Sigma Aldrich,
172 UK) as a growth indicator. One hundred microlitres of essential oil dilutions was added
173 into 96-well plates (Corning Incorporated, USA) and each well was inoculated with 100 µl
174 of bacterial suspension (OD_{600}=0.132). Three microlitres of bacterial suspension (diluted
175 1/20 in sterile water) was plated in MHA and incubated at 28°C for 48h. Streptomycin
176 sulphate salt (Sigma-Aldrich, UK) was used as a positive control at 1 mg/ml and 10 mg/ml
177 concentrations, and MHB as negative control.

178 Absorbance (OD_{600}) was measured at t=0h and plates were incubated at 28°C. Absorbance
179 was measured at 20, 24 and 48h using an Infinite M200 TECAN plate reader. Three
180 microlitres from each clear well was plated into MHA and incubated at 28°C for 48h. All
181 tests were performed in triplicate. MIC and MBC were obtained from this test.

182 **2.5 Synthesis of MSNPs**

183 Mesoporous silica nanoparticles (MSNPs) were synthesised using a sol-gel hot aqueous
184 solution as previously described (Hom et al., 2010) with some modifications. Briefly, 100
185 mg cetyltrimethylammonium bromide (CTAB, 99%; Sigma Aldrich, UK) was dissolved in 48
186 mL deionised distilled water and 350 µl 2M NaOH (Fisher Scientific, UK) in a 250 mL round-
187 bottom flask, stirring at 500 rpm. The solution was heated to 80°C and after temperature
188 stabilisation, 500 µl tetraethylorthosilicate (TEOS; Sigma Aldrich, UK) was added. After

189 stirring for 2h at 80°C, nanoparticles were washed twice with methanol (99+%, Acros
190 Organics, UK). To ensure that the resulting particles could be loaded with hydrophobic
191 molecules, the surfactant CTAB was subsequently removed by distillation by refluxing
192 overnight in acidic methanol (20 mL methanol, 1 mL 37% hydrochloric acid) at 80°C.
193 Particles were dried in a vacuum dessicator and collected. These nanoparticles have been
194 previously characterised by our group and determined to have a diameter of ~100 nm
195 with pores of ~2-2.8 nm in diameter (for full characterization details see Huang et al.,
196 2014).

197 **2.6 Loading of Essential Oils into MSNPs**

198 Cinnamaldehyde (CNAD, 1.5 ml), allyl isothiocyanate (AIT, 750 µl) and Ajwain oil (750 µl)
199 were dissolved in dimethyl sulfoxide (DMSO, Sigma Aldrich, UK) to a total volume of 6 ml.
200 The mixture was added to 21 ml PBS with 60 mg MSNPs and sonicated with a probe
201 (Vibra-Cell VCX 130, Sonics & Materials Inc., USA) for 3 minutes at 5 second intervals,
202 before stirring at 250 rpm for 24h at room temperature.

203 After the MSNPs had been loaded with EO, they were capped with a lactose derivative
204 synthesised as described by (Bernardos et al., 2009) with a few modifications. Briefly, 5.85
205 ml of 25mmol 3-aminopropyltriethoxysilane (APTES, Sigma Aldrich, UK) was added to a
206 suspension of 5.4 g D-lactose monohydrate (Sigma Aldrich, UK) in ethanol (final volume of
207 250 ml). The mixture was stirred at 500 rpm and room temperature for 24h before being
208 heated at 60°C for 30 minutes. The solution was transferred to falcon tubes and
209 centrifuged at 8,500 rom for 8 minutes. The supernatant was discarded and the pellet was

dried in a vacuum dessicator overnight. The lactose alkoxysilane derivative was grounded with a mortar and pestle to a fine powder and collected.

Loaded MSNPs were centrifuged at 8,500 rpm for 7 minutes and the pellet was resuspended in 60 ml DW (empty MSNPs were used as control). The as-synthesised lactose derivative (1.5 g) was dissolved in 30 ml DW, added to the MSNPs solution and stirred for 5h. After stirring, the solution was centrifuged and the pellet was washed with PBS and dried in the vacuum dessicator overnight.

2.7 Effect of Essential Oil Loaded MSNPs *in vitro*

To obtain a final nanoparticle concentration of 2 mg/ml (concentration used during our previous studies (Chan et al., 2017) and shown to be effective as antimicrobial), 10 mg of each MSNPs treatment (Table 1) was added to 5 ml of bacterial culture. All treatments were incubated at 28°C and 220 rpm for 24 h. The Miles and Misra method (Miles et al., 1938) was performed to determine the effect of each treatment against the bacterial strains by plating 20 µl of serial dilutions (10^{-4} to 10^{-7}) in triplicate onto LBA plates, which were incubated at 28°C for 24 h. Serial dilutions were prepared using PBS and calibrated pipettes. Incubation parameters for *E. coli* and *P. aeruginosa* were 37°C and 150 rpm.

2.8 Fourier-Transform Infrared Spectroscopy (FT-IR)

Infrared spectroscopy was carried out using an Agilent Digilab Excalibur Fourier Transform Spectrometer. A permanently aligned attenuated total internal reflection sampling attachment (Golden Gate ATR with diamond window) was used to measure the IR spectrum of the samples directly with no sample preparation. The samples; lactose

231 derivative, AIT, CNAD and the combination of AIT or CNAD with the lactose derivative,
232 were placed directly on the diamond window and the spectra ratioed to the blank
233 diamond.

234 **2.9 Liquid Chromatography**

235 To determine the amount of CNAD released from uncapped and lactose-capped MSNPs,
236 10 mg of loaded MSNPs were added to 5 ml PBS and incubated at 30°C and 200 rpm
237 during 48h. A sample of 200 µl was analysed every hour *via* liquid chromatography (LC)
238 using an Agilent Technologies 1120 Compact LC, equipped with a Zorbax Eclipse Plus C18
239 column (Agilent Technologies, UK). Samples were analysed using an isocratic ratio 80:20
240 (acetonitrile:H_2O), UV detector (λ = 270 nm), flow of 1 ml/min and an injection volume of
241 10 µl over 4 minutes.

242 **2.10 Statistical Analyses**

243 Statistical analyses were performed using Microsoft Excel and Minitab® version 18.1. To
244 determine statistically significant differences between samples, two-factor analysis of
245 variance (ANOVA) and t-tests followed by Tukey's HSD (honest significant difference) test
246 were applied when appropriate. *P* values of 0.05 and 0.01 were used and are specified for
247 each analysis.

248 **3. Results**

249 **3.1 Antimicrobial activity and selectivity of essential oils against phytopathogens**

250 Conventional Treatment.

To be able to determine the efficacy of the EOs in comparison to currently used industry standards, we first assessed the effect of streptomycin against our test microorganism, *P. syringae* pv. *pisi* race 2 strain 203 (NCPPB 2585). A logarithmic increase in antimicrobial activity was observed, reaching maximum inhibition at ~10 mg/ml (Figure 1). The use of higher concentrations of antibiotic did not result in greater inhibition of bacterial growth. Therefore, concentrations of 1 mg/ml and 10 mg/ml streptomycin sulphate salt were selected as positive controls for subsequent experiments.

Essential Oils as Antimicrobials.

To assess whether natural biocides could compare to the conventional antimicrobials used in agriculture, the activity of 41 EOs was investigated (Figure 2). Mustard and cinnamon oils showed greater antimicrobial activity than a high dose of streptomycin (10 mg/ml) against all three bacterial species, while oregano, ajwain and thyme oils also displayed high antimicrobial activity. For *P. syringae* pv. *pisi,* the antimicrobial effect of mustard (31.67±2.89 mm inhibition zone diameter including 6mm disc) was comparable to that of streptomycin at 10 mg/ml (30 mm); lemongrass (20.67±1.15 mm) also demonstrated high efficacy, similar to a lower dose (1 mg/ml) of the antibiotic (23±1 mm). Cinnamon (15.67±1.15 mm) and oregano (13.33±0.58 mm) also produced large inhibition zones compared to other EOs tested. The 41 EOs were also tested against *P. fluorescens* NCPPB 1964 and the phytopathogen *P. carotovorum* subsp. *carotovorum* NCPPB 1274. For *P. fluorescens,* mustard (>90 mm) and cinnamon (25.33±1.53 mm) were more potent antimicrobials than 10 mg/ml of streptomycin (24.33±0.58 mm). Furthermore, *P. carotovorum* subsp. *carotovorum* was more susceptible to EOs than the other species

tested; particularly to mustard (>90 mm), cinnamon (39.33±2.31 mm), and ajwain (26.33±1.15 mm) which were more potent than 10 mg/ml of streptomycin (26±1 mm).

Specificity of Essential Oils.

The antimicrobial effect of the EOs observed was species-dependent. Highly antimicrobial EOs such as mustard, cinnamon, oregano or thyme produced significantly different inhibition zones ($p<0.01$) for the microorganisms tested (Figure 2) demonstrating the specificity of EOs and the difference in bacterial species susceptibility towards EOs. To further illustrate the specificity of EOs, Figure 3 represents the fold-change of the antimicrobial effect of EOs on pathogenic *P. syringae* pv. *pisi* compared to non-pathogenic *P. fluorescens*. Mustard oil, despite being a potent biocide more effectively inhibited growth in the non-pathogenic *P. fluorescens* rather than *P. syringae* pv. *pisi*. In contrast, lemongrass showed a strong specificity towards *P. syringae* pv. *pisi*. More than 67% of the EOs tested demonstrated specificity towards the pathogenic species over the non-pathogenic.

Eight EOs that resulted in the largest inhibition zones on the growth of *P. syringae* pv. *pisi* (mustard, lemongrass, cinnamon, oregano, marjoram, sage, ajwain and thyme) as well as the four EOs with a higher selectivity towards *P. syringae* pv. *pisi* over *P. fluorescens* (lavender, *Eucalyptus citrodora*, coriander and grapefruit; lemongrass had already been selected for its efficacy) were selected for further investigation to confirm the results obtained using the disk diffusion test by a different methodology.

293 The minimum inhibitory concentration (MIC; lowest EO concentration resulting in clear
294 wells) and the minimum bactericidal concentration (MBC, lowest EO concentration able to
295 kill 99.9% of microorganisms, calculated by plating the original culture and 3 µl from clear
296 wells) of EOs against *P. syringae* pv. *pisi* were obtained using the broth microdilution test
297 (Table 2), which was performed with and without 2,3,5-Triphenyltetrazolium chloride
298 (TTC) as a growth indicator. Streptomycin was used as positive control (1 mg/ml and 10
299 mg/ml) and culture media with and without ethanol 3% (v/v) were used as negative
300 controls.

301 Cinnamon, mustard, oregano, ajwain, thyme and lemongrass EOs were the most effective
302 of the compounds tested in terms of antimicrobial activity against *P. syringae* pv. *pisi* at
303 low concentrations, whilst the other oils tested were only able to inhibit growth at
304 concentrations of ≥0.25% at 24h and ≥1% at 48h (Table 2). The oils that demonstrated
305 specificity towards the phytopathogen required much greater concentrations to be
306 effective, with the exception of lemongrass (MIC 0.125% at 48 h) and coriander (MIC 1%
307 at 48 h).

308 **3.2 Effect of essential oil-loaded MSNPs *in vitro***

309 To determine if the inhibitory effect and the stability of EOs could be enhanced, three of
310 the most potent antimicrobials; cinnamaldehyde (CNAD, ~70% of cinnamon EO, solubility
311 in water: 1420 mg/L), allyl isothiocyanate (AIT, >90% of mustard EO, solubility in water:
312 2000 mg/L), and Ajwain EO (solubility in water of main component, thymol: 900 mg/L);
313 were encapsulated into mesoporous silica nanoparticles (MSNPs). CNAD and AIT were

used as we had previously reported their encapsulation (0.74 mg and 0.095 mg per mg of MSNPs, respectively) and demonstrated their efficacy against bacterial biofilms (Chan et al., 2017). The loading capacity of the MSNPs for ajwain EO was approximately 0.70 mg per mg of MSNPs.

EO-loaded MSNPs were added to bacterial cultures (OD_{600} = 0.132) to evaluate the antimicrobial effect of this system. Lactose-capped MSNPs and uncapped MSNPs were used to compare the efficacy of the system with and without a sugar capping. Bacterial cultures without the addition of MSNPs, empty nanoparticles and nanoparticles treated with DMSO (used during the encapsulation protocol) were employed as controls.

The effect of each treatment on three bacterial species is illustrated in Figure 4a. Controls treated with empty silica nanoparticles resulted in a bacterial growth increase of 222%, 25%, and 45% of *P. syringae* pv. *pisi, P. fluorescens,* and *P. carotovorum* subsp. *carotovorum*, respectively, compared to the untreated controls (pure bacterial culture without any nanoparticles added).

Uncapped AIT-loaded MSNPs decreased bacterial viability, demonstrating the effect of AIT as a potent biocide. However, uncapped CNAD- and Ajwain MSNPs did not induce any significant decrease in viability compared to the negative and empty MSNPs controls. Furthermore, lactose-capped AIT-MSNPs and lactose-capped Ajwain-MSNPs resulted in a bacterial increase, particularly of *P. carotovorum* subsp. *carotovorum*. Interestingly, lactose-capped CNAD-loaded MSNPs resulted in the elimination of >99.9%, >99.8%, and

334 >95% of *P. syringae* pv. *pisi, P. fluorescens* and *P. carotovorum* subsp. *carotovorum*,
335 respectively; compared to the results from non-capped CNAD-MSNPs.

336 To further confirm the efficacy of lactose-capped CNAD-loaded MSNPs, these
337 nanoparticles were tested against two additional bacterial species. Figure 4b illustrates
338 the effects of lactose-capped and uncapped AIT- and CNAD-loaded MSNPs on *Escherichia*
339 *coli* NCIMB 8879 and *Pseudomonas aeruginosa* NCIMB 950. The results demonstrate that
340 lactose-capped CNAD-loaded MSNPs reduced *E. coli* growth by >98% and *P. aeruginosa* by
341 >89% compared to the uncapped CNAD-MSNPs treated cultures.

342 To determine the mechanism underlying the heightened antimicrobial activity of the
343 lactose-capped CNAD-loaded MSNPs, FT-IR analysis of the compound was undertaken.
344 The FT-IR spectrum of the combination of CNAD with the lactose derivative used for
345 capping revealed a peak at 2362 cm^{-1} that was not present in the FT-IR spectra of the
346 individual components (Figure 5A). However, the FT-IR spectrum of lactose (without any
347 modifications) also showed this peak at 2360 cm^{-1} (Figure 5B). This suggested that the
348 lactose, originally attached to the MSNPs *via* APTES (3-aminopropyltriethoxysilane), was
349 released from the MSNPs and replaced by CNAD. The potential grafting of CNAD onto the
350 MSNPs surface though the APTES molecule would have immobilised the EO and increased
351 its antimicrobial potency. This was only observed for CNAD, and not for AIT or Ajwain EO,
352 since the binding of CNAD to APTES occurred through the aldehyde group naturally
353 present on CNAD which was absent on the other two essential oils tested.

To verify the grafting of CNAD onto the surface of MSNPs, the release profiles of CNAD from uncapped or lactose-capped MSNPs were evaluated and found to be consistent with the FT-IR findings. A total of 35.63 mg/L CNAD was readily released from uncapped MSNPs after 24h of incubation; with 49% of that amount being released only after one hour of incubation (Figure 6). In contrast, no CNAD was observed from lactose-MSNPs, even after 48h of incubation (Figure 6); suggesting that the immobilisation of the EO onto the nanoparticle surface protected the biocide from a fast release and volatilisation, enabling a longer-lasting presence of the antimicrobial. Assuming the concentration of CNAD released from uncapped MSNPs (35.63 mg/L) was the same as that grafted onto the surface of the capped-MSNPs, at least 35.63 µg/ml was available to reduce *P. syringae* pv. *pisi* growth by 99.99%. In contrast, 325.5 µg/ml of free cinnamon EO (0.031% v/v, Table 2) without nanoparticle encapsulation was needed to achieve a similar reduction in bacterial growth (99.9%). This demonstrates that the MSNP-encapsulated CNAD is ten-times more potent antimicrobial than the free essential oil.

4. Discussion

The aim of this study was to determine and compare the antimicrobial activity and bacterial species-specificity of a range of EOs against several bacterial phytopathogens and to determine the potential to enhance and prolong their kill potency by delivery *via* nanoparticle encapsulation and capping.

Forty one essential oils were tested against three bacterial species to determine their effectiveness as antimicrobials. Mustard oil was found to be the most potent; completely

inhibiting the growth of *P. carotovorum* subsp. *carotovorum* and *P. fluorescens* yet it failed to do so against *P. syringae* pv. *pisi.* It may be possible that specific genes were involved in this reduced sensitivity, as other *P. syringae* pathovars such as *P. syringae* pv. m*aculicola* and pv. *tomato* can overcome aliphatic isothiocyanate-based defences of *Arabidopsis* and elicit a disease outcome due to the presence of *sax* genes (Fan et al., 2011).

Only a few studies have reported the antimicrobial effects of a small number of EOs: garlic (Verma and Agrawal, 2015), cumin and caraway (Iacobellis et al., 2005), coriander and fennel (Lo Cantore et al., 2004), *Satureja hortensis* and *Calamintha nepeta* (Gormez et al., 2015), against *P. syringae* pv. *pisi.* As with this study, *P. syringae* pv. *pisi* appeared to be more resistant towards more essential oils than *P. carotovorum* subsp. *carotovorum.* In fact, Gormez et al (2012) demonstrated that *P. syringae* pv. *pisi* was the most resistant of the 20 microorganisms tested against *Nepeta nuda* EO (Gormez et al., 2012).

The results presented confirm the antimicrobial activity of essential oils and demonstrate their ability to solicit species-specific bactericidal effects. Mustard, cinnamon, oregano, ajwain and thyme showed a strong effect against all species tested but particularly *P. carotovorum* subsp. *carotovorum*, which was the most susceptible, with *P. fluorescens* being the most resistant. *P. fluorescens* is known to have a heightened ability to acquire resistance against antimicrobials, and previous studies have demonstrated its lower susceptibility to a number of EOs compared to other microorganisms (Mith et al., 2014; Sarac and Ugur, 2008).

Mustard oil had the lowest MIC at 24h, together with cinnamon oil, however, at 48h cinnamon oil still had a low MIC whilst mustard oil failed to prevent bacterial growth at the lower concentrations. This may have been due to the greater volatility of mustard oil when compared to that of cinnamon oil, as we concluded in an earlier study (Chan et al., 2017). Furthermore, previous studies have demonstrated that after 48h in water, allyl isothiocyanate (AIT) is degraded to three major products (diallylthiourea, diallylurea and diallyl disulphide), none of which inhibit bacterial growth, not even in combination with sub-lethal doses of AIT. This suggests that AIT may only be antimicrobial in its original form, before degradation (Luciano and Holley, 2009) and may also explain why the antimicrobial activity of AIT decreased after 48h of incubation.

Consequently, the high volatility and rapid degradation of essential oils could limit their exploitation potential but their encapsulation into MSNPs could increase the stabilisation of compounds and overcome this limitation. The main components of mustard and cinnamon oils; AIT and CNAD were selected for nanoparticle encapsulation due to their strong antimicrobial effect against all bacterial species tested. Additionally, ajwain oil was selected since it has a highly antimicrobial effect and has not been previously studied as extensively as mustard or cinnamon. Oregano and thyme oils were not selected since they have been extensively studied as antimicrobials and their effect was comparable to that of the selected oils.

The encapsulation of EOs into MSNPs was evaluated to enhance their antimicrobial activity. Interestingly, the adition of empty nanoparticles increased bacterial viability. This might have been due to the microorganisms utilising the silica or its by-products as a

417 nutrient source or to the MSNPs altering the environment and making other nutrients
418 more available (Umamaheswari et al., 2016; Wainwright et al., 1997). Other studies have
419 shown that bacteria and fungi can solubilise insoluble silicates (Duff et al., 1963) and that
420 silicon compounds can increase bacterial (Price, 1932; Umamaheswari et al., 2016) or
421 fungal growth (Wainwright et al., 1997). In agreement with these findings, our results
422 demonstrated that the addition of empty MSNPs increased bacterial growth of *P. syringae*
423 pv. *pisi, P. fluorescens,* and *P. carotovorum* subsp. *carotovorum* by 222%, 25%, and 45%,
424 respectively, when compared to the untreated controls.

425 The effect on bacterial growth of Ajwain-, CNAD- and AIT-loaded MSNPs was evaluated
426 with and without a lactose derivative capping. Regarding the uncapped MSNPs, only AIT-
427 loaded MSNPs decreased bacterial viability, indicating the strong antimicrobial effect of
428 AIT. Moreover, CNAD and Ajwain may not have escaped the encapsulation as readily as
429 AIT due to their lower volatility, which could explain why these treatments did not show a
430 significant decrease in bacterial viability. The effect of AIT- and CNAD-loaded MSNPs
431 against *E. coli* and *P. aeruginosa* has been previously reported by our group (Chan et al.,
432 2017).

433 The EO-loaded MSNPs were capped with a lactose derivative to enhance EOs protection
434 and prolong the release of the antimicrobials. In most cases, the lactose-capping increased
435 bacterial growth, particularly of *P. carotovorum* subsp. *carotovorum,* possibly due to its
436 utilisation as a carbon source by the microorganism. Interestingly, CNAD-loaded lactose-
437 capped MSNPs were shown to be the best system to reduce bacterial growth, eliminating
438 up to >99.99% of bacteria of five different bacterial species.

FT-IR analyses suggested that the observed increase in potency may have been due to the grafting of CNAD onto the surface of the MSNPs *via* the APTES molecule, which was initially designed to be used as a bridge to attach the lactose onto the MSNP surface. The FT-IR spectrum of the mixture of CNAD and lactose derivative (Figure 5A) presented peaks that belonged to either of the individual components, except for a peak at 2362 cm^{-1}, which was attributed to the lactose before attachment to APTES (Figure 5B). This suggested that after adding CNAD to the lactose derivative, the APTES molecule was released from the lactose to bind the CNAD molecule *via* its aldehyde group (Figure 7). The resulting silanised APTES-CNAD was grafted onto the surface of MSNPs, preventing volatilisation and allowing a strong and long-lasting antimicrobial effect of the EO, which resulted in 89-99.99% bacterial reductions, with >99.99% of *P. syringae* pv. *pisi* being killed compared to treatment with uncapped CNAD-MSNPs. Additionally, liquid chromatography results confirmed that the potential grafting of CNAD onto MSNPs surfaces, rather than adsorption within the pores, prevented the volatilisation of the EO during at least 48h. It may also be possible that the resulting CNAD-APTES molecule was itself bioactive, enhancing the biocidal effect of the EO; further studies would be necessary to evaluate this. Ruiz-Rico *et al* have similarly demonstrated that carvacrol, eugenol, thymol and vanillin can be grafted onto silica supports thus enhancing their antimicrobial activity through the incorporation of an aldehyde group or its natural presence, as in the case of vanillin (Ruiz-Rico et al., 2017). This result is consistent with our findings where the presence of an aldehyde group in cinnamaldehyde enabled the immobilisation of the compound onto the surface of the MSNPs, increasing the biocidal activity significantly.

5. Conclusions

In conclusion, this study evaluated the specificity and effectivity of 41 essential oils as potential antimicrobials against phytopathogen *P. syringae* pv. *pisi* in comparison to *P. carotovorum* subsp. *carotovorum* and *P. fluorescens.* Additionally; the encapsulation of mustard, cinnamon, and ajwain oils into MSNPs was evaluated to enhance their antimicrobial effect. Cinnamaldehyde appeared to be grafted onto the surface of MSNPs *via* its aldehyde group, increasing its antimicrobial activity by 10-fold and eliminating up to 99.99% of bacterial growth of five different bacterial species, demonstrating the effectiveness of MSNPs as delivery vehicles for volatile compounds. This significant result confirms that MSNPs hold great promise as a means of exploiting the antimicrobial potential of essential oils previously overlooked because of the limitation of their physical characteristics. To demonstrate the practical application of this work, the resulting CNAD-MSNPs have been evaluated *in planta* as a seed treatment to protect peas against bacterial blight (Bravo Cadena et al, *unpublished data*).

Acknowledgements

Marimar Bravo Cadena is funded by the National Council of Science and Technology (Conacyt, Mexico, grant no. 384507), the Schlumberger Foundation FFTF Award and the Public Secretariat of Education (SEP DGRI, Mexico). Renier Van der Hoorn received funding from ERC Consolidator grant 616449 'GreenProteases'. Helen E. Townley is grateful to the Williams Fund for ongoing support. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

We would like to thank the Oxford Materials Characterisation Service of the Department of Materials, Oxford University, for access to the FT-IR facilities.

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615 **Figure Captions**

616 **Figure 1**. Streptomycin calibration curve. Effect of increasing concentrations of
617 streptomycin sulphate salt on the growth of *Pseudomonas syringae* pv. *pisi*. Diameter of
618 inhibition zone increases logarithmically as antibiotic concentration increases. Data shows
619 mean ± Standard Deviation, n = 3.

620 **Figure 2** Efficacy of 41 essential oils against *Pseudomonas syringae* pv. *pisi*,
621 *Pectobacterium carotovorum* subsp. *carotovorum* and *Pseudomonas fluorescens.* Essential
622 oils are listed from least to most inhibitory against *P. syringae* pv. *pisi* followed by the four
623 controls: two streptomycin concentrations as positive controls and empty disk and water
624 as negative controls. Y-axis starts at 6 mm (disc diameter). Horizontal dotted lines
625 represent inhibition zones produced by streptomycin at 10mg/ml on *P. syringae* pv. p*isi*
626 (30 mm), *P. carotovorum* subsp. *carotovorum* (26 mm) and *P. fluorescens* (24.33 mm).
627 Results represent the mean inhibition zone diameter of three replicates ± standard
628 deviation of the mean.

629 **Figure 3** Selectivity of essential oils against pythopathogen *P. syringae* pv. *pisi*. EOs with
630 positive Log_2 FC (fold-change) showed selectivity against *P. syringae* pv. *pisi* over the
631 ubiquitous organism *P. fluorescens.* Four controls are included: two streptomycin
632 concentrations as positive controls and empty disk and water as negative controls. Results
633 represent the mean of three replicates +/- standard deviation of the mean.

634 **Figure 4** Effect of Essential-oil loaded MSNPs *in vitro.* Two mg/ml of Essential oil-loaded
635 MSNPs were added to bacterial cultures to evaluate their antimicrobial effect (decrease in

colony forming units per ml, CFU/ml). Lactose-capped and uncapped MSNPs were used to determine the efficacy of a sugar coating. CNAD-loaded lactose-capped MSNPs (pointed by arrows) completely inhibited bacterial growth demonstrating a potential application as an antimicrobial system. a) Treatments against *P. fluorescens, P. carotovorum* subsp. *carotovorum* and *P. syringae* pv*. pisi*. Negative controls without MSNPs and DMSO (dimethyl sulfoxide) controls were included. b) Treatments against *E. coli* and *P. aeruginosa* to compare efficacy of CNAD-loaded lactose-capped MSNPs. Data shows mean ± Standard Deviation, n = 3.

Figure 5 FT-IR analysis of lactose, lactose derivative and cinnamaldehyde. A) FT-IR of CNAD, lactose derivative and their combination. A peak can be observed at wavenumber 2362 cm^{-1} on the IR spectrum of the combination, which was not present in either of the individual components. B) FT-IR of lactose containing the observed peak at 2360 cm^{-1}.

Figure 6 CNAD release from uncapped and lactose-capped MSNPs. Liquid chromatography results showing the concentration of CNAD released into solution from uncapped and capped MSNPs after 24h. CNAD was immobilised onto the surface of MSNPs after capping protocol, preventing the loss or volatilisation of the essential oil from the nanoparticles. Error bars represent ± Standard Deviation (SD) of triplicates (all SD are <0.5 mg/L).

Figure 7 CNAD-APTES formation. The APTES (3-aminopropyltriethoxysilane) molecule originally attached to lactose was released and attached to CNAD through its aldehyde group. The resulting silanised CNAD-APTES molecule could be immobilised onto the surface of MSNPs.