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

Marimar Bravo Cadena[a], Gail M. Preston[b], Renier A.L. Van der Hoorn[b], Helen E. Townley[a,c], Ian P. Thompson[a,*]

[a] Department of Engineering Science, University of Oxford, Parks Road, Oxford OX1 3PJ, UK
[b] Department of Plant Sciences, University of Oxford, S Parks Road, Oxford OX1 3RB, UK
[c] Nuffield Department of Women's and Reproductive Health, University of Oxford, Level 3, Women's Centre, John Radcliffe Hospital, Oxford, OX3 9DU, UK

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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 24 h 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.

## 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

* Corresponding author.
*E-mail addresses:* marimar.bravocadena@eng.ox.ac.uk (M. Bravo Cadena), ian.thompson@eng.ox.ac.uk (I.P. Thompson).

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

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

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

An additional advantage of MSNPs is that they can be capped to further protect the 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 min 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, turmeric and ajwain essential oils were obtained from Herbalveda (UK). The remaining 29 essential oils were obtained from Oils4Life (UK).

### 2.3. Antimicrobial susceptibility tests (ASTs) – disk diffusion assay

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

### 2.4. Antimicrobial susceptibility tests (ASTs) – broth microdilution test

The broth microdilution test was carried out to study the effect of varying concentrations of twelve selected essential oils on