Application of Mesoporous Silica Nanoparticles for Biocide Delivery to Plants to Prevent Pre-Harvest Losses

A thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

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Abstract

The ever-increasing human population, expected to reach over 9 billion people by 2050, is creating a growing need for new technologies that increase agricultural productivity and yields. The use of mesoporous silica nanoparticles (MSNPs) as biocide delivery vehicles in agriculture has only recently started receiving attention, as these nanoparticles have the potential to revolutionise agriculture.

MSNPs are biocompatible, biodegradable, have a low-cost synthesis and their surface can be easily functionalised or modified according to the desired application. For this reason, they represent a promising approach to encapsulate antimicrobials which enable longer-lasting, more targeted and controlled delivery. The encapsulation of biocides into MSNPs has the benefit of protecting the compounds from degradation or evaporation whilst enhancing the antimicrobial activity by providing a heightened and more localised killing effect precisely where it is required. The reduction of the quantities of biocides needed to obtain an efficient antimicrobial effect consequently has the added benefit of reducing the amount of chemical released into the environment and so any potential hazardous unwanted consequences.

This thesis aims to evaluate the application of MSNPs as delivery vehicles to encapsulate natural antimicrobial agents such as essential oils to address bacterial diseases affecting agriculture. Forty-one essential oils were evaluated for their efficacy and specificity against three bacterial species, including two plant pathogens. The most effective antimicrobials were encapsulated into MSNPs, which prevented their rapid volatilisation and degradation whilst maintaining a slow-release and prolonged effect of the biocide. The biocide-loaded MSNPs were demonstrated to be effective *in vitro* against plant pathogens. Particularly, cinnamaldehyde-MSNPs were shown to decrease bacterial counts (CFUs) by up to 99.9% and the encapsulation increased the antimicrobial activity by 10-fold, compared to free cinnamon essential oil.

The loaded nanoparticles were also incorporated into an alginate seed coating to evaluate the use of a seed treatment to prevent bacterial infection of plants. Different alginate formulations were assessed and the beneficial effects of the seed coat were demonstrated, resulting in taller plants (42.2%-57.6% taller) that germinated and developed faster (25%-62.5% more germinated plants by the third day after sowing) than the controls. To determine the protective effect of the alginate seed treatment containing essential oil-loaded MSNPs, *in planta* experiments were carried out. As a proof of concept, this study focused on phytopathogen *Pseudomonas syringae* pv. *pisi*, the causative agent of pea bacterial blight. The number of symptomless plants after treatment increased by 143.58% compared to the control, demonstrating the efficacy of this treatment at decreasing the incidence of bacterial diseases in plants.

This study demonstrates the efficacy of mesoporous silicates as delivery vehicles for volatile compounds. The encapsulation of highly antimicrobial essential oils into MSNPs prevented the rapid loss or degradation of the biocides and enabled a controlled slow-release of the compounds achieving targeted delivery towards the seed-borne pathogens commonly present on the seed coat. The use of MSNPs also permitted very low concentrations of essential oils, up to 90,000-fold lower, to be effective antimicrobials. The results of this study demonstrate the enormous potential of MSNP in agriculture for stealth delivery of killing agents for reducing crop losses and increasing agricultural yields whilst reducing the unwanted consequences of pesticide release in the environment.

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Abbreviations

AFM – Atomic Force Microscope

AIT – Allyl isothiocyanate

ANOVA – Analysis of variance

APTES – 3-aminopropyltriethoxysilane

APTMS - 3-aminopropyltrimethoxysilane

AST – Antimicrobial Susceptibility Test

PBS – Phosphate buffered saline

CFU – Colony forming unit

Chl – Chlorophyll

CMC – Critical micellar concentration

CNAD – Cinnamaldehyde

CTAB – Cetyl trimethyl ammonium bromide

CVD – Chemical Vapour Deposition

DCM – Dichloromethane

Dpi – Days post infection

DMSO – Dimethyl sulfoxide

ENPs – Engineered Nanoparticles

EOs – Essential oils

FAO – Food and Agriculture Organisation

FITC – Fluorescein isothiocyanate

FT-IR – Fourier transform infrared spectroscopy

GC-MS – Gas Chromatography-Mass Spectroscopy

GRAS – Generally Recognised as Safe

HSD – Honest significant difference

LB – Luria-Bertani broth

LBA – Luria-Bertani agar

LC – Liquid Chromatography

LCT – Liquid crystal templating

LD – Lethal dose

MHA – Mueller-Hinton agar

MHB – Mueller-Hinton broth

MIC – Minimum inhibitory concentration

MBC – Minimum bactericidal concentration

MCM41 – Mobil Composition of Matter No. 41

MS – Murashige and Skoog

MSNPs – Mesoporous silica nanoparticles

NCPPB – National Collection of Plant Pathogenic Bacteria

nm - Nanometre

NPs – Nanoparticles

OD – Optical density

PBS – Phosphate buffered saline

PEG – Polyethylenglycol

PMOs – Periodic Mesoporous Organosilicas

ROS – Reactive Oxygen Species

rpm – Revolutions per minute

SEM – Scanning Electron Microscope

TEM – Transmission Electron Microscope

TEOS – Tetraethylorthosilicate

TTC – 2,3,5-Triphenyltetrazolium chloride

USD – United States Dollars

USDA – United States Department of Agriculture

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Publications and Presentations

Publications

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- Parts of Chapter 1, Chapter 2, Chapter 3 and Chapter 6 have been published in:

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- Parts of Chapter 1, Chapter 4, and Chapter 7 have been published in:

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Oral presentations

- Parts of Chapter 2 were presented orally at the "Bioactive Natural Products: Translating Promise into Practice" Conference in Oxford, UK.
- Parts of Chapter 2, Chapter 4 and Chapter 5 were presented orally at the "7th International Conference on Nanotechnology: Fundamentals and Applications" in Budapest, Hungary

Poster Presentations

- Parts of Chapter 3, Chapter 4, Chapter 6 and Chapter 7 were presented at the "Science Protecting Plant Health" Conference in Brisbane, Australia

Chapter 1
Introduction

The human population is expected to reach over 9 billion people by 2050. This rapid increase in population is creating a growing need for new technologies to enhance agricultural productivity. It has been estimated by the Food and Agriculture Organisation (FAO) that global food production will need to increase by 70% in order to satisfy the dietary requirements of the growing population (Alexandratos & Bruinsma 2012). Unfortunately, the natural resources available for food production, such as water and land, are limited. Hence, 90% of the necessary growth in crop production has to come from increased crop intensity and yields (FAO 2009). An approach to increase agricultural yields is to target one of the main causes of crop losses: pests and diseases. This thesis focuses on bacterial diseases affecting agriculture and the use of nanoparticles as delivery vehicles for natural biocides such as essential oils.

1.1. Nanotechnology

Nanotechnology is a rapidly developing technology which opens up many avenues of opportunity for improving agricultural practices. This is defined as “the design, characterisation, production and application of structures, devices and systems by controlling shape and size at the nanometre (nm, 10^{-9} m) scale” (The Royal Society & The Royal Academy of Engineering 2004). The distinguishing characteristic of nanomaterials is that they are found in the range of 0.1-100 nm since at this size materials show different properties than their bulk counterparts. This is due to a higher surface area per unit mass, which increases chemical reactivity, and the role of quantum effects that change optical, magnetic or electrical properties. Characteristics such as surface tension and Brownian motion also play a role since the former can alter physical and chemical properties while the latter affects the control of individual atoms (The Royal Society & The Royal Academy of Engineering 2004). While nanomaterials are characterised by having at least one dimension of less than 100 nm, nanostructures can have different dimensions: Nanoparticles (NPs) are considered zero-dimensional (0D), nanotubes are 1D, nanosheets are 2D and arrangements made of combinations of these structures are 3D nanomaterials. For the purpose of this thesis, only nanoparticles will be discussed.

1.1.1. Engineered Nanoparticles

In addition to high surface area, NPs have other unique physicochemical properties including high reactivity, tuneable pore size, particle morphology and a large surface area to volume ratio providing better opportunities for interaction (Siddiqui *et al.,* 2015; Rai *et al.,* 2014).

The different types of NPs (Table 1.1) can be categorised as natural, incidental or engineered. Humans have always been exposed to natural NPs present in the environment (Dennekamp *et al.,* 2002) as well as to incidental NPs such as those produced during fires and volcanic dust. Engineered nanoparticles (ENPs) are those which are manufactured for a specific purpose. This thesis will focus on a specific type of ENPs; silica (SiO_2) NPs.

Table 1.1 Types of nanoparticles (The Royal Society & The Royal Academy of Engineering 2004; Norwegian Pollution Control Authority 2008; Peralta-Videa *et al.,* 2011).

Type of nanoparticles	Description
- Natural	Originated in nature such as soil colloids or sea salts nanocrystals.
- Incidental	Created as a by-product of either natural (mainly pyrogenic) or man-made processes such as carbon black formed during fires.
- Engineered (ENPs)	Manufactured to have specific characteristics and properties. Engineered NPs (ENPs) can be carbon-based (carbon nanotubes, fullerenes and graphene) or metal-based (zero-valent metal, metal oxides, metal salts).
Fullerenes	60 Carbon atoms organised as 20 hexagons and 12 pentagons to form a sphere of ~1nm diameter. Applications include electronic circuits and drug delivery.
Organic Polymers	Dendrimers – Spherical polymeric molecules formed by self-assembly. Applications include coatings, drug delivery, inks and environmental remediation.
Quantum Dots	Semiconductor nanoparticles with quantum effects that limit the energies within the particle. Applications include solar cells, fluorescent biological labels, composites and chemical reagents.
Mineral ENPs	Can be made of single elements such as silver, gold, copper, iron, etc., of metal oxides such as Al_2O_3, TiO_2, ZnO, **SiO_2** etc., or of multi-element oxides such as $MgAl_2O_4$ or $SrTiO_3$

NPs have a wide range of applications in a variety of industries including cosmetics, textiles, medicine, chemical, food industry and agriculture. It is estimated that more than 1,800 products based on nanotechnology are already commercially available in the market worldwide in products such as paints, batteries, lubricants, cosmetics, food additives, disinfectants, and many others. This number has increased significantly from 54 products registered in 2005 to 1,317 products in 2010 and 1,827 in 2018 on the Consumer Products Inventory (The Project on Emerging Nanotechnologies 2015). The use of NPs as delivery vehicles and their application in agriculture will be the main focus for this thesis.

1.1.1.1. Mesoporous Silica Nanoparticles

Silicon dioxide (SiO_2), also known as silica, is one of the nanomaterials that has been studied in recent years due to its chemical stability, versatility and biocompatibility (Slowing *et al.,* 2008); reducing the risks and concerns regarding the release of nanoparticles to the environment. Silica nanoparticles can be easily functionalised with molecules for targeted delivery, are stable due to their Si-O bonds (Liong *et al.,* 2008), have tuneable pore size and porosity (Trewyn *et al.,* 2007), and a simple and low-cost synthesis (Kwon *et al.,* 2013). The application and synthesis of MSNPs is described in more detail in Section 1.3.2 and in Chapter 3, Section 3.1.

1.2. Agriculture: In need of novel approaches

Agriculture constitutes the backbone of several countries' economies; driving growth and aiming to satisfy basic nutritional needs of human populations. Over the last 50 years, global crop production has increased 300% mainly due to higher production yields and crop intensification (FAO 2013). However, natural resources are limited and currently decreasing. Every year ~10 million hectares of cropland are lost due to erosion, 10-40 times faster than the rate of soil formation (Pimentel & Burgess 2013). Additionally, the continuously growing population requires more water yet around 70% of fresh water is currently consumed by agriculture (Pimentel *et al.,* 2004), severely reducing potable water availability. It is essential to further increase agricultural productivity and yields, while reducing production and environmental costs, to fulfil the population's demands (Misra *et al.,* 2013) and achieve sustainable agriculture.

A promising approach to increase agricultural productivity and food production is to tackle one of the main problems causing yield losses: crop diseases. Every year, more than 40% of global food production (estimated at $500 billion USD) is lost to diseases (Peshin 2002). This is despite the annual use of over two million tonnes of pesticides (Pimentel 1997; Grube *et al.,* 2011). It is estimated that up to 90% of pesticides do not reach their desired target since they can be lost during application, as run-off, leaching or by decomposition (Pimentel *et al.,* 1992; Mogul *et al.,* 1996). This causes a decrease in fertiliser/pesticide efficacy, and an increase in the quantity needed, the cost of the treatment and the amount of biocide being released into the environment. As a result, there is an urgent need for novel technologies to deliver biocides more effectively and reduce the percentage of crops being lost to diseases every year.

1.2.1. Bacterial Phytopathogens

Pests and pathogens such as bacteria, fungi, and insects constantly affect agricultural yields. In the United States alone, crop losses due to plant pathogens have been estimated to result in a 33 billion dollars loss per year (Sankaran *et al.,* 2010). *Pseudomonas syringae* pathovars, as well as *Erwinia* species have been identified as some of the most important plant pathogenic bacteria affecting agriculture (Mansfield *et al.,* 2012).

Pseudomonas syringae pv. *pisi* was used throughout this thesis as a proof of concept to evaluate the use of NPs to deliver biocides to plants to prevent and control bacterial infections in agriculture. The bacterium mainly infects common peas (*Pisum sativum*) and causes pea bacterial blight. The pathogen is seed-borne and seed-transmitted, which was an essential characteristic leading to its selection for this study to develop a seed treatment against this bacterial disease. Two additional bacterial species were used in Chapter 2 and Chapter 6 as a comparison to *P. syringae* pv. *pisi. Pectobacterium carotovorum* subsp. *carotovorum*, also known as *Erwinia carotovora* pv. *carotovora*, causes soft rot of different plant tubers and storage organs (Lim *et al.,* 2013); resulting in wilting, lesions and discolouration symptoms. In contrast, *Pseudomonas fluorescens* is generally non-pathogenic and a ubiquitous organism widely used in industries such as food, bioremediation or as biocontrol agents against plant pathogenic bacteria (Governement of Canada 2015). These bacterial species are discussed in further detail in Chapter 2, Section 2.1.1.

1.2.2. Control and Treatment Measures

Bacterial diseases are currently difficult to control. The available treatments are limited to antibiotics and copper compounds, or Bordeaux mixture (copper sulphate and lime), none of which are compatible with modern day issues. These compounds are toxic to human and animal health and have negative effects on the environment since they can accumulate high levels of toxic residues in crops and result in antibiotic resistance (Bajpai *et al.,* 2011; Rodriguez *et al.,* 1997).

Additionally, the application of antibiotics to crop plants in the US is regulated by the Environmental Protection Agency and is restricted to two compounds: streptomycin and oxytetracycline. The first is used on 12 species, mainly apple and pear trees to treat *Erwinia amylovora,* while the latter is mostly used on peach, nectarine and pear trees. Moreover, antibiotics used in the US are banned in agriculture in many European countries to prevent horizontal gene transfer to animals and humans (Bajpai *et al.,* 2011) and copper products are limited as well in Europe by rule 473/2002 due to their potential long-term negative impacts on the environment due to soil accumulation (Commission Regulation 2002).

Due to the restrictions and potentially hazardous effects of treatment measures, the control of bacterial diseases currently relies on preventive strategies: using resistant plants (plant breeding), disease-free seeds, hygiene measures (disinfect and clean equipment and machinery), crop rotations, use of beneficial bacteria, and isolation and burial or destruction of infected plants (Vidaver & Lambrecht 2004).

There is currently no treatment for pea bacterial blight (the main bacterial disease studied in this thesis). Seed treatments and fungicides are aimed at fungal diseases and are ineffective against bacterial pathogens. Copper based products have been used to treat this disease but the evidence for their efficacy is limited and inconclusive (Richardson & Hollaway 2012). Consequently, this disease is usually controlled by crop rotation, crop hygiene, sowing time, and use of disease-free seeds and resistant cultivars. Infected crops result from sowing infected seeds and bacteria spreading from infected plants to healthy plants through contact or water splashing (Richardson & Hollaway 2012) (Figure 1.1).

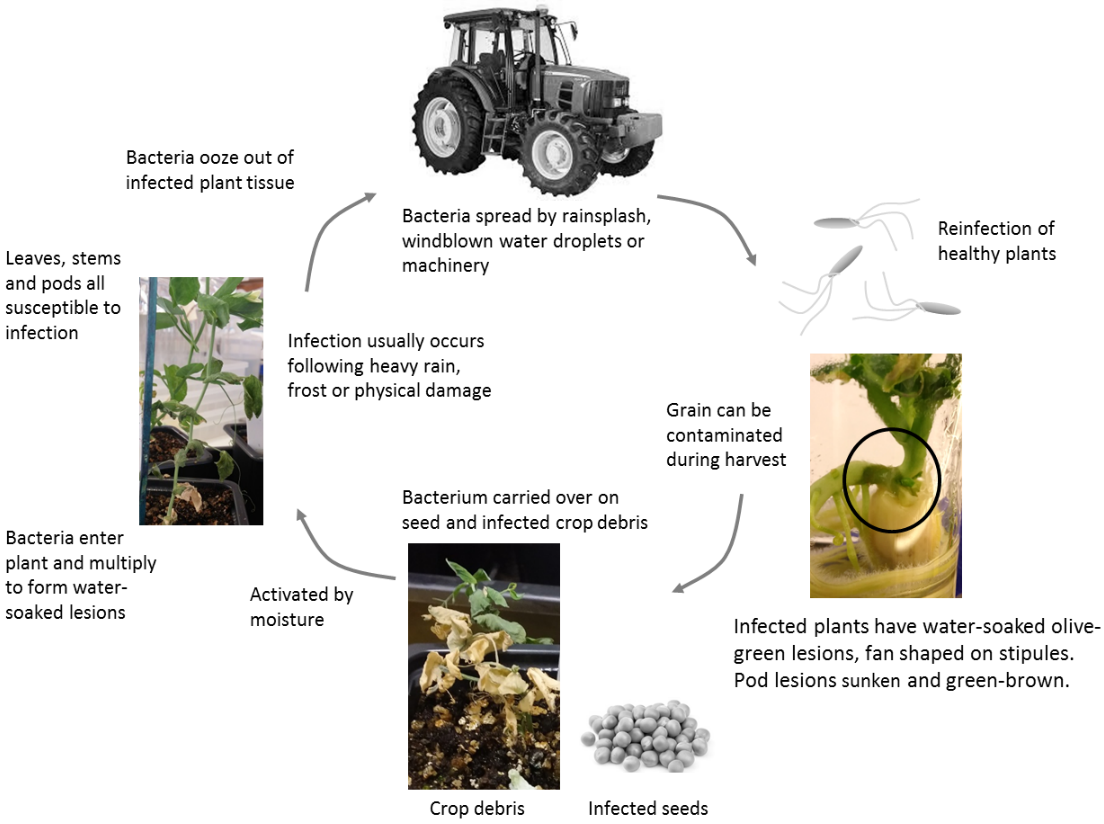

Figure 1.1 Disease cycle of pea bacterial blight. Figure adapted from (CropPro 2014).

Regarding antibiotic use, a streptomycin seed treatment has been demonstrated to reduce disease of almost 90% of infected seeds. However, soaking the seeds in a streptomycin solution has been shown to be toxic for many of the pea seeds tested (Taylor & Dye 1976). Further to the negative effect that the antibiotic caused on the pea seeds, the use of antibiotics in agriculture is strictly regulated and results in antibiotic resistance, causing additional agricultural challenges.

P. syringae pv. *pisi* can remain viable on seeds for at least three years (Lawyer & Chun 2001), so seed testing is usually performed to ensure seeds are disease-free. In the UK, bacterial blight is mainly controlled through a seed certification scheme initiated in 1987 (The Plant Health (Great Britain) SI 1987/1758 1987). However, a negative seed test result does not necessarily guarantee a seed lot is free of the pathogen and only one infected seed in 10,000 (0.01%) is enough to initiate an epidemic and under favourable conditions destroy an entire crop (Hollaway *et al.,* 2007; Taylor & Dye 1976). Therefore, it is important to develop treatment measures that are both effective and will not present potential risks such as bacterial resistance, or human and environmental hazards.

1.2.2.1. Seed Treatments

Seed treatments are a $6.11 billion USD (2016) industry, estimated to reach $11.31 billion USD by 2022 (Markets and Markets 2018). Seed treatments can be classified by function, application technique, or type (Figure 1.2).

Function: Seed treatments can be used for enhancement or protection. Enhancement refers to seed performance improvements such as enhanced germination and uniformity, or increased nitrogen fixation, among others (Copeland & McDonald 2001). Protection is related to disease control against pests and pathogens. An ideal protective seed treatment should reduce the transmission of the pathogen from seed to shoot, not reduce germination or plant development, have low toxicity and not present a risk towards human/animal health nor to the environment (Koch & Roberts 2014). For the purpose of this study, only protection seed treatments will be considered.

Application technique: Seed dressings refer to the application of a dry or wet formulation to dress the seed; they are the easiest type of treatment and can be applied both on-farm or in treatment facilities. Seed coatings utilise a special formulation to enhance the attachment and have an impact on the seed size and shape. This type of treatment requires specific application technologies. Seed pelleting is the most complex technology consisting of a coating (usually of clay and other inert materials) that changes the physical shape of seeds to enhance handling. This technology requires specific machinery and it is the most expensive of the types of seed treatments (International Seed Federation 2007). The current market is dominated by the seed coating technology due to the increasing demand for multipurpose and controlled-delivery formulations (Markets and Markets 2018). More information about seed coatings is presented in Chapter 4, Section 4.1.1.

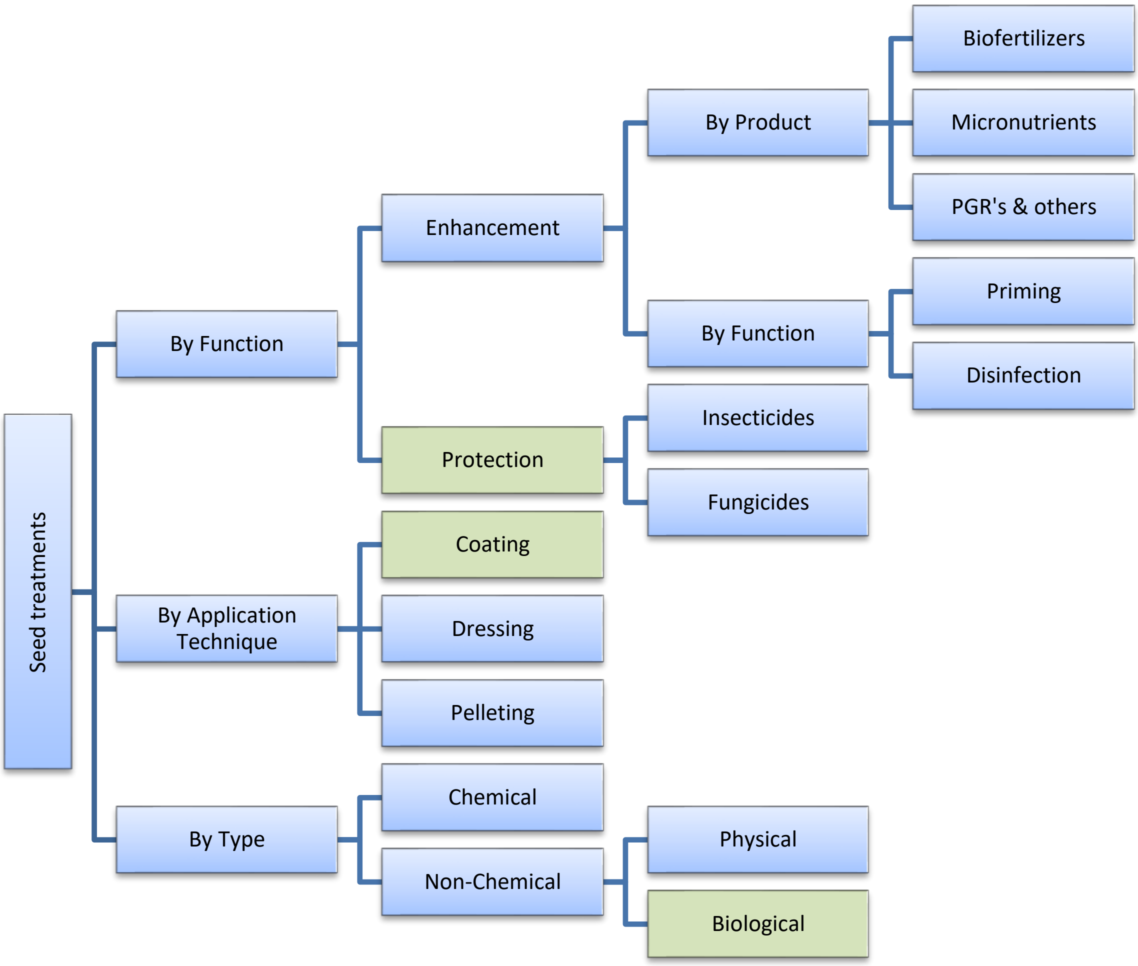

Figure 1.2 Classification of seed treatments by type, function or application technique. PGR's = Plant growth regulators. Green indicates the classification of the seed treatment that will be investigated throughout this thesis.

Types: Chemical, physical, or biological. Even though the majority of seed treatments are chemical, there is an increasing concern towards agrochemicals and their impact on the environment and health. Additionally, chemical seed treatments are mainly targeted towards fungal pathogens and are ineffective against bacterial diseases.

Non-chemical seed treatments include physical or biological methods. The main physical treatments involve heat (thermotherapy); such as hot water, aerated steam and dry air. There are disadvantages associated with these treatments, including the requirement for post-drying, limited success against bacterial diseases and reductions in seed germination (Koch & Roberts 2014). Other physical treatments include the application of low-energy electrons (e-ventus®), which frees the seed from adhered pathogens but does not prevent it from getting re-infected; and other physical effects like microwaves which can negatively affect germination and viability (Han 2010; Ambrose *et al.,* 2015).

Another type of seed treatment is biological, such as application of microorganisms or other natural products. Microorganisms can protect plant hosts by competing with pathogens, producing antibiotics or other enzymes/metabolites, and by eliciting plant host defences. There are only a few microbial seed treatments available for seed-borne pathogens due to commercial constraints such as development costs, registration of products, and mass-production feasibility (Koch & Roberts 2014). With respect to plant-derived products, particularly essential oils (EOs), antimicrobial activities against bacterial of fungal pathogens have been previously reported (see Chapter 2, Section 2.1). However, a disadvantage of plant-derived products, such as EO preparations, for seed treatments is the requirement for high concentrations, which often have phytotoxic properties and negatively affect germination (Lo Cantore *et al.,* 2009; Van Der Wolf *et al.,* 2008).

A broad variety of compounds have been used as seed treatments over the history of plant protection (Figure 1.3). Some of the first recorded seed treatments include plant-derived products such as onion juice and cypress extract, which have been used since the Roman and Egyptian periods (International Seed Federation 2007). This exemplifies the potential use of natural plant extracts as seed treatments. However, as it will be discussed in section 1.3.3, the volatility, immiscibility and easy degradation of EOs, together with the potential negative effects that high concentrations can have on the seeds, hinders the efficient use of these compounds as seed treatments in mainstream agriculture.

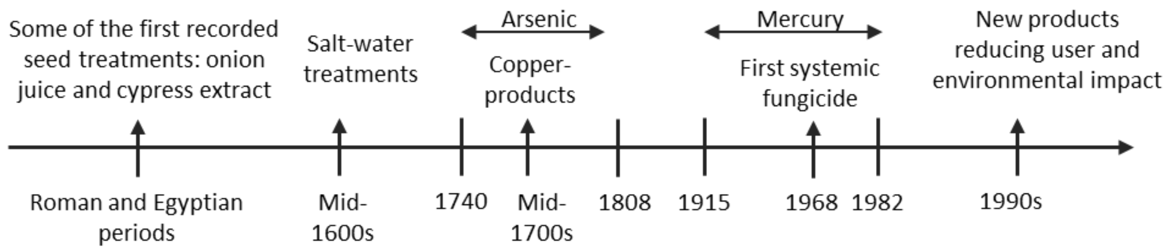

Figure 1.3 Compounds used for seed treatments in agriculture throughout time.

1.2.2.2. Natural Biocides – Essential Oils

Essential oils (EOs) are natural biocides that can be used as antimicrobials to substitute chemicals or antibiotics in agriculture. They are produced by secondary metabolism in aromatic plants and are therefore natural volatile liquids that are biologically active (Bajpai *et al.,* 2011). EOs can be obtained from different plant parts including flowers, buds, seeds, leaves, twigs, roots, fruits, herbs or wood (Guenther 1949) through different processes such as distillation (water, steam or hydro diffusion), expression (cold pressed method mainly used for citrus) or solvent extractions using hypercritical CO_2, solvents (hexane, methanol), or hot oil for maceration (Esoteric Oils 2015).

There are approximately 3,000 EOs 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 (Grand View Research 2018). These compounds have a wide variety of applications in agriculture, food and beverage, cosmetics, and medicine, amongst other industries.

A promising application of EOs is as biocides to target the vast number of diseases affecting crops. EOs are safe and publicly accepted, many being classified as GRAS (Generally Regarded as Safe) and appearing in the EAFUS list (Everything Added to Food in the US) (Smoley 1993). Many of the individual components or EOs are also used in industry and can be either extracted from plants or manufactured synthetically (Oosterhaven *et al.,* 1995).

Most EOs possess antimicrobial properties since they play an important role in plants' defence mechanisms against pathogens (Pradhanang *et al.,* 2003). EOs are comprised of a multifaceted mixture of different volatile compounds, mainly terpenes and terpenoids or aliphatic compounds (Bajpai *et al.,* 2011). This complexity results in a reduced potential of resistance development against their antimicrobial effect (Yap *et al.,* 2014), since the likelihood of several mutations spontaneously developing endowing the ability to resist several biocides is very low. Certainly, when compared to generation of single mutation leading to resistance to a single biocide, which is the current situation for most applications. Specific components of EOs such as thymol, carvacrol and eugenol have been shown to be important contributors to their antimicrobial activity as they are also antimicrobial on their own (Conner 1993; Didry *et al.,* 1993; Horváth *et al.,* 2004).

The mechanism of action of EOs against microorganisms has not been studied in detail. However, hydrophobicity is an important characteristic that helps EOs disrupt microbial structures (Knobloch *et al.,* 1986; Sikkema *et al.,* 1995) since it enables them to partition the lipids of cell membranes, leading to cell disruption, leakage and death (Boire 2013). It has been observed that the EOs that have the most antimicrobial effects have a high percentage of phenolic compounds (carvacrol, thymol and eugenol) (Farag *et al.,* 1989; Juliano *et al.,* 2000; Lambert *et al.,* 2001). Phenols disrupt cell membranes and result in the dissolution of the proton motive force and thus in the decrease of ATP synthesis (Sikkema *et al.,* 1995), which can then lead to reduced production of enzymes and toxins.

In addition to the broad activity of EOs against cell membranes, specific targets have also been proposed due to the different effects observed against a variety of microorganisms (Burt 2004). It has been suggested that Gram-negative organisms are more resistant to EOs due to an additional barrier provided by the outer membrane (Vaara 1992). However, the susceptibility of Gram-negative microorganisms against EOs varies, possibly due to the presence or absence of specific targets. Moreover, other mechanisms of action of EOs have been suggested including interference or inhibition of quorum sensing (Rasmussen *et al.,* 2005), disruption of cellular division, respiration and sporulation (Inouye *et al.,* 1998), and anti-efflux activity (Garvey *et al.,* 2011).

Furthermore, the chemical structure of the individual components of the EOs has an effect on the antimicrobial properties; a hydroxyl group or a phenolic ring have been shown to be important (Dorman & Deans 2000; Ultee *et al.,* 2002). Alcohols, and particularly terpene alcohols, are suggested to have a strong antibacterial effect due to different mechanisms, including denaturation of proteins, dehydration of cells or dissolution of cell membranes (Dorman & Deans 2000). In contrast, aldehydes are thought to interfere with electron transfer and central metabolism, leading to cell death (Langeveld *et al.,* 2014).

Even though EOs hold promising potential as antimicrobials in agriculture, they present an inevitable problem; that of their controlled and effective delivery. To counteract this issue, the encapsulation of EOs into nanoparticles incorporated to a seed treatment will be addressed in the following chapters.

1.3. Nanotechnology in Agriculture

Nanotechnology has the potential to address major agricultural issues and to help achieve a sustainable agriculture. The use of nanotechnologies in agriculture and food industries was first addressed in 2003 by the United States Department of Agriculture (USDA) (The United States Department of Agriculture 2003). The importance of the application of these new technologies in agriculture can be observed through the increasing investments made by the USDA into the US

National Nanotechnology Initiative starting from $0 in 2001 and rising to over $12 million by 2013 (Sargent Jr 2014) and $28.4 million in 2016 (NSTC/COT/NSET 2017).

Nanotechnology is attracting increasing interest for agriculture applications due to the unique physicochemical properties of nanomaterials that make them suitable to be used as delivery vehicles (Siddiqui *et al.,* 2015). Nanoparticles can be used to deliver fertilizers, pesticides, herbicides, growth promoters and nutrients to agricultural crops. The use of nanoparticles as nano-pesticides could prevent the current loss of up to 90% of agricultural products either during application, as run-off or by decomposition (Mogul *et al.,* 1996).

The use of nanotechnology in agriculture has three main objectives: a) to achieve targeted delivery of products such as fertilisers, growth regulators and biocides, b) to provide a controlled release of compounds triggered by a specific response to stimuli or signals and c) to provide products with higher efficacy attained by increased solubility, stability and effectiveness of compounds (ObservatoryNANO 2010).

There are a wide variety of nanotechnology-based products that can be applied to agriculture. Nano-clay capsules containing growth stimulants or biocontrol agents; nano-formulations, nano-emulsions or nano-encapsulated herbicides and pesticides; nano-sensors for monitoring of soil conditions and growth conditions and for precision farming; nanoscale systems for disease diagnosis, 'smart' fertilisers and pesticides that respond to environmental stimuli to achieve controlled release of active ingredients; and DNA and chemicals delivery to plants or pathogens (Anandaraj *et al.,* 2011).

1.3.1. Nanoparticles as Delivery Systems in Agriculture

Nanoparticles (NPs) can be used as delivery systems in agriculture for disease prevention and treatment (by delivering pesticides and biocides) as well as for nutrient management (by delivering fertilizers) (Siddiqui *et al.,* 2015). Efficient delivery of herbicides, pesticides, fertilizers, plant growth regulators, can be achieved through different mechanisms such as encapsulation and entrapment or surface attachments (Rai *et al.,* 2014; Siddiqui *et al.,* 2015)

Nanoparticles can influence plant growth, cell structure and physiological and biochemical structures in plants, both positively and negatively. Despite many investigations being carried out about the effects of nanoparticles to plants, only a few studies have been conducted to elucidate the mechanisms by which the nanoparticles affect plant systems (Siddiqui *et al.,* 2015). The effects that NPs can have on plants depend on the properties of the nanomaterial such as chemical composition, size, coating, reactivity, dosage, concentration, among others (Khodakovskaya *et al.,* 2013); as well as on the plant species, developmental stage and growth media used (Ma *et al.,* 2010; Oberdörster *et al.,* 2007) .

Up to 90% of pesticides applied to plants are lost due to runoff, leaching, degradation and decomposition (Siddiqui *et al.,* 2015), requiring reapplication and higher doses of chemicals to achieve the desired effect, which increases costs and chemicals being released to the environment, increases pathogen resistance and reduces soil biodiversity (Ghormade *et al.,* 2011). Therefore, there is an increasing need for a new delivery system that can protect the biocide in order to achieve highest efficiency of the product. Nano-formulations need to have an effective concentration (stable and soluble), controlled release, enhanced targeted activity and reduced toxicity (Siddiqui *et al.,* 2015); so these factors need to be taken into consideration when developing a nano-pesticide. Examples of nanopesticides include encapsulated Avermectin (Li *et al.,* 2007) and commercial pesticide 'Karate®ZEON' (encapsulation breaks when in contact with leaves) from Syngenta.

Nanoformulations can be delivered to plants using different methods. They can be delivered *via* soil or foliar applications or *via* the roots either by spraying them while suspended in the air (aeroponics) or by immersing them in the solution (hydroponics) (Rai *et al.,* 2014). An alternative delivery method, which will be presented in this thesis, can be *via* a seed treatment.

The incorporation of nanoparticles into a seed treatment has only recently started to gain attention and could present a novel alternative to address seed-borne diseases. However, the effects of the NPs on seed germination and plant development need to be considered. For instance, the use of metal nanoparticles such as silver or zinc has been shown to result in plant stress (wheat) and decreased germination (sesame), respectively (Belava *et al.,* 2017; Narendhran *et al.,* 2016). In contrast, other studies have reported that silver NPs can enhance germination of plants such as rice, watermelon or courgette (Mahakham *et al.,* 2017; Almutairi & Alharbi 2015) and NPs such as TiO_2 or FeS_2 can also have a beneficial effect on plants (Okupnik & Pflugmacher 2016; Srivastava *et al.,* 2014). Silica nanoparticles have been shown to have beneficial effects on plants due to the natural role of silica in plant protection. For this reason, this type of nanoparticles will be the focus of this work and will be described in more detail in the following sections.

1.3.2. Mesoporous Silica Nanoparticles in Plants and Agriculture

Silicon is naturally present in the soil as silica (SiO_2) and has an important physiological role in plants. It is deposited as hydrated amorphous silica in the endoplasmic reticulum, cell wall and intercellular spaces and its deficiency increases the plant's susceptibility to lodging and infections (Rai *et al.,* 2014). Silica's characteristics and its natural presence in the environment and plant systems, make this material a promising candidate to be used in agriculture.

Mesoporous silica nanoparticles (MSNPs) have mainly beneficial effects on plants; increasing seed germination, seedling and root growth, and nutrient availability (Table 1.2). To evaluate the effect of nanoparticles on plants, different parameters are taken into consideration such as seed germination, seedling growth, root elongation, plant height, nutrient uptake and transportation, chlorophyll content, gene expression, enzymatic activity and yield and quality. Still, the effects on plants due to bioaccumulation, bio-magnification and biotransformation of nanomaterials have not been elucidated (Siddiqui *et al.,* 2015).

Silica nanoparticles can improve plants' tolerance to abiotic stress and enhance plant growth and development (Table 1.2). Furthermore, silica nanoparticles that are absorbed through the plant roots have been shown to form films at cell walls that enhance the plant's stress resistance and to improve yields (DeRosa *et al.,* 2010).

The use of MSNPs in agriculture consequently presents comparatively low risks regarding toxicity and safety to the environment and food chain introduction. In addition, adjusting the size of the nanoparticles can control or prevent internalisation of MSNPs into the plant system. Plant walls typically have pores between 3-8nm (Carpita & Gibeaut 1993), so only NPs smaller than the largest pores will be able to penetrate the cell wall into the plasma membrane.

Microscopic techniques have been used to study the fate and localisation of NPs in plants. Sun *et al.,* (2014) localised and quantified MSNPs (20nm) in lupin, wheat, maize and *Arabidopsis* and demonstrated that MSNPs penetrate into the roots and are transported to aerial parts of the plants showing no negative effects on seed germination or when MSNPs were transported to different plant organs (Sun *et al.,* 2014).

MSNPs possess several characteristics that make them attractive as drug delivery vehicles. Silica is stable, both chemically and thermally, inexpensive and harmless (Iskandar *et al.,* 2001). MSNPs have the potential to aid sustainable agriculture with optimised yields that can be protected by controlled delivery of biocides that are effective and safe for both the environment and plant, animal and human health.

Table 1.2 Effect of silica nanoparticles on plant growth and development on different plant species

Nanoparticles	Plant species	Effect	Reference
Nano-SiO_2	Tomato	Improved seed germination in low concentration	(Siddiqui & Al-Whaibi 2014)
Nano-SiO_2	Maize	Increased seed germination by providing better nutrient availability and pH and conductivity to growing medium, enhanced plant dry weight and levels of proteins, chlorophyll and phenols.	(Suriyaprabha *et al.,* 2012)
Nano-SiO_2	Changbai larch	Improved seedling growth and quality (mean height, root collar diameter, main root length, number of lateral roots) and induced synthesis of chlorophyll with exogenous application.	(Bao-shan *et al.,* 2004)
Nano-SiO_2	Tomato	Enhanced seed germination and stimulated antioxidant system under NaCl stress.	(Haghighi *et al.,* 2012)
Nano-SiO_2	Squash	Enhanced seed germination and stimulated antioxidant system under NaCl stress.	(Siddiqui *et al.,* 2014)
Nano-SiO_2	Spruce	Increase in height, root length and diameter, number of lateral roots, chlorophyll content and plant stress resistance.	(Lin *et al.,* 2004)
Silica, palladium, gold and copper NPs	Lettuce	Significant influence on lettuce seeds and no significant effect on microbial communities.	(Shah & Belozerova 2008)
Nano-SiO_2	Soybean	Improved seed germination by increasing nitrate reductase by exogenous application.	(Lu *et al.,* 2002)
Quantum Dots (QD) and Silica coated with QDs	Rice	Silica coated with QDs promoted root growth.	(Wang *et al.,* 2014)
Nano-SiO_2	Rice	MSNPs of 25nm labelled with fluorescein isothiocyanate (FITC) have no effect on germination at concentrations up to 50mg/L while QD stopped germination.	(Nair *et al.,* 2011)
Nano-SiO_2	Lupin, wheat and *Arabidopsis*	MSNPs (20nm) functionalised with amine cross-linked fluorescein isothiocyanate were absorbed and transported without affecting germination.	(Hussain *et al.,* 2013)
Nano-SiO_2	Basil	Increased leaf dry and fresh weight, chlorophyll and proline content under NaCl stress and reduced pollution caused by salinity	(Kalteh *et al.,* 2014)
Nano-SiO_2	Bean	Alleviate negative effects caused by salinity	(Alsaeedi *et al.,* 2017)

Nanoparticles have been used to deliver pesticides, fertilizers, chemicals and DNA into plants. Torney *et al.* (2007) used gold-capped MSNPs to deliver DNA and chemicals into plant cells (Torney *et al.,* 2007). Porous hollow silica nanoparticles have also been used to encapsulate active compounds including pesticides validamycin (Liu *et al.,* 2006) and 1-naphthylacetic acid (Ao *et al.,* 2013), fungicide prochloraz (Zhao *et al.,* 2018), phytohormones (Sun *et al.,* 2018) and insecticide chlorfenapyr (Song *et al.,* 2012). Moreover, nano-silica has been used as insecticide on its own due to its ability to penetrate the cuticular layer of insects that acts as a protective barrier (Barik *et al.,* 2008). Likewise, porous silica nanoparticles have been used to protect pesticides from UV light degradation, enhancing their stability (Li *et al.,* 2007).

There are a limited number of patents regarding the use of nano-silica in agriculture such as a Korean patent describing the addition of colloidal silica (5-60 nm) to a NPK fertilizer to promote plant growth and resistance against pathogenic bacteria (Kim 2007). Similarly, Bignozzi *et al.* (2008) have patented anti-microbial compositions based on TiO_2, SiO_2, ZnO, SnO, ZrO_2 nanoparticles modified with fatty acids (Bignozzi *et al.,* 2008). Other patents using nanosilica include SiO_2 and TiO_2 nanoparticles for fertilizer or pesticide controlled delivery (Chen 2002) and a production process to synthesise SiO_2, Al_2O_3, kaolin and fermentation residue nanoparticles to be used as water-retaining additives or as controlled-release coating (Zhang *et al.,* 2005).

MSNPs increase stability of cargo by providing controlled and targeted release while protecting the compounds from degradation or volatilisation. Studies of urea encapsulation into MSNPs (~150 nm) demonstrated a five-fold improvement in the release profile of urea in soil (Wanyika *et al.,* 2012). In addition to providing efficient delivery and stability of cargo, MSNPs have been shown to be safe to humans as well as plants. Silica nanoparticles containing nitric oxide (NO) were demonstrated to be effective against *Pseudomonas aeruginosa* while being nontoxic to human fibroblast cells, demonstrating silica NPs are safe to use as antimicrobial agents (Shin *et al.,* 2007).

Even though the effects of MSNPs have been studied to some level in plants and microorganisms, there is still a research gap regarding the mechanism of action and the effect and their fate on the environment and soil rhizosphere that needs to be explored further (Siddiqui *et al.,* 2015). The direct application of NPs on land can come in contact with soil microbes and enzymes and start the mobilisation of NPs through food chains (Holden *et al.,* 2013) extending the impact of the NPs to higher organisms. It has also been demonstrated that NPs can potentially be transferred to the progeny of plants, such as CeO_2 NPs in soybean (Hernandez-Viezcas *et al.,* 2013), and this should be considered when releasing NPs to the environment. It is therefore important to study the effect and fate of MSNPs on the environment, food chain and plant, animal and human health. Nanoparticles that are biodegraded or incorporated into the soil after use are extremely desirable (Rai *et al.,* 2014) as delivery vehicles for biocides in agriculture.

1.3.3. Essential Oils as Antimicrobial Agents in Agriculture

The use of EOs as seed treatments for agriculture (Table 1.3) has mostly focused on fungal pathogens and there is a lack of investigations regarding their use against bacterial phytopathogens.

Despite the potential antimicrobial activity of EOs and their use as free agents in agriculture and other fields, 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, EOs can be encapsulated in nanoparticles, so providing protection and preventing volatilisation of the oils, whilst improving their stability, long-term effects, and immiscibility in aqueous solutions.

A few EOs have been encapsulated into different types of nanoparticles with potential use in agriculture (Table 1.4). NP encapsulation can efficiently reduce the rapid evaporation, degradation, and loss of activity of EOs while offering controlled release from the nanoparticles (Lai *et al.,* 2006; Bernardos *et al.,* 2014; Hosseini *et al.,* 2013; Yang *et al.,* 2009). Additionally, it presents an alternative to current control measures in agriculture that is not only natural but also safe, non-toxic, biodegradable, biocompatible and highly effective.

Table 1.3 Examples of essential oils investigated as seed treatments

Essential Oil	Pathogen	Host	Reference
Hyptis marrubioides, Aloysia gratissima and *Cordia verbenacea*	Fungus (*Colletotrichum truncatum*)	Soybean seeds	(Costa da Silva *et al.,* 2012)
Cinnamon, clove, oregano, savoury and thyme	Fungi (*Penicillium, Fusarium* and *Pythium*)	Corn seeds	(Christian 2007)
Cymbopogon citratus, Ocimum gratissimum and *Thymus vulgaris*	Fungi (*Alternaria padwickii, Bipolaris oryzae* and *Fusarium moniliforme*)	Rice seeds	(Nguefack *et al.,* 2008)
Cymbopogon citratus, Ocimum gratissimum	Fungus (*Fusarium moniliforme*)	Maize seeds	(Tagne *et al.,* 2013)
Maleluca alternifolia (tea tree), *Thymus vulagris* (thyme), *Laurel nobilis* (laurel), *Mentha piperita* (peppermint), *Origanum vulgare* (oregano), *Syzygium aromaticum* (clove) and *Rosmarinus officianalis* (rosemary)	Fungi (*Peyronellaea pinodella, P. pinodes, Diaporthe phaseolorum* var. *caulivora, Phomopsis longicolla, Ascochyta lentis* and *Colletotrichum gloeosporioides*)	Legumes	(Marinelli *et al.,* 2012)
*Cymbopogon citratus(*Lemongrass*)* *Eucalyptus camaldulensis,* (Eucalyptus) *and* *Azadirachta indica* (Neem)	Fungi (*Colletotrichum graminicola, Phoma sorghina* and *Fusarium moniliforme*)	Sorghum seeds	(Somda *et al.,* 2007)
Lemongrass, thyme and rose (together with bioagent *Bacillus subtilis*)	Fungus (*Aspergillus niger*)	Peanut seeds	(Abdel-Kader *et al.,* 2013)
Savoury and thyme (together with *Pseudomonas* spp.)	Fungus (*Alternaria radicina*)	Carrot seeds	(Lopez-Reyes *et al.,* 2015)
Eucalyptus (*Eucalyptus globules* Labill.) and rosemary (*Rosmarinus officinalis* L.)	Bacteria (*Xanthomonas* spp.)	Tomato seeds	(Mbega *et al.,* 2012)
Thyme, oregano, clove and lemongrass	Fungi (*Aspergillus parasiticus, A. flavus, A. clavatus*)	Oats	(Božik *et al.,* 2017)

Table 1.4 Examples of encapsulated essential oils with potential application in agriculture

Essential Oil	Encapsulation	Target Organism	Reference
Artemisia arborescens	Solid Lipid nanoparticles	Insects	(Lai *et al.,* 2006)
Essential Oil components: carvacrol, cinnamaldehyde, eugenol and thymol	Mesoporous Silica nanoparticles	Fungus (*Aspergillus niger*)	(Bernardos *et al.,* 2014)
Origanum vulgare	Chitosan nanoparticles	Not specified	(Hosseini *et al.,* 2013)
Allium sativum L.	Polyethylene glycol (PEG) coated nanoparticles	Red Flour beetles (*Tribolium castaneum*)	(Yang *et al.,* 2009)
Melaleuca alternifolia	Poly(ε-caprolactone) (PCL) nanocapsules	Fungus (*Trichophyton rubrum*)	(Flores *et al.,* 2013)
Gaultheria procumbens L.	Chitosan-cinnamic acid	Fungus (*Aspergillus flavus*)	(Kujur *et al.,* 2017)

1.3.4. Controlled Release of Biocides in Agriculture

MSNPs can function as vehicles to deliver essential oils to treat bacterial pathogens in agriculture. Specific compounds can be used to cap NPs and ensure the biocide is released only after the capping agent has been physically, chemically or biologically removed. This capping agent could also function as a targeting mechanism.

There are a variety of capping agents available. Chen *et al.* (2013) used hyaluronic acid (HA) to cap and target MSNPs. The MSNPs release the cargo after the HA has been enzymatically degraded (Chen *et al.,* 2013). Similarly, MSNPs have been capped using protamine to selectively release a drug targeting cancer cells. The release is triggered by trypsin, which is over-expressed during inflammation and cancer (Radhakrishnan *et al.,* 2014). Ma *et al.* (2013) capped MSNPs with oligonucleotides that release the encapsulated compound after the capping DNA is denatured thermally or a disulphide bond cleavage is redox-induced (Ma *et al.,* 2013). A gelatin derived from collagen has also been used as a capping agent, which allows the controlled intracellular release triggered by acidic endosomal pH (Zou *et al.,* 2013). Collagen has been used as well to cap MSNPs to achieve controlled release of cargo by disulphide bond cleavage (Luo *et al.,* 2011). Other examples of capping agents include galacto-oligosaccharide (Agostini *et al.,* 2012), heparin and lactobionic acid (Dai *et al.,* 2014), PEGylated-phospholipids (Wang *et al.,* 2010) and cytochrome c (Zhang *et al.,* 2014).

Most capped NPs have been investigated for medical applications such as drug delivery to cancer cells. The exploitation of capping agents for biocide delivery in agriculture has not received as much attention. Chan *et al.* (2013) utilised carbohydrate capping of MSNPs to exploit the target microorganisms' metabolic activity. Capping MSNPs with lactose enabled the EO-loaded NPs to be targeted towards lactose-degrading *Escherichia coli,* which metabolised the sugar causing the release of the biocide (Chan *et al.*, 2013).

1.4. Aims and Objectives

The research reported in this thesis focuses on the evaluation of MSNPs as delivery vehicles for natural biocides in agriculture. The overall aim was to develop a seed treatment containing EO-loaded MSNPs capable of decreasing the incidence of bacterial infections affecting agriculture. Figure 1.4 summarises the structure of this thesis as well as the relationship between the chapters and the main outcome of each chapter. The specific objectives of this study were to:

- Evaluate the efficacy and specificity of a variety of essential oils as antimicrobials against three bacterial species
- Select the most effective EOs for nanoparticle encapsulation
- Synthesise and characterise MSNPs
- Study the volatility as well as the loading and unloading behaviour of the selected EOs into MSNPs
- Develop an alginate seed coating and the best formulation to be used as a seed treatment
- Investigate the effect of the resulting seed coat on the plants' germination, growth and development

- Obtain a seed coat formulation able to incorporate EO-loaded MSNPs to be used as a seed treatment against microbial pathogens
- Determine the effect *in vitro* of EO-loaded MSNPs against three bacterial species
- Assess the effect of capping the MSNPs with a sugar cap to enhance the protection of the EOs and study if targeting of the antimicrobial can be achieved
- Determine the effect *in planta* of an alginate seed treatment containing EO-loaded MSNPs against bacterial phytopathogen *Pseudomonas syringae* pv. *pisi*
- Quantify the reduction in bacterial infection of peas obtained by the alginate-EO-MSNPs seed treatment developed.

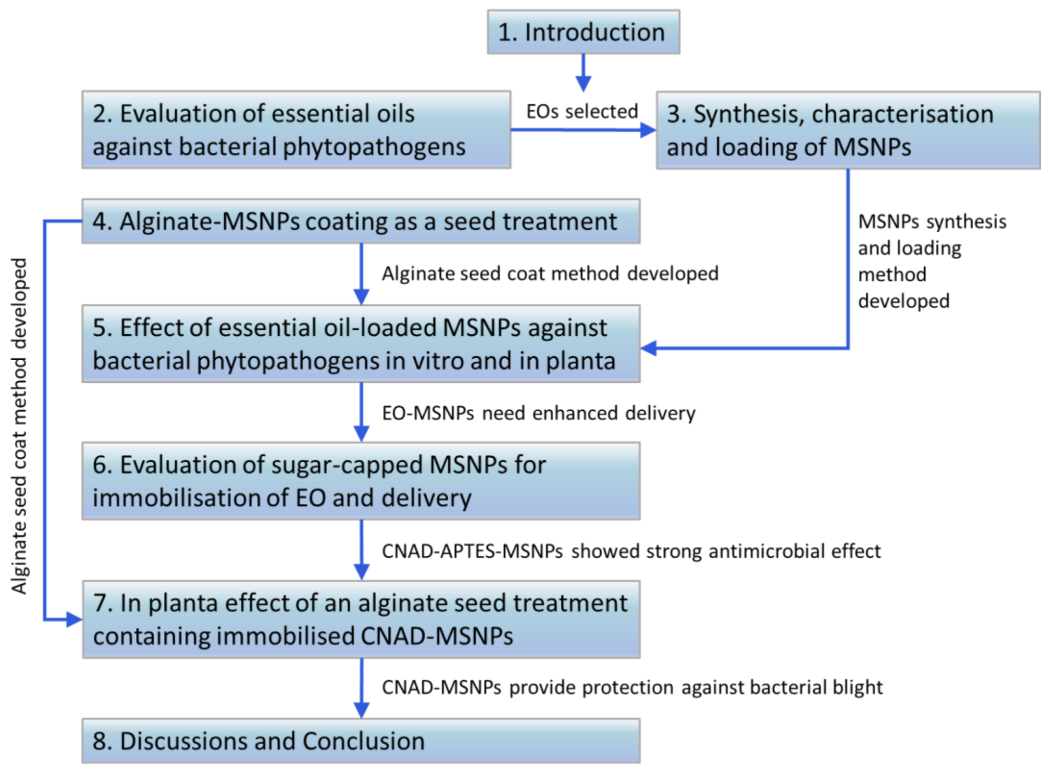

Figure 1.4 Flow diagram illustrating the structure of this thesis, the relationship between the chapters and the main outcome of each chapter.

Chapter 2
Evaluation of Essential Oils against Bacterial Phytopathogens

2.1. Introduction

This chapter focuses on the evaluation of the antimicrobial effect of a variety of natural biocides against three different bacterial species. Natural biocides such as essential oils are strong antimicrobials that are also environmentally-friendly and are less susceptible to the development of microbial resistance than antibiotics. This is because they are multifaceted and less susceptible to single mutation resistance developing (Yap *et al.,* 2014). However, their volatile nature (tendency to go into vapour phase due to a high vapour pressure) prevents their effective use as free agents in different fields including agriculture or medicine. The potential encapsulation of such volatile biocides into nanoparticles could enhance their effectivity and potency. To determine the specific oil to be encapsulated into nanoparticles, the experiments presented in this chapter aim to evaluate the specificity and potency of a variety of essential oils as antimicrobials against agriculturally important phytopathogens.

2.1.1 Bacterial Phytopathogens

This chapter focuses on the antimicrobial effect of essential oils against the phytopathogens *Pseudomonas syringae* pv. *pisi* and *Pectobacterium carotovorum subsp. carotovorum* (also known as *Erwinia carotovora* pv. *carotovora*), compared to the ubiquitous *Pseudomonas fluorescens.*

Pseudomonas syringae pv. *pisi* was the main microorganism used throughout this study as a proof of concept to evaluate the development of a seed treatment against bacterial phytopathogens. It is a seed-borne and seed-transmitted pathogen that causes bacterial blight of peas (*Pisum sativum*), but can also infect other leguminous hosts (Afonin *et al.,* 2008; CABI 2017).

P. syringae pv. *pisi* cells are straight rods (0.6-0.8 x 1.1-3.2 μm), Gram-negative and motile (one polar flagella) that grow under aerobic conditions, at an optimum temperature of 27-28°C (maximum of 37°C and minimum of 7°C) (Afonin *et al.,* 2008). Seven races of this pathovar are currently recognised (Taylor *et al.,* 1989), and the interaction between pathogen races and plant cultivars is controlled by a genetic interaction between avirulence genes in the pathogen and resistance genes in the host (Bevan *et al.,* 1995). The ‘Kelvedon Wonder’ cultivar (used in Chapters 4-7) is susceptible to all seven races and all pea cultivars are susceptible to race 6 (Taylor *et al.,* 1989).

Bacterial blight was first recorded in the United States in 1915 (Sackett 1916) and has since been reported to occur in most pea growing areas worldwide (Figure 2.1) causing devastating effects reducing yield and seed quality. Up to 71% pea yield reductions (Kelvedon Wonder and Solara cultivars) have been reported after repeated bacterial inoculations under glasshouse conditions (Roberts 1993).

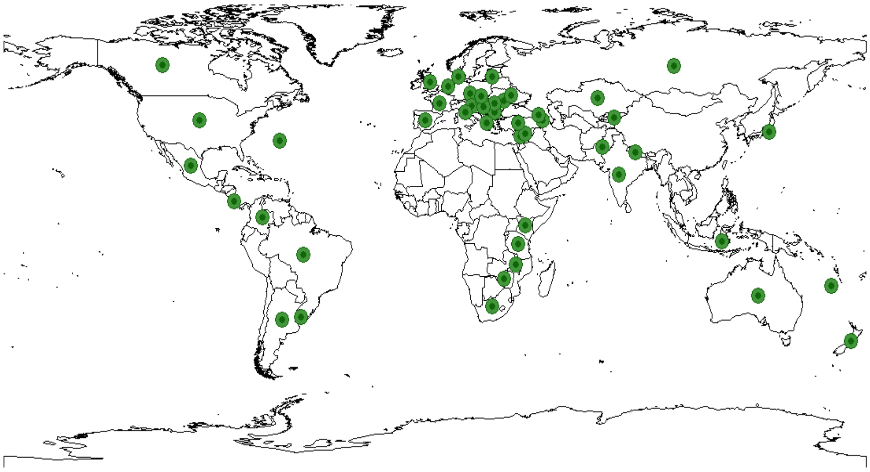

Figure 2.1. Global distribution of *Pseudomonas syringae* pv. *pisi,* compiled by the Plantwise knowledge bank based on published reports in the scientific literature. Reproduced by kind permission of CABI. ©CABI 2017. www.plantwise.org/KnowledgeBank

Pectobacterium is a bacterial genus of Gram-negative, facultatively anaerobic, fermentative, motile (peritrichous flagella) straight rods of 0.5-1 × 1-3 μm that occur singly or in pairs. Their optimal temperature is 27-30°C with a maximum of 40°C (Holt *et al.,* 1994). *Pectobacterium carotovorum* subsp. *carotovorum*, also known as *Erwinia carotovora* pv. *carotovora*, causes soft rot of tubers and storage organs of a variety of plants such as potato, carrot, tobacco, and cabbage (Lim *et al.,* 2013), by secreting enzymes responsible for plant cell wall degradation. It exists in plant surfaces and soil and can enter its host though wounds or natural openings (Toth *et al.,* 2003). Symptoms include water-soaked lesions, maceration and rotting of affected tissues, wilting, discolouration and unusual odours.

Pseudomonas fluorescens are straight or slightly curved rods (0.5-1.0 x 1.5-5.0 µm), Gram negative, aerobic and motile (one or several polar flagella) that grow at an optimum temperature of 25-30°C. It is a generally non-pathogenic and a ubiquitous organism that can thrive on soil, plants and aqueous surfaces. It is used in industrial and commercial sectors such as the food industry, paper and textile processing, wastewater treatment, bioremediation, and household cleaning products (Government of Canada 2015). This species is closely related to *P. syringae* (Government of Canada 2015), despite their opposite effect on plants. *P. fluorescens* can enhance and benefit plant growth due to the production of siderophores; iron chelating compounds that scavenge iron from the environment and make it available to the cell (Neilands 1995). Besides acting as a plant growth promoter, *P. fluorescens* is important to control frost damage and can act as a biocontrol agent against different organisms including weeds, insects, pests and microorganisms (Government of Canada 2015).

Type strain ATCC 13525 (biovar I) was used throughout this study to compare the effect of the treatments studied on pathogenic and non-pathogenic bacterial species. This strain was isolated in 1951 from water works pre-filter tanks in Reading, UK (Hugh *et al.,* 1964). It is considered unlikely to be toxic to any aquatic or terrestrial organisms and the environmental hazard and human health hazard severities are considered low (Governement of Canada 2015).

2.1.2 Prevention, Control and Treatment

As discussed in Chapter 1 Section 1.2.2, treatment of bacterial diseases in agriculture is mainly restricted to two antibiotics, streptomycin and oxytetracycline; and copper compounds or Bordeaux mixture (copper sulphate and lime). However, their use is tightly regulated due to the emergence of bacterial resistance together with negative environmental and health impacts (Bajpai *et al.,* 2011; Rodriguez *et al.,* 1997). Therefore, it is imperative to find new and improved alternative agents and natural antimicrobials such as essential oils are promising candidates.

2.1.3 Essential Oils

Essential oils (EOs) are naturally occurring odorous, volatile, oily liquids that are biologically active and are produced by secondary metabolism in aromatic plants (See Chapter 1, Section 1.2.2.2 and 1.3.3 for more information about EOs). Many EOs, as well as their individual compounds, can be used to substitute chemicals or antibiotics used in agriculture due to their public acceptance, safe use and low toxicity, fewer effects on the environment, and most importantly, their known antimicrobial properties (Bajpai *et al.,* 2011).

A wide range of EOs has been reported as antimicrobial against different microorganisms. Table 2.1 and Table 2.2 summarise the effect of a variety of EOs on *Pectobacterium* and *Pseudomonas* species, respectively. Nevertheless, the minimum inhibitory concentrations are not always reported, so the results are not directly comparable.

Table 2.1. Examples of the effects of essential oils on *Pectobacterium carotovorum*, also known as *Erwinia carotovora*. MIC - Minimum inhibitory concentration. *Per Petri dish containing 10 ml agar media.

Essential Oil	Target Microorganism	MIC	Ref.
Allium sativum L.	*P. carotovorum*	*Not reported*	(Curtis *et al.,* 2004)
	P. carotovorum	*Not reported*	(Ngadze *et al.,* 2012)
Azadirachta indica A.Juss	*P. carotovorum*	*Not reported*	(Ngadze *et al.,* 2012)
Coriandrum sativum	*P. carotovorum*	*435 µg* *	(Lo Cantore *et al.,* 2004)
Foeniculum vulgare	*P. carotovorum*	*435 µg* *	
Cuminum cyminum	*P. carotovorum*	*1840 µg* *	(Iacobellis *et al.,* 2005)
Carum carvi	*P. carotovorum*	*910 µg* *	
Artemisia judaica		*550 mg/L*	(Badawy & Abdelgaleil 2014)
Artemisia monosperma		*525 mg/L*	
Calistemon viminals		*600 mg/L*	
Citrus aurantifolia		*625 mg/L*	
Citrus lemon		*600 mg/L*	
Citrus paradisi		*400 mg/L*	
Citrus sinensis		*700 mg/L*	
Cupressus macrocarpa		*525 mg/L*	
Cupressus sempervirens	*P. carotovorum*	*575 mg/L*	
Myrtus communis		*>1000 mg/L*	
Origanum vulgare		*400 mg/L*	
Pelargonium graveolens		*400 mg/L*	
Rosmarinus officinalis		*550 mg/L*	
Schinus molle		*700 mg/L*	
Schinus terebinthifolius		*750 mg/L*	
Syzygium cumini		*450 mg/L*	
Thuja occidentalis		*350 mg/L*	
Vitex agnus-castus		*425 mg/L*	

Table 2.2. Examples of the effects of essential oils on *Pseudomonas* spp. MIC – Minimum inhibitory concentration. *Per Petri dish containing 10 ml agar media.

Essential Oil	Target Microorganism	MIC	Ref.
Melaluca alternifolia	*P. aeruginosa, P. putida*	5%, 2.5%	(Kavanaugh & Ribbeck 2012)
Myroxylon balsamum		2.5%	
Syzygium aromaticum		>5%	
Cinnamomum aromaticum		0.2%	
Lavandula	*P. aeruginosa, P. putida*	>5%	(Kavanaugh & Ribbeck 2012)
Lavandula angustigolia	*P. syringae* pv. *syringae*	Not reported	(Amlai Todi Poswal & Witbooi 1997)
Thymus vulgaris	*P. aeruginosa, P. putida*	>5%, 2.1+/-0.4%	(Kavanaugh & Ribbeck 2012)
		Not reported	(Kokoskova *et al.,* 2011)
	P. syringae pv. *syringae*	Not reported	
	P. fluorescens		
Artemisia afra	*P. syringae* pv. *syringae*	Not reported	(Amlai Todi Poswal & Witbooi 1997)
Coriandrum sativum	*P. syringae* pathovars	870-6960 μg *	(Lo Cantore *et al.,* 2004)
	P. syringae pv. *pisi*	2610 μg *	
Foeniculum vulgare	*P. syringae* pathovars	3840 μg *	(Lo Cantore *et al.,* 2004)
	P. syringae pv. *pisi*	Not applicable	
Cuminum cyminum	*P. syringae* pathovars	920-7360 μg *	(Iacobellis *et al.,* 2005)
Carum carvi		910-5460 μg *	
Origanum vulgare	*P. syringae* pv. *syringae*	Not reported	(Kokoskova *et al.,* 2011)
	P. fluorescens	5 μl/ml	(de Sousa *et al.,* 2013)
Origanum compactum	*P. syringae* pv. *syringae*	Not reported	(Kokoskova *et al.,* 2011)
	P. fluorescens		
Rosmarinus officinalis	*P. fluorescens*	40 μl/ml	(de Sousa *et al.,* 2013)
Mentha arvensis	*P. fluorescens*	0.567 mg/ml	(Tyagi & Malik 2010)
Mentha piperita		1.125 mg/ml	
Eucalyptus globulus		2.25 mg/ml	
Cymbopogon citratus		0.567 mg/ml	
Allium sativum	*P. syringae* pv. *tomato*	Not reported	(Curtis *et al.,* 2004)
	P. syringae pv. *pisi*	Not reported	(Verma & Agrawal 2015)
Tremineli achebula *Withania somnifera* *Azadirachta indica* *Emblica officinalis* *Zinziber officinalis*	*P. syringae* pv *pisi*	Not reported	(Verma & Agrawal 2015)

Following chapters report on investigations on the encapsulation of the most antimicrobial EOs against *P. syringae* pv. *pisi* to protect the oils from evaporation and degradation and to enable an improved antimicrobial activity.

2.1.4 Aims and Objectives

The first objective of the research presented in this chapter was to determine the specificity and effectivity of 41 EOs against the seed-borne pathogen *Pseudomonas syringae* pv. *pisi* in comparison to *Pectobacterium carotovorum* subsp. *carotovorum* and *Pseudomonas fluorescens*. The second was to determine the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) for the most effective oils to evaluate their potential delivery *via* nanoparticle encapsulation. The overall aim of this chapter was to select the oil(s) for encapsulation into mesoporous silica nanoparticles to potentially treat pea bacterial blight caused by phytopathogen *P. syringae* pv. *pisi*.

2.2. Materials and Methods

2.2.1. 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 and incubating overnight at 30°C (28°C for *P. syringae* pv. *pisi*) before inoculating 25 ml of Mueller-Hinton Broth (MHB; Oxoid Ltd, UK) with one colony and incubating at 30°C (28°C for *P. syringae* pv. *pisi*) and 120 rpm overnight. Overnight cultures were used to inoculate 100 ml MHB in 250 ml Erlenmeyer flasks, which were incubated until mid-exponential phase was achieved (mid-exponential phase time was estimated using growth curves of all bacterial strains, see Section 2.3 Results).

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. The obtained bacterial suspension was used for the antimicrobial susceptibility tests using essential oils as natural biocides.

2.2.2. Natural Biocides – Essential Oils

All essential oils 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).

2.2.3. Antimicrobial Susceptibility Tests (ASTs)

Forty-one essential oils were selected to evaluate their antimicrobial activity against *P. syringae* pv. *pisi*, *P. carotovorum* subsp. *carotovorum* and *P. fluorescens*. The essential oils selected have been previously reported to possess antimicrobial properties against different microorganisms and were therefore selected for evaluation against the three species studied in this thesis.

2.2.3.1. Disk Diffusion Assay

The disk diffusion assay, also known as the Kirby-Bauer method, is one of the oldest and most commonly used antimicrobial susceptibility tests (ASTs). This technique is simple to perform, efficient, reliable, standardised and cost-effective (Engelkirk & Duben-Engelkirk 2008; EUCAST 2017; Scholar & Pratt 2000). MHA is the standardised media for the disk diffusion assay since it is a non-selective, non-differential medium that supports the growth of most non-fastidious pathogens, it shows reproducibility, and it allows better diffusion of the antimicrobials compared to other media (Hudzicki 2009).

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 essential oils against *P. syringae* pv. *pisi*, *P. fluorescens* and *P. carotovorum* subsp. *carotovorum*. Bacterial suspensions (OD_{600}=0.132) were homogenously streaked onto MHA plates using sterile cotton swabs. Whatman antibiotic assay discs (6mm 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 minutes 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 hours 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.

2.2.3.2. Broth Microdilution Test

The broth microdilution test is a widely used AST to determine the MIC (minimum inhibitory concentration) and the MBC (minimum bactericidal concentration) of an antimicrobial. The MIC is the lowest concentration of the antimicrobial that will inhibit the visible growth of the microorganism; while the MBC is the lowest concentration of the antimicrobial that is required to kill >99.9% of the microorganism. The broth microdilution test is more costly and labour-intensive than the disk diffusion test, but it provides a better estimate of the MIC (Scholar & Pratt 2000). Hence, this test was carried out solely for the twelve most antimicrobial EOs against *P. syringae* pv. *pisi,* which were selected from the disk diffusion test results.

To solubilise the essential oils in the culture media, it was necessary to test different compounds that increase the oils' solubility. Tween 20 (0.5% v/v final concentration; Sigma Aldrich, UK), Tween 80 (0.5% v/v final concentration; Sigma Aldrich, UK), dimethyl sulfoxide (DMSO, 20% v/v; Sigma Aldrich, UK), skimmed milk (0.1% fat) and ethanol (>99.8%; Sigma Aldrich, UK) were evaluated as solubilising agents. The most effective method to solubilise the essential oils was to dilute with ethanol before dissolving in culture media. The effect of ethanol on bacterial growth was assessed by employing a control of culture media with ethanol inoculated with *P. syringae* pv. *pisi*.

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

Absorbance (OD_{600}) was measured at t = 0h and plates were incubated at 28°C. Absorbance was measured at 20, 24 and 48 hours using an Infinite M2000 TECAN plate reader. Three µl from each clear well was plated into MHA and incubated at 28°C for 48 hours. All tests were performed in triplicate. MIC and MBC were obtained from this test.

2.2.4. Statistical Analyses

Statistical analyses were performed using Microsoft Excel and Minitab® version 17.2.1. To determine statistically significant differences between samples, two-factor analysis of variance (ANOVA) was evaluated when appropriate. *P* values of 0.1 and 0.05 were used and are specified for each analysis.

2.3. Results

2.3.1. Test Microorganisms and Growth Conditions

The antimicrobial activity of a variety of essential oils was evaluated against the seed-borne phytopathogen *P. syringae* pv. *pisi* and compared to bacterial pathogen *P. carotovorum* subsp. *carotovorum* and the non-pathogenic *P. fluorescens* species. Bacterial growth curves were prepared (Figure 2.2) to determine the incubation time needed to achieve mid-exponential phase of bacterial cultures. The data points obtained were fitted using the Boltzmann sigmoidal fit since it has been demonstrated that microbial growth follows Boltzmann statistics of exergy distribution, which establishes a relationship between microbial growth and available energy (Desmond-Le Quéméner & Bouchez 2014). The incubation time to achieve mid-exponential phase of bacterial cultures was determined to be 5-6 hours.

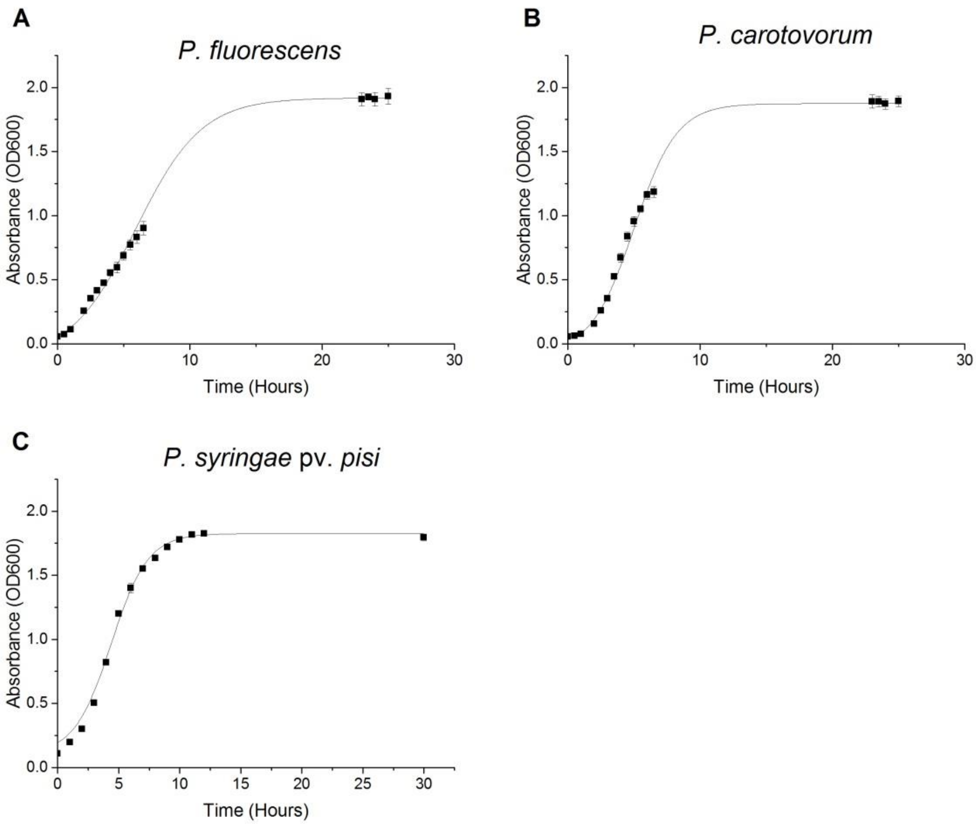

Figure 2.2 Bacterial growth curves of A) *P. fluorescens*, B*)* *P. carotovorum* subsp. *carotovorum* and C) *P. syringae* pv. *pisi*. Data shows mean ± standard deviation; n = 3. Curves fitted to Boltzmann sigmoid equation using OriginPro 9.1.

2.3.2. Disk Diffusion Assay

2.3.2.1. Conventional Treatment

The effect of streptomycin, one of the two antibiotics conventionally used in plant agriculture, was evaluated against *P. syringae* pv. *pisi*. A logarithmic increase was observed, reaching maximum inhibition at ~10 mg/ml (Figure 2.3). The use of higher concentrations of antibiotic did not result in greater inhibition of bacterial growth. In subsequent experiments, concentrations of 1 mg/ml and 10 mg/ml streptomycin sulphate salt were selected as positive controls.

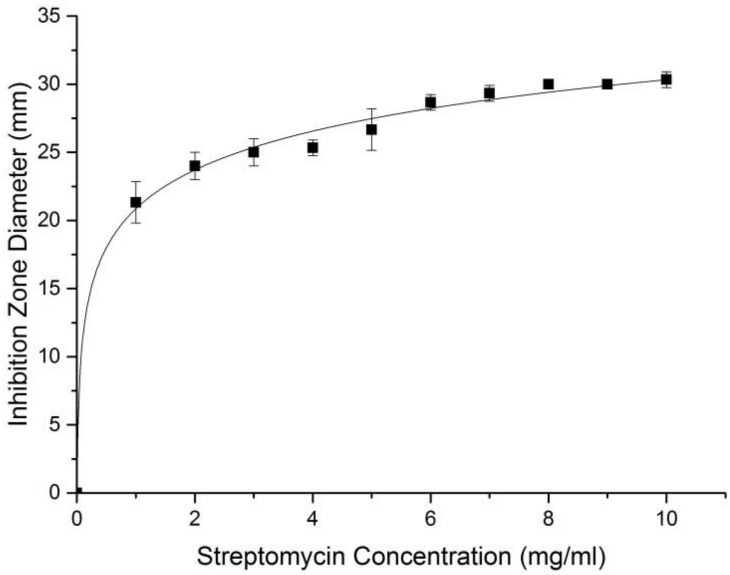

Figure 2.3. Streptomycin calibration curve. Effect of increasing concentrations of streptomycin sulphate salt on the growth of *Pseudomonas syringae* pv. *pisi*. Diameter of inhibition zone increases logarithmically as antibiotic concentration increases. Data shows mean ± standard deviation; n = 3.

2.3.2.2. Essential Oils as Antimicrobials

To assess whether natural biocides could compare to the conventional antimicrobial used in agriculture, the activity of 41 EOs was investigated. A bacterial lawn was spread across Petri plates, placing a disk inoculated with 10 µl of 100% essential oil and incubating overnight (Figure 2.4).

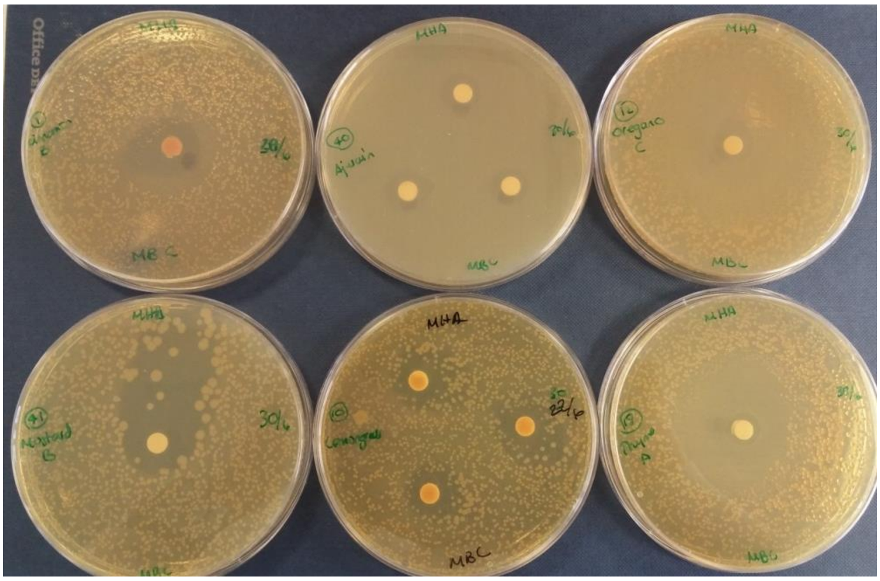

Figure 2.4. Disk diffusion assay for *P. syringae* pv. *pisi*. Inhibition zones can be observed around the disks saturated with essential oils. Top (left to right): Cinnamon, Ajwain, Oregano plates. Bottom (left to right): Mustard, Lemongrass, Thyme plates. Photograph taken five days after inoculation of plates (plate diameter = 90 mm).

Ten microlitres of mustard (ρ = 1.01 g/ml) and cinnamon (ρ = 1.05 g/ml) oils showed greater antimicrobial activity than a high dose of streptomycin (10 mg/ml) against all bacterial species, while oregano, ajwain and thyme oils also displayed high antimicrobial activity against some species (Figure 2.5). 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 effectivity, 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 the rest of the EOs. The EOs were also tested against *P. fluorescens* and the phytopathogen *P. carotovorum* subsp. *carotovorum.* 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) (Figure 2.5).

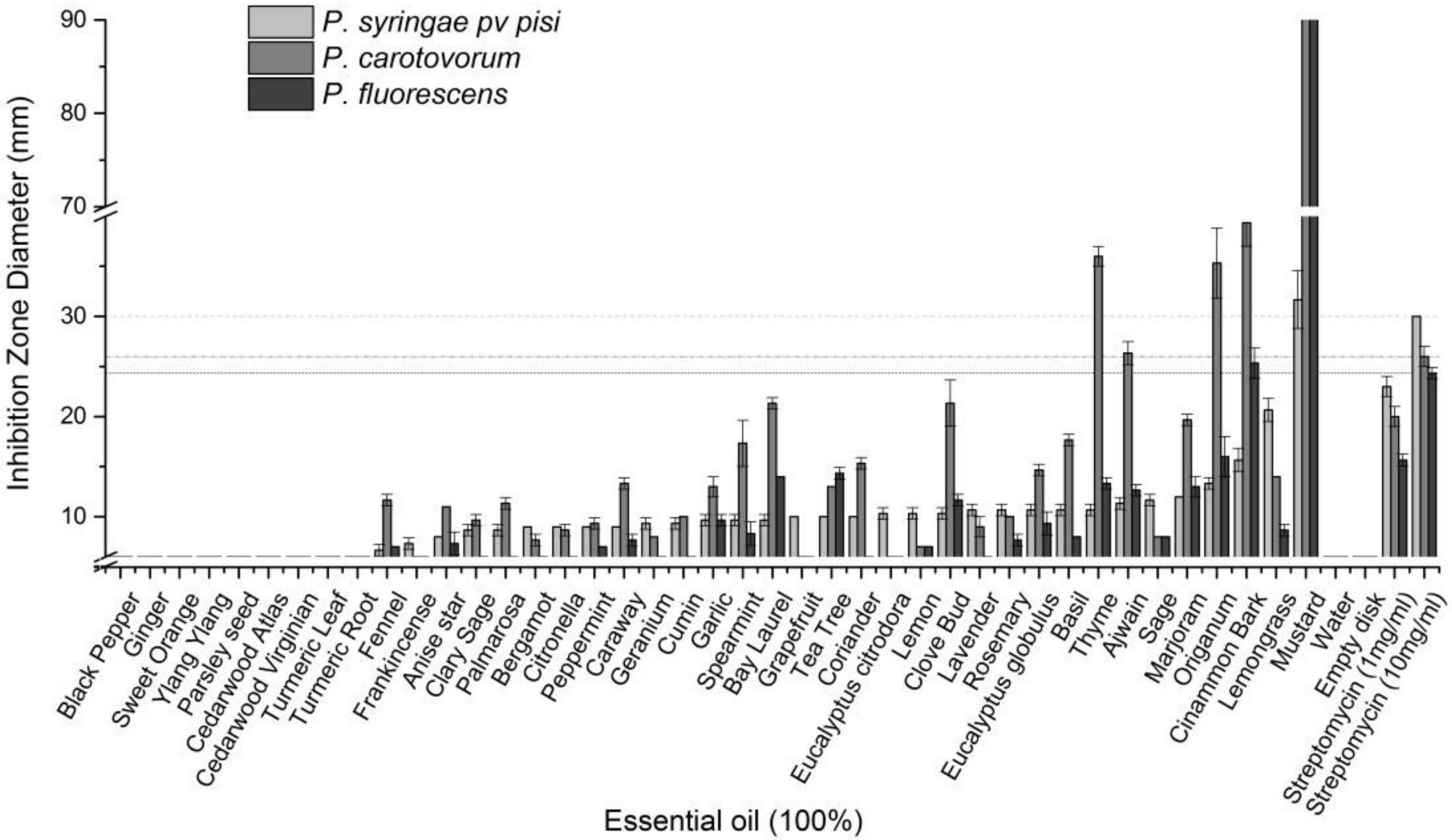

Figure 2.5 Efficacy of 41 essential oils against *Pseudomonas syringae* pv. *pisi*, *Pectobacterium carotovorum* subsp. *carotovorum* and *Pseudomonas fluorescens.* Essential oils are listed from least to most inhibitory against *P. syringae* pv *pisi* followed by the four controls: two streptomycin concentrations as positive controls and empty disk and water as negative controls. Y-axis starts at 6 mm (disc diameter). Horizontal dotted lines represent inhibition zones produced by streptomycin at 10mg/ml on *P. syringae* pv. *pisi* (··· 30 mm), *P. carotovorum* subsp. *carotovorum* (- - - 26 mm) and *P. fluorescens* (– 24.33 mm). Results represent the mean inhibition zone diameter of three replicates ±standard deviation of the mean.

2.3.2.3. Specificity of Essential Oils

The antimicrobial effect of the EOs observed was species-dependent. Highly antimicrobial EOs such as mustard, oregano or thyme produced significantly different inhibition zones ($p<0.01$) for the microorganisms tested (Figure 2.5) demonstrating the specificity of EOs and the difference in bacterial species susceptibility towards EOs. To further illustrate the specificity of EOs, Figure 2.6 represents the fold-change of the antimicrobial effect of EOs on *P. syringae* pv. *pisi* compared to non-pathogenic *P. fluorescens*. Mustard oil, despite being a potent biocide, had a negative specificity towards *P. syringae* pv. *pisi* and more effectively inhibited *P. fluorescens* growth. 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.

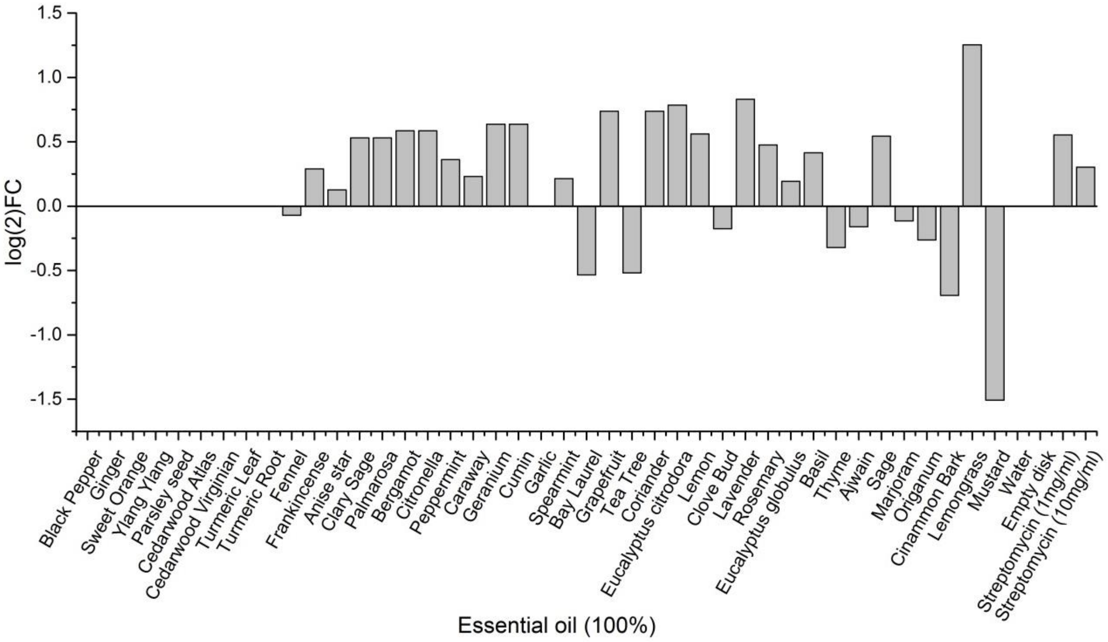

Figure 2.6 Selectivity of essential oils against phytopathogen *P. syringae* pv. *pisi*. EOs with positive Log_2 FC (fold-change) showed selectivity against *P. syringae* pv. *pisi* over the ubiquitous organism *P. fluorescens.* Four controls are included: two streptomycin concentrations as positive controls and empty disk and water as negative controls. Results represent the mean of three replicates ± standard deviation of the mean.

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.

2.3.3. Broth Microdilution Test

The minimum inhibitory concentration (MIC; lowest EO concentration resulting in clear wells) and the minimum bactericidal concentration (MBC, lowest EO concentration able to kill 99.9% of microorganisms, calculated by plating the original culture and 3 µl from clear wells) of EOs against *P. syringae* pv. *pisi* were obtained using the broth microdilution test (Table 2.3), which was performed with and without 2,3,5-Triphenyltetrazolium chloride (TTC) as a growth indicator; a colourless and water-soluble compound that can be enzymatically reduced by a variety of organisms to 1,3,5-triphenylformazan (TPF) which is water-insoluble and red coloured; indicating metabolic activity (Chang *et al.,* 1999). Streptomycin was used as positive control (1 mg/ml and 10 mg/ml) and culture media with and without ethanol 3% (v/v) were used as negative controls (Figure 2.7).

Absorbance (OD_{600}) was measured at 24h and 48h (Figure 2.8). Cinnamon, mustard, oregano, ajwain, thyme and lemongrass EOs were the most effective of the compounds tested in terms of antimicrobial activity against *P. syringae* pv. *pisi* at low concentrations, whilst the other oils tested were only able to inhibit bacterial growth at concentrations of ≥0.25% at 24h (Figure 2.8A) and ≥1% at 48h (Figure 2.8B).

The oils that demonstrated specificity towards the phytopathogen required much higher concentrations to be effective, with the exception of lemongrass and coriander. Lemongrass had an MIC of 0.25% after 48h and coriander of 1%, which despite being higher than other effective oils, was still able to inhibit *P. syringae* pv. *pisi* bacterial growth.

Table 2.3. MIC and MBC determination of twelve essential oils tested against *Pseudomonas syringae* pv. *pisi*

Essential Oil	MIC at 24 h	MIC at 48 h	MBC
Mustard	0.016%	0.063%	0.25%
Lemongrass	0.031%	0.125%	0.25%
Cinnamon	0.016%	0.031%	0.031%
Oregano	0.031%	0.031%	0.031%
Marjoram	0.25%	1%	1%
Sage	1%	>1%	>1%
Ajwain	0.031%	0.063%	0.063%
Thyme	0.031%	0.063%	0.063%
Lavender	1%	>1%	>1%
Eucalyptus citrodora	0.5%	>1%	>1%
Coriander	0.5%	1%	1%
Grapefruit	0.5%	>1%	>1%

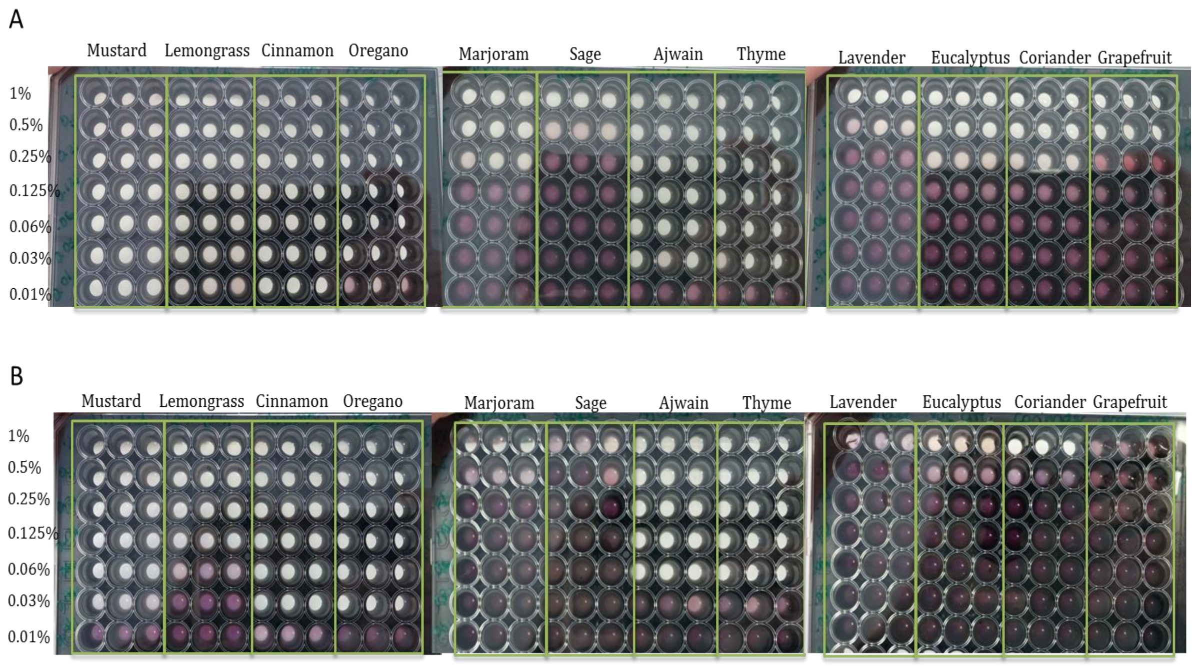

Figure 2.7. Broth dilution test to evaluate the antimicrobial effect of twelve essential oils (EOs) on the growth of *Pseudomonas syringae* pv. *pisi*. TTC is used as growth indicator, giving a red-purple colouring of wells when microorganism's growth is not inhibited. Clear wells indicate effective antimicrobial activity of the EO at the tested concentration. Concentrations from 1% to 0.01% of each oil were tested in triplicate. A) Results after 24 hours of incubation. B) Results after 48 hours of incubation.

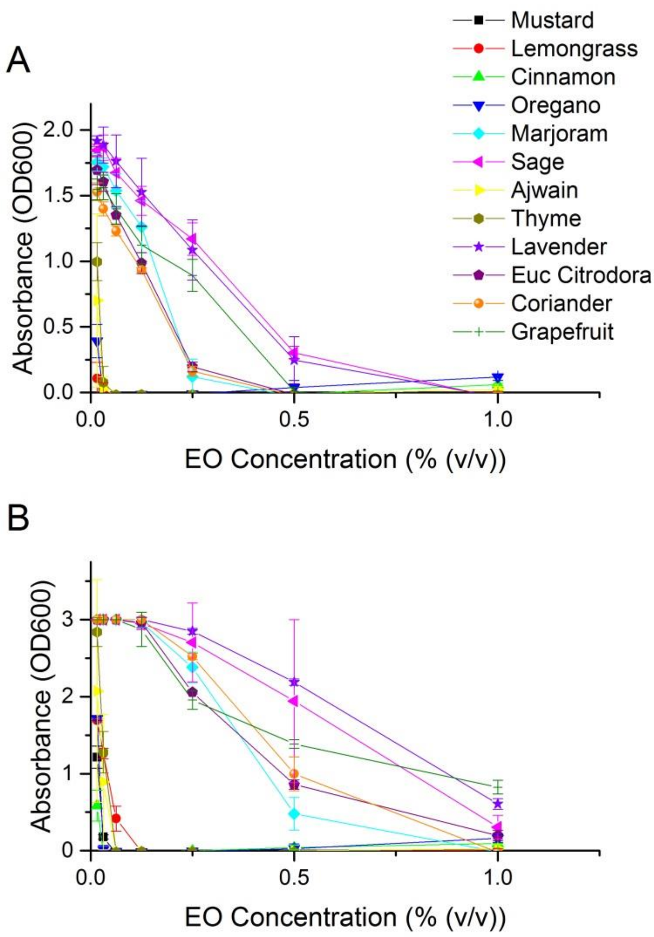

Figure 2.8. Absorbance (OD600) after A) 24 and B) 48 hours of incubation with TTC (growth indicator) showing *Pseudomonas syringae* pv. *pisi* growth after treatment with essential oil concentrations. Data shows mean ± standard deviation; n = 3.

2.4. Discussion

The research detailed in this chapter demonstrates the antimicrobial activity and species-specificity of a range of EOs against *Pseudomonas syringae* pv. *pisi*, *Pectobacterium carotovorum* subsp. *carotovorum,* and *Pseudomonas fluorescens.* The experiments revealed the most potent antimicrobial EOs against *P. syringae* pv. *pisi* as well as their MIC and MBC and provided the bases to determine the best oils for future nanoparticle encapsulation and delivery to potentially treat and prevent pea bacterial blight.

All the EOs 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 decreased sensitivity, as it has been shown to occur in other *P. syringae* pathovars, such as *P. syringae* pv. m*aculicola* and pv. *tomato,* which can overcome aliphatic isothiocyanate-based defences of *Arabidopsis* and elicit a disease outcome due to the presence of *sax* genes (Fan *et al.,* 2011). Furthermore, strains of *P. carotovorum* subsp. *carotovorum* isolated from *Brassica* and wasabi plants have been shown to have copies of *saxA* genes that confer resistance to isothiocyanates, but for the strains that are isolated from potato, only some have saxA genes (Welte *et al.,* 2016). This is consistent with the findings from this chapter where the *P. carotovorum* strain investigated (originally isolated from potato) did not exhibit any resistance against mustard oil (allyl isothiocyanate) suggesting it does not contain any *saxA* copies in its genome.

Even though the antimicrobial activity of EOs against phytopathogens has been reported (Table 2.1 and 2.2), there are only a few studies specifically related to *P. syringae* pv. *pisi.* The antimicrobial effects of garlic (Verma & 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 a number of phytopathogens, including *P. syringae* pv. *pisi* and *P. carotovorum* subsp. *carotovorum*, have been demonstrated*.* As with this study, *P. syringae* pv. *pisi* appeared to be more resistant to a greater number of EOs 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 few studies evaluating *P. syringae* pv. *pisi* only considered the activity of a few compounds; thus, this chapter provides a more comprehensive and inclusive analysis of the effect of a wider variety of EOs against it when compared to other pathogenic and non-pathogenic bacterial species.

The results presented in this chapter confirm the antimicrobial ability of EOs and demonstrate their ability to cause species-specific bactericidal effects. When comparing the results from the three bacterial species; mustard, cinnamon, oregano, ajwain and thyme showed a strong effect against all species 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 & Ugur 2008). This could be associated with an active efflux mechanism and the barrier function of the outer membrane (Cox & Markham 2007). In the case of *P. syringae* pv. *pisi*, the effect of mustard was the strongest, comparable to that of streptomycin at 10 mg/ml, while lemongrass and cinnamon also exhibited a greater antimicrobial effect than the remaining 38 oils.

Furthermore, of the four pathogen-specific oils tested, coriander was the only one capable of inhibiting bacterial growth at a concentration of 1% (v/v). The antimicrobial activity of coriander oil is greater than that of its main constituent, linalool; and its mechanism of action is by membrane damage leading to cell death (Silva *et al.,* 2011). Lo Cantore *et al.* (2004) demonstrated that *P. carotovorum* was at least six times more susceptible to coriander oil than *P. syringae* pv. *pisi* (Lo Cantore *et al.,* 2004), which is consistent with the findings of this study. Even though coriander exhibited a species-specificity towards *P. syringae* pv. *pisi*, the concentration needed to kill 99.9% of the bacterial population was more than 32 times higher than the concentration of cinnamon or oregano needed.

Mustard oil had the lowest MIC at 24 hours, together with cinnamon oil, however, at 48 hours cinnamon oil still had a low MIC while 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, which is detailed in Chapter 3, section 3.3.3.3 and has been reported by our research group (Chan *et al.,* 2017). Furthermore, previous studies have demonstrated that after 48h in water, allyl isothiocyanate (AIT, main component of mustard EO) 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 & Holley 2009) and may also explain why the antimicrobial activity of mustard EO 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. This could improve the effect of the oils at lower concentrations and decrease the MIC and MBC significantly and optimise the utilisation of such potent antimicrobials for prolonged periods of time.

For subsequent experiments, mustard and cinnamon oils were selected due to their strong antimicrobial effect against all species tested. Ajwain oil also exhibited one of the strongest antimicrobial effects 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.

2.5. Conclusions

In conclusion, investigations in this chapter evaluated the specificity and effectivity of 41 essential oils against three bacterial species and determined the minimum concentrations needed to inhibit bacterial growth and kill 99.9% of the bacterial population. Additionally, as a result from this chapter; mustard, cinnamon and ajwain essential oils will be evaluated in the following chapters as potential candidates for nanoparticle encapsulation and pea bacterial blight treatment.

Chapter 3
Synthesis, Characterisation and Loading of MSNPs

3.1. Introduction

The great potential of essential oils (EOs) as antimicrobials, and their application in fields such as agriculture, has been obstructed by the nature of these compounds. Nanotechnology presents a promising alternative to encapsulate, stabilise and protect volatile compounds, allowing a controlled release of the cargo. By encapsulating EOs into nanoparticles, these compounds could potentially be used in agriculture to protect seeds and plants from bacterial or fungal infections due to their strong antimicrobial properties. This chapter focuses on the synthesis of mesoporous silica nanoparticles (MSNPs) and their use as nanocarriers to encapsulate and deliver antimicrobials such as EOs. The experiments presented in this chapter aim to determine the loading and unloading behaviours of MSNPs using three of the most effective antimicrobial EOs studied in Chapter 2.

3.1.1. Mesoporous Silica Materials

The synthesis of mesoporous silica materials was first reported in 1990 by Japanese scientists using kanemite layers ($NaHSi_2O_5 \cdot 3H_2O$) as a template for the intercalation of alkyltrimethylammonium chloride (Yanagisawa *et al.,* 1990). Afterwards, in 1992, researchers from the Mobil Oil Corporation reported the synthesis of ordered mesoporous molecular sieves, MCM-41 (Mobil Composition of Matter No. 41) (Kresge *et al.,* 1992).

MCM-41 has a hexagonal array of uniform pores and channels and it belongs to a family of mesoporous materials known as M41S. The proposed synthesis for these materials was a 'liquid crystal templating' (LCT) mechanism; which consists of surfactant molecules organised into micellar liquid-crystals that act as templates for silicate condensation (Beck *et al.,* 1992). The pore diameters of these materials can be modified by altering the length of the alkyl chains of the surfactants or by adding hydrocarbons during the synthesis (Kresge *et al.,* 1992).

MCM-41 is still widely used today. In fact, the first report of mesoporous materials as drug delivery vehicles used MCM-41 nanoparticles loaded with ibuprofen (Vallet-Regi *et al.,* 2001).

3.1.2. Nanoparticles synthesis

Nanomaterials can be produced either by a 'top-down' or 'bottom-up' approach. The top-down methods usually result in imperfections and impurities and have problems achieving the necessary size reductions. Hence, nanoparticles are usually prepared *via* bottom-up methods that can produce more homogeneous particles at the nanometre scale (Singh *et al.,* 2014; Cao 2004).

There are a variety of methods to obtain silica nanoparticles (Figure 3.1); for the purpose of this thesis, only sol-gel synthesis will be studied.

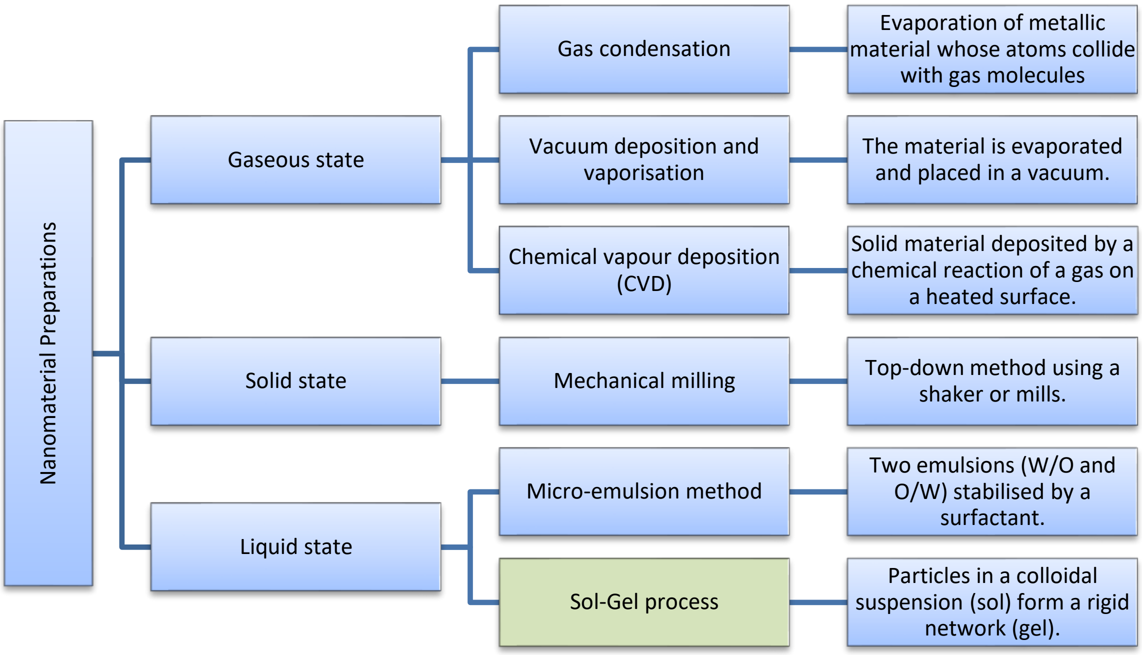

Figure 3.1 Methods for nanomaterial preparations. (Singh *et al.,* 2014).

3.1.2.1. Sol-gel synthesis

The synthesis of MSNPs is most commonly carried out by using a surfactant template and a sol-gel condensation of a precursor around such template. The sol-gel method (Figure 3.2) is the most commonly used to due to its simplicity, scalability and controllability and it was therefore used throughout this thesis.

The cationic surfactant cetyl trimethyl ammonium bromide (CTAB) is one of the most commonly used for MSNPs synthesis. The surfactant dissolves into a solution, and when the surface has been fully covered by the surfactant, the critical micellar concentration (CMC) is reached. Further addition of surfactant results in the formation of micelles which are grouped into micellar rods that later get organised into a hexagonal arrangement as the surfactant concentration increases (Figure 3.2) (Cao 2004).

As a simultaneous process to the template formation by the surfactant molecules, a precursor (usually a metal alkoxide) is added, together with a catalyst that will trigger the hydrolysis and condensation of the precursor around the template (Cao 2004).

Hydrolysis: $M(OEt)_4 + xH_2O \leftrightarrow M(OEt)_{4-x}(OH)_x + xEtOH$

Condensation: $M(OEt)_{4-x}(OH)_x + M(OEt)_{4-x}(OH)_x \leftrightarrow (OEt)_{4-x}(OH)_{x-1}MOM(OEt)_{4-x}(OH)_{x-1} + H_2O$

This process involves two phases. The first phase, or 'sol' phase, consists of particles suspended in a liquid, forming a stable colloidal suspension known as 'sol'. During the second phase, the particles in the suspension react with each other to create a 3D network or 'gel' (Singh *et al.,* 2014).

The two most common silicon alkoxides used during the sol-gel synthesis of MSNPs are TMOS (tetramethyl orthosilicate; $Si(OCH_3)_4$) and TEOS (tetraethyl orthosilicate; $Si(OC_2H_5)_4$); the latter was used in this chapter for NP synthesis. Since silicon alkoxides are not very sensitive to hydrolysis, acid or basic catalysts can be added to the process. Acid catalysts increase hydrolysis while basic ones enhance condensation (Livage 2004). For the protocol used in this chapter, the basic catalyst NaOH was used since silica is more soluble in alkaline conditions and this triggers the inter-linking of silica (Singh *et al.,* 2014).

After the condensation of the precursor around the template, the latter is removed either by calcination or by acidic refluxing to form hollow MSNPs. Variations to the synthesis process such as temperature, pH, silica precursor, surfactants and concentrations result in changes of the characteristics of MSNPs including particle and pore size as well as NP morphology. Figure 3.2 illustrates the general sol-gel process for the synthesis of MSNPs.

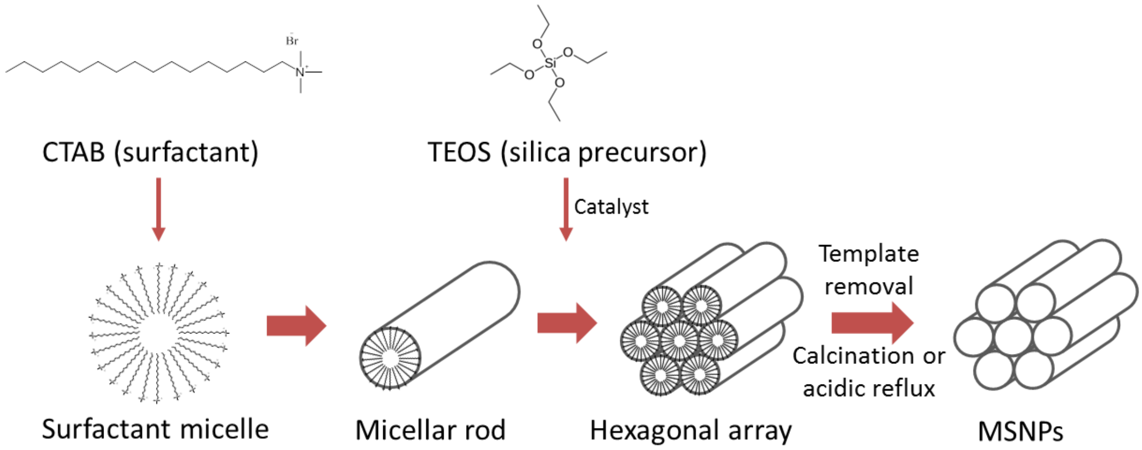

Figure 3.2 Sol-gel synthesis of MSNPs. A surfactant (CTAB) is added above its critical micellar concentration (CMC) to form micelles that are grouped into micellar rods that can organise into a hexagonal array that will function as template. A silica precursor (TEOS) is added and its hydrolysis and condensation around the template are triggered by a catalyst. The template is removed by calcination or acidic reflux to obtain the hollow MSNPs.

3.1.3. Aims and Objectives

The studies in this chapter aim to evaluate the potential application of MSNPs as biocide delivery vehicles in agriculture. The first objective was to synthesise MSNPs *via* the sol-gel method with the physicochemical characteristics needed to encapsulate the biocides. The second was to load the MSNPs with allyl isothiocyanate (AIT), cinnamaldehyde (CNAD) and Ajwain oil and estimate the loading and release capabilities of the nanoparticles. The resulting EO-loaded MSNPs will be used in the following chapters as biocide carriers against bacterial phytopathogens.

3.2. Materials and Methods

3.2.1. Synthesis of MSNPs

Mesoporous silica nanoparticles (MSNPs) were synthesised using a sol-gel hot aqueous solution as previously described (Hom *et al.,* 2010) with some modifications. Briefly, 100 mg cetyltrimethylammonium bromide (CTAB, 99%; Sigma Aldrich, UK) was dissolved in 48 ml deionised distilled water and 350 µl 2M NaOH (Fisher Scientific, UK) in a 250 ml round-bottom flask, stirring at 500 rpm. The solution was heated to 80°C and after temperature stabilisation, 500 µl tetraethylorthosilicate (TEOS; Sigma Aldrich, UK) was added. To avoid agglomeration, 127 µl of 3-(trihydroxysilyl)propyl methylphosphonate (Sigma Aldrich, UK) were added 15 minutes after the addition of TEOS. This step was included only for some syntheses, while other nanoparticles were synthesised without the addition of 3-(trihydroxysilyl)propyl methylphosphonate to compare the resulting MSNPs. Samples were stirred for 2 hours at 80°C before nanoparticles were washed twice with methanol (99+%, Acros Organics, UK). To ensure that the resulting particles could be loaded with hydrophobic molecules, the surfactant CTAB was subsequently removed by distillation by refluxing overnight in acidic methanol (20 ml methanol, 1 ml 37% hydrochloric acid) at 80°C. Particles were dried in a vacuum desiccator and collected.

3.2.2. MSNPs Characterisation

Synthesised MSNPs were characterised using SEM, TEM, disc centrifuge and zetasizer. The complete characterisation of hexagonal symmetry MSNPs has been previously reported by our research group (Huang *et al.,* 2014).

3.2.2.1. Scanning Electron Microscope (SEM)

Scanning electron microscopy (SEM) was used to visualise and analyse the size and morphology of the synthesised MSNPs and to evaluate the effect of the addition of 3-(trihydroxysilyl)propyl methylphosphonate on the morphology of the nanoparticles. SEM was performed using a Carl Zeiss Evo LS15 VP-SEM using a primary energy of 15 kV.

3.2.2.2. Transmission Electron Microscope (TEM)

Synthesised MSNPs were imaged *via* transmission electron microscopy (TEM) with JEOL JEM 2100 operating at 200 kV for bright field imaging. Specimens were prepared by drop-casting MSNPs onto holey carbon-coated TEM copper grids (carbon film on 3 mm 300 mesh, Agar Scientific, UK), allowing them to air-dry overnight before imaging.

3.2.2.3. Size Distribution and Zeta potential

The size distribution of the synthesised MSNPs was evaluated using a CPS Disc Centrifuge, Model DC24000 (CPS Instruments Inc, UK). Aqueous sucrose solutions (8% and 24%) were prepared with distilled water and a sucrose density gradient was formed inside the disc. The instrument was set to 24,000 rpm and calibrated using particles of known size (0.359 µm). MSNPs suspensions (100 µl) were injected into the disc centrifuge using a flat needle syringe for measurement.

A Malvern Zetasizer Nano ZS (Malvern Panalytical, UK) was used to determine the zeta potential of MSNPs. Nanoparticles were dissolved in distilled water (5 mg/ml) and sonicated during 15 minutes before analysis. The sample was injected into a previously cleaned disposable capillary cell DTS1070 (Malvern Panalytical, UK). Measurement was performed in triplicate.

3.2.3. Gas Chromatography – Mass Spectroscopy (GC-MS)

The twelve essential oils selected in Chapter 2 for analyses (mustard, cinnamon, ajwain, lavender, grapefruit, coriander, lemongrass, marjoram, eucalyptus, sage, oregano and thyme) were prepared for Gas chromatography – Mass spectrometry (GC-MS) analysis by dissolving in methanol at 0.1% (v/v). Samples were analysed using a Waters GCT Classic GC-MS and the conditions summarised in Table 3.1.

Table 3.1 GC-MS instrument conditions to analyse the chemical composition of essential oils.

GC-MS Conditions	
Instrument	Waters GCT Classic GC-MS
Ionisation	Electron impact Chemical Ionisation
Analyser	Time of Flight (TOF)
Injector temperature	300°C
Injection volume	0.5 µl
Injection mode, Split ratio	Split, 10:1
Flow rate	1.2 ml/min
Column	HP-5, 30 m x 250 µm x 0.24 µm
Oven programme	50°C for 3 min 10°C/min to 320°C
Solvent	Methanol

3.2.4. Loading of EOs into MSNPs

CNAD (1.5 ml) or AIT (750 µl) was dissolved in dimethyl sulfoxide (DMSO, Sigma Aldrich, UK) to a total volume of 6 ml. The mixture was added to 21 ml PBS with 60 mg MSNPs and sonicated with a probe (Vibra-Cell VCX 130, Sonics & Materials Inc., USA) for 3 minutes at 5 second intervals, before stirring at 250 rpm for 24 hours in dark conditions.

Loaded MSNPs were centrifuged at 8,500 rpm for 7 minutes. The concentration of AIT and CNAD in the supernatant was analysed by liquid chromatography (see section 3.2.5) and compared to a calibration curve to calculate the loading capacity of the nanoparticles. A control in the absence of MSNPs was used to account for the oil that was lost from the system *via* evaporation.

After this, MSNPs were washed twice with 50:50 (v/v) methanol: distilled water and once with PBS to remove any excess oil and to ensure the removal of DMSO, which can be potentially toxic to bacterial cells. Washed MSNPs were dried in a vacuum desiccator and collected.

For Ajwain oil, 48 µl of EO dissolved in 1 ml DMSO were added to 4 ml PBS containing 60 mg MSNPs. The mixture was sonicated with a probe for 3 minutes at 5 second intervals before stirring at 900 rpm for 24 hours in dark conditions. The solution was frozen at -80°C and freeze-dried in a vacuum lyophiliser for 24 hours or until all liquid had evaporated and loaded-MSNPs were collected.

3.2.5. Loading and volatility studies

The volatility of AIT, CNAD and Ajwain oils was evaluated by incubating 10 µl of each EO in 10 ml PBS (with or without the presence of 20 mg empty MSNPs) at 30°C and 150 rpm for 24 hours.

Volatility and loading tests were carried out *via* liquid chromatography (LC) analysis, using an Agilent Technologies 1120 Compact LC, equipped with a Zorbax Eclipse Plus C18 column (Agilent Technologies, UK). Samples were analysed using an isocratic ratio 80:20 (acetonitrile: H_2O), UV detector (λ = 270 nm), flow of 1 ml/min and an injection volume of 10 µl (3 for loading tests) over 4 minutes. Calibration curves for both compounds were constructed using these LC parameters to determine the loading capacity of the MSNPs.

To estimate the loading capacity of the MSNPs, the oil that remained in the supernatant after the loading process was analysed *via* LC. Owing to the volatility of the compounds, a control sample was also set up in parallel which contained essential oils in the same solvent system, but in the absence of nanoparticles. The difference in the amount of essential oils in the supernatant between the control sample and the experimental sample was the amount loaded into the MSNPs.

3.2.6. Release studies

Resulting EO-loaded MSNPs, resuspended in 5 ml of PBS, were incubated at 30°C and 150 rpm to allow the molecules to be released *via* diffusion. Release was monitored every hour for 4h and then again at 24h, *via* LC using the instrument and method described in Section 3.2.5.

3.3. Results

3.3.1. MSNPs Characterisation

MSNPs with and without the addition of 3-(trihydroxysilyl)propyl methylphosphonate were initially prepared. SEM imaging confirmed a significant difference in size and morphology between the two types of nanoparticles (Figure 3.3). MSNPs synthesised without the addition of 3-(trihydroxysilyl)propyl methylphosphonate were agglomerated but presented a homogenous morphology where all nanoparticles were spherical and measured approximately 100nm in diameter (Figure 3.3 A&B). In contrast, samples synthesised with the addition of 3-(trihydroxysilyl)propyl methylphosphonate to reduce agglomeration resulted in both spherical and elongated particles ranging between 100nm and 1µm in diameter (Figure 3.3 C&D).

The differences between the two types of nanoparticles could play a significant role in the biocide encapsulation as well as on their subsequent effect on the seeds, plants and bacterial pathogens. To ensure a more homogeneous sample, MSNPs synthesised without the addition of phosphonates were selected for future experiments.

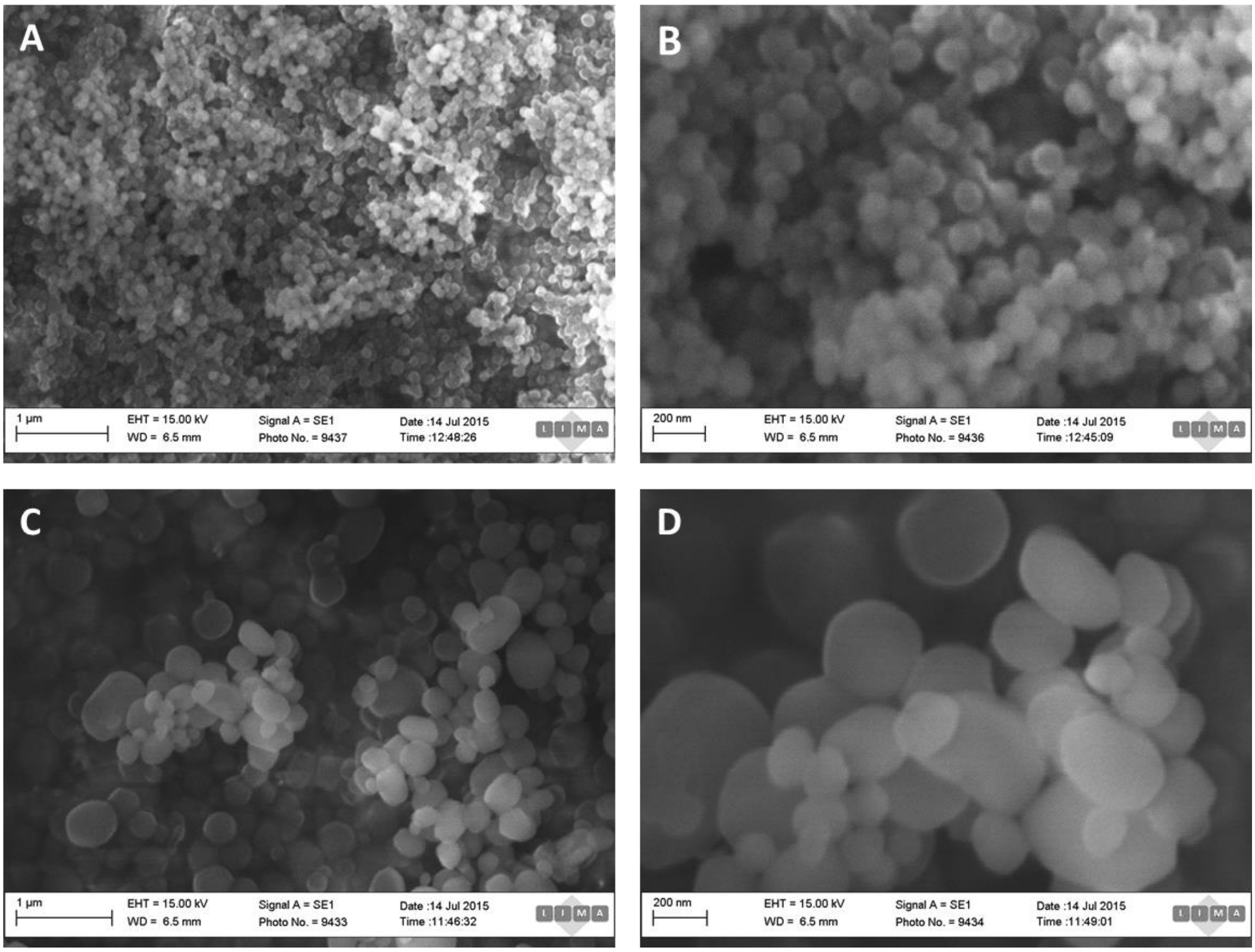

Figure 3.3 SEM Characterisation of MSNPs. A) and B) MSNPs synthesised via sol-gel method. C) and D) MSNPs synthesised with the addition of phosphonate groups to prevent agglomeration of particles. Addition of phosphonates resulted in larger and less homogeneous MSNPs.

MSNPs were then observed using a TEM to determine shape and dimension of the nanoparticles as well as pore characteristics (Figure 3.4). MSNPs appeared spherical, with a diameter close to 100 nm, and depending on the orientation of the nanoparticles during imaging, both pores (Figure 3.4A) and channels (Figure 3.4B) could be visualised.

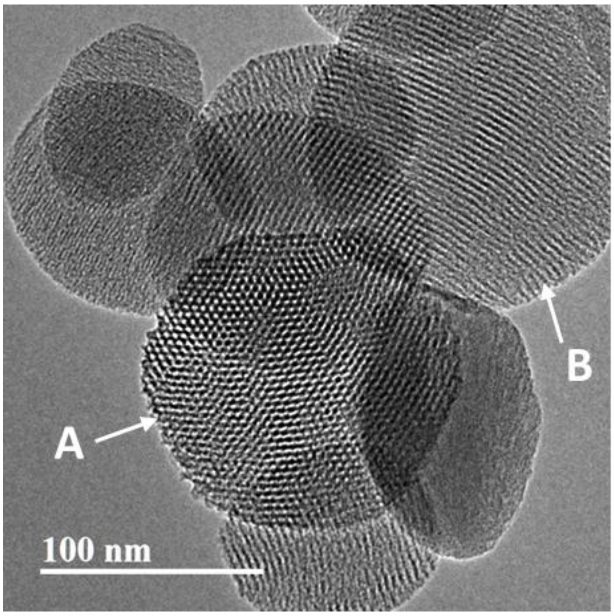

Figure 3.4 TEM Imaging of MSNPs. Pores (A) and channels (B) can be observed according to the nanoparticle orientation during imaging.

The zeta potential of MSNPs was -28.5 ± 1.02 mV at physiological pH. The size distribution of the nanoparticles (Figure 3.5) showed that almost half of the total MSNPs (47.1%) had a diameter between 89.9 – 149.8 nm, with 75.13% of the total MSNPs having a diameter <249.4 nm.

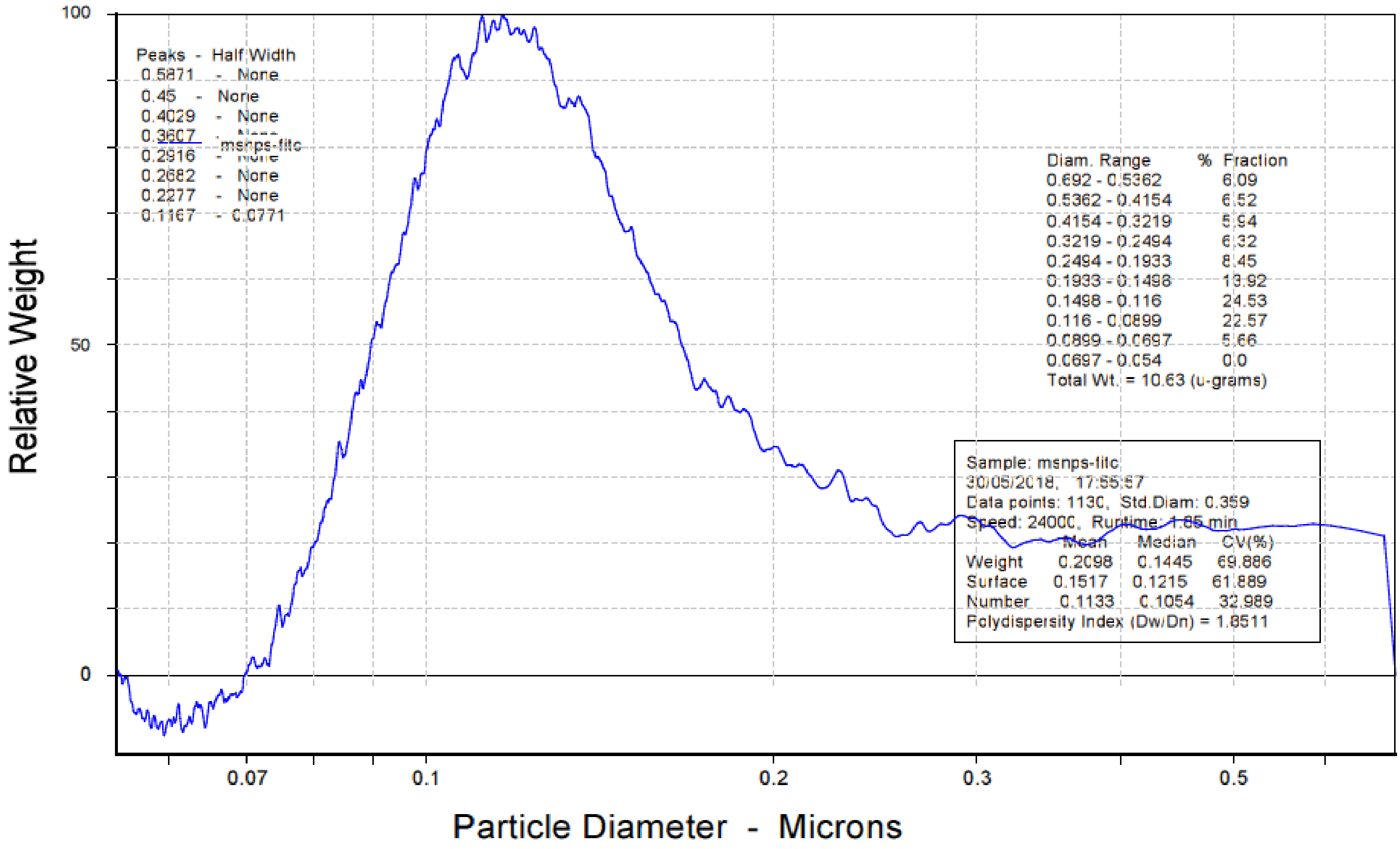

Figure 3.5 Size distribution of nanoparticles analysed *via* CPS Disc Centrifuge.

3.3.2. Analysis of essential oil composition (Gas Chromatography-Mass Spectroscopy)

The twelve EOs previously selected in Chapter 2 due to their antimicrobial activity were analysed *via* GC-MS to determine their chemical composition.

Table 3.2 summarises the percentage composition of EOs while Figure 3.6 and Figure 3.7 illustrate the chromatograms for each EO with its main component labelled. Allyl isothiocyanate (AIT), cinnamaldehyde (CNAD) and Ajwain oil were selected for encapsulation into MSNPs.

Table 3.2 GC-MS. Percentage of the main constituents of twelve EO analysed by GC-MS.

Constituent / Essential Oil	1-Methylverbenol	Allyl isothiocyanate	Benzyl benzoate	Iso-Borneol	Endo-Borneol	Bornyl Acetate	Camphene	Camphor	trans-3-Caren-2-ol	3-Carene	Carveol	Caryophyllene	Cinnamaldehyde
Mustard		96.27											
Cinnamon Bark			1.16						1.1				65.73
Ajwain													
Lavender					2.56			6.97		0.7		2.49	
Grapefruit													
Coriander							1.18	7.01	4.09				
Lemongrass	16.78											3.68	
Marjoram									3.97	6.04	1.63	1.41	
Eucalypto												1.49	
Sage				1.14	3.96	1.78	6.52	20.02	0.99			7.74	
Oregano												2.33	
Thyme					1.44		0.54			2.45		0.93	

Constituent / Essential Oil	Cinnamyl acetate	Citral	Citronellal	Citronellol	β-Ocymene	o-Cymene	Eucalyptol	Eugenol	Geraniol	Humulene	D-Limonene	Linalool	Linalyl acetate
Mustard													
Cinnamon Bark	9.43					2.2	3.1	6.08					
Ajwain						8.89							
Lavender													42.19
Grapefruit											97.88		
Coriander						2.83						70.27	2.2
Lemongrass		29.07							8.35				
Marjoram					1.72	11.12							
Eucalypto			85.31	8.14			0.71						
Sage						0.9	11.42			3.76			
Oregano						15.07							
Thyme						35.06							

Constituent / Essential Oil	Linalyl anthranilate	cis-p-Mentha-2,8-dien-1-ol	α-Phellandrene	α-Pinene	β-Pinene	iso-Pulegol acetate	Terpinen-4-ol	4-Terpinenyl acetate	γ-Terpinene	α-Terpineol	Thujone	Thymol	cis-Verbenol
Mustard													
Cinnamon Bark			0.33	0.74			0.46			1.73			
Ajwain												91.11	
Lavender	32.18						2.45			1.19			
Grapefruit				0.59	0.54								
Coriander				6.97	1.17				3.84	0.44			
Lemongrass													24.72
Marjoram		1.92	0.32	0.57	1.02		24.03	21.98	13.9	5.2			
Eucalypto					0.56								
Sage				7.09	1.97						30.13		
Oregano				1.15					1.44			79.08	
Thyme				1.66		0.71				11.67		45.54	

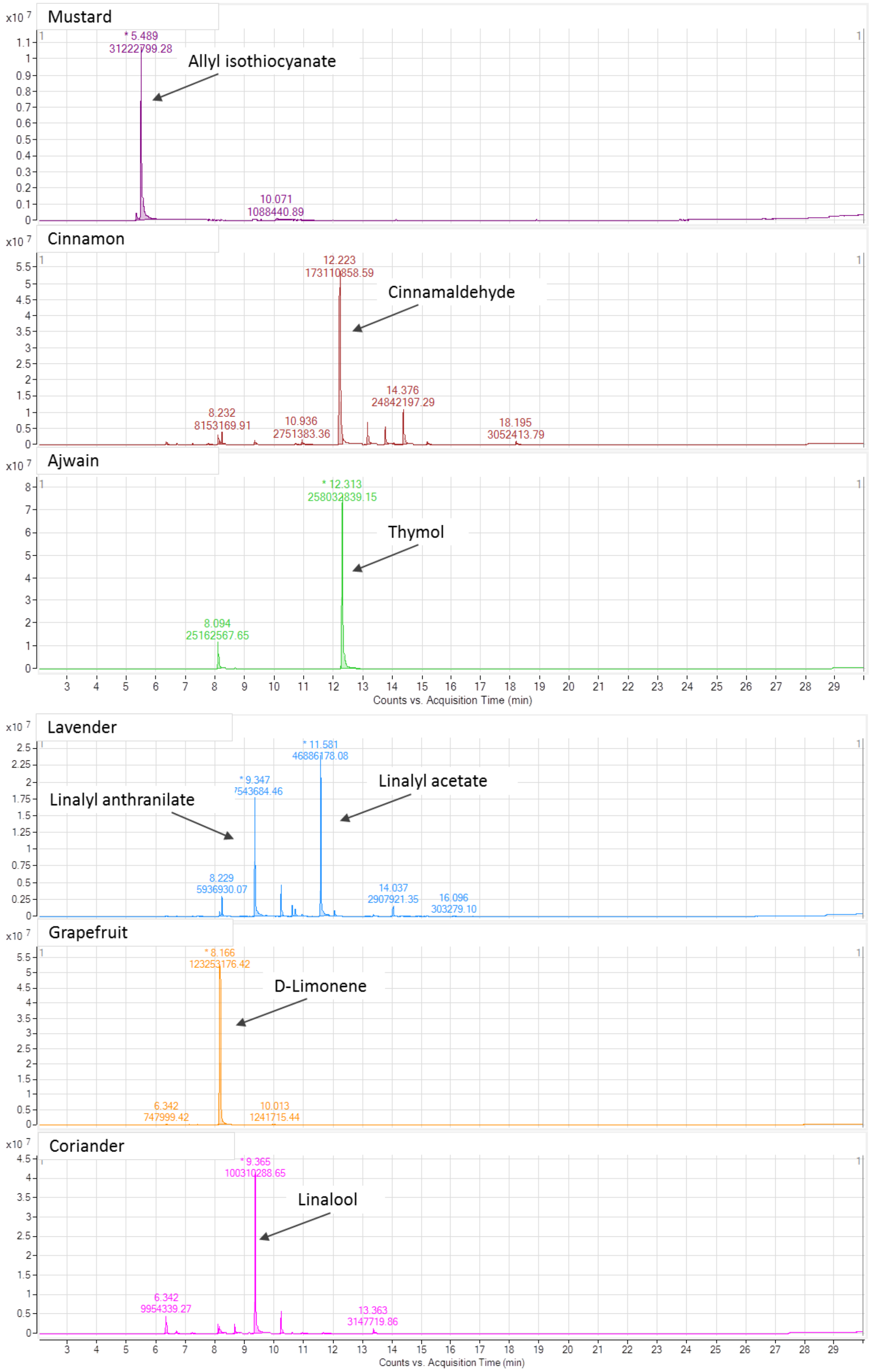

Figure 3.6 Essential oil chromatograms for mustard, cinnamon, ajwain, lavender, grapefruit and coriander EOs. X-axis shows retention time and y-axis shows the intensity (abundance) of each compound.

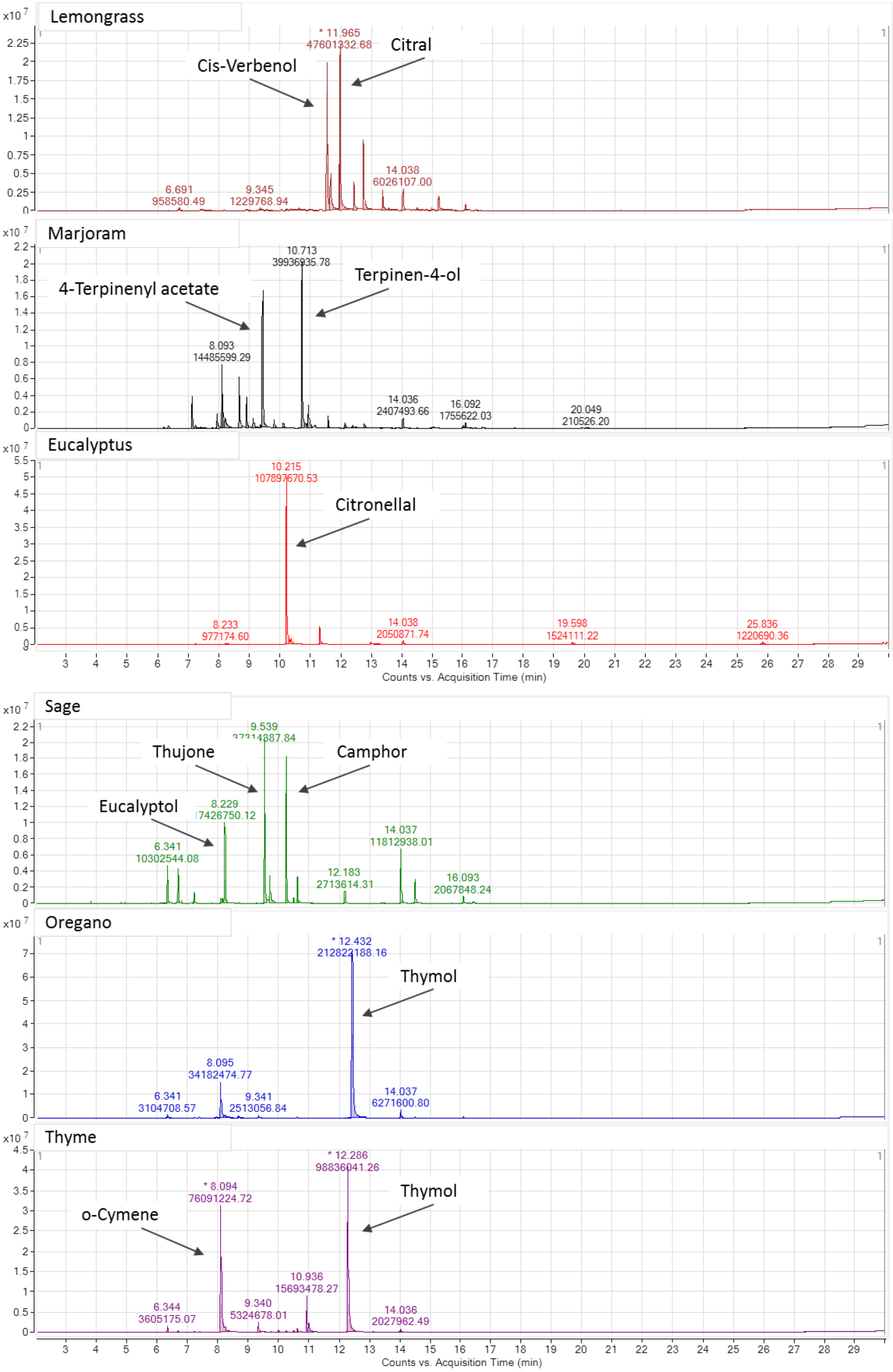

Figure 3.7 Essential oil chromatograms for lemongrass, marjoram, eucalyptus, sage, oregano and thyme EOs. X-axis shows retention time and y-axis shows the intensity (abundance) of each compound.

3.3.3. Loading, release and volatility studies (Liquid Chromatography)

Calibration curves for AIT, CNAD and Ajwain oil were prepared *via* LC analysis (Figure 3.8) and were used to quantify volatility, loading and release of the oils from MSNPs. AIT and CNAD had a retention time of 2.2 and 1.98 minutes, respectively. In the case of Ajwain, since it is made up of several compounds, the calibration curve was prepared for its main component (thymol), which had a retention time of 2.43 minutes.

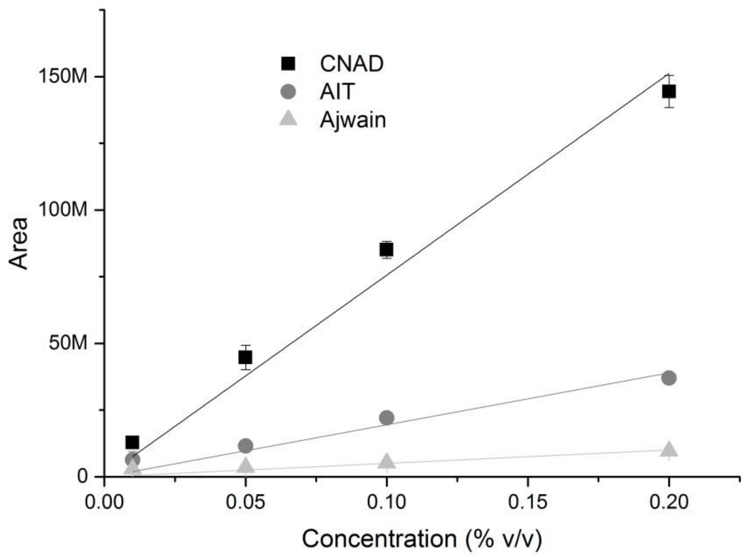

Figure 3.8 Calibration curves. Liquid chromatography calibration curves for CNAD, AIT and Ajwain oils. For Ajwain oil, only its main component (thymol) was used to produce the calibration curve, assuming its concentration was representative of that of the whole oil. R^2 = 0.99 (CNAD), R^2 = 0.98 (AIT) and R^2 = 0.93 (Ajwain). Data represents mean ± standard deviation, n = 3.

3.3.3.1. Loading of EOs into MSNPs

The loading capacity of the MSNPs assessed *via* LC was approximately 7.4 mg AIT, 0.95 mg CNAD and 7.03 mg Ajwain EO for every 10 mg MSNPs and was calculated as follows:

$$Control\ average\ Area - NPs\ average\ Area = Difference\ (loaded)$$

AIT (ρ = 1.01 g/ml) loaded into 10 mg MSNPs suspended in 4.5 ml PBS:

$$Area\ (\mathrm{y}) = 125{,}426{,}438 - 93{,}679{,}987 = 31{,}746{,}452$$

$$Using\ calibration\ curve\ for\ AIT: y = 1.95 \times 10^8 x$$

$$x = 0.163\% \frac{v}{v} = 1{,}646 \frac{mg}{L}$$

$$\underline{= 0.74\ mg\ AIT\ loaded\ into\ 1\ mg\ MSNPs}$$

CNAD (ρ = 1.05 g/ml) loaded into 10 mg MSNPs suspended in 4.5 ml PBS:

$$Area\ (\mathrm{y}) = 623{,}895{,}450.3 - 608{,}715{,}140.3 = 15{,}180{,}310$$

$$Using\ calibration\ curve\ for\ CNAD: y = 7.56 \times 10^8 x$$

$$x = 0.02\% \frac{v}{v} = 210 \frac{mg}{L}$$

$$\underline{= 0.0945\ mg\ CNAD\ loaded\ into\ 1\ mg\ MSNPs}$$

For Ajwain EO, MSNPs were not washed after the loading process and instead, the loaded-MSNPs were freeze dried to maintain most of the oil in the NPs. Since there was no supernatant left to estimate the loading capacity, the highest concentration of oil released from the NPs was calculated and considered as the amount of Ajwain oil loaded into the NPs (see section 3.3.3.2):

$$x = 0.156\% \frac{v}{v} = 1405 \frac{mg}{L}$$

$$\underline{= 0.703\ mg\ Ajwain\ EO\ loaded\ into\ 1\ mg\ MSNPs}$$

3.3.3.2. Release studies (Unloading)

The release of EOs from MSNPs was measured during 24h of incubation (Figure 3.9). The concentration of CNAD reached 35.63 mg/L after 24h (Figure 3.9A). AIT concentration increased during the first two hours, reaching its highest concentration of 187.59 mg/L after 2h and then declining to 113.06 mg/L after 24h due to the rapid evaporation of this oil (Figure 3.9B). For Ajwain oil, since the drying process after the loading of this oil comprised freeze-drying the loaded-MSNPs instead of washing them and vacuum-drying, the amount of oil present on the MSNPs was released immediately after resuspending the MSNPs. The highest concentration of ajwain oil measured was of 1405.88 mg/L at t = 0h and slowly decreased during incubation due to the evaporation of the oil (Figure 3.9C); even though the evaporation was not as rapid as that of AIT.

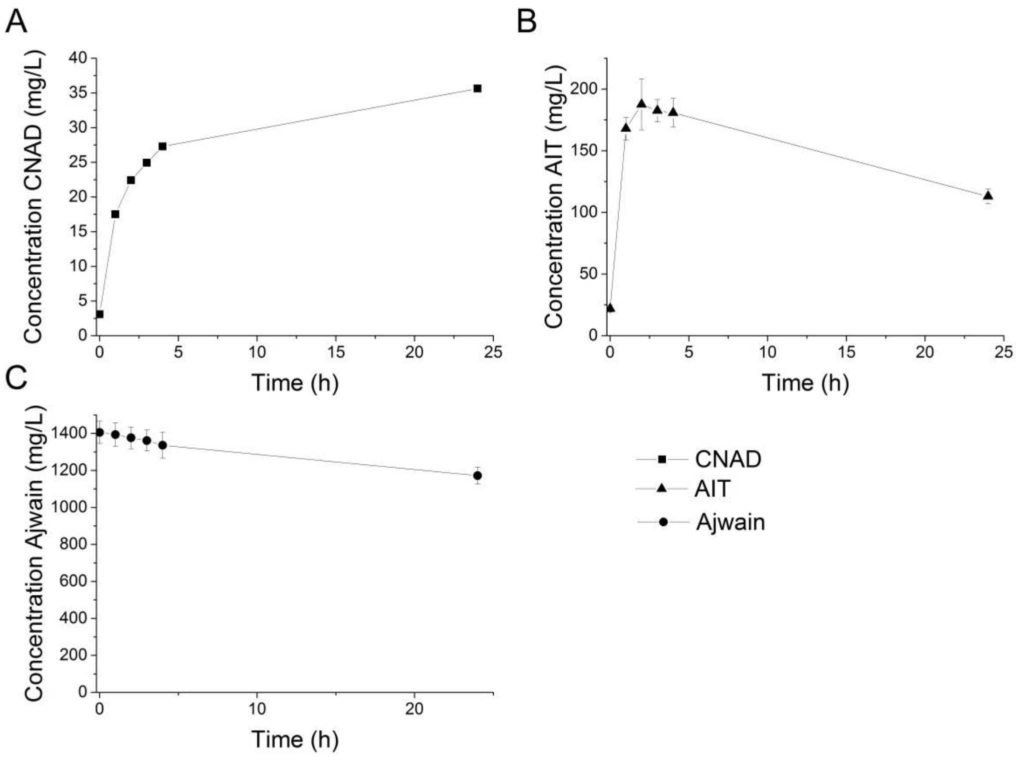

Figure 3.9 Biocide unloading behaviour from MSNPs. Concentration of A) CNAD, B) AIT, and C) Ajwain EO released from 10 mg MSNPs resuspended in 5 ml of water during 24h of incubation. For Ajwain oil, only its main component (thymol) was measured, assuming its concentration was representative of that of the whole oil. Data shows mean ± standard deviation, n = 3.

3.3.3.3. Volatility studies

To evaluate the volatility of the oils, AIT, CNAD and Ajwain oils were incubated with and without empty nanoparticles. This confirmed that AIT was highly volatile, and up to 65% of the oil in solution evaporated within 24h (Figure 3.10). CNAD was less soluble than AIT and therefore the concentration of oil in solution increased during the first hour of incubation. However, after the first hour, a slight decrease in concentration demonstrated the volatility of this compound with <10% of the oil in solution lost from the system after 24h (Figure 3.10). Ajwain oil behaved in a similar manner to CNAD, with only around 10% of the oil having evaporated after 24h (Figure 3.10).

The concentration of each compound and rate of evaporation were not affected by the presence of unloaded nanoparticles in the solution, suggesting that no oil became immobilized by irreversible adherence to the nanoparticles. Taken together, it can be inferred that the decline in concentrations of oil released from the MSNPs represents a quasi-steady-state equilibrium between continued release from the MSNPs and evaporation.

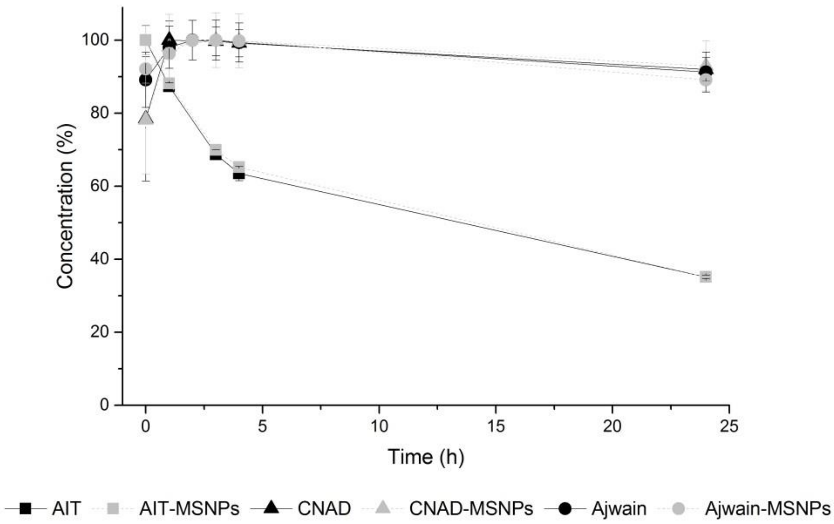

Figure 3.10 Volatility profiles of essential oil compounds. Volatility of 10 µl of free AIT, CNAD, and Ajwain oil incubated in 10 ml PBS as estimated for the decrease in concentration in the absence and presence of unloaded MSNPs. Data shows mean ± standard deviation, n = 3.

3.4. Discussion

The main objective of this chapter was to synthesise MSNPs and load them with specific EOs, so that they could be used as antimicrobial delivery vehicles. The loading and unloading capabilities of the synthesised MSNPs were evaluated since the focus of the subsequent chapters was to deliver antimicrobials to bacterial phytopathogens *in planta*.

In the previous chapter, twelve EOs selected for their high antimicrobial activity against phytopathogen *Pseudomonas syringae* pv. *pisi* were investigated. In this chapter, they were analysed *via* GC-MS to determine their chemical composition and to select the compounds that would be encapsulated into the MSNPs. Some EOs were composed mainly of one compound, such as mustard EO (96.27% AIT) or grapefruit (97.88% D-limonene) but most contained one main component and several other compounds in much smaller quantities. Chemical compounds like caryophyllene, o-cymene, α-pinene, β-pinene or α-terpineol, were present in most EOs tested.

Research presented in the previous chapter demonstrated that mustard, cinnamon and ajwain essential oils would be promising candidates for nanoparticle encapsulation. For the first two EOs, their main components were selected for encapsulation: AIT (96.27%) and CNAD (65.73%), respectively. These compounds were selected as they make up most of the essential oil, and have a strong antimicrobial activity on their own (Chan *et al.,* 2017). Also, previous studies in our research group had involved these two compounds and their loading into MSNPs, providing results that could be compared to those from this study (Chan *et al.,* 2017). Ajwain oil was selected for encapsulation due to its high antimicrobial properties and since it has not been as extensively studied as AIT and CNAD, there are only a few reports of its potential use in agriculture (Dwivedi & Singh 1998; Sahaf *et al.,* 2007; Shojaaddini *et al.,* 2008). GC-MS analysis of Ajwain oil revealed that thymol makes up 91.11% of this oil; however, thymol usually makes up 35-60% of Ajwain oil (Bairwa *et al.,* 2012). The increased percentage of thymol in the Ajwain oil sample was possibly due to the fast evaporation of other minor components before analysis.

MSNPs were synthesised to encapsulate the selected EOs. The size and morphology of the obtained nanoparticles was consistent with previous reports of this type of MSNPs (Huang *et al.,* 2014). The resulting MSNPs were used to encapsulate AIT, CNAD and Ajwain oil. The loading of MSNPs was carried out with excess oil to maximize the loading efficiency. EOs were adsorbed onto the silica surface and in the nanoparticles' pores by hydrogen bonds and van der Waals forces that can be broken by water to enable the release of the biocide (Jia *et al.,* 2013).

AIT and CNAD have been encapsulated into MSNPs by our research group (Chan *et al.,* 2017). Other studies have encapsulated essential oil components, including AIT and CNAD, into mesoporous materials such as MCM-41 but the loading and unloading efficiency is not always reported (Janatova *et al.,* 2015). Bernardos *et al.* (2014) encapsulated EO components such as CNAD and thymol (Ajwain's main component) into MCM-41 and demonstrated loading of 0.537 mg and 0.496 mg per mg MCM-41, respectively (Bernardos *et al.,* 2014). The results from this chapter revealed a greater loading for thymol (Ajwain oil) but this was 5-fold lower for CNAD. The encapsulation of AIT into MCM-41 or SBA-15, another mesoporous material, has previously been reported to have a loading efficiency of 0.8 and 0.9 mg/mg_{NPs}, similar to the results presented in this chapter of 0.74 mg/mg_{NPs} (Park & Pendleton 2012; Siahaan *et al.,* 2013).

The concentration of EO released from the MSNPs was evaluated to determine the unloading behaviour of the nanoparticles. The high initial rates of release over the first hour for AIT and CNAD may have been due to the steep concentration gradient established from within and outside the pores in the external solution, or because of release of compounds bound to the external surface of the nanoparticle (see Huang *et al.,* 2014 for a discussion of the ratio of external to internal surface area).

Subsequently, there was a slight and gradual decrease in AIT concentration from 1 to 24 h. This may have been due to the highly volatile nature of the plant compound, which is easily lost from the aqueous phase *via* spontaneous vaporization. Even though there was a decrease in concentration of AIT in solution, the decrease observed during 24 hours was 39.73%, while during the volatility studies up to 65% of AIT evaporated within the same timeframe. This suggested that even though there was a decrease in the concentration of AIT in the solution, AIT was still being released from the MSNPs at a rate slower than the evaporation rate. On the contrary, for CNAD, the evaporation rate was slower than the release rate which would explain the continuous concentration increase during the 24 hours of incubation. In the case of Ajwain EO, results show that all the biocide (or most of it) was released at t=0h. Hence, the slight decrease in concentration during the 24h was solely due to the evaporation rate while no oil, or only a very small amount, was released after the initial unloading.

Henry's Law volatility constant for AIT is 2.4 x 10^{-4} atm-m^3/mole (PubChem 2018a), for CNAD it is 1.6 x 10^{-6} atm-m^3/mole (PubChem 2018b) and for thymol (Ajwain EO's main component) is 3.5 x 10^{-6} atm-m^3/mole (PubChem 2018c). This means that AIT has a greater tendency than the other oils to escape from the water phase into the atmosphere. This could explain why the decrease in concentration from 1h to 24h was much greater for AIT than for CNAD or Ajwain EO, which behaved in a similar manner.

Much greater concentrations of AIT were detected in solution than of CNAD (approximately 5-fold) as a result of diffusive release from the same amount of MSNPs. This may have been due to a combination of several factors: firstly, AIT is a smaller molecule than CNAD and may be able to load at a higher density. Considering the longest inter-atomic distance that occurs within each molecule (5.77 Ǻ (angstrom, 1 Ǻ = 0.1 nm) for AIT and 8.5 Ǻ for CNAD) and adding the Van der Waals radii (for AIT 1.2 Ǻ (hydrogen) and 1.8 Ǻ (sulphur); for CNAD 1.2 Ǻ (hydrogen) and 1.55 Ǻ (oxygen)), the smallest cube that could contain one CNAD molecule, would have dimensions of approximately 6.50 × 6.50 × 6.50 Ǻ and a volume of approximately 274.02 $Ǻ^3$. By contrast, the smallest cube containing an AIT molecule would have dimensions of approximately 5.06 × 5.06 × 5.06 Ǻ and a volume of approximately 129.81 $Ǻ^3$. As the AIT molecules are smaller than the CNAD molecules, approximately twice as many AIT molecules could be housed in the mesopores of MSNPs as compared to CNAD molecules. Secondly, as AIT is a smaller molecule than CNAD, it may have been released more readily from pore openings due to less physical self-obstruction. Thirdly, AIT is more soluble in water (2000 mg/L) (Yalkowsky *et al.,* 2010) than CNAD (1420 mg/L) (Valvani *et al.,* 1981) or than thymol (900 mg/L) (Yalkowsky *et al.,* 2010) so it could be more easily unloaded from the MSNPs into the aqueous PBS.

For Ajwain EO, the concentration detected in solution was much greater than that of AIT or CNAD. This could have been due to the difference in the loading protocol. During the loading of Ajwain oil, the MSNPs were freeze-dried instead of being washed since the density of the oil prevented the efficient washing of the MSNPs. This resulted in a higher concentration of the oil being encapsulated and, in particular, adsorbed onto the surface of the MSNPs. However, due to this excess loading, the release of Ajwain oil was a lot faster than for the other compounds, with most of the oil being released immediately after the MSNPs were added to the solution.

AIT- and CNAD-loaded MSNPs resulted in a more controlled unloading of the cargo. In contrast, even though Ajwain-MSNPs released the biocide immediately after being added to the solution, they were loaded with a much higher concentration of the antimicrobial.

3.5. Conclusions

In conclusion, the research detailed in this chapter aimed to synthesise MSNPs and to quantify the EOs released from the system. The composition of EOs was assessed to select the compounds that would be encapsulated into MSNPs. Furthermore, the amount of EO loaded into 10 mg MSNPs was calculated for AIT, CNAD and Ajwain EO; as well as the concentration of biocide released into solution from the loaded-MSNPs. The results from this chapter demonstrate the loading and unloading capabilities of MSNPs and function as the foundation for the following chapters, where these loaded-MSNPs will be used to deliver biocides to bacterial phytopathogens both *in vitro* and *in planta.*

Chapter 4
Alginate-MSNPs Coating as a Seed Treatment

4.1. Introduction

This chapter centres on a key challenge in terms of chemical engineering; that of controlled delivery of chemicals. In this instance, the development of a seed coating incorporating biocide-loaded mesoporous silica nanoparticles (MSNPs) for the protection of plants against bacterial pathogens. Seed treatments and coatings are one of the most promising ways of controlling bacterial infection of plants, reducing chemical loss and environmental contamination. Seed treatments reduce the contact area from 10,000 m^2 (foliar applications) or 500 m^2 (furrow applications) to only 50 m^2 (International Seed Federation 2007). This method accomplishes a high efficacy in crop protection while reducing the quantity of chemical required and consequently reducing application costs as well as the potential environmental impact. A coating containing MSNPs loaded with essential oils as antimicrobials would be able to act as a protective barrier, releasing the essential oils and killing the pathogen before it can invade and colonise the plant.

4.1.1. Seed Treatments and coatings

The delivery of biocides to plants in agriculture can be applied directly on the seed *via* a 'seed coat' to enable a more effective delivery when compared to land applications (Scott 1989). Seed coatings can be used to protect seeds, supply nutrients and growth factors, control germination, and modify the seed morphology to facilitate handling.

There are three types of seed coatings: i) film coating, which is a thin layer surrounding the seed, ii) encrusting, which is when the seed weight is increased up to 100-500% but the shape is not significantly altered, and iii) pelleting, which completely alters the shape of the seeds (Pedrini *et al.,* 2017). This chapter focuses on the development of a film coat.

Different materials can be used for seed coating and the market for such materials is rapidly increasing, already expected to reach $1.63 billion USD by 2020 (Markets 2016). The three materials used for seed coating are binders, fillers and active ingredients. Binders or adhesives are liquids that have adhesive properties in order to fix the active ingredient onto the seed surface. The most commonly reported binders include methylcellulose, gelatine, polyethylene glycol, chitosan, polyvinyl alcohol, ethyl cellulose, polyvinyl acetate and gum Arabic (Pedrini *et al.,* 2017). The most commonly used binder is methyl cellulose, mainly due to its low-cost, ease of use at very low rates (1-5% w/v) and low-toxicity (Scott 1989; Dow Industrial Cellulosics n.d.). Fillers are inert powders that are used to alter the shape or size of the seeds. Some examples of fillers are bentonite, pumice, calcium carbonate, clays, montmorillonite, vermiculite, diatomaceous earth, talc, sand and wood dust (Pedrini *et al.,* 2017; Scott 1989). The active ingredients can be nutrients, microorganisms, chemicals, colours, or adjuvants.

Seed treatments and coatings are one of the most promising and feasible approaches to prevent and control bacterial diseases in agriculture such as pea bacterial blight. A seed treatment can reduce the biocide contact area by 200-fold (International Seed Federation 2007) compared to foliar or furrow applications. This enables much lower concentrations of chemicals to be effective; decreasing application costs and any potential environmental impact. For more information about seed treatments, refer to Chapter 1, Section 1.2.2.1.

Most seed treatments currently available are targeted to fungal diseases and are ineffective against bacterial pathogens. Silica nanoparticles loaded with essential oils and embedded in an alginate layer have the potential to fulfil the need for effective and environmentally friendly seed treatments that can enable a controlled delivery of the antimicrobial in a timely-manner.

Furthermore, a foliar application of nanoparticles could cause stomatal obstructions in certain plants, leading to overheating and triggering cellular and physiological changes (Fernandez & Eichert 2009). For this reason, by incorporating the nanoparticles into a seed treatment, issues related to foliar applications could be avoided, resulting in a promising NP delivery alternative. This chapter evaluates the use of alginate for the development of a seed coating that is cheap to produce, easy to apply on seeds and effective as a delivery method for loaded-MSNPs.

4.1.2. Alginate

Alginate, or alginic acid, is a natural polysaccharide widely present in brown algae (Phaeophyceae), which occurs in two forms, acids and salts. The acid is known as alginic acid whilst the salt is present in brown seaweeds as a major constituent of the cell wall, representing up to 40-47% of dry weight of algal biomass (Nalamothu *et al.,* 2014). Alginate is a linear co-polymer that consists of β- 1,4-linked D-mannuronic acid (M) and α- 1,4-linked L-guluronic acid (G) residues arranged in homo-polymeric blocks (MM and GG) together with alternating sequence blocks (MG) (Smidsrød 1974; Joint FAO/WHO Expert Committee on Food Additives 1997) (Figure 4.1).

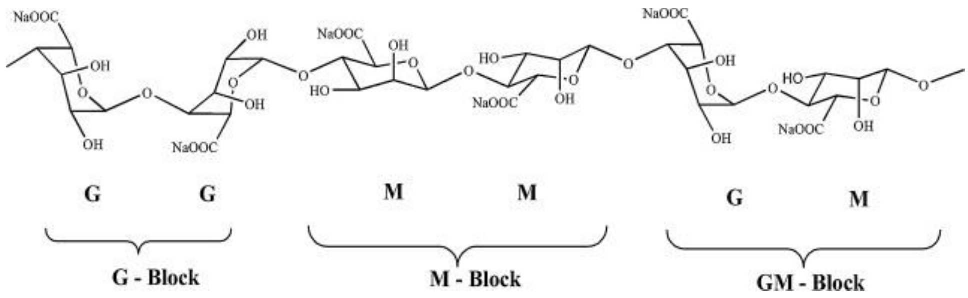

Figure 4.1. Chemical structure of sodium alginate showing GG, MM and GM blocks. Reprinted from (Daemi *et al.,* 2013) Copyright 2013 with permission from Elsevier.

In the presence of divalent cations such as Ca^{+2}, an alginate solution forms a hydrogel (Figure 4.2a) due to the selective binding of the ions to the GG blocks (the other blocks have a much lower selectivity for calcium ions) (Simpson *et al*., 2004; Smidsrød 1974). The junction zones are terminated by mixed regions and poly(M) sequences (Nilsson 1992), resulting in a 3D network. Morris *et al*. (1978) showed that calcium ions induce inter-chain association that constitute the junction zones responsible for gel formation (Morris *et al*., 1978). The formation of these junction zones in alginate gelation is described by the egg-box model developed by Grant *et al*. (1973) (Figure 4.2b).

The carboxylate groups in alginate's main structure make alginate a negatively charged polyelectrolyte at neutral pH, having one charge per repeating unit; and the interaction between the alginate coil and the ions can be modified by adding inert salts to the solution (Nilsson 1992). The physical properties of the alginate hydrogel depend on the composition and order of the residues. A higher content of guluronic acid as well as longer G-blocks results in a stronger and more porous gel that can maintain its form for a longer period of time. In contrast, greater amounts of mannuronic acid residues generate more elastic gels that undergo swelling and shrinking during cationic cross-linking, affecting their integrity (De Vos *et al.,* 1996; Martinsen *et al.,* 1989). Additional to the alginate composition, greater concentrations of $CaCl_2$ during the gelation process create a thicker "egg-box" model (Martinsen *et al.,* 1989) (Figure 4.2c), allowing more aggregation of the dimers to occur.

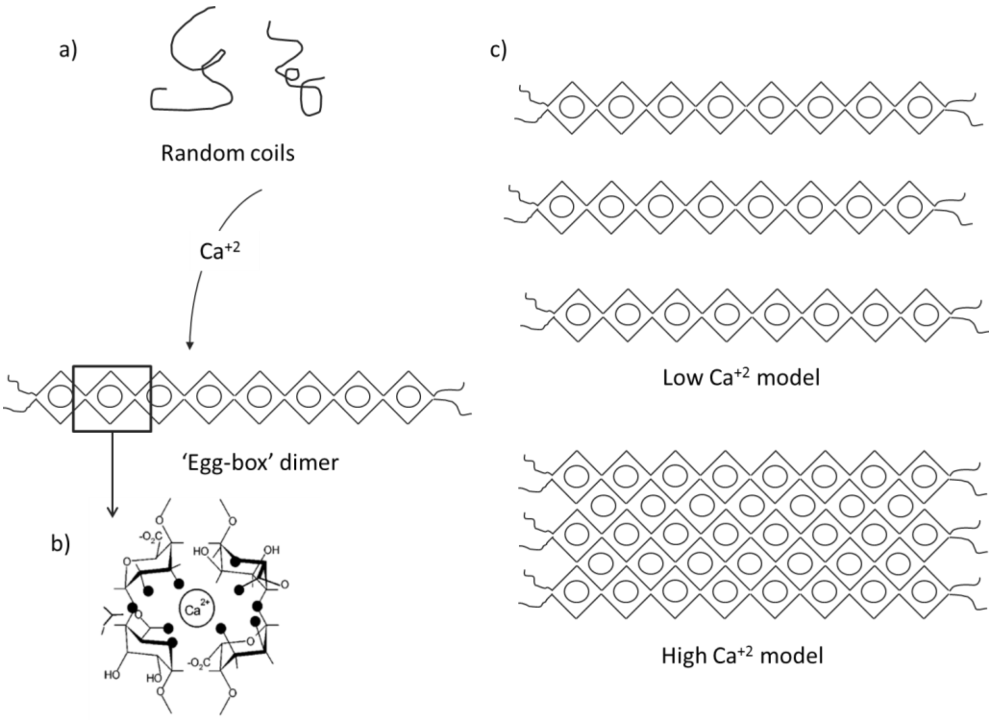

Figure 4.2. 'Egg-box' model. a) Schematic representation of Ca^{+2} chelation to G sequences to form the 'egg-box' dimer (Adapted from (Morris *et al.,* 1978) Copyright 1978 with permission from Elsevier). b) Calcium coordination of the 'egg-box model' illustrating the pair of G chains in the calcium alginate junction zones. Dark circles represent the oxygen atoms involved in the coordination of the calcium ion (Adapted with permission from (Braccini & Pérez 2001) Copyright 2001. American Chemical Society). c) 'Egg-box' model in low and high concentration of $CaCl_2$. Circles represent calcium ions in the junction zones between the G sequences while M residues are illustrated as lines at the end of the 'egg-box' (Adapted from (Simpson *et al.,* 2004) Copyright 2004 with permission from Elsevier).

4.1.2.1. Uses and Applications: Alginate in Industry and Agriculture

Alginate is commonly used as a stabiliser, thickener, emulsifier or gelling agent in a variety of industries as it is a biocompatible, biodegradable and safe compound that is widely used. In the field of medicine, alginates have been used for drug delivery and as smart hydrogels with controlled drug release (Mano 2008; Abd El-Ghaffar *et al.,* 2012), as anti-ulcers (Bogentoff 1981) and in many commercial products including acid reflux treatments such as Gaviscon® and Gastralgin® or dressing wounds due to their absorption and haemostatic properties (Algicell™, AlgiSite™, and Tegagen™). Alginates have also been shown to prevent obesity, hypocholesterolemia, and diabetes in rats (Kimura *et al.,* 1996) and to have anti-inflammatory and anti-oxidant properties (Sarithakumari *et al.,* 2013).

Alginate also has important pharmaceutical and tissue engineering applications including controlled and targeted release of chemical drugs and protein therapeutics (Colinet *et al.,* 2009; Ishak *et al.,* 2007; Wells & Sheardown 2007; Chan & Neufeld 2010) as well as cell support, immobilisation and encapsulation and tissue regeneration (Venkatesan *et al.,* 2015; Martinsen *et al.,* 1989). In the food industry, alginates are used as stabilisers, additives, emulsifiers, gelling agents. They are widely present in a variety of foods including soft drinks, pasta, dressings and sauces, ice cream, beer, among others (Nalamothu *et al.,* 2014); supporting the biocompatibility, safety and biodegradability of this compound as well as its GRAS (Generally Recognised as Safe) status. Alginate is also used in other fields such as the textile industry (dye thickener), paper industry (stabiliser) (FAO 1987) and cosmetics (thickener and moisture retainer) and there are some companies such as ©Zhermack producing cosmetic alginates.

In the agricultural field, alginate has been used as fertilizers (Guo *et al.,* 2006), biofertilizers (Ivanova *et al.,* 2005), and soil conditioner (Abd El-Rehim 2006). Additionally, it has been used in agricultural research to produce artificial or synthetic seeds by encapsulating somatic embryos and meristematic tissues (Redenbaugh 1983; Slade *et al.,* 1989; Pintos *et al.,* 2008; Sakhanokho *et al.,* 2013; Sharifeh *et al.,* 2011; Khor *et al.,* 1998; Asmah *et al.,* 2011). Furthermore, true seeds have also been encapsulated by hydrogels. Sarrocco *et al.* (2004) encapsulated wheat, cabbage, basil and radish seeds in calcium alginate by immersing seeds in $CaCl_2 \cdot 2H_2O$ 2M before immersing them in a 1% (w/v) sodium alginate solution (Sarrocco *et al.,* 2004).

Alginate can also be employed to encapsulate biocontrol agents (Fravel *et al.,* 1985) for agricultural and environmental applications. In fact, combinations of alginate derivatives with nutrients, microorganisms, stabilisers, surfactants, adhesives, cryo-protectants, etc., are the preferred polymers to be used for the encapsulation of microorganisms due to their biodegradability, non-toxicity, low production cost ($2 USD/kg) and slow release into the soil (Bashan & de-Bashan 2016). Moreover, alginate has been used in agriculture for other plant protective processes such as solarisation; the use of solar energy to kill pathogens, pests and weeds (Russo *et al.,* 2001).

4.1.2.2. Effect of Alginate on Plants

Alginate and other natural polymers, such as chitosan, elicit plant activities such as germination, root and shoot elongation and growth (Abd El-Rehim 2006; Luan *et al.,* 2002). A degraded product of alginate has been shown to act as a plant growth promoter, a phytoalexin-induction trigger (antimicrobials and antioxidants) and an enhancer of enzymatic activity. Consequently, the use of radiation to obtain degraded alginate has been studied and shown to result in a beneficial treatment on plant growth and development by enhancing metabolism and nitrogen assimilation, improving photosynthesis, physiological activities and alkaloid production (Le *et al.,* 2003; Naeem *et al.,* 2011; González *et al.,* 2013; Sarfaraz *et al.,* 2011; Idrees *et al.,* 2011).

4.1.3. Aims and Objectives

The primary aim of the work reported in this chapter was to evaluate the use of alginate as a seed treatment for peas and determine its effect on the plant's germination, growth and development. In addition, it was to determine the best combination of sodium alginate and calcium chloride that would enable the optimal plant germination and growth. The third and last aim was to evaluate the effect of calcium chloride molarity on alginate gelation and properties. As a result of this chapter, an appropriate alginate seed treatment able to incorporate empty or loaded MSNPs to protect seeds from bacterial pathogens will have been developed.

4.2. Materials and Methods

4.2.1. Preparation of fluorescein-labelled MSNPs

Fluorescein isothiocyanate (FITC) – 3-aminopropyl-triethoxysilane (APTES) was prepared by adding 100 ml APTES (Sigma Aldrich, UK) to 25 mg FITC mixed isomers (Sigma Aldrich, UK) in 5 ml absolute ethanol in a dry N_2 atmosphere and stirring for 12 hours. MSNPs were synthesised as described in Chapter 3 Section 3.2.1 with one modification; to label MSNPs, 75 µl of FITC-APTES was added to synthesis at the same time of the TEOS addition.

4.2.2. Development of an Alginate Seed Coating

Two protocols to obtain an alginate coating on pea seeds were studied. First, alginate coatings were prepared as described by Sarrocco *et al.* (2004). Briefly, previously sterilised pea seeds (see Section 4.2.3) were immersed in a 2 M $CaCl_2 \cdot 2H_2O$ solution (≥99.0% Sigma Aldrich, UK) for 30 minutes at room temperature. Seeds were allowed to dry before immersing them in a stirred 1% (w/v) sodium alginate (Sigma Aldrich, UK) solution for 20 minutes. Seeds were dried for 24 hours under airflow. Second, the inverse protocol was performed where peas were initially immersed in a 1% or 3% (w/v) sodium alginate solution and swirled for 2 minutes to remove any bubbles. Seeds were then directly transferred into a 0.1 M or 2 M $CaCl_2 \cdot 2H_2O$ solution and left for 30 minutes, swirling occasionally, to allow the coat to form around the seeds. After coating, seeds were placed in a sterile petri dish and allowed to dry overnight.

4.2.3. *In planta* Evaluation of Alginate as a Seed Coating

Pea seeds ("Kelvedon Wonder" cultivar) were sterilised by immersing seeds in 70% ethanol (Sigma Aldrich, UK) in a sterile flask during 1 minute before washing seeds with sterile water for an additional minute. After this, seeds were immersed in a 2% sodium hypochlorite (Sigma Aldrich, UK) solution for 5 minutes and washed 10 times with sterile water (1 minute per wash). Seeds were drained and placed in a sterile petri dish and allowed to dry completely in a laminar flow hood before use. Only seeds that remained wrinkled after sterilisation were used to avoid using seeds that have absorbed ethanol or sodium hypochlorite during the sterilisation process.

Sterile, dry seeds were coated as described in Section 4.2.2. Four different alginate coating preparations (1% and 3% sodium alginate with 0.1 M and 2 M $CaCl_2$) were studied to evaluate the best pea seed coating formulation that would serve as the scaffold for the loaded MSNPs. To evaluate the effect of MSNPs as part of the seed coating, 25 mg/ml MSNPs-FITC (fluorescein isothiocyanate labelled MSNPs) were suspended in 3% sodium alginate solutions before immersing pea seeds.

Five pea seeds were grown in sterile Magenta™ vessels (Sigma Aldrich, UK) with ~100 ml Murashige and Skoog (MS) medium. MS medium was prepared by mixing 4.4 g/L MS basal (Sigma Aldrich, UK), 10 g/L sucrose (Sigma Aldrich, UK) and 2.5 g/L Phytagel™ (Sigma Aldrich, UK). Fifteen seeds were used per treatment to have a triplicate set up with 5 seeds grown in each vessel. Seeds with no alginate coating were used as a control. Vessels were incubated in controlled light and temperature conditions, which were monitored using a LI-250A Light meter (LI-COR Biosciences Ltd., UK) and a MyPCLab thermocouple (Novus Automation, UK), respectively. Germination, shoot emergence and plant growth were evaluated daily and after 2-3 weeks of incubation; plant height, main root length, secondary roots number, number of nodes, and number of leaves were measured for each plant.

4.2.4. Microscopy Analyses

4.2.4.1. Scanning Electron Microscope (SEM)

SEM was used to visualise the alginate layer on the seed surface. A pea seed coated with an alginate layer containing MSNPs was observed using a JEOL JSM-5510 SEM (Jeol, UK) with a tungsten filament operated at 15 kV.

4.2.4.2. Atomic Force Microscope (AFM)

Alginate layers on glass slides were analysed using an Atomic Force Microscope (Agilent 5400 AFM/SPM; Agilent Technologies, UK) to evaluate the hardness of the different coating formulations. A hydrophobic marker was used to define an area in a glass slide to be covered with

100 µl sodium alginate before submerging the slide into $CaCl_2 \cdot 2H_2O$ for 30 minutes. The alginate coating was allowed to dry before microscopy analyses were carried out. Force-Distance (F-D) curves were made through the Spectroscopy feature of the instrument's Contact mode using MikroMasch cantilevers (HQ:NSC35/Al BS) with resonance frequency of 150-300 kHz (or 4.5-14 N/m).

4.2.4.3. Fluorescence Microscope

To evaluate the presence and internalisation of FITC-labelled MSNPs in alginate/MSNPs coated seeds, different plant tissues were observed under a fluorescence microscope (Motic AE31, UK). Sections from the seed coat, stem, leaves and roots were observed using a standard fluorescence filter (FITC Ex D480/30x, DM 505DCI, BA D535/40m; Motic, UK) and fluorescence intensity was compared between control seeds without any alginate coating and test seeds coated with alginate/MSNPs-FITC.

4.2.5. Statistical Analyses

Statistical analyses were performed using Excel Microsoft and Minitab® version 17.2.1. To determine statistically significant differences between samples, analysis of variance (ANOVA) and t-tests followed by Tukey's HSD (honest significant difference) test were applied when appropriate. *P* values of 0.05 and 0.1 were used and are specified for each analysis.

4.3. Results

4.3.1. Development of an Alginate Seed Coating

The first protocol to coat pea seeds with alginate as described by (Sarrocco *et al.,* 2004) where seeds are initially soaked in a calcium chloride solution before immersion in sodium alginate did not produce an alginate layer on wrinkled seeds. Only those seeds that had absorbed liquid either during the sterilisation process (ethanol or sodium hypochlorite) or during the coating process developed an alginate coating; however, the coating was not smooth and it peeled off easily. Additionally, the use of seeds that absorb liquid and are not wrinkled should be avoided. Therefore, an inverse protocol was evaluated where the seeds were initially immersed in the sodium alginate solution before immersion in calcium chloride. This inverse protocol allowed the formation of a smooth alginate coating on wrinkled peas (Figure 4.3). The inverse protocol was selected for further experiments due to the formation of a smooth alginate coating surrounding the seeds.

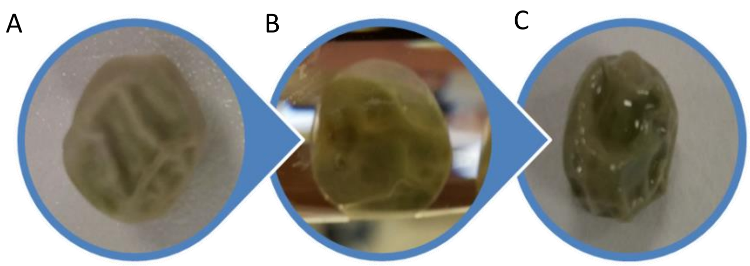

Figure 4.3. Alginate coating formation around pea seed using the inverse protocol. A) Pea seed, 'Kelvedon Wonder' cultivar. B) Pea seed in calcium chloride solution during gelation of alginate. The alginate coat around the seed is clearly visible. C) Pea seed after coating and drying.

4.3.2. *In planta* Evaluation of Alginate as a Seed Coating

To determine the effect of alginate on pea germination, growth and development, seeds coated with 1% or 3% sodium alginate and 0.1 M or 2 M calcium chloride were incubated for two weeks. Incubation parameters (light and temperature) were monitored during light and dark hours. Light was measured at six points inside the growth chamber (the four corners, centre-back and centre-front) at two heights: top and bottom of Magenta vessels. Light at the bottom of the growth chamber was 67.16 ± 12.93 µmol s^{-1} m^{-2} per µA, while light intensity at the top of the vessels was 88.36 ± 16.42 µmol s^{-1} m^{-2} per µA. Light periods were established from 9am to 5pm daily, having the remaining incubation time in darkness. Temperature recordings (Figure 4.4) demonstrate temperature during light hours was ~24°C while temperature during dark hours was ~19.5°C.

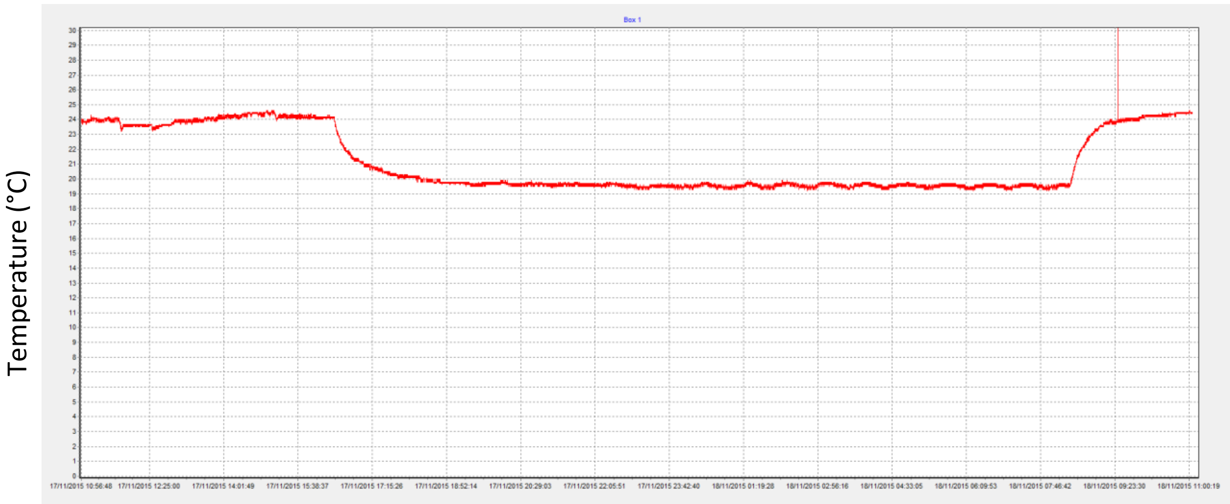

Figure 4.4. Temperature recordings in plant incubation chamber during light and dark hours. Recordings show the temperature during light hours is ~24°C while temperature drops to ~19.5°C during dark hours.

Seeds were coated using four different formulations to study their effect on germination and plant growth. Two concentrations of sodium alginate (1% and 3%) were combined with two concentrations of calcium chloride (0.1M and 2M). Two experiments were performed, the first to evaluate all four formulations and the second to determine if the addition of MSNPs to the alginate caused any significant difference. During the first test, seeds coated with a higher calcium chloride molarity germinated more slowly. Five days after sowing, 80% of control seeds (no coating) had germinated, compared to 100% of seeds coated using 0.1 M $CaCl_2$ and 60% using 2 M $CaCl_2$, suggesting that a higher concentration of $CaCl_2$ delayed and possibly prevented germination by trapping the seed inside the alginate coating (Figure 4.5), while a lower concentration enabled germination even faster than control seeds.

Plant height was measured 20 days after sowing and a higher $CaCl_2$ molarity yielded the tallest plants, even though it initially hindered and delayed germination. All coated seeds yielded significantly taller plants than the control (Figure 4.6).

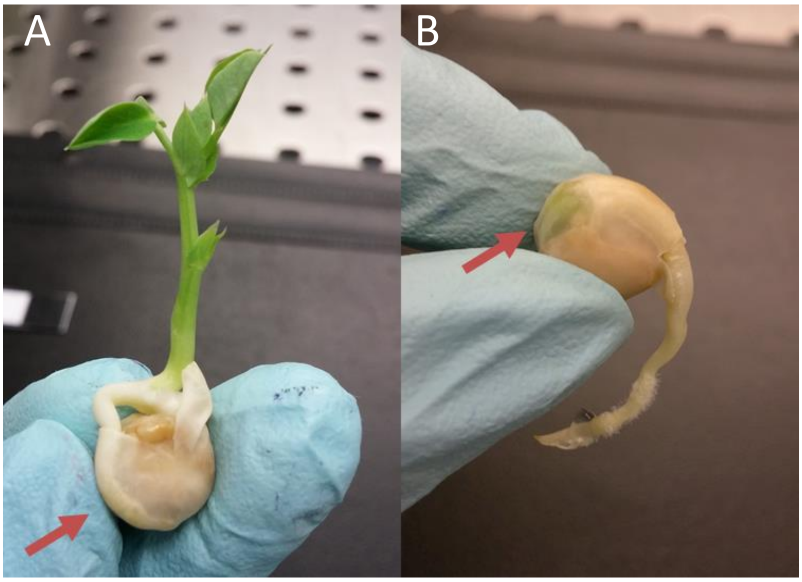

Figure 4.5. Alginate coating prepared using a high calcium chloride molarity (2 M) prevents pea germination and development. Red arrows point towards trapped area inside alginate coat. A) Root is trapped inside alginate coating. B) Even though some roots are able to penetrate the alginate layer, the shoot can also get trapped inside the alginate coating.

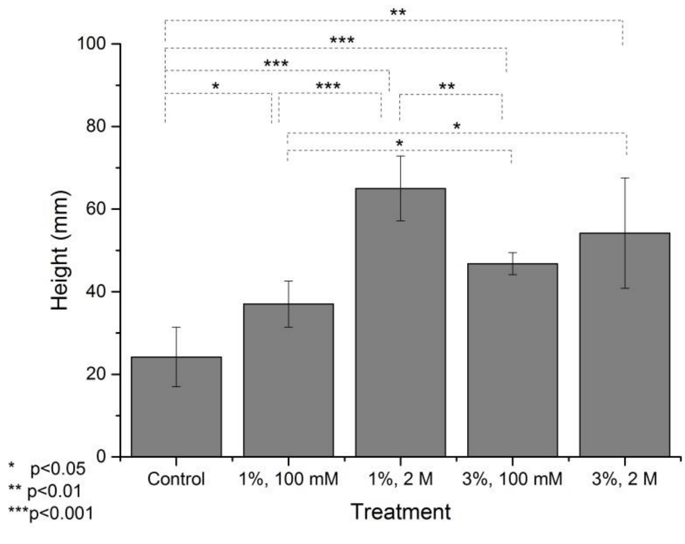

Figure 4.6 Plant height of germinated seeds twenty days after sowing. All four alginate coating treatments produced significantly taller plants than the control. Seeds coated using 2M $CaCl_2$ resulted in statistically significant taller plants than those coated with 0.1 M $CaCl_2$. Statistical significance obtained by Student's T-test comparison. Data shows mean ± standard deviation, n=15 seeds per treatment. *p<0.05, **p<0.01, ***p<0.001.

After studying the effect of all four formulations on plant growth, a second experiment was performed to include a coating containing MSNPs to evaluate its effect on plant germination and growth compared to coatings without nanoparticles. MSNPs-FITC (25 mg/ml) was added to 3% sodium alginate before coating the seeds with calcium chloride to study if the addition of nanoparticles had a positive or negative effect on seed germination. The coating appeared to be smoother using 2 M $CaCl_2$ while a lower molarity produced a coating that peeled off more easily. Furthermore, as previously observed, seeds coated with a low calcium chloride molarity (0.1 M) were able to germinate faster than the control seeds, and seeds coated with 3% alginate and 2 M $CaCl_2$ with added MSNPs were able to germinate at a rate similar to the control seeds (Figure 4.7a). Seeds germinated within five days, except for those coated with 2 M $CaCl_2$ that did not have any MSNPs added to the formulation, of which only 73% and 80% of the seeds germinated (for 3% and 1% sodium alginate, respectively) after 15 days. This suggested that the addition of MSNPs changed the properties of the coating, allowing the roots to penetrate and break through the alginate (see AFM results for more details). Statistical analysis (Two-way Analysis of Variance, ANOVA) 15 days after incubation of the seeds demonstrated that the effect on germination by sodium alginate concentration was not significant, the same as the interaction between alginate and $CaCl_2$ molarity; however, the effect of $CaCl_2$ molarity on its own on germination is indeed significant ($p<0.1$) as a higher molarity of calcium chloride negatively affects the germination of peas.

Shoot emergence was also evaluated (Figure 4.7b). Shoot appearance from seeds coated with 3% sodium alginate and 0.1M $CaCl_2$ containing MSNPs was faster than all other treatments, including the control. Besides the control, only treatments with low $CaCl_2$ molarity resulted in 100% shoot emergence.

Fifteen days after incubation, the plant height, root length, and the number of secondary roots, leaves and nodes were measured for all germinated plants (Figure 4.8). Statistical analyses (ANOVA and Tukey's HSD test) revealed that there was a significant difference (*$p<0.05$, **$p<0.01$) between the plant height of control seeds and seeds coated with a low molarity of calcium chloride (Figure 4.8a). The main root length was also significantly greater (*$p<0.05$, **$p<0.01$) for some treatments with 0.1M $CaCl_2$ compared to the higher molarity of 2M, however, no significant difference is observed compared to the control roots (Figure 4.8b).

Similarly, there is no significant difference ($p>0.05$) between the treatments and control regarding the number of secondary roots, but seeds coated with 3%, 2M and MSNPs had significantly fewer secondary roots than seeds coated with other treatments (Figure 4.8c). Additionally, seeds treated with low molarity $CaCl_2$ developed a significantly higher ($p<0.05$) number of leaves and nodes when compared to the control seeds (Figure 4.8d&e).

Further statistical analysis (Two-way ANOVA) showed that the effect on germination by sodium alginate concentration was not significant, this was also the case for the interaction between alginate and $CaCl_2$ molarity; however, the effect of $CaCl_2$ molarity on its own on germination was more significant ($p < 0.13$), since higher molarity of calcium chloride prevented the germination of peas.

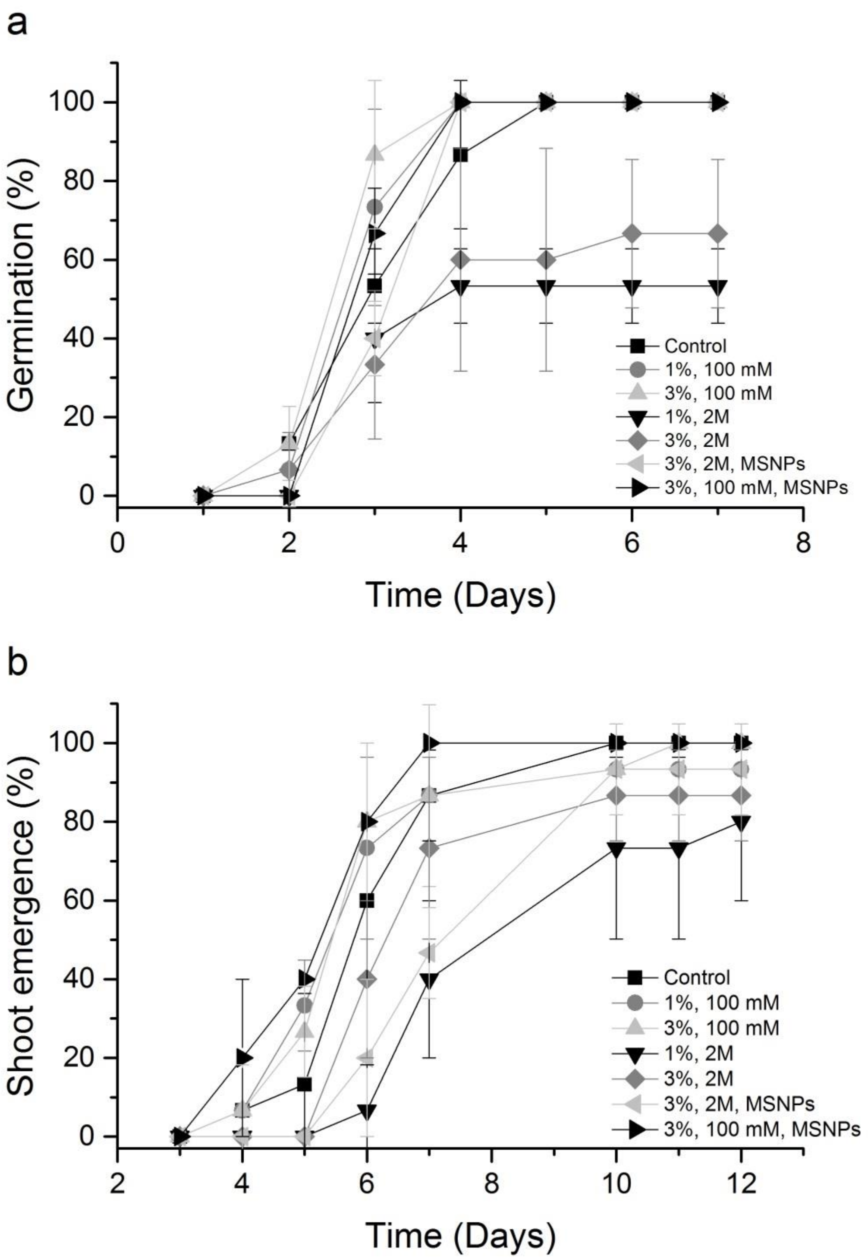

Figure 4.7. Seed germination and shoot emergence of alginate-coated peas compared to control seeds. a) Seed germination. Only some seeds coated using 2M $CaCl_2$ without MSNPs were not able to germinate. The addition of MSNPs caused a change on the coating that allowed the root to penetrate the alginate layer for germination. b) Shoot emergence. A low $CaCl_2$ molarity resulted in an increased shoot emergence. The addition of MSNPs caused a change on the coating that allowed the shoot to penetrate the alginate layer for emergence. Data shows mean ± standard deviation, n=15 seeds per treatment.

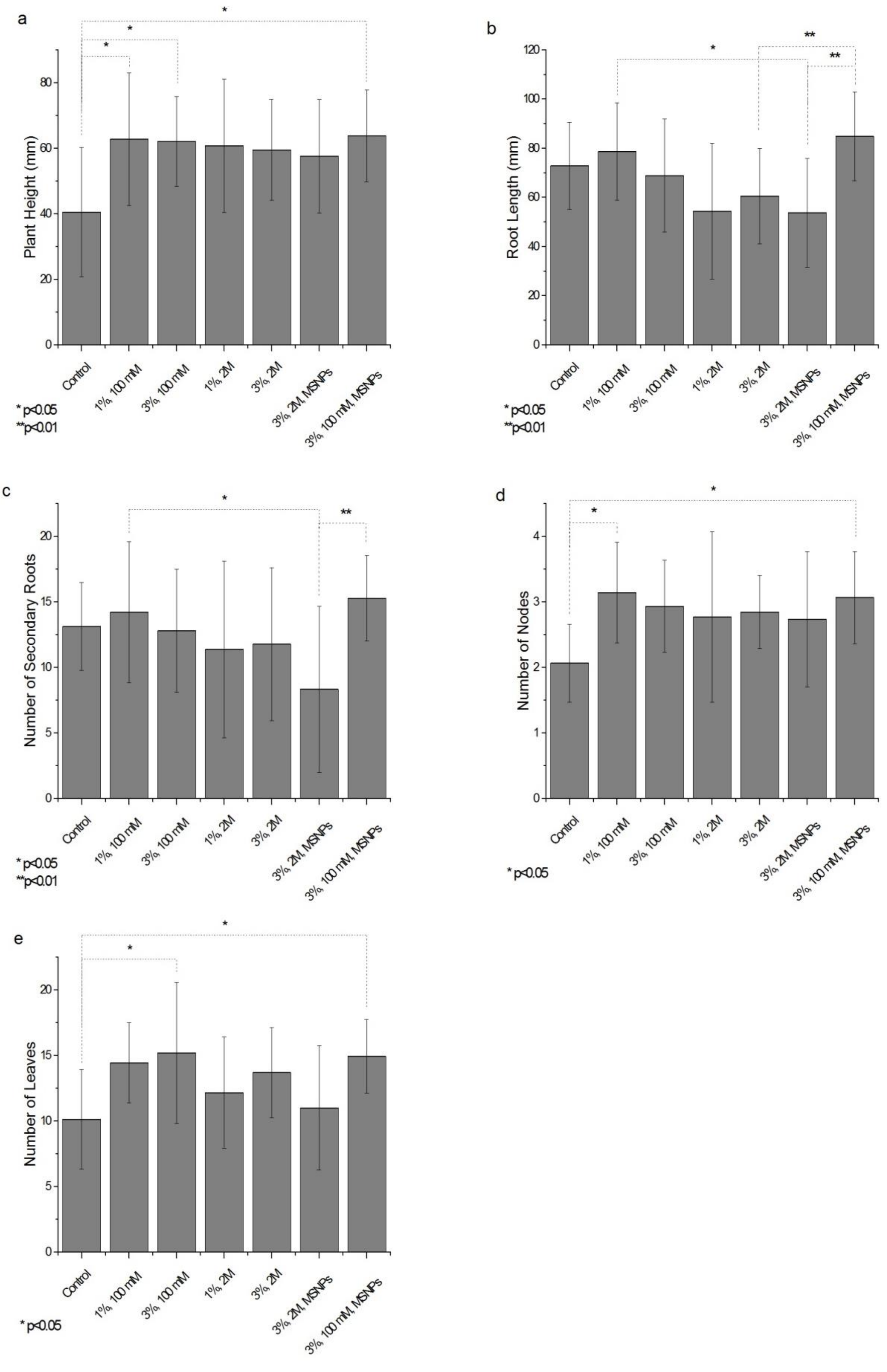

Figure 4.8. Results of germination test 15 days after sowing of pea seeds. a) Average plant height (mm), b) average main root length (mm), c) average number of secondary roots, d) average number of nodes, and e) average number of leaves per germinated seed in each treatment. Data shows mean ± standard deviation. Statistically significant differences between treatments: Tukey's HSD test $*p<0.05$, $**p<0.01$. n = 15 seeds per treatment.

4.3.3. Microscopy Analyses

4.3.3.1. Scanning Electron Microscope (SEM)

SEM images were obtained to observe the alginate coating on pea seeds. The alginate layer coating the seed is illustrated in Figure 4.9 (B,C,D). Energy from the electron beam caused holes and ruptures of the alginate layer but it was still possible to appreciate the presence of the alginate coating on the seed surface. In general, the alginate coat was smooth and covered the majority of the extent of the seed surface with some areas having less coverage while others presented a few alginate aggregations (Figure 4.9B). MSNPs incorporated in the alginate layer could not be visualised in any of the obtained images due to the scale of the image.

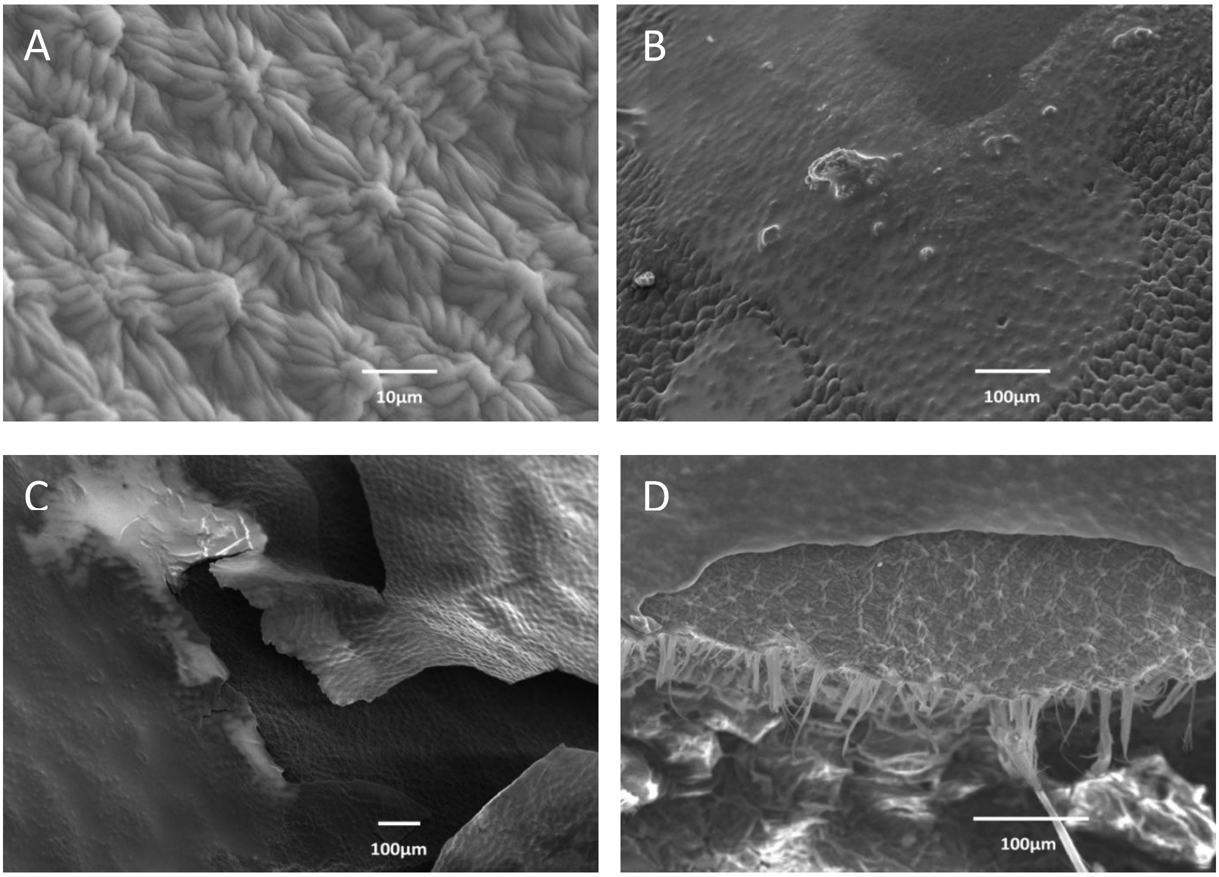

Figure 4.9. SEM images of pea seed coated with sodium alginate layer. A) Control seed with no alginate. Individual plant cells can be observed. B) Seed surface coated with alginate. Coating looks smooth in some areas, while aggregations of alginate can also be observed. Plant cells are seen under alginate layer. C) Broken alginate layer on seed surface. Energy from electron beam caused the alginate to rupture. D) Side view of seed surface and inner seed. The alginate layer is observed on top of the surface. The place of seed breakage can be appreciated, showing ruptured plant tissue under the alginate layer.

4.3.3.2. Atomic Force Microscope (AFM)

In planta experiments consistently demonstrated that the use of a higher molarity of calcium chloride hindered the germination of seeds by trapping the root and/or shoot inside the alginate coating. Moreover, the incorporation of MSNPs into the alginate coating, even with a high concentration of calcium chloride, improved germination of the seeds. These results suggest that the addition of MSNPs to the sodium alginate changed the properties of the coating. To evaluate this, alginate layers on glass slides were analysed using an Atomic Force Microscope (Agilent 5400 AFM/SPM; Agilent Technologies, UK).

Alginate layers were prepared with 1% sodium alginate and 0.1 M calcium chloride and with 3% sodium alginate and 2 M calcium chloride, to obtain what was hypothesised to be the softest and hardest (respectively) of the formulations. AFM results (Figure 4.10) demonstrated that what was initially thought to be a 'hard' coating that was preventing the germination of seeds (2 M $CaCl_2$) was indeed a softer but more flexible coating that would not break as easily and was therefore trapping the root and shoot within the alginate. In contrast, the alginate layer synthesised with a lower calcium chloride molarity (0.1 M), produced a harder but more brittle coating that was more easily broken by the roots and shoots (Figure 4.10). Furthermore, when MSNPs were added to the alginate layer with 2 M $CaCl_2$, the hardness of the coating was modified, and the alginate layer became even more brittle and harder than the layer with 0.1 M $CaCl_2$, positively influencing germination.

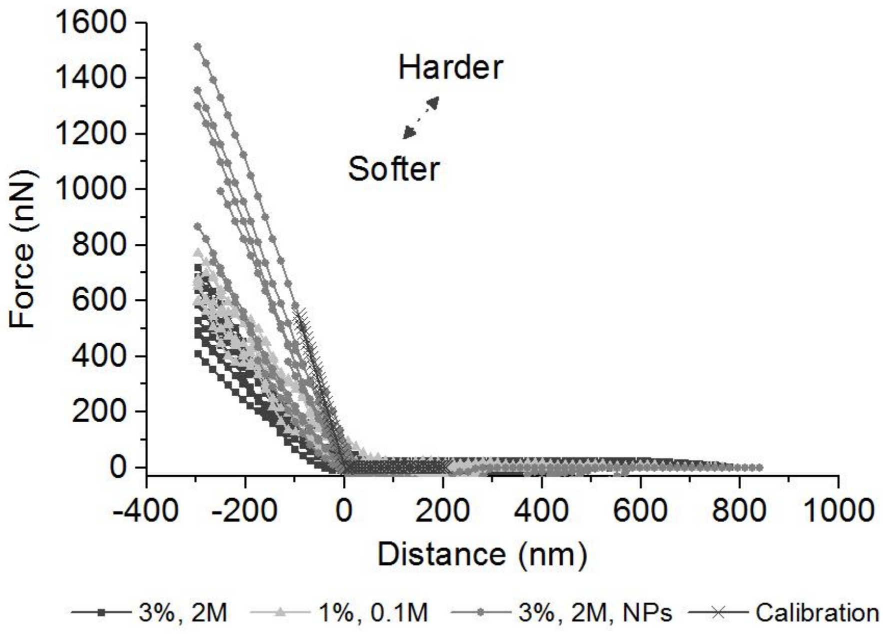

Figure 4.10. AFM results. Force-Distance (F-D) curves of alginate layers. Each line represents one measurement at one specific point on alginate layer. Percentage of sodium alginate and $CaCl_2$ molarity are indicated, as well as the addition of nanoparticles (NPs). A higher molarity of $CaCl_2$ produced a softer and more flexible alginate layer that was difficult to be penetrated by the roots during germination. A lower molarity produced a harder and more brittle layer that allowed the seeds to germinate. Addition of MSNPs changed the properties of the alginate coating, making it more brittle, allowing the root and shoot to penetrate the alginate coating.

4.3.3.3. Fluorescence Microscope

Plant tissues from seed coats, stems, leaves and roots were observed under a fluorescence microscope to evaluate the presence and internalisation of FITC-labelled MSNPs present in the alginate coating (Figure 4.11). Naturally present pigments from pea plants caused all plant tissues to have a degree of fluorescence. However, the brightness of the seed coat area, especially of the alginate layer, was considerably greater than that of any other plant tissue. This suggested that the labelled MSNPs were present only on the seed surface where the alginate coating was applied and that MSNPs were not internalised by the plant and transported into other plant tissues. Nevertheless, due to the presence of natural fluorescence from all plant tissues, it is possible that a negligible amount of MSNPs were present in other plant tissues and could not be detected due to the background natural fluorescence.

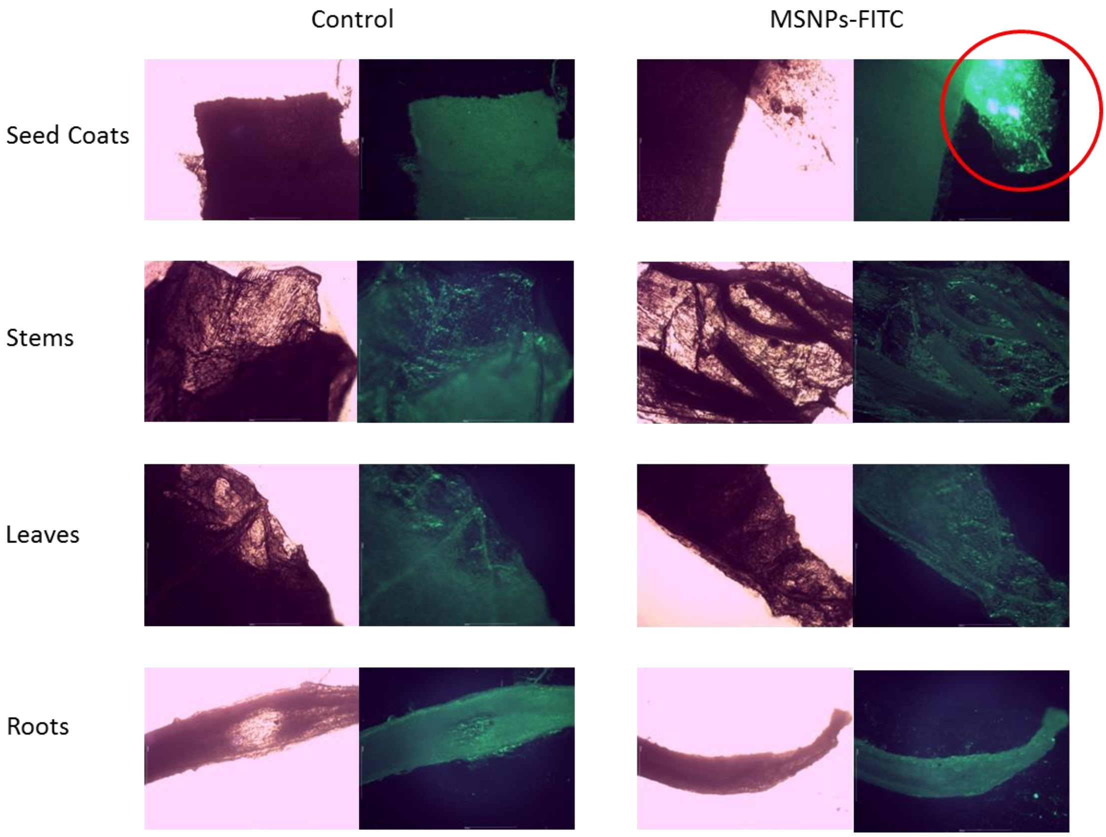

Figure 4.11. Fluorescence microscope images of different plant tissues of control plants and plants coated with alginate containing fluorescent MSNPs. A 'white balance' image is presented for each tissue sample to compare with the fluorescent image. The red circle shows the brightest section of all plant tissues which was the alginate layer on top of the natural seed coat.

4.4. Discussion

In this chapter, the effect of four different alginate formulations on plant growth and development was evaluated, enabling the development of a seed treatment containing nanoparticles to protect pea seeds against bacterial pathogens.

The deployment of alginate in agriculture is mainly focused on the production of synthetic seeds by encapsulating meristematic tissues or somatic embryos, or to encapsulate microorganisms and nutrients, to be used as fertilizers. Sarrocco *et al.* (2004) evaluated the encapsulation of true seeds (cabbage, basil, radish and wheat) and showed that germination was similar to controls, with the exception of wheat. The study evaluated the use of 1% sodium alginate and 2 M calcium chloride to encapsulate seeds, with and without the addition of solid substances (Sarrocco *et al.,* 2004). The protocol used by Sarrocco *et al.* (2004) was replicated in this chapter using pea seeds but was not successful in producing smooth coatings. Hence, an inverted protocol with initial immersion in sodium alginate before immersion in calcium chloride was developed to produce alginate coated pea seeds. The inverse protocol allowed a smooth layer of alginate to be formed around the wrinkled seeds.

To evaluate the best alginate coating formulation, four different combinations of sodium alginate (1% and 3%) and calcium chloride (0.1 M and 2 M) were studied. A high molarity of calcium chloride was shown to slow down and hinder the germination of peas, which corroborates previous reports where some seeds remained entrapped in the gel (Sarrocco *et al.,* 2004). In contrast, a low molarity of calcium chloride resulted in a germination rate faster than control seeds and in general, all coated seeds produced taller plants than the controls, regardless of $CaCl_2$ concentration. Similar results were demonstrated by Simpson *et al.* (2004) where a greater concentration of calcium chloride increased the strength of the gel, impeding the growth characteristics of encapsulated βTC3 mouse insulinoma cells. This effect was observed for alginate gels with a high content of guluronic acid, but not for gels high in mannuronic acid since those have a low percentage of consecutive guluronic acid residues to interact with the calcium ions. Therefore, depending on the use and application of the alginate, the optimum formulation needs to be determined: a weak alginate gel is advantageous when cell growth and expansion is desired whereas a stronger gel network would be more beneficial if these effects are not wanted (Simpson *et al.,* 2004).

The experiments in this chapter also evaluated the effect of the alginate coating (with and without MSNPs) on plant height, shoot emergence, root length, secondary roots, and number of nodes and leaves. Germinated seeds demonstrated no significant difference in height, nodes and leaves but 2 M $CaCl_2$ resulted in shorter main roots and less secondary roots. The most important differences between all alginate formulations were germination rate and shoot emergence. This was caused by the entrapment of the roots and shoots within the alginate gel. If the roots and shoots were able to penetrate the alginate coating, plant development was similar to the control and in some instances even better due to the beneficial effect of alginate on plants. Also, during the coating process, seeds undergo a wetting/drying cycle that resembles that of seed priming (Scott 1989), which involves soaking seeds in solutions with high osmotic pressure; this stimulated germination and resulted in a reduction between the sowing time and the emergence time.

In general, all coated seeds able to germinate produced taller plants than the controls, some having more roots, leaves and nodes (with low $CaCl_2$ molarity). Alginate is a plant growth promoter and alginate derivatives have been shown to trigger antimicrobial and antioxidant production, to enhance enzymatic activity and metabolism and to improve physiological activities (Le *et al.,* 2003; Naeem *et al.,* 2011; González *et al.,* 2013; Sarfaraz *et al.,* 2011; Idrees *et al.,* 2011). The results of this study are in agreement, the beneficial effect of alginate on plants has been demonstrated in this chapter, with all germinated and coated plants showing better growth and development compared to control pea seeds.

The addition of MSNPs to the alginate coating allowed an improved germination of seeds that had been coated with a high molarity of $CaCl_2$. AFM analyses of alginate layers with and without MSNPs demonstrated that the formulations with a low $CaCl_2$ molarity were harder and more brittle than the high molarity formulations, which were softer and more flexible. The increased flexibility of 2 M $CaCl_2$ alginate prevented the germination of seeds since the layer was not brittle enough to be broken and penetrated by the root/shoot, which then became entrapped in the gel. It has been previously suggested that an unsuitable elasticity or strength of gel can prevent the emergence of encapsulated embryos from alginate beads (Onishi *et al.,* 1994). This was confirmed through AFM analysis of sodium alginate layers, where a difference in elasticity and hardness of gels was demonstrated to be dependent on $CaCl_2$ concentration. Moreover, AFM analysis of an alginate coating containing MSNPs demonstrated that MSNPs altered the properties of the gel, making it harder and more brittle (similar to a gel prepared with less calcium chloride), allowing an improved germination of seeds. This may have been due to the MSNPs interfering between the sodium alginate coils and the calcium ions and reducing the formation of 'egg-box' dimers, thereby creating a structure more similar to a gel produced with a lower concentration of $CaCl_2$.

A seed treatment containing alginate as a binder (instead of soaking the seeds in a biocide solution) would enable a longer exposure to the antimicrobial due to the incorporation of the EO-MSNPs into the alginate layer. Also, alginate provides added benefits due to its beneficial effects on plants, as previously demonstrated. Furthermore, alginate can be used in very low concentrations such as 1% (w/v) used in this study. By contrast, methyl cellulose (also used in low concentrations) is commonly applied at 3-5% (w/v) while other binders such as gum Arabic require a rate of 45% (w/v) (Renugadevi & Jayanthi 2010). Consequently, alginate could be used as a scaffold to deliver MSNPs to seeds while allowing an improved seed germination and plant development.

SEM images revealed the presence of a smooth alginate layer covering the seed's surface. Since the nanoparticles were not visible using SEM, FITC-labelled MSNPs were added to the alginate coating on pea seeds and different plant tissues were visualised using a fluorescence microscope to evaluate the presence of MSNPs. Even though natural plant pigments fluoresced, the intensity of the fluorescence was considerably brighter on the seed of coated peas. The MSNPs fluoresced mainly in the alginate layer. The lack of increased fluorescence in any other plant tissues suggested that the MSNPs remained in the alginate coating and were not being internalised and transported to other plant tissues.

Peas (as well as other dicotyledons) do not produce many phytoliths (silica deposits) and it has been suggested that this is due to the presence of a mechanism of silica rejection, made up of a fatty substance on the root hairs that prevents the entry of soluble substances (Parry & Winslow 1977). In contrast, grasses like wheat and barley, which are very efficient at producing phytoliths, do not possess such fatty substances; demonstrating that plants have control over the amount of silicic acid that is absorbed from the environment to produce phytoliths (Piperno 2006). The results from this chapter suggest the silica nanoparticles were not being internalised and transported by the peas, which was expected as the plant possessed a mechanism to reject silica absorption. Additionally, if any of the nanoparticles were to be internalised, the effect of silica on plants has been previously shown to be beneficial.

Silicon is the second most abundant element on the crust of the Earth, occurring as silica or silicate, and available to plants as mono-silicic acid and it is regarded as a quasi-essential element for plant growth (Tripathi *et al.,* 2015; Epstein 1999). Silicon performs an essential function in plant health, inducing resistance against stress, pathogens and disease in addition to minimising toxicity levels on soils (Sahebi *et al.,* 2015). It has been demonstrated that silicon nanoparticles protect peas against Cr(VI) toxicity by reducing its accumulation and oxidative stress, and by up-regulating the plant's defence system (Tripathi *et al.,* 2015). Therefore, even though the results suggest there is no internalisation of MSNPs, the absorption of silica by the plant would not present any risk and would potentially only have beneficial effects on its growth and development.

4.5. Conclusions

In conclusion, this chapter evaluated the use of alginate as a seed coating for peas. It was demonstrated that the use of low calcium chloride allowed an improved germination and plant development while a higher molarity of this compound prevented seed germination, unless supplemented with MSNPs, which modified the gelling properties, improving germination. Alginate is a potential material to be used to develop a seed coating to incorporate biocide-loaded MSNPs to protect pea seeds against phytopathogens. The technology evaluated in this chapter was used in subsequent studies as a scaffold to embed the nanoparticles loaded with essential oils to be delivered on pea seeds to control bacterial infections.

Chapter 5
Effect of Essential Oil-Loaded MSNPs against Bacterial Phytopathogens *in vitro* and *in planta*

5.1. Introduction

The main objective of this part of the study was to develop a treatment to protect seeds from bacterial diseases. Essential oils have been demonstrated to have strong antimicrobial properties; and their encapsulation into mesoporous silica nanoparticles (MSNPs) can aid in their delivery to the target pathogen while protecting the oils from rapid evaporation and degradation. By incorporating MSNPs loaded with essential oils into the alginate coating developed in the previous chapter (Chapter 4), the protective effect of this system was investigated. In this chapter, Ajwain-loaded MSNPs were evaluated both *in vitro* and *in planta* to determine their potential application as a seed treatment to protect plants from microbial diseases. The research detailed here focused on bacterial blight pathogen *P. syringae* pv. *pisi* and its plant host, common pea (*Pisum sativum*), as a proof of concept; but this treatment could be modified in the future to protect a broad range of plant hosts from multiple microorganisms.

5.1.1. Ajwain essential oil

Ajwain (*Trachyspermum ammi*) is an annual herb widely distributed throughout Egypt, Iran, Afghanistan, Pakistan and India. It is commonly used in Indian cuisine as well as in aromatherapy and Ayurvedic medicines against inflammatory or digestive disorders due to its anti-inflammatory (Thangam & Dhananjayan 2003), anxiolytic (Rajput *et al.,* 2015), carminative, analgesic (Dashti-Rahmatabadi *et al.,* 2007), antihypertensive and antispasmodic (Gilani *et al.,* 2005), effects. Additionally, Ajwain EO has antimicrobial, antiviral (Hussein *et al.,* 2000), antihelminthic (Lateef *et al.,* 2006; Apte *et al.,* 2014), antifilarial (Mathew *et al.,* 2008), spermicidal (Buch *et al.,* 1988), hypolipidemic (Kumari & Prameela 1992), antioxidant (Singh *et al.,* 2004; Chatterjee *et al.,* 2013), antiplatelet (Srivastava 1988), antihistaminic (Boskabady & Shaikhi 2000), antitussive (Boskabady *et al.,* 2005), antilithiasis and diuretic (Ahsan *et al.,* 1990) effects.

Ajwain fruits accumulate ~5% EO in special compartments (Minija & Thoppil 2002), even though the accumulation of up to 9% has been reported (Balbaa *et al.,* 1973). The main constituent of this EO is thymol (35-60%) (Bairwa *et al.,* 2012), which is also present in other highly antimicrobial oils such as thyme or oregano. Ajwain essential oil has been demonstrated to have a strong antimicrobial activity. At concentrations of 0.03% (v/v), it led to 100% inhibition of mycelial growth of ten different fungal species (Singh *et al.,* 2004). Additionally, a study evaluating the EO of seven spices demonstrated that Ajwain was the strongest antimicrobial against eight human pathogenic bacteria, even more than standard antibiotics (Singh *et al.,* 2002).

Ajwain EO has also been evaluated for protection of stored foods from fungal contamination or pests or to increase food shelf-life. It has been demonstrated to be an effective fungicide (12-20 times more effective than synthetic fungicides) against pathogens such as *Fusarium oxysporum, Pythium aphanidermatum*; with a lower MIC than that of its main constituent, thymol (Dwivedi & Singh 1998). As a pesticide for stored food, it has been studied against the Indian meal moth, *Plodia interpunctella* (Shojaaddini *et al.,* 2008) and the beetles *Sitophilus oryzae* (L.) and *Tribolium* *castaneum* (Sahaf *et al.,* 2007). This oil has also been used to increase shelf-life of products. Ajwain EO together with *Lactobacillus plantarum* A7 was used against phytopathogen *Botyris cinerea* to increase the shelf-life of strawberries (Zamani-Zadeh *et al.,* 2013). Likewise, 3% Ajwain seed extract has been utilised to extend the shelf-life of fish products (Raeisi *et al.,* 2016).

As illustrated, Ajwain EO possesses several properties that make it a promising antimicrobial that could be used to treat bacterial diseases in crops. Since it has not been as extensively studied as other EOs with antimicrobial properties (such as oregano, thyme, mustard), it was selected to be further investigated in this chapter. Moreover, the results represented in Chapter 2 confirmed the high antimicrobial activity of this oil against *P. syringae* pv. *pisi* at concentrations as low as 0.031% (v/v). This oil is highly effective at very low concentrations and, in addition, it has been shown to be non-phytotoxic in seed germination and seedling growth of beans (*Phaseolus vulgaris* L.) (Dwivedi & Singh 1998), demonstrating its feasible incorporation into a seed treatment. The encapsulation of Ajwain EO into MSNPs and its delivery *via* an alginate seed coating to protect peas against bacterial blight will be evaluated and discussed in the present chapter.

5.1.1.1. Ajwain and nanoparticles

Ajwain seed extract has been investigated for the green synthesis of nanoparticles. Biogenic silver (Chouhan *et al.,* 2017; Kaur *et al.,* 2013; Vijayaraghavan *et al.,* 2012), $AgNO_3$ (Sahu *et al.,* 2015) and ZnO (Saravanakkumar *et al.,* 2016) nanoparticles have been synthesised using Ajwain seed extract as a reducing agent. The resulting metal nanoparticles were effective as antimicrobials against different human pathogens such as *Escherichia coli, Bacillus subtilis, Staphylococcus aureus* and *Pseudomonas aeruginosa*. Ajwain leaf extracts have also been used to synthesise green nanoparticles of different metals (copper, silver, nickel and magnesium) and screened for antifungal activity against the banana pathogen *Colletotrichum musae* (Jagana *et al.,* 2017). Moreover, the effect of iron pyrite (FeS_2) and Molybdenum disulphide (MoS_2) nanoparticles has also been investigated and proved to enhance seedling growth of Ajwain (Ahmad *et al.,* 2017).

5.1.2. *P. syringae* pv. *pisi* infection

As a proof-of-concept, the research presented in this chapter focuses on the protection of pea seeds from bacterial blight infection. The outbreaks of this disease are sporadic and their effects can range from minor damage to complete crop loss (Hollaway *et al.,* 2007). There is a current lack of preventive and treatment measures against bacterial infections in agriculture (Chapter 1, Section 1.2.2), particularly against *P. syringae* pv. *pisi.* This bacterial pathogen is present on the seed surface and seed coat (Skoric 1927; Grondeau, Poutier, *et al.,* 1992), where it can remain viable for over three years (Lawyer & Chun 2001). Infection occurs after germination. When the seedling starts emerging, its surface is contaminated (particularly the outside of the plumule) as it enters into direct contact with the infected seed coat (Skoric 1927) (Figure 5.1).

This pathogen is mainly present on the seed, but it does not penetrate the cotyledons or the embryo (Figure 5.1). Instead, it remains on the seed coat, close to the rootlet, so that it can infect the seedling once it emerges (Skoric 1927). The soil water content plays an important role in bacterial transmission, with a higher water content making transmission more likely (Skoric 1927; Roberts 1992; Hollaway *et al.,* 1996; Roberts *et al.,* 1996). However, sowing into a dry area would not prevent transmission since germination would be delayed (Hollaway *et al.,* 1996).

There are two main routes of bacterial transmission; seed-borne and trash-borne inoculum (Hollaway *et al.,* 2007). The most important is seed-borne inoculum, as this is the route of most cases of infection (Skoric 1927; Lawyer & Chun 2001) since only 1 in 10,000 infected seeds (0.01%) is enough to destroy a crop (Taylor & Dye 1976; Hollaway *et al.,* 2007). Nevertheless, trash-borne inoculum is also important; the pathogen can survive in pea trash from at least 29 weeks (if trash is buried) to more than 78 weeks (on soil surface) (Hollaway & Bretag 1997). Furthermore, heavy rain and strong winds can also support the local spread of the disease (Roberts 1997). Since all aerial plants are susceptible to infection (Hollaway *et al.,* 2007), it is important to control and prevent it before germination when the pathogen presence is restricted to the seed coat.

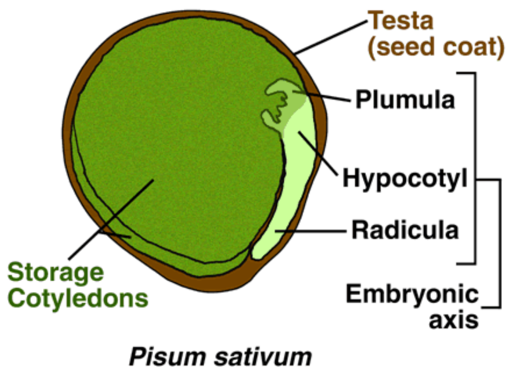

Figure 5.1 Morphology of a mature and endospermless pea (*Pisum sativum*) seed. Image reproduced from 'The Seed Biology' (http://www.seedbiology.de) and reprinted with permission from (Finch-Savage & Leubner-Metzger 2006) and the publisher.

P. syringae pv. *pisi* enters the plant, mainly through wounds or openings. Frost damage is also important since this pathogen has ice nucleating activity that permits frost damage at higher temperatures than it would normally occur without the bacterium (Lindow 1983). Once inside the plant, the bacterium produces extracellular polysaccharides (EPS) (Billing 1982). These EPS help the bacterium to attract nutrients, protect it against the environment and natural plant defences (Leigh & Coplin 1992; Denny 1995; Király *et al.,* 1997), and facilitate the progression of the pathogen (Denny 1995). Moreover, EPS delay the evaporation of leaked water from the plant cells, contributing to the water-soaked lesions that are characteristic of this disease, as well as to the wilting of the plant at later stages of the infection (Billing 1982).

The initial study of this pathogen indicated that control may be possible by disinfection of the seed (Skoric 1927). As this pathogen is present mainly on the seed surface and seed coat (without any indication of infection) that can easily spread and infect other plants; a seed treatment able to control and prevent the infection before germination occurs would be extremely desirable.

5.1.3. Aims and Objectives

The experiments detailed in this chapter aimed to evaluate the effects of essential oil-loaded MSNPs *in vitro* and *in planta* against bacterial phytopathogen *P. syringae* pv. *pisi*, causative agent of pea bacterial blight. The experiments in this chapter focused on the use of Ajwain essential oil as a biocide to determine the feasibility of the application of a seed treatment containing such EO-loaded MSNPs to protect plants from bacterial infections.

5.2. Materials and Methods

5.2.1. Loading of MSNP with Ajwain EO

Sixty microliters of Ajwain essential oil was dissolved in 1.25 ml dimethyl sulfoxide (DMSO, Sigma-Aldrich, UK) and then loaded into 75 mg MSNPs suspended in 5 ml phosphate-buffered saline (PBS, Sigma-Aldrich, UK). The mixture was sonicated with a probe (Vibra-Cell VCX 130, Sonics & Materials Inc., USA) for 1.5 minutes at 5 second intervals, then left to stir at 500 rpm during 48 hours in dark conditions. Loaded MSNPs were freeze dried for 24 hours.

5.2.2. 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 and incubating overnight at 28°C before inoculating 25 ml of Mueller-Hinton Broth (MHB; Oxoid Ltd, UK) with one colony and incubating at 28°C and 220 rpm overnight. Turbidity was measured and adjusted to OD_{600} = 0.05 using MHB.

5.2.3. *In vitro* effect of Ajwain-MSNPs

Falcon tubes containing 25 ml of *P. syringae* pv. *pisi* cultures (OD_{600} = 0.05) were treated as described in Table 5.1. Bacterial cultures were incubated at 28°C and 220 rpm. Turbidity was measured after 24 and 48 hours. Negative controls without nanoparticles and MSNPs controls with empty nanoparticles were included. Empty nanoparticles underwent the loading protocol but without the addition of the essential oil to consider the possible effect that the compounds used during loading could have on the bacterial cultures. All treatments were performed in triplicate.

Table 5.1 Treatments added to *P. syringae* pv. *pisi* cultures to determine the effect of Ajwain-loaded MSNPs (at a final concentration of 2 mg/ml).

Treatment	**Microorganism**	**Nanoparticles**	**Biocide**
Negative Control	*P. syringae* pv. *pisi*	None	None
MSNPs Control	*P. syringae* pv. *pisi*	MSNPs (freeze dried)	None
Test	*P. syringae* pv. *pisi*	MSNPs (freeze dried)	Ajwain Essential Oil

5.2.3.1. Live/Dead test

A live/dead test was performed to determine the viability of bacteria treated with Ajwain-loaded MSNPs (1.5 mg/ml). Bacterial cultures were incubated overnight after treatment. Samples were centrifuged at 4,000 rpm and 15°C for 15 minutes and resuspended in 2 ml 0.85% NaCl. For samples, 1 ml of this suspension was added to 20 ml 0.85% NaCl. To prepare a calibration curve, 1 ml of the suspension was added to each of two centrifuge tubes with either 20 ml of 0.85% NaCl (for live bacteria) or 20 ml of 70% isopropyl alcohol (for dead bacteria). All samples were incubated at room temperature for one hour, mixing every 15 minutes before centrifugation at 4,000 rpm for 10 minutes. Pellets were resuspended in 20 ml 0.85% NaCl and centrifuged again. Pellets were resuspended in 10 ml 0.85% NaCl and optical density at 600 nm was determined.

The staining protocol was prepared for microscopy and for the microplate reader. The staining dye was prepared by mixing equal volumes (21 µl each) of components A and B from the LIVE/DEAD BacLight Bacterial Viability Kit (Thermo Fisher Scientific, UK) and mixing thoroughly. For microscopy, 3 µl of the dye mixture were added for each ml of bacterial suspension and mixed thoroughly before incubating at room temperature in dark conditions for 15 minutes. After incubation, 5 µl were placed in a glass slide and observed under a fluorescence microscope (Motic AE31, UK). For plate reader analysis, bacterial optical density was adjusted to OD_{600} = 0.06. The calibration curve was prepared using five different proportions of cells (Table 5.2) in culture tubes to a total of 2 ml. Microplates were prepared by adding 100 µl of each bacterial suspension mixture (for calibration curve) or 100 µl of each bacterial sample (in triplicate) into separate wells. One hundred microliters of 2x stain solution (6 µl component A + 6 µl component B, suspended in 2 ml distilled water) were added to each well and mixed thoroughly. Microplates were incubated at room temperature in dark conditions for 15 minutes. With excitation centred at 485 nm, the fluorescence intensity was measured at 530 nm ($F_{cell,em1}$ = emission 1; green) and at 630 nm ($F_{cell,em2}$ = emission 2; red).

Table 5.2 Proportion of Live:Dead cells used to construct calibration curve.

Ratio of Live:Dead Cells	ml Live-Cell Suspension	ml Dead-Cell Suspension
0:100	0	2.0
10:90	0.2	1.8
50:50	1.0	1.0
90:10	1.8	0.2
100:0	2.0	0

5.2.4. *In planta* effect of Ajwain-MSNPs

To evaluate the effect of Ajwain EO-loaded MSNPs, an alginate seed coating containing 1, 10 and 20 mg/ml Ajwain-MSNPs was used to coat sterile "Kelvedon wonder" pea seeds before sowing.

5.2.4.1. Seed Coating

Pea seeds ("Kelvedon Wonder" cultivar) were sterilised as previously described in Chapter 4 Section 4.2.3. Briefly, seeds were immersed in 70% ethanol (Sigma Aldrich, UK) for 1 minute before washing with sterile water. Subsequently, seeds were immersed in a 2% sodium hypochlorite (Sigma Aldrich, UK) solution for 5 minutes and washed 10 times with sterile water (1 minute per wash). Seeds were drained and dried, and only wrinkled seeds were used.

Sterile, dry seeds were coated as described in Chapter 4 Section 4.2.2. Initial concentrations of 3% (w/v) sodium alginate (Sigma Aldrich, UK) and 1 M $CaCl_2 \cdot 2H_2O$ solution (≥99.0%, Sigma Aldrich, UK) were used. However, this alginate coating prevented seed germination and all future coatings were carried out utilising 1% sodium alginate and 0.1 M $CaCl_2$. Table 5.3 and Table 5.4 summarise the seed treatment used in the first two experiments and in the third experiment, respectively. A total of 15 seeds were used for each condition. Coat control seeds were coated solely with alginate while test seeds were coated using alginate containing 1, 10 or 20 mg/ml CNAD-MSNPs, dissolved in the sodium alginate before the coating protocol. After coating, seeds were placed in a sterile petri dish and allowed to dry overnight.

Table 5.3 Seed treatments used during the first and second experiments to evaluate the effect *in planta* of Ajwain EO-loaded MSNPs against *P. syringae* pv. *pisi*. MSNPs – mesoporous silica nanoparticles, EO – essential oil, *Psp* – *P. syringae* pv. *pisi.*

Treatment	**Bacterial inoculum**	**Alginate coat (alg %, $CaCl_2$ M)**	**MSNPs (mg/ml)**	**Biocide**
Control – Negative	None	None	None	None
Control – Positive	*Psp*	None	None	None
Coat – Control	None	3%, 1 M (1st test) 1%, 0.1M (2nd test)	None	None
EO-MSNPs – Control	None	1%, 0.1M	1, 10, 20	Ajwain EO
EO-MSNPs – Test	*Psp*	1%, 0.1M	1, 10, 20	Ajwain EO

Table 5.4 Seed treatments used during the third experiment to evaluate the effect *in planta* of Ajwain EO-loaded MSNPs against *P. syringae* pv. *pisi*. MSNPs – mesoporous silica nanoparticles, EO – essential oil, *Psp* – *P. syringae* pv. *pisi.*

Treatment	Bacterial inoculum	Alginate coat (alg %, $CaCl_2$ M)	MSNPs (mg/ml)	Biocide
Control – Negative	None	None	None	None
Control – Positive	*Psp*	None	None	None
Coat – (-)Control	None	1%, 0.1M	None	None
Coat – (+)Control	*Psp*	1%, 0.1M	None	None
EO-MSNPs – Control	None	1%, 0.1M	20	Ajwain EO
EO-MSNPs – Test	*Psp*	1%, 0.1M	20	Ajwain EO
EO-MSNPs2x – Control	None	1%, 0.1M double coating	20	Ajwain EO
EO-MSNPs2x – Test	*Psp*	1%, 0.1M double coating	20	Ajwain EO

5.2.4.2. Seed Inoculation

Alginate-coated seeds were inoculated with *P. syringae* pv. *pisi* using an adapted version of the method described by (Roberts *et al.,* 1996). Briefly, seeds were immersed in 50 ml *P. syringae* pv. *pisi* inoculum (OD_{600} = 0.361, 0.185 and 0.066 for the first, second and third trials, respectively) for 5 minutes, transferred to sterile Petri dishes and left to dry. All controls without inoculum were immersed in sterile water for the same amount of time.

5.2.4.3. Seed Incubation and Growth

Five pea seeds were grown in each sterile Magenta™ vessel (Sigma Aldrich, UK) containing 100 ml of Murashige and Skoog (MS) medium. MS media was prepared by dissolving 4.4 g of MS basal salt mixture (Sigma Aldrich, UK), 10 g of sucrose (Sigma Aldrich, UK) and 2.5 g of Phytagel (Sigma Aldrich, UK) in 1 L of distilled water. Fifteen seeds were used per treatment to have a triplicate set up with 5 seeds grown in each vessel. Magenta vessels were incubated in a Sanyo growth cabinet with a photoperiod of 16h at 21/20°C, under fluorescent light (80 µmol photons $m^{-2}s^{-1}$). Germination, plant height and appearance of bacterial blight signs were monitored for 2-3 weeks.

After three weeks of incubation, three representative symptomless plants from each treatment were transferred to soil and incubated for a further two weeks. A mixture of Pot and Bedding Compost (Levington Advance, UK) and Vermiculite (Sinclair Pro, UK) with 0.28 g/L of Imidasect (Sinclair Pro, UK) to control sciarid fly, was used to grow plants. Plants were watered three times a week and incubated under the same initial conditions.

5.2.4.4. Increased sample size study

To evaluate if a larger sample size could more reliably demonstrate the efficacy of the treatment, a study with a larger sample size was carried out. A total of 78 seeds per treatment (three replicates with 26 seeds each) were sterilised and coated as described in Section 5.2.4.1 using 1% sodium alginate and 0.1 M $CaCl_2$ with 2 mg/ml Ajwain-loaded MSNPs. After coating had dried, seeds were inoculated as described in Section 5.2.4.2 using a bacterial culture adjusted to OD_{600} = 0.025. Control seeds were only coated with alginate without the addition of Ajwain-MSNPs, and inoculated with *P. syringae* pv. *pisi*. Finally, seeds were individually placed in sterile culture tubes containing 20 ml of MS medium, and incubated as described in Section 5.2.4.3.

5.2.5. Statistical Analyses

Statistical analyses were performed using Microsoft Excel and Minitab® version 18.1. To determine statistical significance of differences between samples, two-factor analysis of variance (ANOVA) and t-tests were undertaken. These were followed by Tukey's HSD (honest significant difference) or a Chi-squared test when appropriate for quantitative or categorical data, respectively. *P* values of 0.05 and 0.01 were used and specified for each analysis.

5.3. Results

5.3.1. *In vitro* effect of Ajwain-MSNPs

The effect of Ajwain-loaded MSNPs was evaluated by adding 2 mg/ml of loaded MSNPs (0.703 mg_{Ajwain} / mg_{MSNPs}, see Chapter 3, Section 3.3.3.1) to 25 ml of *P. syringae* pv. *pisi* cultures (OD_{600} = 0.05 = 1.23 × 10^7 CFU/ml). After 24 h, control bacterial cultures were more turbid (Figure 5.2 A&B) than Ajwain-MSNPs treated cultures, where aggregated cells well visible (Figure 5.2C).

Ajwain-treated bacterial cultures showed a bacterial growth reduction of 32.34% and 47.87% compared to MSNPs controls and untreated controls, respectively (Figure 5.3). After 48h of incubation, the reduction was of 41.42% and 40.82%, respectively (Figure 5.3). This demonstrated the effectivity of the EO-loaded MSNPs as antimicrobials.

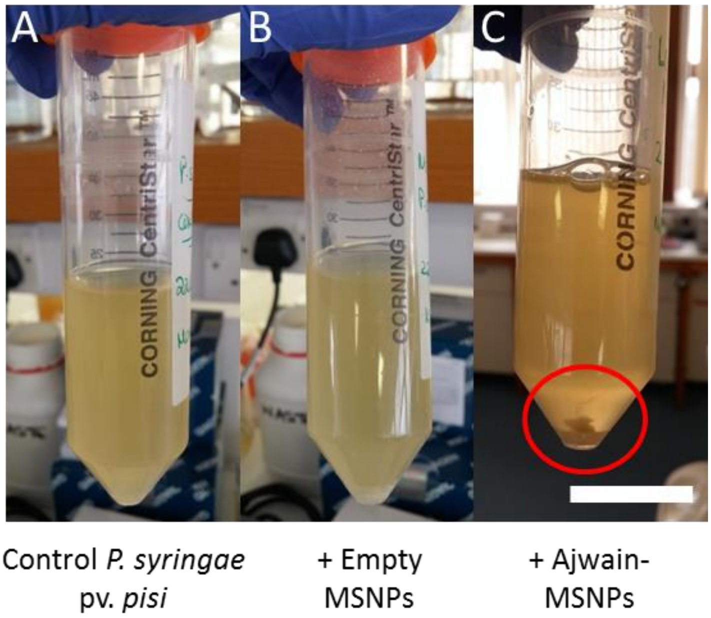

Figure 5.2 *In vitro* effect of Ajwain EO-loaded MSNPs on *P. syringae* pv. *pisi* bacterial cultures after 24 h. Control untreated bacterial cultures as well as controls treated with empty MSNPs were used. Circle shows the bacterial sediment formed after EO-MSNP treatment. Images are representative of triplicates. Scale bar = 3 cm.

Furthermore, controls treated with empty MSNPs resulted in a bacterial growth increase of 29.8% compared to the untreated controls after 24 h. This suggested that the addition of MSNPs initially accelerated bacterial growth. However, after 48h both controls displayed a similar optical density (Figure 5.3).

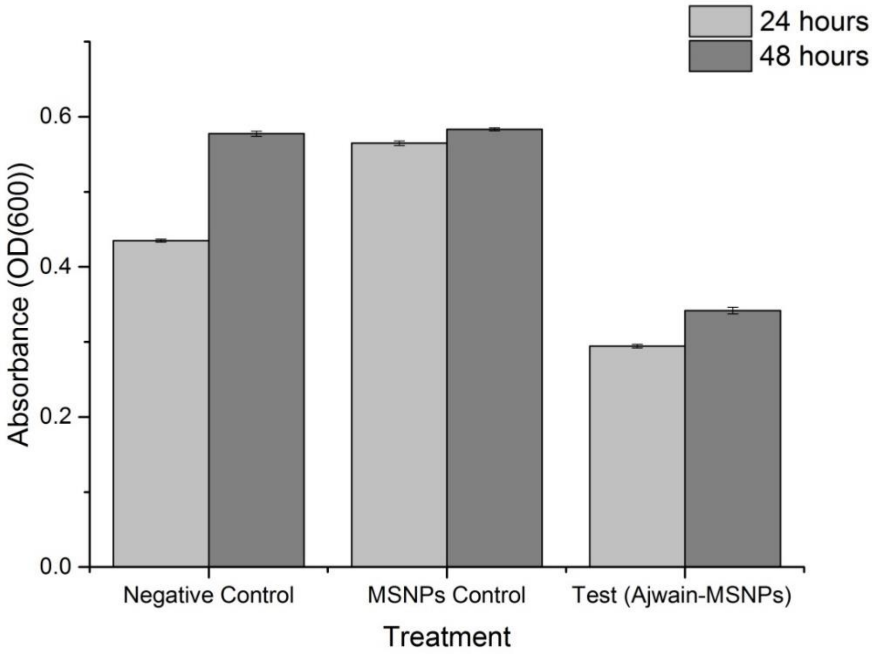

Figure 5.3 *In vitro* effect of Ajwain EO-loaded MSNPs on *P. syringae* pv. *pisi.* Results after 24 and 48 hours of incubation with empty or Ajwain EO-loaded MSNPs. Negative controls (untreated bacterial culture) and MSNP controls were included. Data shows mean ± standard deviation, n = 3.

The measurement of absorbance as an indication of bacterial viability has a key drawback, as it only considers cell density and does not take into account the percentage of cells in the sample that are dead or alive. Therefore, to obtain a more accurate quantification of the antimicrobial effect of the Ajwain-loaded MSNPs on *P. syringae* pv. *pisi*, a Live/Dead test was performed.

5.3.1.1 Live/Dead Test

Bacterial cells were stained after EO-MSNPs treatment using the LIVE/DEAD BacLight Bacterial Viability Kit. Control bacterial cultures (live cells, dead cells, empty MSNP-treated) as well as control samples of empty and EO-loaded MSNPs were stained for comparison. All stained samples were observed under a fluorescence microscope to determine bacterial viability after treatment. Control live bacteria were predominantly green (Figure 5.4A) while control dead bacteria showed only red fluorescence (Figure 5.4B). Samples treated with empty MSNPs displayed green live cells together with red aggregations that were initially thought to be clusters of dead bacteria (Figure 5.4C); however, after observing a stained sample of empty MSNPs without any bacterial inoculation (Figure 5.4D) it became evident that the MSNPs were aggregated and had been stained with the red dye. Furthermore, samples of Ajwain-loaded MSNPs without any bacterial inoculation did not present these nanoparticle aggregations (Figure 5.4E), suggesting the essential oil loaded into the nanoparticles prevented their accumulation and the emission of red fluorescence that could be misinterpreted as dead bacterial cells. For the test bacterial cells treated with essential oil-loaded MSNPs, both dead and live cells could be observed, although most cells appeared to have died after the treatment (Figure 5.4F).

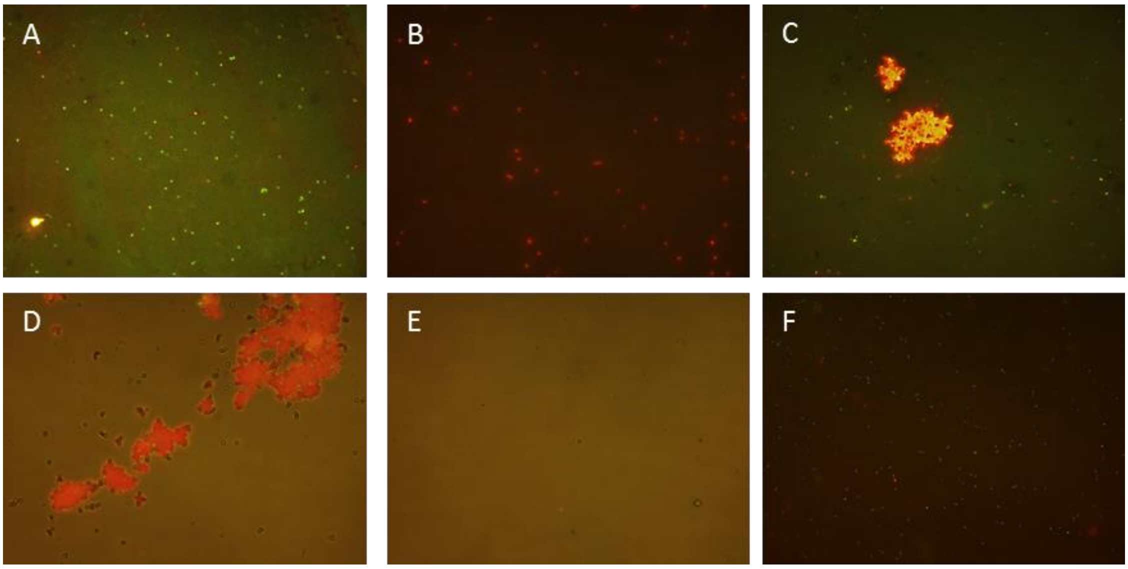

Figure 5.4 Live/Dead Test microscope imaging. A) Live *P. syringae* pv. *pisi* cells, B) dead *P. syringae* pv. *pisi* cells, C) *P. syringae* pv. *pisi* cells incubated with empty MSNPs, D) Empty MSNPs, E) Ajwain EO-MSNPs, F) Test sample: *P. syringae* pv. *pisi* cells incubated with Ajwain EO-MSNPs. Images are representative of triplicates.

To quantify the percentage of live and dead cells after EO-MSNPs treatment, two calibration curves were prepared (Figure 5.5). The first calibration curve indicates the ratio of green/red fluorescence in relation to the percentage of live cells (Figure 5.5A). Control empty MSNPs aggregated and emitted red fluorescence, affecting the ratio of green to red fluorescence due to live and dead bacterial cells, respectively. Therefore, a second calibration curve was constructed considering only the emitted green fluorescence in relation to the live cells percentage (Figure 5.5B).

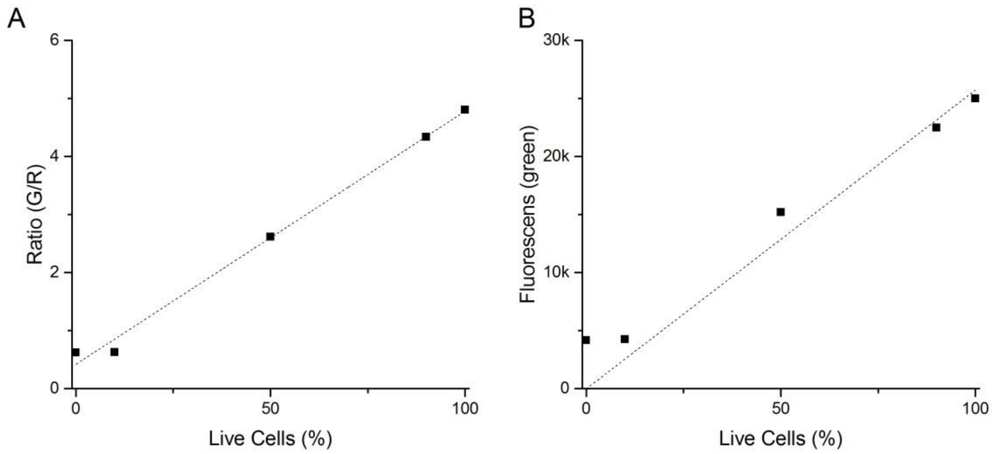

Figure 5.5 Live/Dead Test Calibration Curves. A) Ratio of green (G) to red (R) fluorescence in relation to the percentage of live cells. Linear relationship with R^2 = 0.9922 B) Green fluorescence in relation to the percentage of live cells. Linear relationship with R^2 = 0.97634. Data shows average measurement of duplicates.

The percentage of live bacterial cells in the control treated with empty MSNPs and the test treated with Ajwain-loaded MSNPs was estimated using the green fluorescence calibration curve. EO-loaded MSNPs resulted in a 23% live cells reduction compared to the empty MSNPs-treated controls (Figure 5.6). The large error bar of the control may have been due to the high intensity of red fluorescence emitted from the empty nanoparticles. Even so, there was a clear difference between the treatments, demonstrating that the Ajwain-MSNPs have a strong antimicrobial effect *in vitro*.

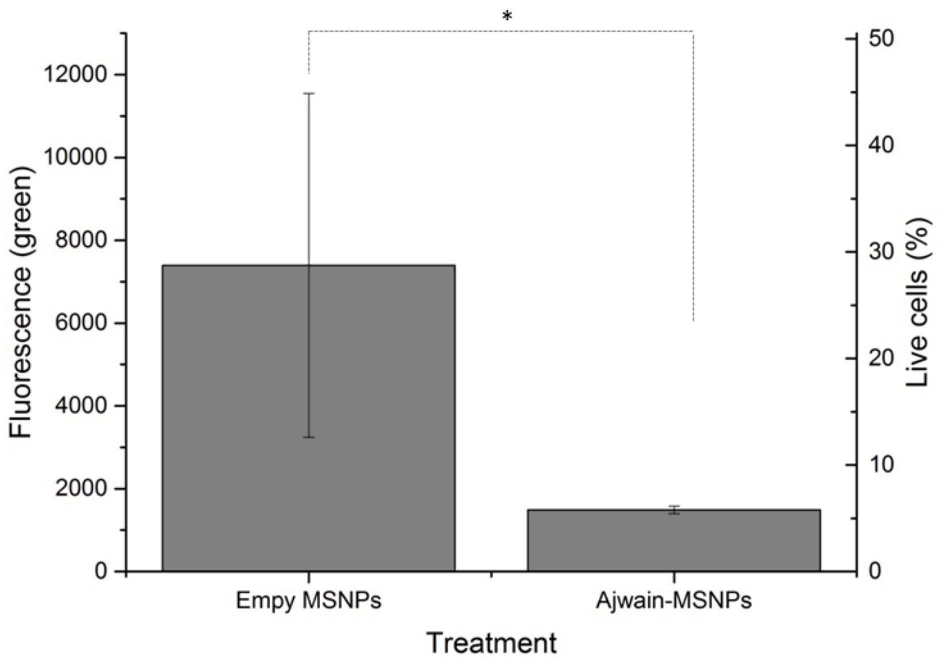

Figure 5.6 Quantification of live *P. syringae* pv. *pisi* cells after treatment with empty MSNPs or with Ajwain-loaded MSNPs. Fluorescence and percentage of live cells compared to bacterial culture control. Data shows mean ± standard deviation; n=3, *p<0.1.

5.3.2. *In planta* Effect of Ajwain-MSNPs

To confirm the potential antimicrobial effect of essential oil-loaded MSNPs, Ajwain-MSNPs were evaluated *in planta* against bacterial blight phytopathogen *P. syringae* pv. *pisi*. Initially, a total of 15 seeds per treatment, divided into three vessels, were coated with an alginate layer (developed in Chapter 4) containing varying quantities of Ajwain-MSNPs (1, 10 or 20 mg/ml).

The first test was performed using a 3% sodium alginate (since the percentage was shown not to be significant in the previous chapter) and 1 M $CaCl_2$ seed coating (to test an intermediate concentration). All uncoated control seeds germinated (Figure 5.7 A&B) and the infected controls presented signs of bacterial blight, including water-soaked lesions, wilting and bacterial ooze (Figure 5.7B). Two weeks after sowing, most test seeds coated with alginate containing 1 mg/ml

(Figure 5.7 C&D), 10 mg/ml (Figure 5.7 E&F) and 20 mg/ml (Figure 5.7 G&H) Ajwain-MSNPs were still unable to germinate. Bacterial ooze was visible surrounding the infected seeds; however, the test seeds that were able to germinate did not appear to have blight symptoms (Figure 5.7 D,F,H).

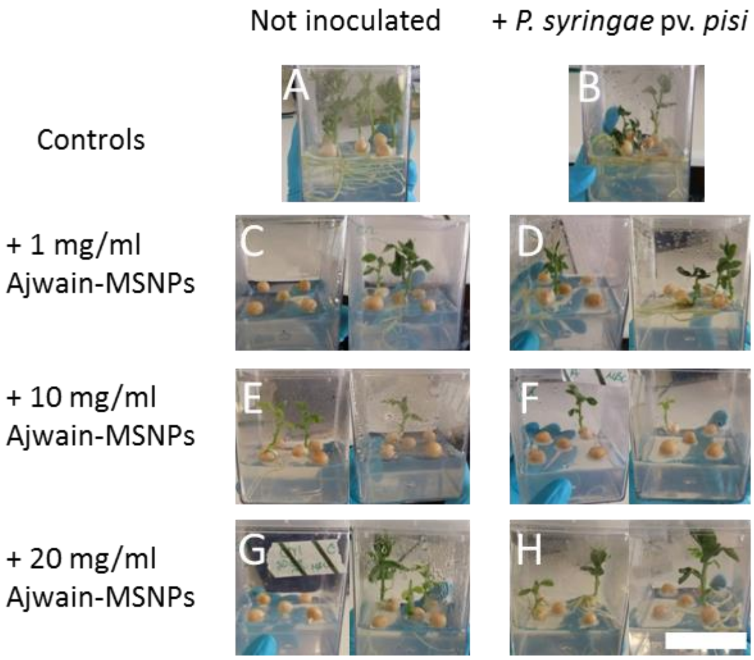

Figure 5.7 Results of initial experiment using a 3% sodium alginate and 1M $CaCl_2$ seed coating. Uncoated control seeds A) with or B) without inoculation were used. Test seeds were coated with alginate containing C&D) 1 mg/ml, E&F) 10 mg/ml or G&H) 20 mg/ml Ajwain-loaded MSNPs. Seeds in B,D,F,H were inoculated with *P. syringae* pv. *pisi.* Images are representative of triplicates. n = 15 seeds per treatment. Scale bar = 5 cm.

A second experiment was performed using a 1% sodium alginate solution with 0.1 M $CaCl_2$ to overcome the effect of alginate on germination. This lower concentration alginate solution was used for all subsequent experiments. Three weeks after sowing, there was an observable difference between the treated plants and the controls. Treated plants resulted in 6.67-13.33% more symptomless plants than the control (Figure 5.8A). Additionally, symptomless plants treated with 1 mg/ml MSNPs were significantly taller than the control ($p<0.01$) and plants treated with 10 mg/ml ($p<0.05$) (Figure 5.8B) and all coated seeds grew faster than the controls. Nevertheless, the difference in symptomless plants was not statistically significant.

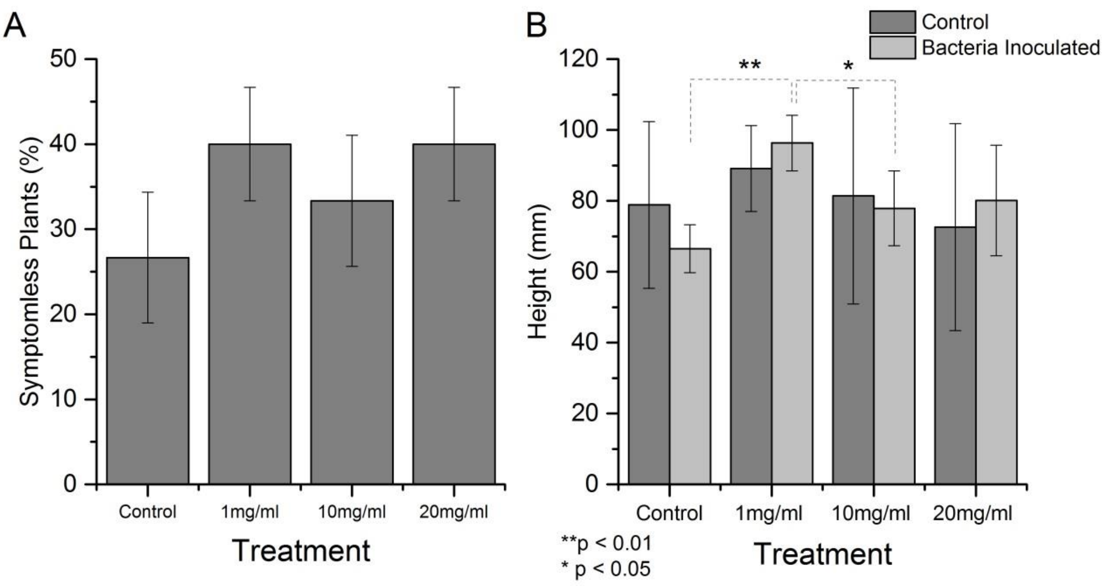

Figure 5.8 Effect of Ajwain-loaded MSNPs *in planta* against bacterial blight causing agent *P. syringae* pv. *pisi* during second experiment. A) Percentage of symptomless plants for each treatment three weeks after *P. syringae* pv. *pisi* infection with uncoated seeds as controls. B) Height of symptomless plants three weeks after sowing with controls not having been inoculated. Data shows the mean ± standard deviation of triplicates. *p<0.05, **p<0.01, n = 15 seeds per treatment.

For each of the treatments, three representative symptomless plants were transferred to soil and incubated, with all control plants in one tray and all protected plants in another. After two more weeks of incubation, 55% of plants previously treated with Ajwain-MSNPs remained symptomless, while the remaining 45% developed signs of infection or died. Furthermore, one month after transferring the representative plants to soil, only 20% of Ajwain-MSNPs treated plants had survived while all control plants (without bacterial inoculation) were healthy (Figure 5.9).

Finally, a third experiment was performed using a lower inoculum concentration (OD600 = 0.066) and treating seeds with an alginate coating without nanoparticles, as a coat control, or with a single or double coating (coated twice to investigate if this increased seed protection) containing 20 mg/ml Ajwain-MSNPs (Figure 5.10).

Figure 5.9 Control without bacterial infection nor treatment (right) and infected Ajwain-MSNPs treated plants (left) one month after being transferred to soil. Fifteen representative plants were used for each treatment. Scale bar = 5 cm.

All coated seeds resulted in a larger percentage of symptomless plants two weeks after sowing (Figure 5.11A) and symptomless plants were visibly taller than the controls (Figure 5.11B). However, a double alginate coating occasionally trapped the rootlets/shoots inside the coating and provided less protection than a single coating. As with the previous results, there was an observable difference between the treatments but it was not statistically significant (Chi-squared test).

To determine if the lack of statistical significance (despite the observable differences between treatments) could be due to the small sample size used; an additional experiment was carried out using a larger sample size of 78 seeds per treatment. Again, a difference was observed between test and control seeds, with and without the addition of Ajwain-MSNPs. Seeds treated with alginate containing Ajwain-MSNPs resulted in 60.26% symptomless plants three weeks after sowing. Control seeds coated only with alginate without the addition of Ajwain-MSNPs resulted in 50% symptomless plants (Figure 5.12). Still, there was no statistically significant difference between treatments ($p>0.1$).

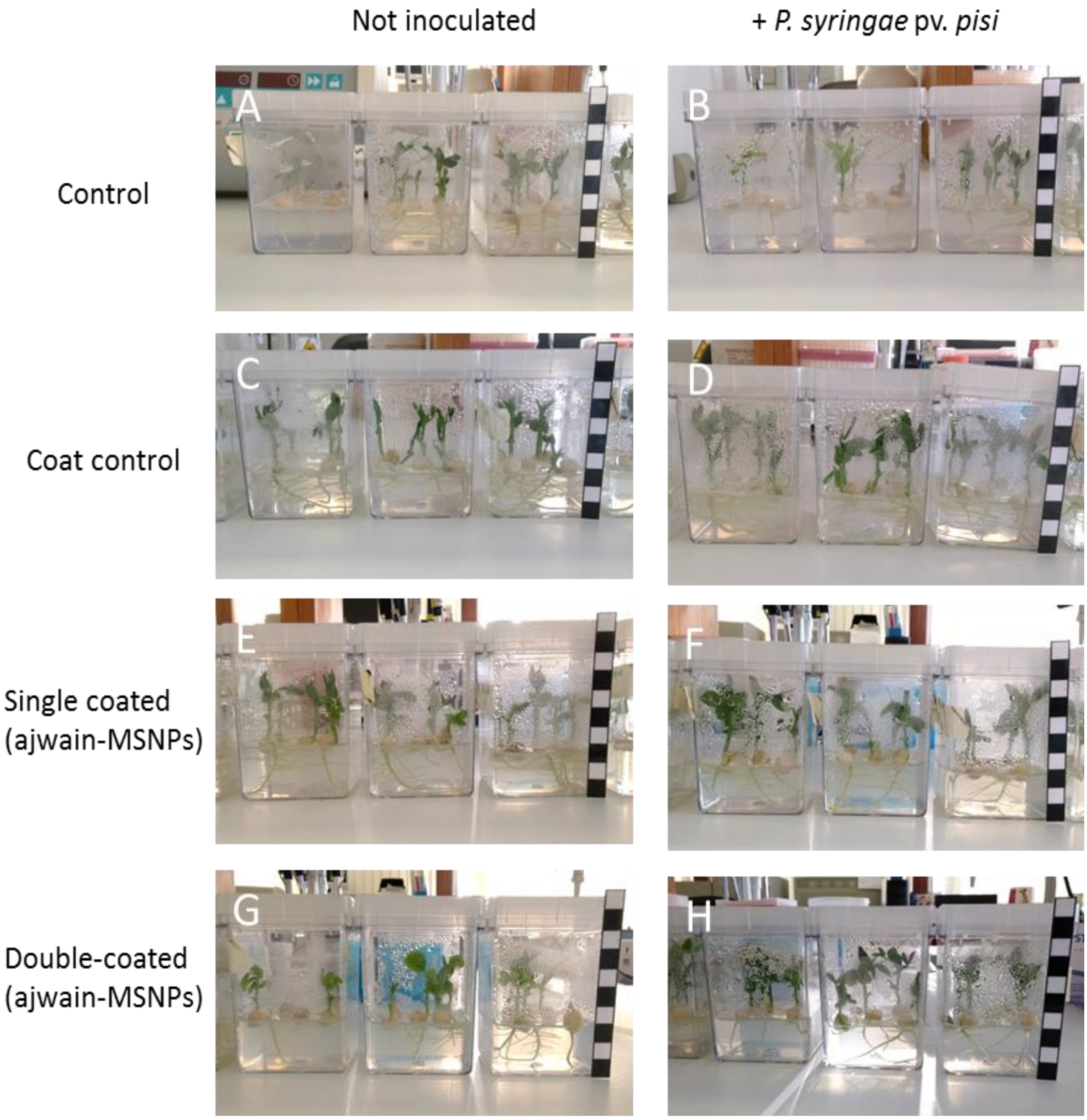

Figure 5.10 Effect of single and double alginate coating two weeks after sowing. Uncoated control seeds A) without or B) with bacterial inoculation were used. A coat control C) without or D) with inoculation was also evaluated. Test seeds were E&F) single or G&H) double-coated with alginate (1% alginate and 0.1M $CaCl_2$) containing 20 mg/ml Ajwain-MSNPs. Seeds in B,C,E,G were inoculated with *P. syringae* pv. *pisi.* n = 15 seeds per treatment. Scale bar: each square = 1 cm.

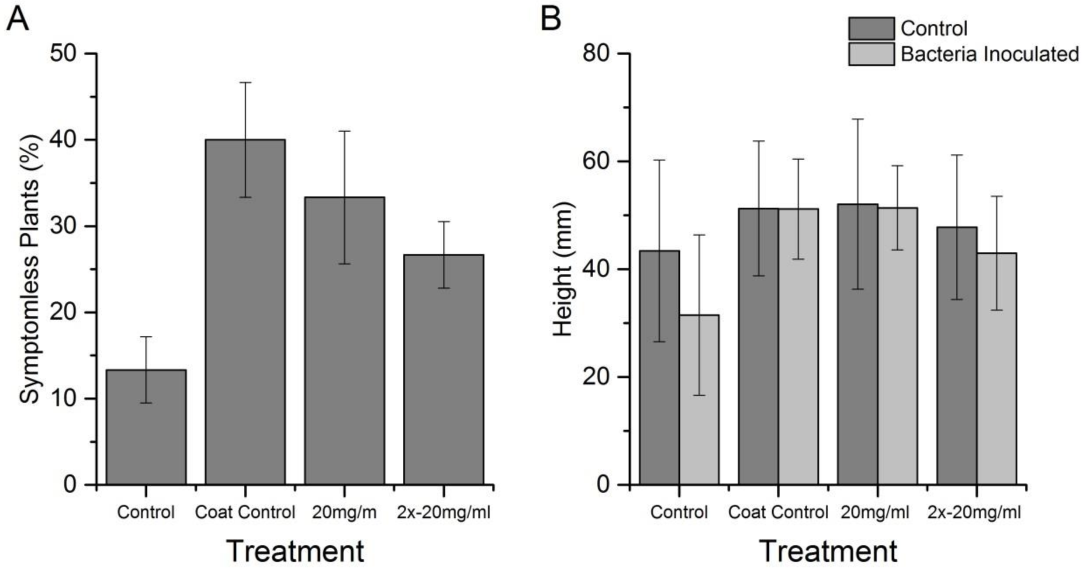

Figure 5.11 Effect of Ajwain-loaded MSNPs *in planta* against bacterial blight causing agent *P. syringae* pv. *pisi* during third experiment. A) Percentage of symptomless plants for each treatment two weeks after *P. syringae* pv. *pisi* infection with uncoated seeds as controls. B) Height of symptomless plants two weeks after sowing with control plants not having been inoculated. Data shows mean ± standard deviation of triplicates. n = 15 seeds per treatment. No significant differences between treatments using ANOVA, Tukey's HSD test and Chi-squared test.

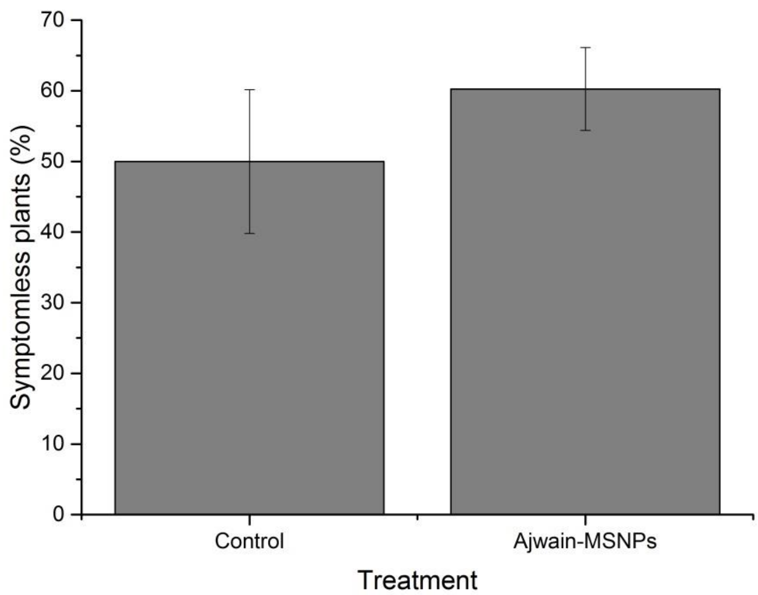

Figure 5.12 Effect of Ajwain-loaded MSNPs *in planta* against *P. syringae* pv. *pisi*. Percentage of symptomless plants for each treatment three weeks after bacterial infection. Control seeds were coated only with alginate, without the addition of Ajwain-MSNPs, and infected. Data shows the mean ± standard deviation; n = 78 seeds per treatment. No significant difference between treatments using Chi-squared test.

5.4. Discussion

The research detailed in this chapter evaluated the effect of Ajwain-loaded MSNPs against *P. syringae* pv. *pisi,* both *in vitro* and *in planta*. The main objective was to assess the potential incorporation of these biocide-loaded nanoparticles into an alginate seed coating that would serve as a seed treatment to protect plants from bacterial infections.

Research in Chapter 4 demonstrated that alginate was a promising material to incorporate biocide-loaded silica nanoparticles as a seed treatment. Results in Section 4.3.3.3 suggested that the alginate coating concentrated the EO-loaded MSNPs on the seed surface and seed coat; which was where the test pathogen, *P. syringae* pv. *pisi,* was present before germination (Skoric 1927). To study the practical application of this approach, this chapter assessed Ajwain-loaded MSNPs. Treatment of *P. syringae* pv. *pisi* was investigated as a proof of principle; however, this treatment could potentially be used to treat other bacterial or fungal phytopathogens.

Ajwain was selected as the test oil in this chapter since it was previously demonstrated to have a strong antimicrobial effect against different bacterial species, including *P. syringae* pv. *pisi* (Chapter 2). Shokrian *et al.* (2016) have also shown the antimicrobial effect of this oil against another important phytopathogen, *P. syringae* pv. *syringae* (Shokrian *et al.,* 2016). In addition, despite this oil's high antimicrobial activity, its application in fields such as agriculture has not been as extensively studied as other EOs (Dwivedi & Singh 1998; Sahaf *et al.,* 2007; Shojaaddini *et al.,* 2008).

The effect of Ajwain-MSNPs was evaluated *in vitro* by adding the loaded nanoparticles to *P. syringae* pv. *pisi* bacterial cultures. The controls treated with empty MSNPs appear to have grown faster than the untreated control, suggesting the silica nanoparticles might play a role in accelerating bacterial growth (this will be further discussed in Chapter 6, section 6.4).

Optical density measurements were used as a preliminary test to evaluate if bacterial cell mass and density decreased after incubation with Ajwain-MSNPs. After 48h, an absorbance reduction of 40.82% and of 41.42% was observed compared to the negative controls and to the NPs controls, respectively. Even though this method is rapid, low-cost and non-destructive; optical density has a key limitation, as it only considers cell density and does not differentiate live and dead cells, or even debris particles, in the sample (Pan *et al.,* 2014). Also, its sensitivity is usually limited to 10^8-10^{10} bacteria/ml (Hazan *et al.,* 2012; Lehtinen 2007). Hence, a change in turbidity does not accurately represent bacterial viability. For that reason, a Live/Dead test was performed to obtain a more accurate quantification of the antimicrobial effect of the Ajwain-loaded MSNPs on *P. syringae* pv. *pisi*.

The Live/Dead staining microscope images illustrated the antimicrobial effect of Ajwain-MSNPs on *P. syringae* pv. *pisi.* A high percentage of dead cells was clearly visible in the samples treated with Ajwain-MSNPs, which resulted in at least 23% less live cells than the controls treated with empty nanoparticles. This decrease in viability may have actually been underestimated due to the red fluorescence emitted by the empty nanoparticles.

The Live/Dead test uses two dyes; SYTO® 9 and propidium iodide (Figure 5.13). The green-fluorescent SYTO® 9 nucleic acid stain passively diffuses through the membranes of both live and death cells. In contrast, the red-fluorescence nucleic acid stain, Propidium Iodide (PI), can only penetrate those cells with a damaged cell membrane and causes a reduction in the total green fluorescence (Molecular Probes Inc 2004). Once PI has bound its target, its fluorescence is enhanced 20- to 30- fold. The results in this chapter indicate that empty MSNPs were stained by PI, resulting in a very high emission of red fluorescence. This red fluorescence given by the MSNPs reduced the total green fluorescence emitted by the live cells. Hence, the percentage of live cells in the control samples, as well as the antimicrobial effect of Ajwain-MSNPs, was underestimated. PI can be loaded into silica nanoparticles (Neumeyer *et al.,* 2011; Théron *et al.,* 2014; Théron *et* *al.,* 2015), and due to its permanently positive charge, the dye could be loaded into the control empty MSNPs during the staining protocol, resulting in an increased red fluorescence emission. Furthermore, the positive charge of the dye, together with the negatively charged MSNPs, would cause the accumulation of the nanoparticles observed in Figure 5.4 C&D; increasing concentrations of PI have been shown to lead to nanoparticle aggregation (Müller *et al.,* 1991). It is important to emphasise that the green fluorescence observed in the Ajwain-MSNPs-treated samples represented more accurately the percentage of live cells, since the EO-MSNPs were already loaded with Ajwain EO, preventing their aggregation or PI-loading. Kaur *et al.* (2013) have previously reported that biogenic silver nanoparticles synthesised using Ajwain extract prevented nanoparticle aggregation due to the Ajwain-coating on the NPs (Kaur *et al.,* 2013). The difference in size of the error bars in Figure 5.6 could also be explained by this loading of PI into previously empty MSNPs affecting the green fluorescence measurements.

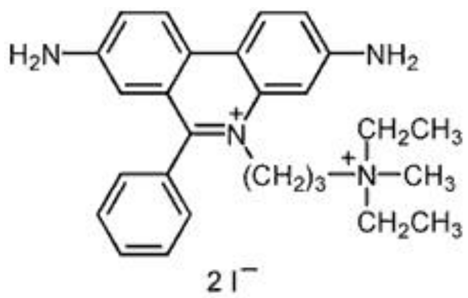

Figure 5.13 Propidium Iodide structure. Permanently positively charged molecule.

After the *in vitro* evaluation of the EO-loaded MSNPs, they were incorporated into an alginate seed coating to determine if they could protect plants against bacterial infection. 'Kelvedon wonder' pea seeds (*Pisum sativum*) were used for this study due to this cultivar's susceptibility to all seven races of *P. syringae* pv. *pisi* (Taylor *et al.,* 1989). Grondeau *et al.* (1992) have previously attempted to eliminate bacterial blight from peas using physical treatments such as hot water and moist/dry heat. The treatments were shown to decrease pea bacterial blight incidence, but germination was also affected, resulting in 5-45% less germination than the control (Grondeau, Ladonne, *et al.,* 1992). A reduction in germination was also observed in this study when a high concentration of $CaCl_2$ (1 M) was used, as demonstrated in Chapter 4. The tests using a lower gel concentration (1% sodium alginate and 0.1 M $CaCl_2$) containing EO-MSNPs produced plants that germinated successfully and resulted in more symptomless plants than the controls.

Overall, seeds treated with Ajwain-MSNPs produced more symptomless and better developed plants than controls. Still, even though there was an observable difference in all tests between EO-MSNPs treated seeds and the controls, it was not statistically significant. Since a larger sample size provides more experimental precision due to the decrease in the sample mean's variance (Facco *et al.,* 2016), an experiment with a total of 78 seeds per treatment, instead of only 15, was performed. However, the observable difference was still not statistically significant.

The experimental design presented in this chapter has three important limitations that should be discussed. First, initial experiments were performed using triplicate magenta vessels, each containing five pea seeds. A disadvantage of this experimental design was that contamination and infection was easily spread between individual plants sown in the same vessel. This potentially affected the results and the quantification of symptomless plants. Second, the bacterial inoculum concentration used varied for each of the infection experiments. This was due to the high level of infection obtained even for the control plants. Hence, an LD_{50} test would determine the inoculum concentration needed to obtain a 50% infection incidence in control plants. Finally, only germination, plant height and bacterial blight infection were evaluated. Considering other important parameters, such as wet and dry mass, main root length, chlorophyll content, number of leaves, nodes, flowers and pods; would provide more information about the effect of the treatment on the plants.

The reduction of symptomless plants after transplanting into soil suggested that the protection conferred by the EO-MSNPs was probably more effective in the short-term as a first line of defence. This could be due to the eventual evaporation or degradation of the antimicrobial

(Chapter 3, Section 3.3.3.3); the bacterial growth being quicker than the killing effect of the oil; and/or the localised presence of the EO-MSNPs on the seed's surface not being able to target bacterial infection on aerial parts of the plants. To counteract this, the following chapter (Chapter 6) evaluates the potential capping of EO-loaded MSNPs to prevent the volatilisation/degradation of the EOs and prolong their antimicrobial effect.

5.5. Conclusions

In conclusion, this chapter reports on the evaluation of the antimicrobial and protective effect of EO-loaded MSNPs against bacterial blight phytopathogen, *P. syringae* pv. *pisi,* both *in vitro* and *in planta*. Ajwain-MSNPs demonstrated a promising protection of peas against bacterial blight, but this effect needs to be further enhanced to provide a significant protection against the pathogen. The following chapters will evaluate a capping mechanism to improve the antimicrobial activity of EO-MSNPs against bacterial phytopathogens and to achieve a significant difference in symptomless plants treated with the aforementioned nanoparticles.

Chapter 6
Evaluation of Sugar-Capped MSNPs for Immobilisation of EO and Delivery

6.1. Introduction

Nanoparticle capping of the MSNP delivery system enables controlled release and prolonged effect of the cargo, or the opportunity to direct the system towards a specific target. This chapter focuses on the capping of essential oil-loaded MSNPs to improve the biocide delivery efficiency and it evaluates the potential application of this improved system on agriculture to treat peas against bacterial blight pathogen *P. syringae* pv. *pisi.* The experiments presented in this chapter aim to determine a possible capping agent that will improve the application of the biocide-MSNPs developed in the previous chapters. Additionally, the research summarises investigations into the potential of sugar-capped MSNPs to target a specific bacterial species able to metabolise the sugar cap and release the biocide. Finally, the application of this enhanced system against a range of bacterial species was studied with the final aim of preventing and treating susceptible peas against *P. syringae* pv. *pisi* infection.

6.1.1. Nanoparticles Surface Functionalisation and Ligand Conjugation

The use of nanoparticles (NPs) for targeted or slow-release delivery usually requires chemical modification of their surface, which should possess the adequate functional group needed for conjugation to the target ligand (Friedman *et al.,* 2013). For silica there are different methods to functionalise the surface, including covalent binding, chemisorption, electrostatic, or hydrophobic interactions (Bergman 2014).

The majority of the conjugations used to modify NPs are covalent, which is the most stable and selective type of conjugation. Despite the occurrence of random coupling in conjugation resulting in different ligand orientations and densities, directed coupling can be achieved by introducing desired functional groups that enable site-specific immobilisation of ligands (Friedman *et al.,* 2013).

The functionalisation of silica NPs often relies on the utilisation of functional silanes. MSNPs possess three regions that can be functionalised; the silica framework, channels or pores, and the NP exterior. Since the functional groups on the exterior surface are more accessible, they are the primary functionalisation target (Stein *et al.,* 2000; Bergman 2014).

There are three main methods to synthesise porous hybrid (organic and inorganic) materials (Figure 6.1); (i) grafting (post-synthetic modification of the pore surface, Figure 6.1A), (ii) co-condensation (simultaneous condensation of silica and organosilica precursors, Figure 6.1B), and (iii) synthesis of Periodic Mesoporous Organosilicas (PMOs) by incorporating bridging organic groups directly into the pore walls using bissilylated, $(R'O)_3Si–R–Si(OR')_3$, organosilica precursors (Figure 6.1C) (Hoffmann *et al.,* 2006).

This chapter focuses on the first method, grafting, where the NPs surface silanol groups (SiOH) serve as anchoring groups (Figure 6.1A). Free (≡Si-OH) and germinal ($=Si-(OH)_2$) silanols are active sites for silylation (substitution of a hydrogen atom by a silyl group, R_3Si) and react with organosilanes, whereas hydrogen-bonded silanols are less accessible due to the hydrophilic networks formed amongst themselves (Zhao & Lu 1998). The variation of the organic residue R allows a variety of organic groups to be functionalised onto the NPs (Hoffmann *et al.,* 2006). Most of the commonly used organosilanes, such as 3-aminopropyltriethoxysilane (APTES) or 3-aminopropyltrimethoxysilane (APTMS), have one organic substituent and three hydrolysable substituents in their molecular structure (Bergman 2014). APTES is commonly used as a coupling agent since it can be used for covalent linking onto the MSNP surface while (due to its protonated amino groups) it can also allow electrostatic interactions with negatively charged ligands such as DNA or proteins (Gomes *et al.,* 2016). This chapter looks at the functionalisation of MSNPs with a sugar (lactose) derivative using APTES as a coupling agent. The addition of essential oil components such as cinnamaldehyde (CNAD) onto the surface of MSNPs will also be discussed.

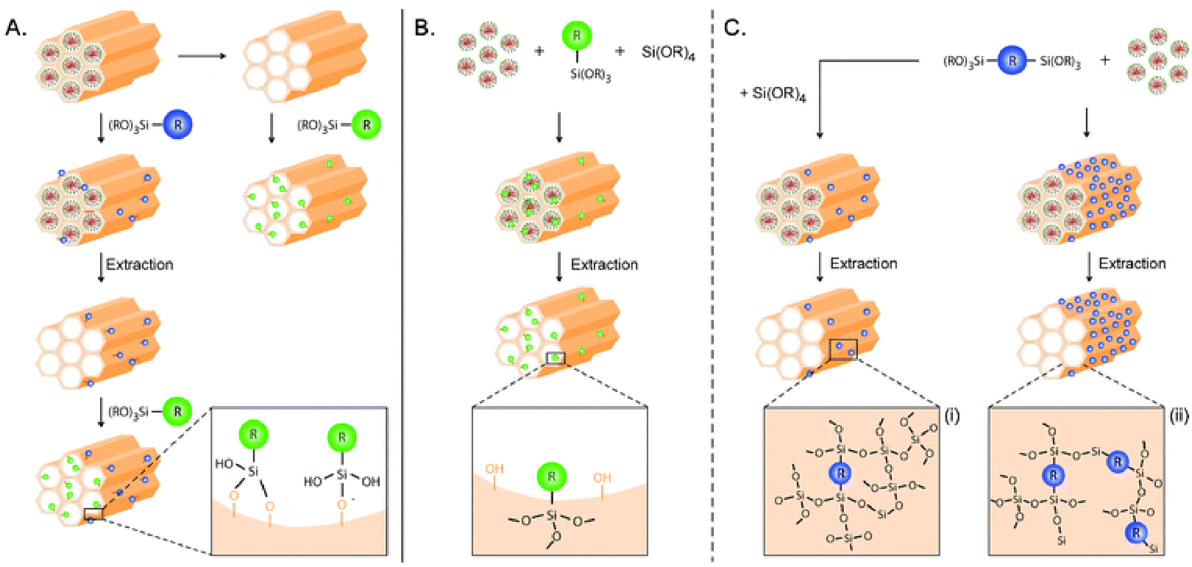

Figure 6.1 Schematic representation of surface functionalisation methods. A) Grafting, B) Co-condensation and C) Co-condensation (i) or condensation (ii) to synthesise Periodic Mesoporous Organosilicas (PMOs). Reproduced from (de Clippel *et al.,* 2013) with permission of The Royal Society of Chemistry.

6.1.2. Sugar-Capped Nanoparticles

Carbohydrates 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). All these characteristics make carbohydrates promising candidates for NP functionalisation. This type of functionalisation has been mainly studied in nanomedicine applications, particularly in cancer diagnostics and therapy.

In cancer studies, NPs have been capped with carbohydrates to serve as glycobiological probes (Lai *et al.,* 2003; Earhart *et al.,* 2008) or to target cancer cells for diagnosis or treatment due to the affinity of cancer cells towards certain carbohydrates. MSNPs capped with mannose (Gary-Bobo *et al.,* 2011) or galactose (Gary-Bobo *et al.,* 2012) targeted cancer cells and enhanced cancer cell death. Silicon NPs capped with carbohydrates have also been demonstrated to result in nontoxic NPs *in vitro* and *in vivo* that targeted cancer cells (Ahire *et al.,* 2013; Ahire *et al.,* 2015).

In diagnostics and detection, mannose-coated magnetic NPs have been demonstrated to detect, quantify and differentiate *E. coli* cells (El-Boubbou *et al.,* 2007) while mannose-capped gold NPs have been shown to bind specifically to *E. coli* (Lin *et al.,* 2002). Manea *et al.* (2008) have capped gold NPs with carbohydrates with potential use as synthetic antigens for immunostimulation (Manea *et al.,* 2008). Also, carbohydrate-capped gold NPs have been shown to enable *E. coli* O157:H7 detection (Wang & Alocilja 2015).

The use of carbohydrates as capping agents has mainly been explored with applications in medicine; diagnostics and cancer targeting with only a few reports as antimicrobials (Sanyasi *et al.,* 2016). However, there are no published reports of sugar-capped NPs used in agriculture for detection or treatment of bacterial infections. Hence, this chapter aimed to explore the possible use of this capping agent to protect the encapsulated biocide from evaporation or degradation, allowing a controlled slow-release of the antimicrobial and to evaluate the potential targeting of specific bacterial species by relying on sugar metabolism differences.

6.1.2.1. Bacterial Carbohydrate Metabolism

Carbohydrates are a major carbon and energy source for microorganisms. The difference in carbohydrate metabolism between bacterial species relies on the production of specific enzymes to degrade sugars (McKee & McKee 2016). Most bacteria are able to utilise simple sugars or monosaccharides, like glucose; whilst fewer can use complex sugars such as disaccharides (e.g. lactose or sucrose) or polysaccharides (e.g. starch). Complex sugars are composed of monosaccharides linked by glycosidic bonds, which need to be broken down by specific bacterial enzymes. The differences in carbohydrate metabolism between bacterial species could be used to identify or target specific bacterial species. For the experiments in this chapter, it was initially hypothesised that metabolic differences of bacterial species could permit targeting of sugar-capped MSNPs to a specific species able to degrade the sugar cap, causing the release of the antimicrobial and its own death.

6.1.3. Aims and Objectives

The main objective of the research summarised in this chapter was to increase the antimicrobial potency of EO-loaded MSNPs to potentially be used to treat bacterial infections in agriculture. The first aim was to evaluate the ability of three bacterial species to grow using specific sugars as sole carbon sources and to determine a potential capping sugar for the MSNPs system previously developed. The second was to cap biocide-loaded MSNPs with the selected sugar and study the effect on different bacterial species to determine if the degradation of the sugar by the targeted species could trigger the release of the biocide from the MSNPs. Finally, to assess the interaction between the loaded biocide and the capping agent which causes an improved antimicrobial effect of the system.

6.2. Materials and Methods

6.2.1. Microorganisms and Growth Conditions

P. syringae pv. *pisi, P. fluorescens* and *P. carotovorum* subsp. *carotovorum* cultures were prepared by inoculating 25 ml of LB (Luria-Bertani medium, Sigma Aldrich UK) with a single colony from previously prepared LBA (LB Agar, Sigma Aldrich, UK) plates and incubating overnight at 28°C and 220 rpm. Overnight cultures were adjusted to an OD_{600} = 0.1 and incubated for 6 hours to achieve mid-exponential growth phase of the microorganisms. Bacterial cultures were centrifuged to harvest cells and washed twice with PBS (phosphate-buffered saline, Sigma Aldrich, UK). Turbidity was adjusted to 0.5 McFarland standard (OD_{600} = 0.132) using PBS to achieve a bacterial density of ~10^8 CFU/ml. *Escherichia coli* and *Pseudomonas aeruginosa* were used to compare results with the previous three species and were cultured as described with incubation parameters of 37°C and 150 rpm.

6.2.2. Sugar Assimilation Studies

Fructose, Raffinose, Xylose, Myo-Inositol, Galactose, Mannitol (VWR Chemicals, Belgium), Sorbitol (Fisher Scientific, UK), Mannose, β-lactose and Cellobiose were evaluated as sole carbon sources for the bacterial growth of *P. syringae* pv. *pisi, P. carotovorum* subsp. *carotovorum* and *P. fluorescens.* All sugars were obtained from Sigma Aldrich, UK unless otherwise specified. M9 minimal medium was prepared in Falcon tubes using 5 ml M9 Salts [5x] (Sigma Aldrich, UK), 50 µl $MgSO_4 \cdot 7H_2O$, 2.5 µl $CaCl_2$, 500 µl of specific sugar and adjusting to a final volume of 25 ml using distilled water.

A 96-well plate was prepared by adding 100 µl of M9 media and 100 µl of bacterial suspension to each well. Control wells containing media without any sugar were used as negative control and wells without the addition of bacterial culture were used as sterility controls. The plates were incubated at 28°C and optical density was read at times = 0, 24 and 48 h. Two Plates were prepared with and without 0.05% (w/v) 2,3,5-Triphenyltetrazolium chloride (TTC; sigma Aldrich, UK) as a growth indicator. The experiment was performed in triplicate.

Bacterial growth curves using glucose or lactose as sole carbon sources were prepared for all species by adding 50µl of bacterial cultures (from Section 6.2.1, OD_{600} = 0.132) to 50 ml M9 media with 2 g/L sugar or no sugar (control). Falcon tubes were incubated at 28°C and growth was monitored using an Infinite M2000 TECAN plate reader. Furthermore, a bacterial growth curve was prepared for *P. carotovorum* subsp. *carotovorum* with two-fold concentrations of lactose ranging from 2000 mg/L to 15.625 mg/L.

6.2.3. Synthesis of Mesoporous Silica Nanoparticles (MSNPs)

MSNPs were synthesised as described in Chapter 3, Section 3.2.1 of this thesis.

6.2.4. Synthesis of Trialkoxysilane Lactose Derivative

A lactose derivative was synthesised as reported by Bernardos *et al.* (2009) with some modifications. Briefly, 5.85 ml of 3-aminopropyltriethoxysilane (APTES, 25 mmol) was added to 5.4 g of D-lactose monohydrate (Sigma Aldrich, UK) dissolved in 244.15 ml absolute ethanol (Sigma Aldrich, UK). The solution was stirred at 500 rpm and room temperature for 24h before being heated to 60°C for 30 minutes and centrifuged at 8,500 rpm for 8 minutes. The supernatant was discarded and the pellet was dried in a vacuum desiccator before being grounded to a fine powder (Bernardos *et al.,* 2009).

6.2.5. Loading and Lactose-Capping of MSNPs

MSNPs were loaded with Ajwain oil, CNAD and AIT by adding 750 µl of ajwain oil (1.5 ml for CNAD and AIT) dissolved in 5.25 ml DMSO (4.5 ml for CNAD and AIT) to 60 mg of MSNPs suspended in 21 ml PBS. The mixture was stirred in dark conditions overnight before centrifugation at 8,500 rpm and 17°C for 7 minutes. The pellet was resuspended in 60 ml distilled water. The lactose-derivative (500 mg) was dissolved in 30 ml DW and added to the MSNPs solution. The mixture was stirred for 5 hours before centrifuging and drying the pellet overnight in a vacuum desiccator. Empty MSNPs were also capped with the lactose derivative to be used as a control. This protocol was modified from the methods reported by Bernardos *et al.* (2009).

6.2.6. *In vitro* Studies of Lactose-Capped MSNPs

To obtain a final nanoparticle concentration of 2 mg/ml, 10 mg of each MSNPs treatment (Table 6.1) was added to 5 ml of bacterial culture previously prepared as described in Section 6.2.1 and tubes were incubated at 28°C and 220 rpm for 24h. The Miles and Misra method (Miles *et al.,* 1938) was performed to determine the effect of each treatment against the bacterial species by plating 20 µl of serial dilutions (10^{-4} to 10^{-7}, vortex tubes previously to homogenise samples) in triplicate onto Luria-Bertani agar (LBA) plates. Serial dilutions were prepared using PBS and calibrated pipettes. The inverted plates were incubated at 28°C for 24h. The experiment was repeated using *E. coli* and *P. aeruginosa* to compare results with the previous three species using CNAD- and AIT-loaded MSNPs. Incubation parameters for *E. coli* and *P. aeruginosa* were 37°C and 150 rpm.

Table 6.1 Treatments added to bacterial cultures to evaluate the antimicrobial effect of essential oil loaded and lactose-capped MSNPs. All treatments had a final nanoparticle concentration of 2 mg/ml. NA – Not Applicable, lac – lactose.

Treatment	Not Capped	Lactose-Capped
Control A - No MSNPs	NA	NA
Control B	Empty MSNPs	Lac-capped empty MSNPs
Control C	Empty MSNPs incubated with DMSO as per loading protocol	Lac-capped, DMSO-treated, empty MSNPs
AIT	AIT-loaded MSNPs	AIT-loaded lac-capped MSNPs
CNAD	CNAD-loaded MSNPs	CNAD-loaded MSNPs lac-capped MSNPs
Ajwain	Ajwain-loaded MSNPs	Ajwain-loaded MSNPs lac-capped MSNPs

6.2.7. Fourier Transform Infrared Spectroscopy (FTIR)

A mixture of 40 mg of lactose derivative and 500 µl essential oil (CNAD or AIT) was prepared and analysed to determine the interaction between lactose and CNAD/AIT. 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 derivative, AIT, CNAD and the combination of AIT or CNAD with the lactose derivative, were placed directly on the diamond window and the spectra ratioed to the blank diamond.

6.2.8. Release of CNAD from MSNPs and lac-MSNPs *via* Liquid Chromatography

To determine the amount of CNAD released from uncapped and lactose-capped MSNPs, 10 mg of loaded MSNPs were added to 5 ml PBS and incubated at 30°C and 200 rpm for 48 hours. A sample of 200 µl was analysed at 0, 1, 2, 3, 4, 24 and 48 hours *via* liquid chromatography (LC) using an Agilent Technologies 1120 Compact LC, equipped with a Zorbax Eclipse Plus C18 column (Agilent Technologies, UK). Samples were analysed using an isocratic ratio 80:20 (acetonitrile:H_2O), UV detector (λ = 270 nm), flow of 1 ml/min and an injection volume of 10 µl over 4 minutes.

6.2.9. Statistical Analyses

Statistical analyses were performed using Microsoft Excel and Minitab® version 18.1. To determine statistically significant differences between samples, two-factor analysis of variance (ANOVA) and t-tests were evaluated when appropriate. *P* values of 0.05 and 0.01 were used and specified for each analysis if applicable.

6.3. Results

6.3.1. Sugar Assimilation Studies

Ten sugars were evaluated as sole carbon sources for three bacterial species: *P. fluorescens, P. carotovorum* subsp. *carotovorum* and *P. syringae* pv. *pisi.* Bacterial growth was measured with the increase in turbidity in each well while bacterial metabolism was estimated using TTC (growth indicator) as a measure of cell respiration. An increased metabolic activity produced a darker purple colour while a transparent well indicated no bacterial respiration (Figure 6.2). The sugar assimilation profiles of all bacterial species tested are summarised in Table 6.2.

After 24 hours of incubation, *P. syringae* pv. *pisi* was only able to slightly grow using fructose out of all the sugars tested as sole carbon sources; nevertheless, more growth was observed after 48 and 72 hours, suggesting this bacterium takes longer to grow and adapt to a lack of sugar sources. All strains were able to grow using Fructose, Myo-inositol, Galactose, Mannitol, Sorbitol, Xylose and Mannose after 72h of incubation. However, only *P. carotovorum* subsp. *carotovorum* demonstrated metabolic activity using Raffinose, Lactose or Cellobiose. In general, *P. carotovorum* subsp. *carotovorum* exhibited more flexibility towards a wider range of sugars as sole carbon sources while *P. syringae* pv. *pisi* was the slowest and more difficult organism to grow with a restricted supply of carbon sources. Figure 6.3 illustrates the bacterial growth and respiration for each species using the ten sugars as sole carbon sources after 24, 48 and 72 hours of incubation.

Table 6.2 Sugar assimilation profiles for *P. fluorescens, P. carotovorum* subsp. *carotovorum, P. syringae* pv. *pisi.* Bacterial growth and metabolic activity (respiration) of three bacterial species after 24h of incubation. a = only after 48h of incubation, b = only after 72h of incubation.

Carbohydrate	*P. fluorescens*		*P. carotovorum* subsp. *carotovorum*		*P. syringae* pv. *pisi*	
	Growth	Respiration	Growth	Respiration	Growth	Respiration
Fructose	+	+	+	+	+	+
Raffinose	+[b]	-	+	+	+[a]	-
Xylose	+	+	+	+	+[a]	+[b]
Myo-inositol	+	+	+	+[a]	+	+[a]
Galactose	+	+	+	+	+	+[a]
Mannitol	+	+	+	+	+	+[a]
Sorbitol	+	+	+[a]	+[a]	+	+[a]
Mannose	+[a]	+	+	+	+	+[b]
β-Lactose	-	-	+	+[a]	-	-
Cellobiose	-	-	+	+	-	+[b]

The main objective of this experiment was to determine if there was a sugar that could only be metabolised by one of the species with the eventual aim of using it to cap loaded-MSNPs and trigger a controlled release of the cargo by the bacteria. Therefore, lactose was selected as a potential capping agent since it was only used as a carbon source by *P. carotovorum* subsp. *carotovorum* and not by the other two strains tested.

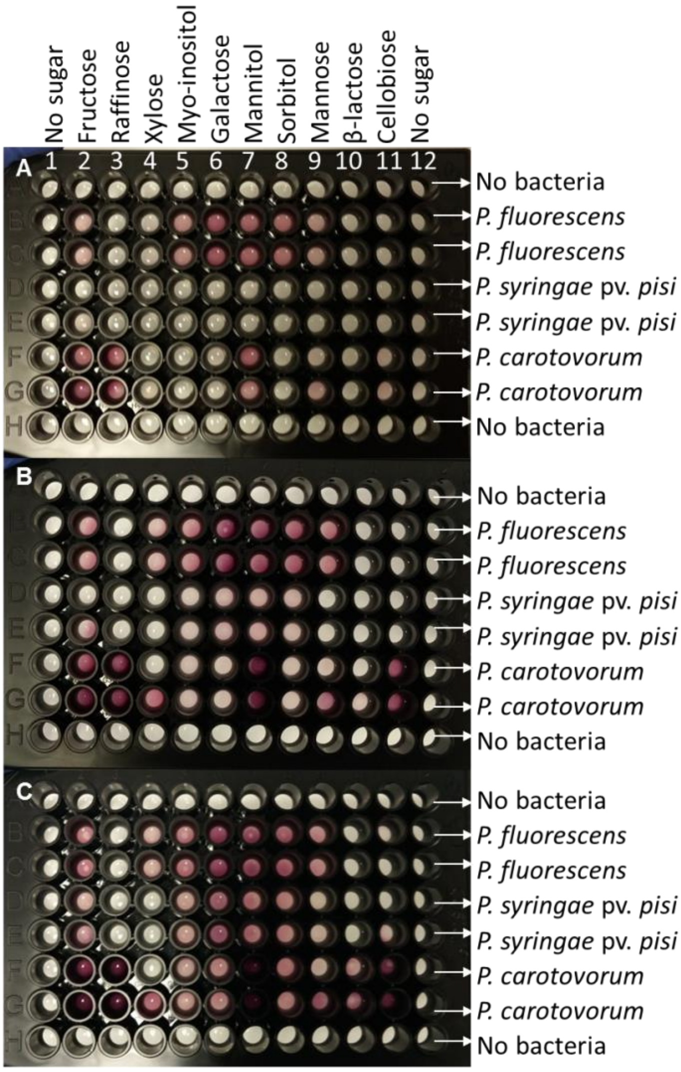

Figure 6.1. Sugar assimilation study plate results after A) 24, B) 48 and C) 72 hours of plate incubation. Each column contains a different sugar as sole carbon source (Columns 1 and 12 Controls without sugar, column 2 Fructose, 3 Raffinose, 4 Xylose, 5 Myo-inositol, 6 Galactose, 7 Mannitol, 8 Sorbitol, 9 Mannose, 10 β-Lactose, and 11 Cellobiose). Lines A and H were sterility controls containing sugars without bacterial culture, lines B and C are *P. fluorescens* , lines D and E are *P. syringae* pv. *pisi,* and lines F and G are *P. carotovorum* subsp. *carotovorum*.

Bacterial growth curves using lactose and glucose as a positive control were constructed for three bacterial species: *P. fluorescens, P. carotovorum* subsp. *carotovorum* and *P. syringae* pv. *pisi* (Figure 6.4a-c). Of the three bacterial species, only *P. carotovorum* subsp. *carotovorum* was able to utilise lactose as a sole carbon source, as expected. After 48h of incubation, *P. carotovorum* subsp. *carotovorum* was able to grow with ≥250 mg/L and after 63h growth was observed at ≥125 mg/L with minimal growth observed even with amounts as low as 62.5 mg/L (Figure 6.4d).

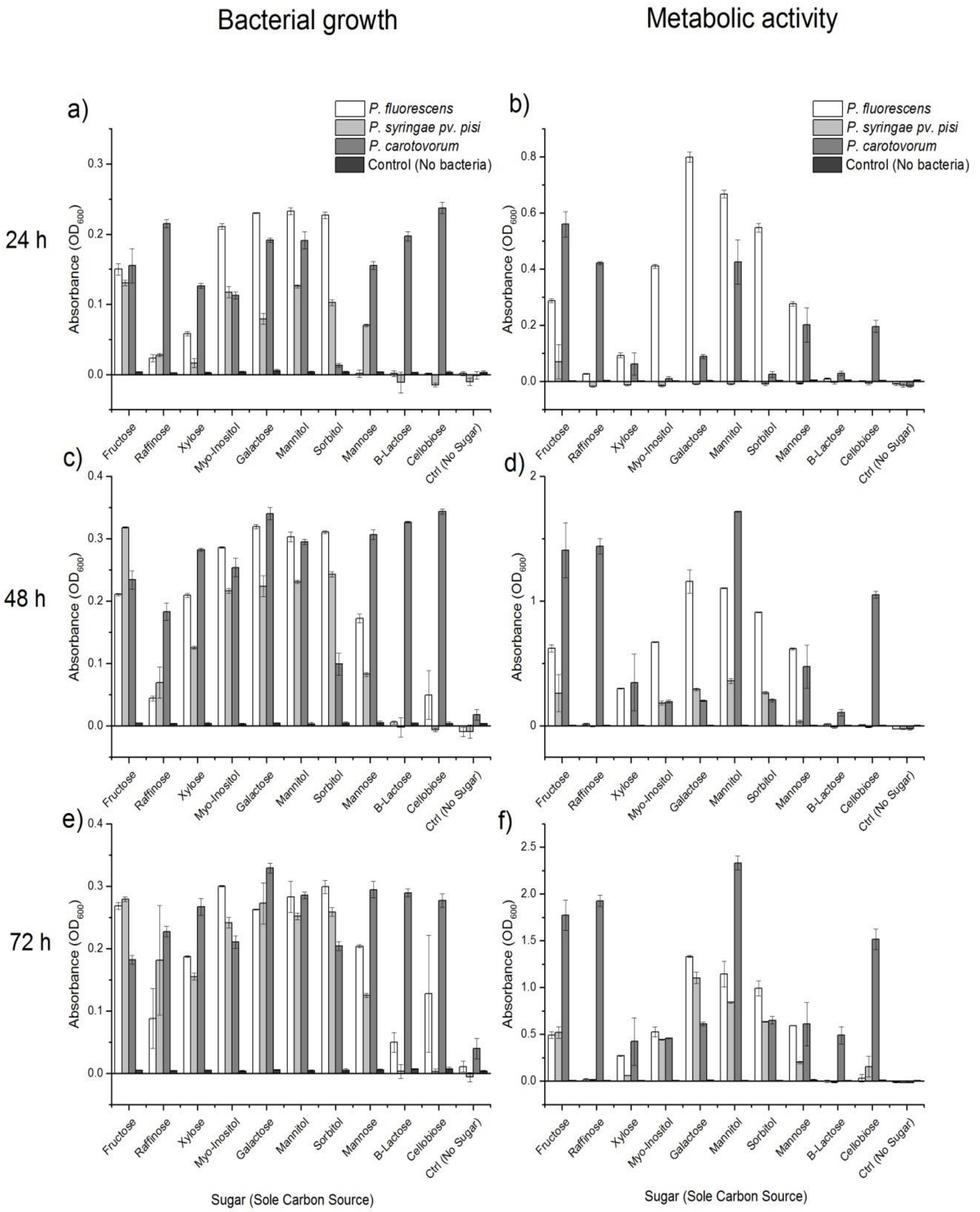

Figure 6.3 Sugar Assimilation Study. Results at 24 hours (a) and (b); 48 hours (c) and (d); and 72 hours (e) and (f) of Bacterial growth (a), (c) and (e) and metabolic activity (b), (d) and (f) of three bacterial species, *P. fluorescens, P. carotovorum* subsp. *carotovorum,* and *P. syringae* pv. *pisi,* using 10 sugars as sole carbon sources. Control samples without bacteria were used for each sugar and control bacteria were grown without the presence of any sugars. Data shows mean ± standard deviation of triplicates.

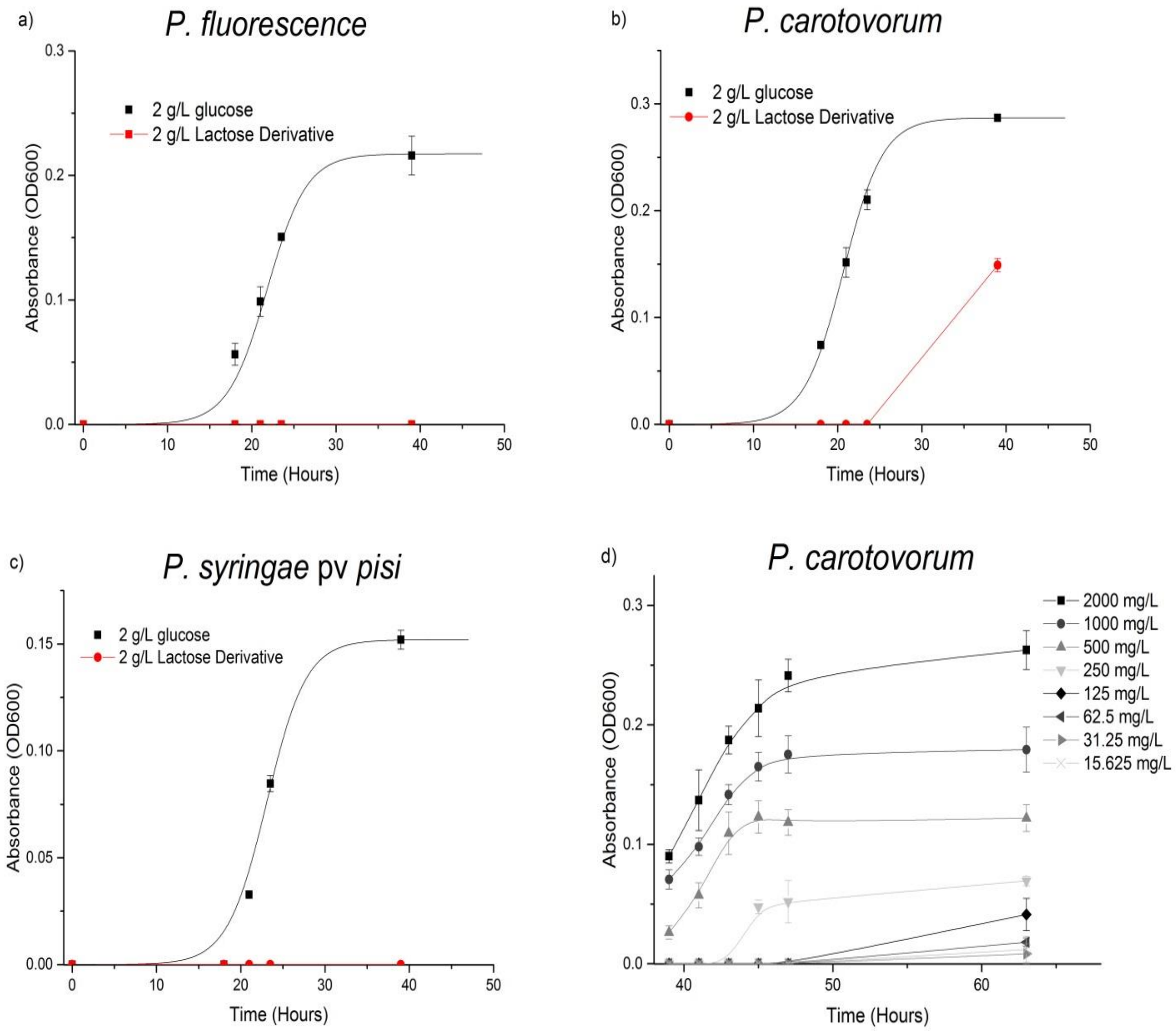

Figure 6.4 Bacterial growth curves of a) *P. fluorescens,* b) *P. carotovorum* subsp. *carotovorum* and c) *P. syringae* pv. *pisi* using lactose and glucose as sole carbon sources. Glucose was used as a positive control. d) Bacterial growth curve of *P. carotovorum* subsp. *carotovorum* using a range of concentrations of lactose as sole carbon source from 2000 mg/L to 15.625 mg/L. Data shows mean ± standard deviation, n = 3.

6.3.2. *In vitro* Studies of Lactose-Capped MSNPs

To determine if the inhibitory effect and the stability of EOs could be enhanced, three of the most potent antimicrobials were encapsulated into MSNPs; cinnamaldehyde (CNAD, main component of cinnamon EO), allyl isothiocyanate (AIT, main component of mustard EO), and Ajwain EO. 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 culture 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 6.5a. Controls with empty nanoparticles appeared to have a higher bacterial growth compared to the untreated controls. A bacterial growth increase (CFU/ml) of 25%, 45% and 222% of *P. fluorescens, P. carotovorum* subsp. *carotovorum* and *P. syringae* pv. *pisi*, respectively, was observed when compared to the untreated controls.

Uncapped AIT-loaded MSNPs decreased bacterial viability (CFU/ml), demonstrating the effect of AIT as a potent biocide. However, uncapped CNAD- and Ajwain MSNPs did not demonstrate any significant decrease in viability compared to the controls. Furthermore, lactose-capped AIT-MSNPs and lactose-capped Ajwain-MSNPs resulted in a bacterial increase, particularly of *P. carotovorum* subsp. *carotovorum* possibly due to the utilisation of lactose as a carbon source by the microorganism. Interestingly, lactose-capped CNAD-loaded MSNPs resulted in the elimination of >99.8%, >99.9% and >95% of *P. fluorescens, P. syringae* pv. *pisi* and *P. carotovorum* subsp. *carotovorum*, respectively, compared to the results from non-capped CNAD-MSNPs. *P. carotovorum* subsp. *carotovorum* demonstrated some growth possibly due to the presence of lactose.

To compare the efficacy of lactose-capped CNAD-loaded MSNPs, the NPs were tested against two additional bacterial species. Figure 6.5b illustrates the effects of lactose-capped and uncapped AIT- and CNAD-loaded MSNPs on *Escherichia coli* and *Pseudomonas aeruginosa*. The results show that lactose-capped CNAD-loaded MSNPs reduced *E. coli* growth by >98% (CFU/ml) and *P. aeruginosa* (CFU/ml) by >89% compared to the uncapped CNAD-MSNPs treated cultures.

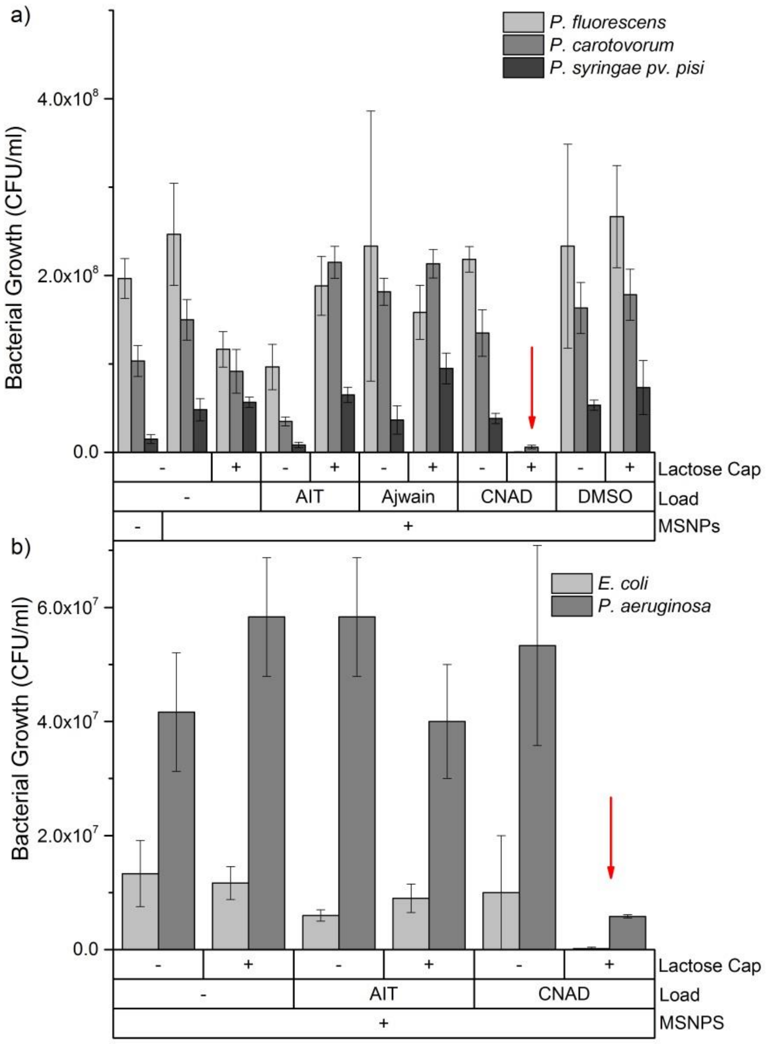

Figure 6.5 Effect of Essential-oil loaded MSNPs *in vitro.* Two mg/ml of Essential oil-loaded MSNPs were added to bacterial cultures to evaluate their antimicrobial effect. Lactose-capped and uncapped MSNPs were used to determine the efficacy of a sugar coating. CNAD-loaded lactose-capped MSNPs (indicated by the red arrow) 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*. b) Treatments against *E. coli* and *P. aeruginosa* to compare efficacy of CNAD-loaded lactose-capped MSNPs. Data shows mean ± standard deviation; n = 3.

6.3.3. Fourier Transform Infrared Spectroscopy (FTIR)

The treatment with lactose-capped CNAD-loaded MSNPs demonstrated significant potential as an antimicrobial system. To understand why this increased potency was observed, FT-IR analyses of CNAD or AIT mixed with the lactose derivative were carried out. The FT-IR spectrum of the combination of CNAD with the lactose derivative used for capping (containing APTES) showed a peak at 2362 cm^{-1} that was not present in the FT-IR spectra of the individual components (CNAD or the lactose derivative) (Figure 6.6A). Interestingly, the FT-IR spectrum of lactose (without any modifications) also showed this peak at 2360 cm^{-1} (Figure 6.6B). This suggested that in the presence of CNAD, the APTES molecules originally attached to lactose were released from the lactose enabling APTES binding onto the CNAD. When this would happen in the presence of MSNPs, the CNAD molecules are probably bound onto the surface of the nanoparticles through APTES, increasing its antimicrobial potency. This was only observed for CNAD, and not for AIT or Ajwain EO, since the binding of CNAD to APTES probably occurred through the aldehyde group naturally present on CNAD and absent on the other two essential oils.

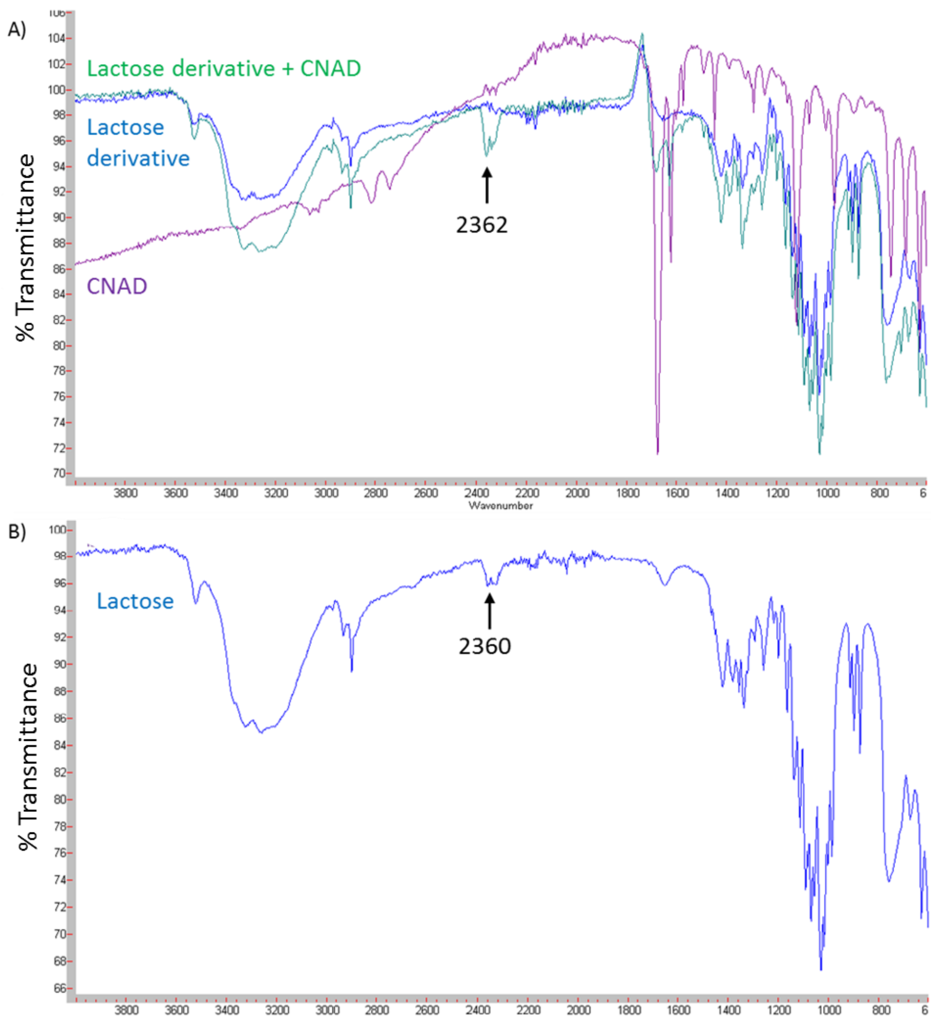

Figure 6.6 FT-IR analysis of lactose, lactose derivative and cinnamaldehyde. A) FT-IR of CNAD, lactose derivative and their combination. A peak can be observed at wavenumber 2362 cm^{-1} on the IR spectrum of the combination, which is not present in either of the individual components. B) FT-IR of lactose containing the observed peak at 2360 cm^{-1}.

6.3.4. Release of CNAD from MSNPs and lac-MSNPs *via* Liquid Chromatography

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 24 hours of incubation; with 49% of that amount being released only after one hour of incubation (Figure 6.7). In contrast, no CNAD was observed from lactose-MSNPs, even after 48 hours of incubation (Figure 6.8); 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. The antimicrobial effect could be achieved by a slow-release or, if the EO is not released due to immobilisation onto the NPs, by proximity to target organisms.

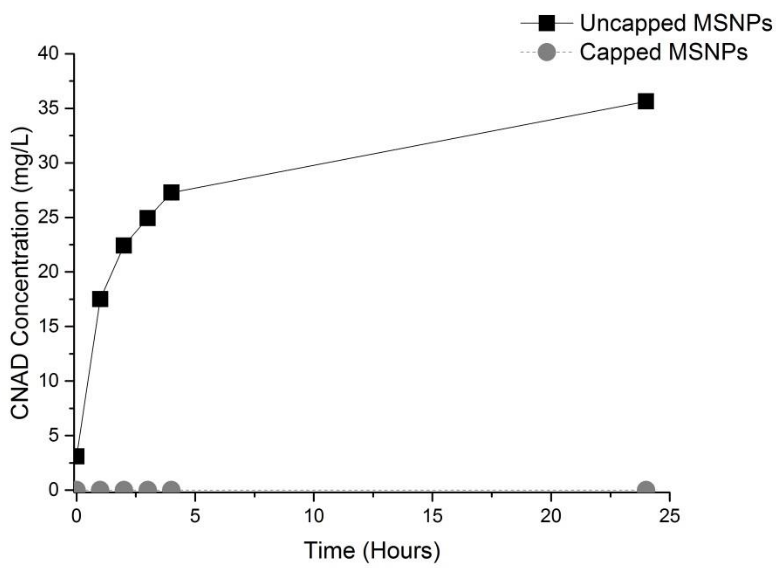

Figure 6.7 CNAD release from uncapped and lactose-capped MSNPs. Liquid chromatography results showing the concentration of CNAD released into solution from uncapped and capped MSNPs after 24 hours. CNAD was immobilised onto the surface of MSNPs after capping protocol, preventing the loss or volatilisation of the essential oil from the nanoparticles. Data shows mean ± standard deviation (SD); n = 3 (all SD are <0.5 mg/L).

6.4. Discussion

The main objective of this chapter was to achieve an increased antimicrobial potency of EO-loaded MSNPs that could lead to a significant protection of plants from bacterial infections. Capping of the pores on MSNPs surfaces can prevent the rapid unloading, leakage or volatilisation of the cargo and allow for a more potent and long-lasting antimicrobial effect. To evaluate the use of sugars as potential capping agents, the ability of three microorganisms to utilise ten sugars as a sole carbon source was investigated.

All tested bacterial species were able to grow using simple sugars (monosaccharides: fructose, galactose, xylose and mannose) or simple sugar alcohols (myo-inositol, mannitol and sorbitol) after 72 hours of incubation. However, complex sugars; raffinose, lactose and cellobiose, were only metabolised by *P. carotovorum* subsp. *carotovorum.* Raffinose is a trisaccharide composed of galactose, glucose and fructose, which needs to be broken down by α-galactosidase (Linden 1982); cellobiose is a disaccharide made of two glucose molecules that can be broken down by cellobiase (β-glucosidase) (Khairudin & Mazlan 2013), and lactose is a disaccharide formed by glucose and galactose that is broken down by the enzyme lactase (beta-galactosidase) (Campbell *et al.,* 2016). The utilisation of these complex sugars by *P. carotovorum* subsp. *carotovorum,* previously reported (Garrity *et al.,* 2005), could provide a differentiating characteristic between this microorganism and the remaining two being tested.

Lactose was selected as a potential MSNP-capping agent to evaluate if its selective use by *P. carotovorum* subsp. *carotovorum* would provide an enhanced and targeted antimicrobial effect. A lactose derivative (lactose attached to organosilane APTES) was used to cap MSNPs, and the effect of lactose-capped and not-capped MSNPs (either empty or EO-loaded) on *P. syringae* pv. *pisi, P. carotovorum* subsp. *carotovorum,* and *P. fluorescens* was evaluated.

First, empty NPs were used as a control. Interestingly, the addition of bare and empty NPs resulted in a greater bacterial viability. This might have been due to the microorganisms utilising the silica or its by-products as a nutrient source or to the MSNPs altering the environment and making other nutrients more available. Other studies have shown that bacteria and fungi can solubilise insoluble silicates (Duff *et al.,* 1963) and that silicon compounds can increase bacterial growth (Price 1932; Umamaheswari *et al.,* 2016) or fungal growth (Wainwright *et al.,* 1997). Wainwright *et al.* (1997) demonstrated that silicon compounds such as silicic acid can increase fungal growth, possibly due to their ability to adsorb carbon and nitrogen from the atmosphere, which then acted as nutrients; or to the use of silicon compounds as energy sources by the microorganisms, enabling them to fix CO_2 from the atmosphere (Umamaheswari *et al.,* 2016; Wainwright *et al.,* 1997). It has also been suggested that silicon compounds adsorb ammonia and CO_2 from the atmosphere and allow bacteria to fix CO_2 by using the energy derived from ammonium oxidation (Bigger & Nelson 1943). In agreement with these findings, the results presented here demonstrated that the addition of empty MSNPs increased bacterial growth of *P. fluorescens, P. carotovorum* subsp. *carotovorum* and *P. syringae* pv. *pisi* by 25%, 45% and 222%, respectively, when compared to the untreated controls.

Secondly, the effect of uncapped Ajwain-, CNAD- and AIT-loaded MSNPs on bacterial growth was evaluated. Only uncapped AIT-loaded MSNPs decreased bacterial viability (CFU/ml) compared to the untreated control (49.15%, 33.87% and 56.67% for *P. fluorescens, P. carotovorum* subsp. *carotovorum* and *P. syringae* pv. *pisi,* respectively); indicating the strong antimicrobial effect of AIT. Moreover, CNAD and Ajwain may not have escaped the encapsulation as readily as AIT due to their lower volatility, which could explain why these treatments did not show a significant decrease in bacterial viability. The effect of AIT- and CNAD-loaded MSNPs against *E. coli* and *P. aeruginosa* has been previously reported by our group (Chan *et al.,* 2017).

Thirdly, the effect of lactose-capped, EO-loaded MSNPs was investigated with the hypothesis that only lactose-degrading bacteria would remove the sugar capping of the MSNPs, releasing the loaded biocide. Bernardos *et al.* (2009) have reported the use of a lactose derivative grafted onto the pores of a mesoporous silica support as a biocontrolled gate whose hydrolysis was triggered by the enzyme β-galactosidase, allowing the release of a loaded dye (Bernardos *et al.,* 2009). In contrast, silver nanoparticles capped with a semi-synthetic polysaccharide-based polymer have been demonstrated to inhibit bacterial growth and biofilm formation of *Bacillus subtilis*, *E. coli* and *Salmonella thyphimurium* but without providing any microbial differentiation or microbial targeting (Sanyasi *et al.,* 2016). In agreement with this, the results reported in this chapter were not as initially hypothesised and the addition of lactose did not result in targeted delivery of the biocide, possibly due to the lack of internalisation of the particles by the bacteria to degrade the sugar (Chan *et al.,* 2017). Instead, it increased bacterial growth, particularly of *P. carotovorum* subsp. *carotovorum,* probably because of the beneficial effect of the available sugar on bacterial growth being greater than the biocidal effect of the EO released from the MSNPs.

To counteract this, cellobiose could be used in the future as a capping agent since it is degraded by the extracellular enzyme β-glucosidase (An *et al.,* 2012), avoiding the need for the internalisation of the particles to achieve the desired targeting. Other possible alternative capping agents that could be evaluated in the future are chitosan and homoserine. The utilisation of homoserine is characteristic of *P. syringae* pv. *pisi* (Garrity *et al.,* 2005) and could provide the possibility of targeting the biocide delivery towards this phytopathogen. Additionally, chitosan could be evaluated as a capping agent that would not only prevent the rapid volatilisation of the biocide, but it could also enhance the plant's immune response and its protection against microbial infections (El Hadrami *et al.,* 2010; Thakur & Sohal 2013).

Despite the lack of targeting, an interesting result was observed with CNAD-loaded MSNPs, capped with lactose, which inhibited the growth of five different bacterial species. CNAD-lactose-

MSNPs were shown to be the best system to reduce bacterial growth, eliminating up to >99.99% of bacteria.

FTIR 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 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 6.6A) 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 6.6B). 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 6.8). The resulting silanised (covered with organofunctional alkoxysilane molecules, $RSi(OMe)_3$) 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 grafting of CNAD onto MSNPs surfaces prevented the volatilisation of the EO during at least 48 hours. Ruiz-Rico *et al* (2017) have similarly demonstrated that carvacrol, eugenol, thymol and vanillin can be grafted onto silica supports to enhance their antimicrobial activity through the incorporation of an aldehyde group or its natural presence, as it is the case of vanillin (Ruiz-Rico *et al.,* 2017). This result is consistent with the findings of this study 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.

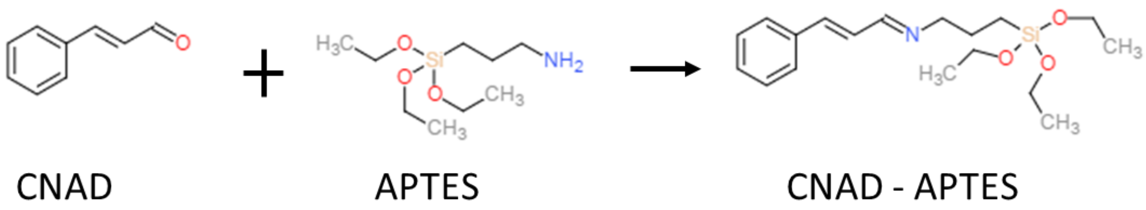

Figure 6.8 CNAD-APTES formation. The APTES originally attached to lactose is released and attached to CNAD through its aldehyde group. The resulting silanised CNAD-APTES molecule can be immobilised onto the surface of MSNPs.

6.5. Conclusions

In conclusion, the research detailed in this chapter aimed to improve the antimicrobial potency of EO-loaded MSNPs and investigated the application of sugar-capped MSNPs loaded with EOs as antimicrobials against a series of bacterial species to evaluate the potential use of this system to treat and control bacterial infections. Cinnamaldehyde was 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 confirms that MSNPs hold great promise as a means of exploiting the antimicrobial potential of volatile compounds previously overlooked because of the limitation of their physical characteristics. Consequently, the experiments detailed in the next chapter focused on the effect of immobilised-CNAD on MSNPs against *P. syringae* pv. *pisi* infection *in planta*.

Chapter 7
In planta effect of an alginate seed treatment containing immobilised CNAD-MSNPs

7.1. Introduction

Mesoporous silica nanoparticles (MSNPs) can encapsulate and protect volatile biocides against degradation or volatility while improving their stability and miscibility in aqueous solutions. Consequently, MSNPs are promising delivery vehicles for volatile antimicrobials. The research reported in this chapter focuses on the evaluation of an alginate seed treatment (developed in Chapter 4) with the addition of cinnamaldehyde (CNAD)-loaded MSNPs (Chapter 6), to protect peas against the bacterial blight causing agent *Pseudomonas syringae* pv. *pisi.* This chapter is an adjunct to Chapter 5; here CNAD is trialled in place of Ajwain which did not protect plants to the expected extent. Ajwain oil had been selected due to its strong antimicrobial activity (Chapter 2) and since it is relatively understudied compared to other EOs. However, the treatment resulted in an observable, but not statistically significant, protection of plants. Therefore, the present chapter aims to re-analyse the system with an alternative biocide with the aim of achieving a statistically significant difference between treated and untreated peas. Results from Chapter 6 demonstrated that CNAD-loaded MSNPs were able to eliminate up to 99.99% of bacterial growth of five different bacterial species and that the MSNP-encapsulation of CNAD greatly increased its antimicrobial activity *in vitro* by 10-fold compared to free cinnamon essential oil. Hence, the experiments presented in this chapter addressed the effectiveness of this treatment *in planta* to control and prevent bacterial infections as well as its potential application in plant agriculture.

7.1.1. Cinnamaldehyde

CNAD is an aromatic aldehyde that occurs naturally, mostly as trans-cinnamaldehyde (Figure 7.1). This compound is widely used in the food and fragrance industries (Environmental Protection Agency 2000a) as well as in cosmetics, medical and hygiene products (National Toxicology Program 2004). It has a GRAS status and has been shown to be not genotoxic or carcinogenic *in vivo* (Kiwamoto *et al.,* 2016; National Toxicology Program 2004). Also, due to its poor miscibility in water (1420 mg/L) and its rapid degradation in soil, CNAD is not considered to present any hazard to non-target organisms and its environmental and ecological effects are considered of low exposure and low toxicity (Environmental Protection Agency 2000a; Valvani *et al.,* 1981).

CNAD can be produced synthetically but it is more economically viable, and therefore more common, to obtain it from the steam distillation of cinnamon bark oil which comprises 65-90% of this essential oil (Vangalapati *et al.,* 2012; Environmental Protection Agency 2000a).

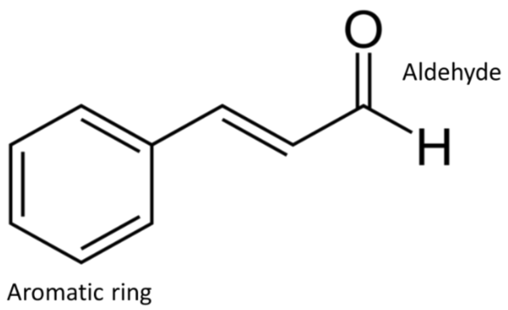

Figure 7.1 Trans-Cinnamaldehyde structure showing an aromatic ring and the aldehyde group.

Besides the commercial uses, cinnamon oil and its main constituent, CNAD, have potential applications in food technology, medicine and agriculture. In food technology, CNAD (1-3%) can be applied onto packaging and films to inactivate foodborne pathogens such as *Salmonella enterica, Listeria monocytogenes* or *E. coli* O157:H7 (Ravishankar *et al.,* 2009; Ouattara *et al.,* 2000). In medicine, it can be used as an anti-diabetic due to its hypoglycemic and hypolipidemic effects (Subash Babu *et al.,* 2007; Kim *et al.,* 2006), as an anti-inflammatory (Tung *et al.,* 2008; Youn *et al.,* 2008), coagulant (Hossein *et al.,* 2013), anti-oxidant (Jayaprakasha *et al.,* 2003), anti-cancer agent (Kwon *et al.,* 1998; Koh *et al.,* 1998; Kwon *et al.,* 2009), anti-mutagenic (Shaughnessy *et al.,* 2001), and in oral hygiene (Gupta *et al.,* 2011). Finally, CNAD and cinnamon oil have potential applications in agriculture as it has antimicrobial, antifungal, insecticidal and nematicidal effects (Rao & Gan 2014). Cinnamon and cinnamon oil are listed as active ingredients eligible for minimum risk pesticide products, along with several other EOs (Environmental Protection Agency 2015). Cinnamaldehyde is commercially available as a broad-spectrum contact fungicide/insecticide/algaecide/miticide. It is sold as wettable powder formulations under the brand names Vertigo Wettable Powder Fungicide (Monterey) and Cinnacure^{TM} A3005 (Pro-guard). However, it is mainly used to control fungal diseases such as powdery mildew, *Pythium*, pitch canker disease, and dollar spot; as well as pests like aphids, mites, or whiteflies (Environmental Protection Agency 2000b) but not to treat or prevent any bacterial diseases.

7.1.1.1. Mechanism of Action – CNAD as an Antibacterial

The mechanism of action of most essential oils is not yet fully understood. Nevertheless, previous studies have revealed a variety of possible mechanisms of action of cinnamaldehyde and other phenylpropenes against bacteria.

Phenylpropenes are organic molecules that consist of a six-carbon aromatic phenol group (benzene ring) attached to a three-carbon propene tail (allyl group) from cinnamic acid (Nazzaro *et al.,* 2013). These molecules are found in many plants and some examples include eugenol, vanillin and cinnamaldehyde. The free hydroxyl groups present in these molecules provide part of their antimicrobial activity (Laekeman *et al.,* 1990). In the case of vanillin, the aldehyde moiety provides the antimicrobial functionality of the compound (Fitzgerald *et al.,* 2003) and this might be the case for CNAD as well, since it does not have a free hydroxyl group (Holley & Patel 2005; Wendakoon & Sakaguchi 1995). As discussed in Chapter 2, the activity of phenylpropenes (as that of EOs in general) depends on the microbial species and the test conditions.

CNAD has been demonstrated to have at least three different mechanisms of action against bacteria. First, low concentrations can inhibit some enzymes and other less important cell functions. CNAD has been proven to inhibit cell separation in *Bacillus cereus* by binding to FtsZ (prokaryotic homolog of tubulin that assembles into a Z-ring at the site of cell division) and disturbing the Z-ring formation and assembly (Domadia *et al.,* 2007); causing CNAD treated cells to appear as elongated structures with incomplete septa (Kwon *et al.,* 2003). Exposure of *E. coli* BL21 cells to CNAD caused the production of different metabolites that could lead to cell stress, such as alkanes, alcohol, acids, esters, dimethyl-sulfoxide; during the mid-logarithmic phase (Hossain *et al.,* 2013).

Secondly, high concentrations of this EO act as ATPase inhibitors. Both cinnamon oil and CNAD have been demonstrated to decrease the intracellular ATP concentration of *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) (Nowotarska *et al.,* 2017). Also, treating *E. coli* and *L. monocytogenes* with EOs such as CNAD inhibited ATP formation and disrupted the cell membrane suggesting CNAD, as well as eugenol or carvacrol, might inhibit the ATPase activity of cells (Gill & Holley 2004). ATPase inhibition may significantly reduce bacterial growth at sub-lethal CNAD concentrations (Gill & Holley 2006).

Thirdly, lethal concentrations of CNAD disrupt the cell membrane. Damage and morphological changes of the cell membrane have been observed after CNAD treatment of bacterial cells. Monolayer model membrane studies have shown that CNAD could cause an increased membrane fluidity by incorporating into the membrane and forming aggregates that reduce the packing effectiveness of the lipids (Nowotarska *et al.,* 2017). Also, *Salmonella typhimurium* and *Pseudomonas* spp. cells treated with CNAD and limonene showed membrane modifications, suggesting these compounds can enter the cell envelope and alter it (Di Pasqua *et al.,* 2006). However, other studies report that CNAD can inhibit the growth of *E. coli* and *S. typhimirium* without disintegrating the outer membrane or depleting intracellular ATP (Helander *et al.,* 1998). The effect of CNAD on the cell membrane might vary depending on the bacterial strain being treated since there might be differences in the ability of the antimicrobial to interact with the outer surface of cells and access the membrane (Nazzaro *et al.,* 2013).

7.1.2. Pea Bacterial Blight – *P. syringae* pv. *pisi*

Pea bacterial blight pathogen *P. syringae* pv. *pisi* has been used for all the experiments detailed in this thesis as a proof of concept to evaluate the effect of EO-loaded MSNPs on bacterial phytopathogens. The work in this chapter focuses on this bacterial disease. For more detailed information about this microorganism and disease refer to Chapter 2, Section 2.1.1 and Chapter 5, Section 5.1.2.

7.1.3. Aims and Objectives

The objective of the research in this chapter was to evaluate the effect of an alginate seed treatment containing CNAD-loaded MSNPs *in planta* against *P. syringae* pv. *pisi.* The overall aim was to evaluate the feasibility of this system as a potential seed treatment to prevent and control the incidence of microbial infections on plants and agriculture.

7.2. Materials and Methods

7.2.1. Loading (immobilisation) of CNAD onto MSNPs

The immobilisation of CNAD onto mesoporous silica was achieved using a modified version of a previously described protocol (Ruiz-Rico *et al.,* 2017). CNAD (2 ml) and APTES (2.3 ml) were dissolved in 20 ml dichloromethane (DCM, Sigma Aldrich, UK) and stirred at 350 rpm for one hour. The mixture was added to 40 ml acetonitrile (Sigma Aldrich, UK) with 1 g MNSPs (previously characterised (Huang *et al.,* 2014)) and stirred for 5.5 hours at room temperature. The solution was centrifuged and washed with acetonitrile and water. The resulting loaded NPs were dried at room temperature under vacuum for 36 hours and collected.

7.2.2. Microorganisms and Growth Conditions

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 Luria-Bertani Agar (LBA; Sigma Aldrich, UK) plates and incubating overnight at 28°C before inoculating 25 ml of Luria-Bertani Broth (LB; Sigma Aldrich, UK) with one colony and incubating at 28°C and 220 rpm overnight. Overnight cultures were used to inoculate 25 ml LB 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 the desired optical density for each test.

7.2.3. LD_{50} Test

To determine the bacterial concentration needed to cause pea bacterial blight symptoms on 50% of the inoculated seeds, an LD_{50} test was performed. Seeds were immersed in 2-fold dilutions of *P.*

syringae pv. *pisi* bacterial cultures (OD_{600} = 0.4, 0.2, 0.1, 0.05, 0.025, 0.0125) for 5 minutes and allowed to dry overnight before sowing in Murashige and Skoog (MS) media. Seeds without any bacterial inoculation and immersed in sterile water were used as negative controls. Germination, shoot emergence, secondary roots, appearance of bacterial blight signs and survival rate were evaluated for each treatment three weeks after infection.

7.2.4. Seed Coating

Pea seeds ("Kelvedon Wonder" cultivar) were sterilised as previously described in Chapter 4 (Section 4.2.2). Sterile, dry seeds were coated as described in Chapter 4 (Section 4.2.2) using 1% (w/v) sodium alginate (Sigma Aldrich, UK) and 100 mM $CaCl_2 \cdot 2H_2O$ solution (≥99.0%, Sigma Aldrich, UK). A preliminary test with 15 seeds per treatment was performed using the treatments displayed in Table 7.1. With the aim of obtaining statistically significant differences between treatments, a second test was carried out using 78 seeds for each treatment in three separate experimental batches (Table 7.2). For test seeds, 2 mg/ml CNAD-MSNPs were dissolved in the sodium alginate before the coating protocol. After coating, seeds were placed in a sterile petri dish and allowed to dry overnight.

Table 7.1 Seed treatments used for the preliminary study to evaluate the protective effect of three types of CNAD-MSNPs on peas against *P. syringae* pv. *pisi.* MSNPs – Mesoporous silica nanoparticles, CNAD – cinnamaldehyde, lac – lactose, APTES - 3-aminopropyltriethoxysilane.

Treatment	Coating – Alginate (%), $CaCl_2$ (M)	Inoculum – Number of seeds	MSNPs	Load
Control	None	15 *Psp*, 15 None	None	None
Coat Control	1%, 0.1 M	15 *Psp*, 15 None	None	None
MSNP-CNAD Test	1%, 0.1 M	15 *Psp*, 15 None	2 mg/ml	CNAD
MSNP-CNAD-lac Test	1%, 0.1 M	15 *Psp*, 15 None	2 mg/ml	CNAD-lactose
MSNP-CNAD-APTES Test	1%, 0.1 M	15 *Psp*, 15 None	2 mg/ml	CNAD-APTES

Table 7.2 Seed treatments used for the final experiments to evaluate the protective effect of three types of CNAD-MSNPs on peas against *P. syringae* pv. *pisi.* MSNPs – Mesoporous silica nanoparticles, CNAD – cinnamaldehyde, lac – lactose, APTES - 3-aminopropyltriethoxysilane.

Treatment	Coating – Alginate (%), $CaCl_2$ (M)	Inoculum – Number of seeds	MSNPs	Load
Control	None	10 *Psp*, 10 None	None	None
Coat Control	1%, 0.1 M	78 *Psp*	None	None
MSNP-CNAD-APTES Test	1%, 0.1 M	78 *Psp*	2 mg/ml	CNAD-APTES

7.2.5. Seed Inoculation

Alginate-coated seeds were immersed in 50 ml *P. syringae* pv. *pisi* inoculum (OD_{600} = 0.025) for 5 minutes, transferred to sterile Petri dishes and left to dry. For control purposes during the final experiments, ten positive controls (without coating) were also inoculated; and ten negative controls (without coating) were immersed in sterile water. Dry seeds were stored for 5 days before sowing in MS media. The experiment was performed in triplicate with 26 seeds per replicate for each treatment.

7.2.6. Seed Incubation and Growth

MS media was prepared by dissolving 4.4 g of MS basal salt mixture (Sigma Aldrich, UK), 10 g of sucrose (Sigma Aldrich, UK) and 2.5 g of Phytagel (Sigma Aldrich, UK) in 1 L of distilled water. Seeds were individually placed in sterile culture tubes containing 25 ml of MS media and incubated with a photoperiod of 16h at 21/20°C, under fluorescent light (80 µmol photons $m^{-2}s^{-1}$). Germination, shoot emergence, secondary roots and appearance of bacterial blight signs were monitored daily for 20 days.

After 20 days of incubation, symptomless plants were transferred to soil and incubated for a further four weeks. A mixture of Pot and Bedding Compost (Levington Advance, UK) and Vermiculite (Sinclair Pro, UK) with 0.28 g/L of Imidasect (Sinclair Pro, UK) to control sciarid fly, was used to grow plants. Plants were watered three times a week and incubated under the same initial conditions.

7.2.7. Evaluation of the long-term effects of MSNPs-alginate coating on peas against *P. syringae* pv. *pisi*

Fifty days after the initial sowing, the growth and development of the remaining symptomless plants was analysed (for the preliminary test, only survival and signs of infection were evaluated). All plants (15 alginate treated controls and 22 alginate-MSNPs treated) were removed from the soil and any loose soil was washed off. Plants were blotted gently to remove any free surface moisture and weighed immediately to record wet mass. Numbers of leaves, flowers, pods, and nodes, as well as height and root length were measured. To evaluate chlorophyll content, two circular sections of leaves (5 mm diameter, ~0.003 g) were cut from all plants from representative leaves. Samples were frozen with liquid nitrogen and kept at -80°C until analysis. Cold methanol (1ml) was added and plant material was homogenised using a MM300 Tissuelyser (Qiagen Retsch, Germany) at 30 Hz for 1.5 minutes. Samples were centrifuged at 10,000 rpm and 4°C for 10 minutes and supernatants were collected and analysed using a spectrophotometer (SmartSpec™ 3000, Bio-Rad) at 665 and 652 nm, subtracting background readings at 750nm. The following equations (Porra *et al.,* 1989) were used to determine the content of chlorophyll a, chlorophyll b and total chlorophylls:

In nmol/ml:

$$Chl\ a = 18.22A^{665} - 9.55A^{652}$$

$$Chl\ b = 33.78A^{652} - 14.96A^{665}$$

$$Chl\ a + b = 24.23A^{652} + 3.26A^{665}$$

In µg/ml:

$$Chl\ a = 16.29A^{665} - 8.54A^{652}$$

$$Chl\ b = 30.66A^{652} - 13.58A^{665}$$

$$Chl\ a + b = 22.12A^{652} + 2.71A^{665}$$

All plants were dried in an oven at 40°C for 14 hours and then at 80°C for 2.5 hours. Plants that were not completely dried after this process were left at 40°C overnight. Total dry mass, root mass and shoot mass were recorded for each sample.

7.2.8. Statistical Analyses

Statistical analyses were performed using Microsoft Excel and Minitab® version 18.1. To determine statistical significance of differences between samples, two-factor analysis of variance (ANOVA) and student t-tests were undertaken. These were followed by Tukey's HSD (honest significant difference) test or a Chi-squared test when appropriate for quantitative or categorical data, respectively. *P* values of 0.05 and 0.01 were used and specified for each analysis.

7.3. Results

7.3.1. LD_{50} Test

An LD_{50} test was performed to determine the bacterial concentration required to achieve 50% survival of pea seedlings infected with *P. syringae* pv. *pisi* (OD_{600} = 0.4, 0.2, 0.1, 0.05, 0.025, and 0.0125). Germination, shoot emergence and appearance of secondary roots were mainly affected by the higher concentrations of bacterial inoculum. The highest concentration of bacteria only allowed 73% of seeds to germinate, 60% of shoot and 43% of secondary roots emergence, compared to 93%, 93% and 73% of the control seeds, respectively.

The highest bacterial inoculum concentration infected half the seeds in less than four days post-infection (dpi), with a final survival rate of only 13%. The three highest concentrations resulted in an accelerated rate of infection, while there was no observable difference between the effects of the three lowest concentrations (Figure 7.2). A final OD_{600}=0.025 resulted in a survival rate close to 50% three weeks after infection and was selected for future experiments.

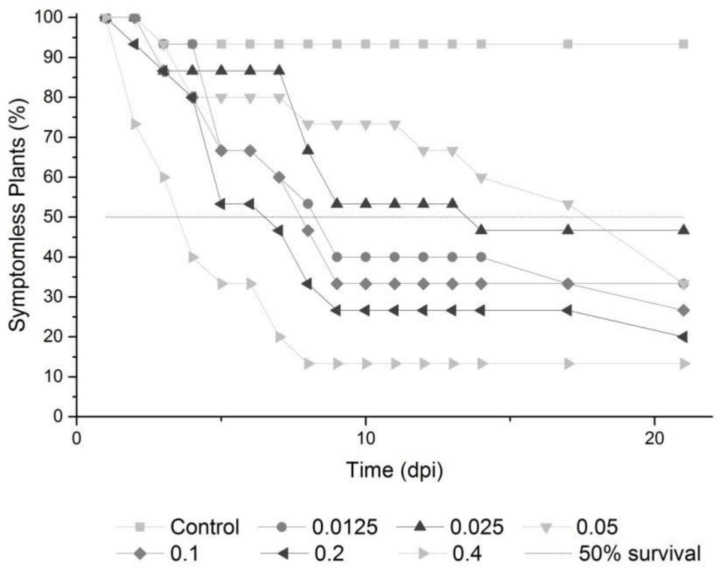

Figure 7.2 LD50 test. Effect of serial concentrations of *P. syringae* pv. *pisi* bacterial culture on pea seedlings. Six different concentrations were evaluated ranging from OD_{600} = 0.0125 to OD_{600} = 0.4 and the percentage of symptomless plants was monitored for 21 days post-infection (dpi). n = 15 seeds for each treatment.

7.3.2. Infection experiments

7.3.2.1. Preliminary infection study

After determining the concentration of the bacterial inoculum required for a 50% survival rate, a preliminary study using 15 seeds per treatment was performed to determine the effect *in planta* of uncapped, lactose-capped or APTES-immobilised CNAD-MSNPs against *P. syringae* pv. *pisi*. Germination, shoot emergence, appearance of secondary roots (Figure 7.3) and bacterial blight infection (Figure 7.4) were assessed.

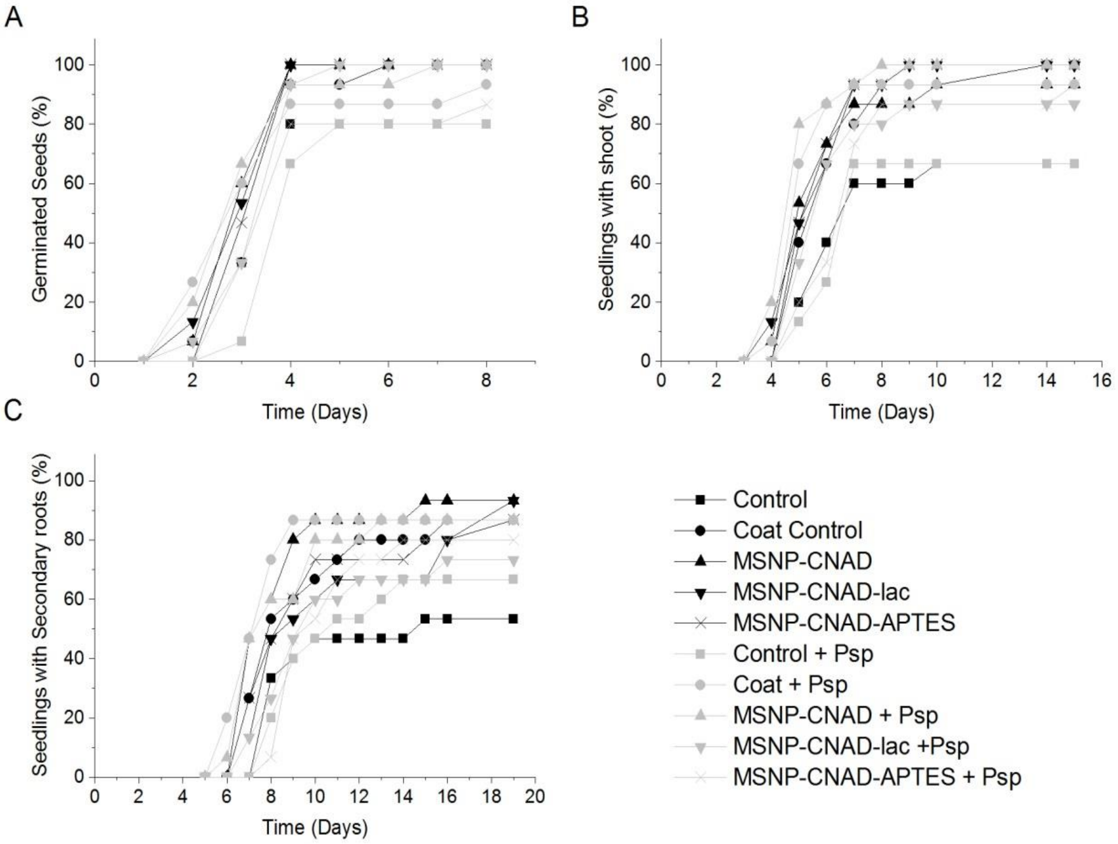

Figure 7.3 Plant growth and development after MSNP-alginate coating treatment. A) Seed germination, B) shoot emergence and C) appearance of secondary roots twenty days after sowing. Not infected (black) and *P. syringae* pv. *pisi* infected (light grey) seeds were evaluated. n = 15 seeds per treatment.

All coated seeds without bacterial inoculation germinated; whereas only 80% of control seeds did. From the infected seeds, those treated with MSNPs-CNAD and MSNPs-CNAD-lac achieved 100% germination. In general, all coated seeds germinated better and faster than the controls (Figure 7.3A). In terms of shoot emergence, all coated seeds resulted in 30-50% more seeds with shoot than the controls, regardless of whether the seeds had been infected or not (Figure 7.3B). Finally, the coat treatment produced 63-75% more seedlings with secondary roots than the controls, for treatments without inoculation, or 10-30% more for treatments with bacterial inoculation (Figure 7.3C).

There was an observable difference between treatments with respect to the appearance of bacterial blight symptoms (Figure 7.4). Uncoated control seeds developed signs of infection much faster than all coated seeds (within the first 5 days after sowing). Furthermore, twenty days after sowing, 66.67% of control seeds showed bacterial blight symptoms. Interestingly, coating only with alginate or with alginate containing CNAD-MSNPs (without capping) resulted in a greater percentage of seeds displaying blight symptoms (73.33%) than the control. However, treatment with alginate containing either MSNPs-CNAD-lac or MSNPs-CNAD-APTES led to 60% and 46.67% infected plants, respectively. This reduction in infection demonstrated that CNAD-immobilised MSNPs were more effective at protecting plants from infection and reduced bacterial blight incidence by 30% compared to the control. Still, this observable difference was not statistically significant. Hence, to confirm whether or not this was simply due to a small sample size, a final study using a larger sample size was conducted.

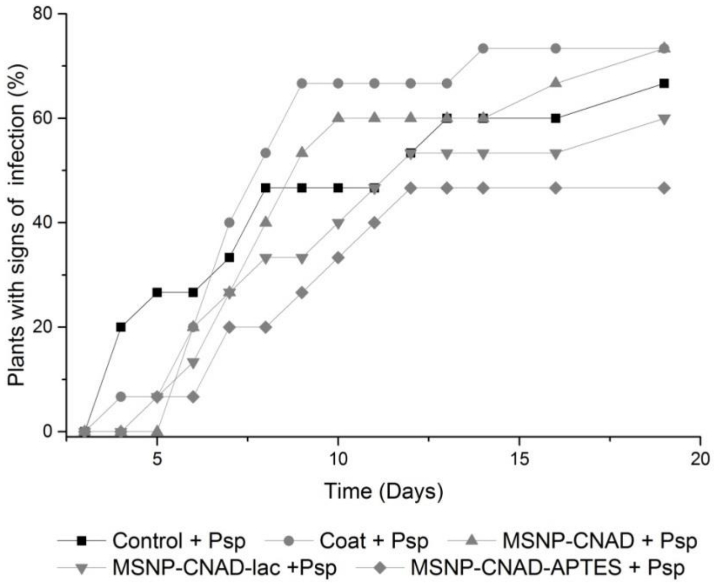

Figure 7.4 Appearance of pea bacterial blight infection during preliminary study. CNAD-immobilised MSNPs provided increased protection against bacterial blight. n = 15 seeds per treatment.

7.3.2.2. Final infection study

Once the MSNPs-CNAD-APTES had been shown to be a promising method to protect pea seeds against bacterial blight, seeds coated with alginate (1% sodium alginate and 0.1 M $CaCl_2$), with or without MSNPs-CNAD-APTES, were sown and incubated. Germination, shoot emergence, secondary roots and appearance of bacterial blight signs were monitored daily over 20 days, to determine the effect of the CNAD-loaded nanoparticles on plant protection against pea bacterial blight.

The appearance of bacterial blight signs on peas was very characteristic. The seeds usually presented no signs unless the level of infection was extremely severe, in which case they looked particularly brown/dark green or had bacterial ooze on the surface (Figure 7.5A), or water-soaked lesions near the hilum. The main indication of infection was observed after the shoot had emerged, when water-soaked lesions appeared on the base of the shoot or on the rootlet and could later appear along the stem or leaves (Figure 7.5B&C). Fan-shaped lesions, wilting, darkening of veins, interveinal tissues drying out and becoming papery (Figure 7.5D&E) are also characteristic signs of this disease. Bacterial ooze could sometimes be observed on lesions (Figure 7.5F) and in some cases whole bacterial colonies were observed on different parts of the plant (Figure 7.5C&D). The signs of infection appeared at different stages of the plants' growth and development (Figure 7.5G) but mainly occurred during the second week after sowing.

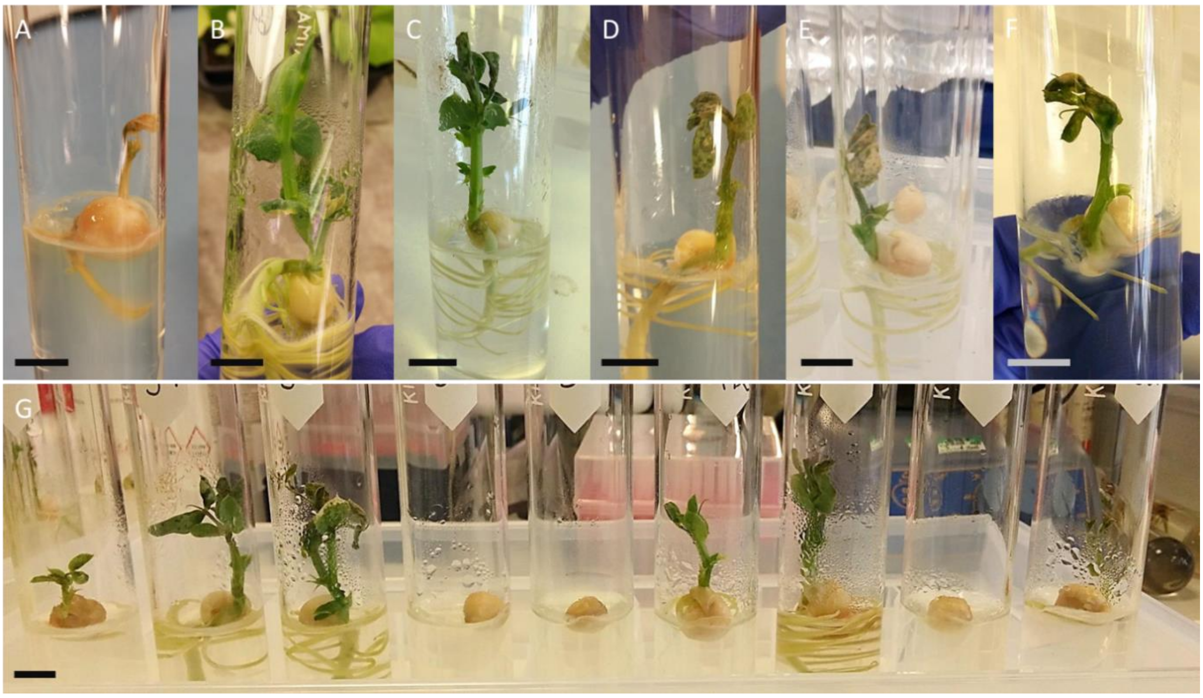

Figure 7.5 Signs of pea bacterial blight infection. A) Bacterial ooze covering seed with papery dead seedling showing severe infection B) Water-soaked lesion (WSL) on rootlet characteristic of bacterial blight infection C) WSL on leaves and rootlet with observable bacterial colonies D) WSL, bacterial colonies on leaves and wilting of plant E) Interveinal tissues drying out and becoming papery F) Bacterial ooze covering seed and plant G) Peas displaying signs of bacterial blight infection. Signs of infection appeared at different times; if the infection level was severe, seeds were not able to germinate. Scale bars = 1 cm.

Germination, shoot emergence and number of seeds with secondary roots were similar in treated seeds and the control. Treated seeds germinated significantly faster ($p<0.01$) than control seeds. Three days after sowing, 87.87% of treated seeds had germinated compared to only 68.75% of the control seeds. However, after four days both treatments reached comparable germination percentages (Figure 7.6A). A similar result was observed for shoot emergence where six days after sowing, MSNP-treated seeds showed a significantly higher ($p<0.05$) percentage of seedlings with shoots than the control (Figure 7.6B). The percentage of seedlings with secondary roots remained slightly higher for MSNP-treated seeds (84.28% after 20 days) than the control (76.89%) but the difference was not statistically significant (Figure 7.6C). The similar results between MSNP-treated and control seeds suggest that the addition of CNAD-MSNPs accelerates plant germination and growth but does not affect the final germination and shoot emergence percentages.

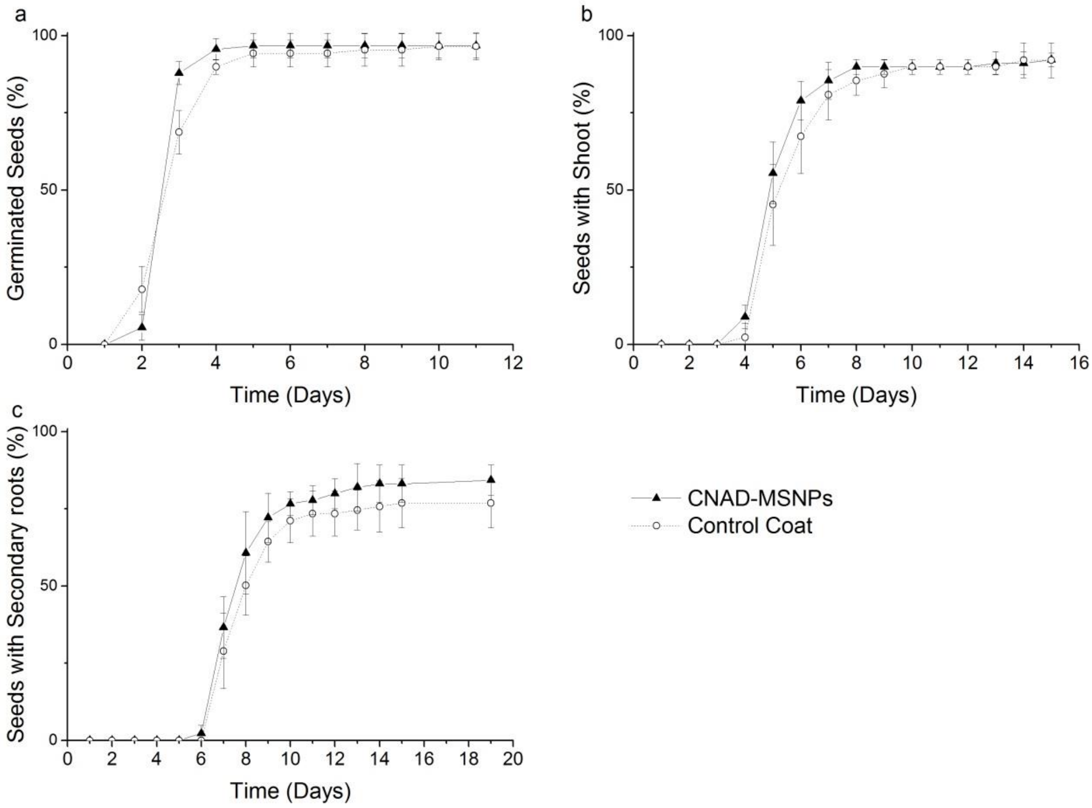

Figure 7.6 Seed germination and growth. a) Germination, b) shoot emergence, and c) appearance of secondary roots of CNAD-MSNPs-treated seeds and control seeds (alginate only coat with 1% sodium alginate and 0.1 M $CaCl_2$). Data shows mean ± standard deviation, n = 78 seeds per treatment (26 seeds per treatment for each of the three replicates).

Nonetheless, statistical analyses demonstrated that there was a significant difference between the number of symptomless and infected plants for each treatment ($p<0.01$) twenty days after sowing. Half of the control seeds coated with alginate but not protected by the nanoparticles showed severe signs of bacterial infection. In contrast, 71.79% of the plants that were protected by CNAD-MSNPs embedded in the alginate coating remained symptomless, demonstrating a significant decrease in infection and the efficacy of the seed coating to protect peas against the bacterial infection (Figure 7.7).

The results during the first week showed that the CNAD-MSNPs were most effective during the first 6 days, when the biocide concentration was higher and able to inhibit bacterial growth almost completely. After 7 days, some bacterial growth was observed and a few seedlings started displaying signs of infection (12.82% of protected vs 30.77% of control). Still, there was a significant difference between both treatments ($p<0.01$, calculated using a Chi-squared Test) demonstrating the effectiveness of the CNAD-MSNPs against pea bacterial blight (Figure 7.7).

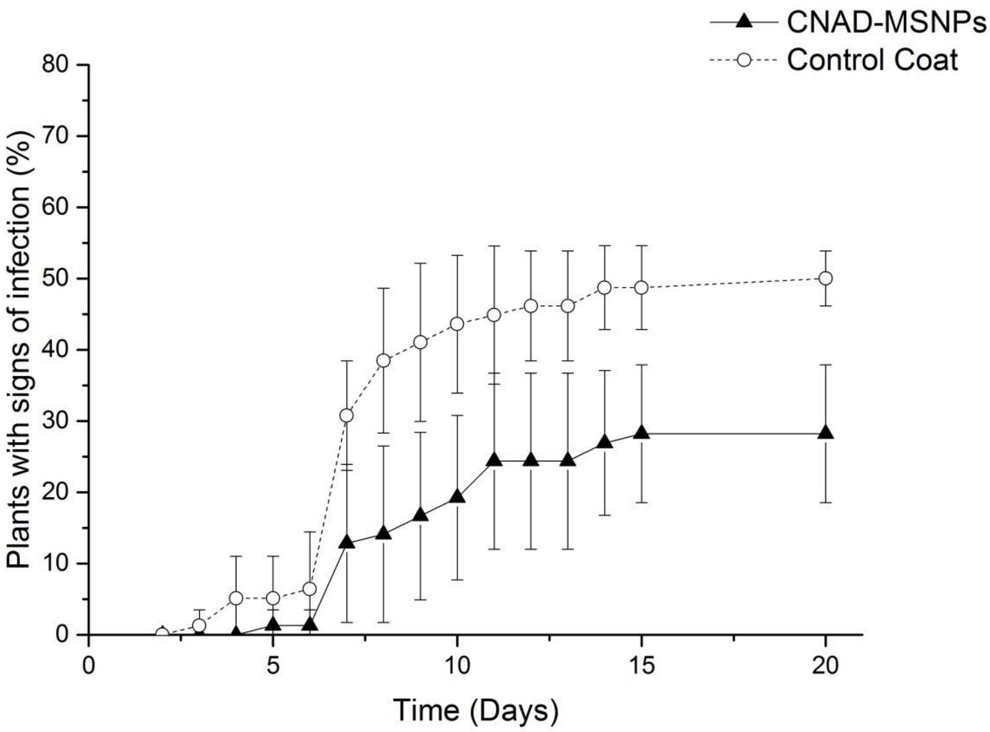

Figure 7.7 Appearance of pea bacterial blight infection. Seeds were monitored for the appearance of pea bacterial blight signs and a statistically significant difference can be observed between MSNP-treated seeds and the control ($p<0.01$). Only 28.21% of MSNP-treated plants showed signs of infection compared to 50% of control plants. Data shows mean ± standard deviation, n = 78 seeds per treatment (26 seeds per treatment for each of the three replicates).

7.3.3. Long term effects of MSNPs-alginate coating on peas against *P. syringae* pv. *pisi*

After 20 days of incubation, plants that presented no signs of infection were transferred to soil and incubated for a further four weeks. Number of leaves, nodes, flowers and pods were recorded for each plant as well as height, wet and dry mass, root/shoot ratio and chlorophyll content.

The total number of leaves and nodes was not significantly different between treatments. However, there was a significant difference ($p<0.01$) with respect to the number of leaves and nodes that were not drying out with those plants treated with nanoparticles which developed healthier nodes and leaves (Figure 7.8 A&B). The number of flowers and pods was not statistically different between treatments; however, the pods from plants whose seeds were treated with alginate/MSNPs were longer than pods from seeds only treated with alginate ($p<0.1$, Figure 7.8C).

Plant height was not significantly different between treatments (Figure 7.8D), but the wet and dry mass of plants treated with MSNPs was slightly greater than the controls ($p<0.1$, Figure 7.8E) and their roots were significantly longer ($p<0.05$, Figure 7.8D). Additionally, seeds treated with alginate/MSNPs resulted in higher shoot mass than the controls ($p<0.1$, Figure 7.8F), whilst there was no significant difference in the root dry mass between treatments (Figure 7.8G). Similarly, there was no significant difference in chlorophyll (Chl) *a* and *b* content between plants whose seeds were only coated with alginate and those coated with alginate containing CNAD-MSNPs. However, plants coated with alginate, with ($p<0.05$) or without nanoparticles ($p<0.01$) have more Chl *a* than the negative control plants whose seeds were not treated (Figure 7.8H and Figure 7.9H). The comparison of all four treatments (including a positive and negative control of uncoated seeds with or without bacterial infection, respectively) is shown in Figure 7.9. Statistical comparison of the positive control with other treatments was not possible due to the low number of surviving untreated plants ($n = 2$). In general, the addition of CNAD-MSNPs to the alginate coat resulted in more symptomless and better developed plants.

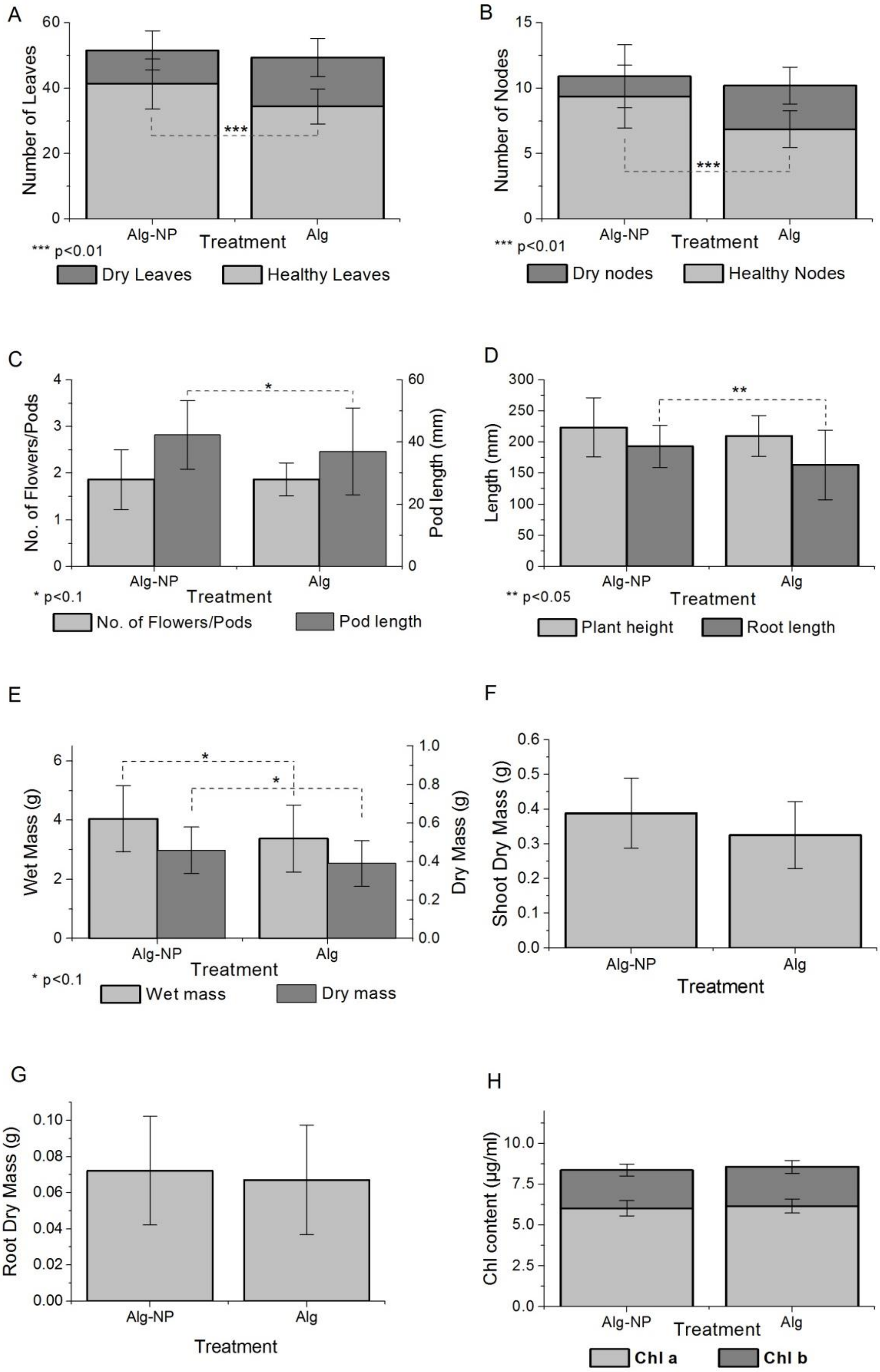

Figure 7.8 Effect of MSNP-alginate coating on peas against pea bacterial blight. Number of leaves (A), nodes (B), flowers/pods and pod length (C), plant height and root length (D), wet and dry mass (E), shoot dry mass (F), root dry mass (G), and chlorophyll content (H) of all pea plants were measured 50 days after initial sowing. Two treatments were compared, peas coated with alginate containing cinnamaldehyde-loaded mesoporous silica nanoparticles (Alg-NPs) and peas coated only with alginate (Alg). Data represents mean ± standard deviation of all replicates. Only symptomless plants that had survived 50 days after sowing were measured, n = 15 (Alg), 22 (Alg-NPs).

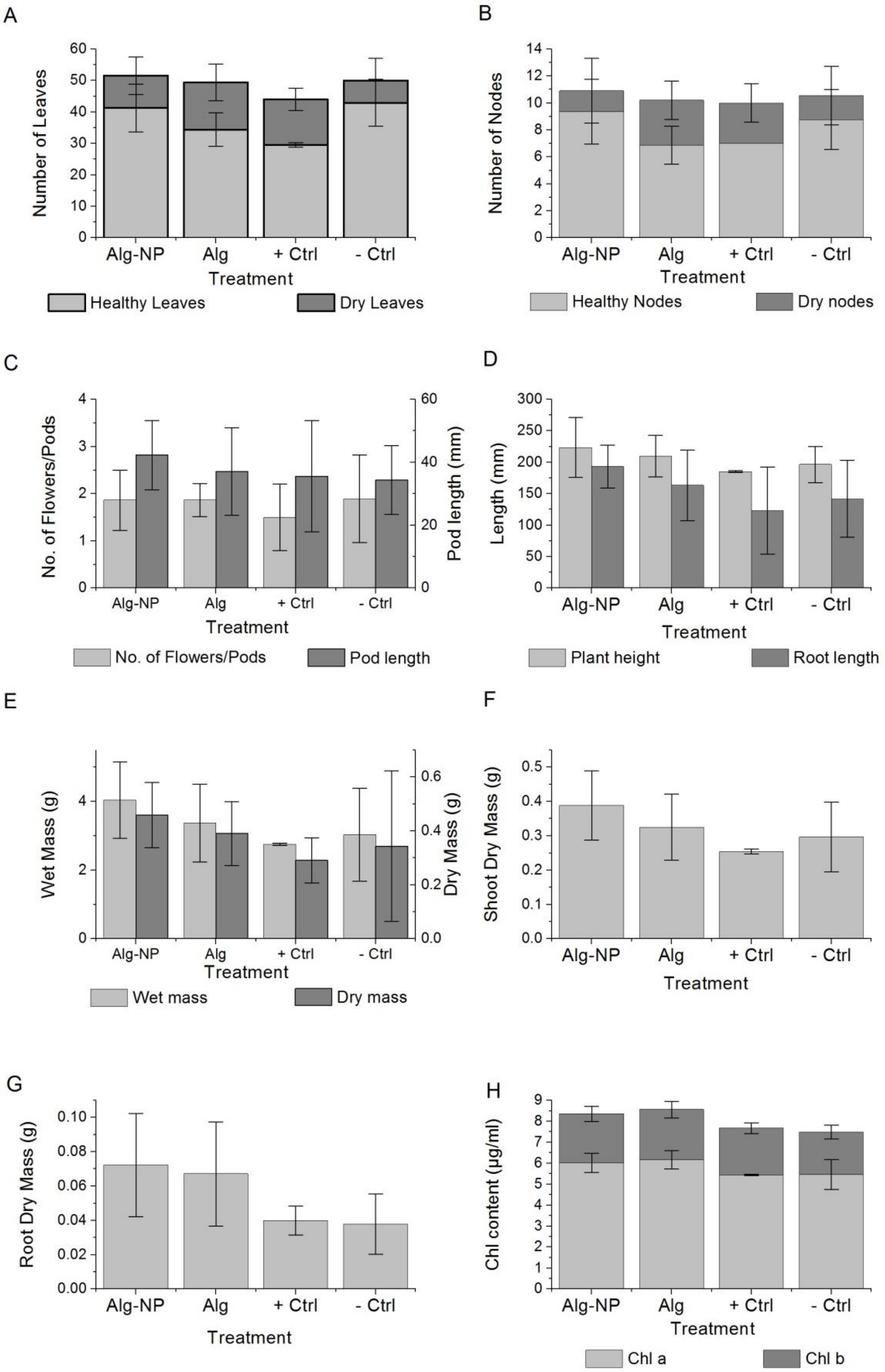

Figure 7.9 Effect of MSNP-alginate coating on peas against pea bacterial blight including control results. Number of leaves (A), nodes (B), flowers/pods and pod length (C), plant height and root length (D), wet and dry mass (E), shoot dry mass (F), root dry mass (G), and chlorophyll content (H) of all pea plants were measured 50 days after initial sowing. Four treatments were compared: peas coated with alginate containing cinnamaldehyde-loaded mesoporous silica nanoparticles (Alg-NPs, n = 22), peas coated only with alginate (Alg, n = 15), positive control of uncoated seeds infected with bacterial blight (+ Ctrl, n = 2), and negative control of uncoated and uninfected seeds (- Ctrl, n = 9). Data represents mean ± standard deviation of all replicates. Only symptomless plants that had survived 50 days after sowing were measured.

7.4. Discussion

The research summarised in this thesis aims to demonstrate the potential of nanoparticles as delivery vehicles to enhance the antimicrobial activity of essential oils by protecting them from evaporation or degradation and enabling the application of volatile biocides in agriculture to prevent microbial diseases. Evidence presented in this chapter demonstrates that an effective seed coating could be produced by embedding cinnamaldehyde-immobilised mesoporous silica nanoparticles (MSNPs-CNAD-APTES) into an alginate matrix. This was tested on the model plant *Pisum sativum* (common pea) since it is a well-studied organism with a short generation time, and ease of laboratory growth.

Investigations presented in Chapter 6 demonstrate that the immobilisation of CNAD onto MSNPs increased its antimicrobial activity by 10-fold compared to free cinnamon essential oil, and was able to reduce *P. syringae* pv. *pisi* growth by 99.99% *in vitro*. In this chapter, the incorporation of CNAD-MSNPs into an alginate coating was evaluated, in order to potentially treat and prevent peas from bacterial blight infection. To determine if the immobilisation of CNAD onto the surface of MSNPs through APTES provided the same heightened antimicrobial effect observed in Chapter 6 from lactose (APTES)-capped CNAD-MSNPs, both types of nanoparticles were used in a preliminary study and further compared to CNAD-loaded MSNPs without immobilisation or capping.

Facco *et al.* (2016) showed that a larger sample size reduces the error percentage and that at least 70 plants were needed to estimate the productive traits of pigeon peas (Facco *et al.,* 2016). To overcome this issue a larger sample size was used in this chapter with a total of 78 seeds per treatment. Furthermore, seeds were sown individually in sterile culture tubes instead of using magenta vessels to prevent any cross-contamination.

Seeds treated with CNAD-MSNPs germinated and developed faster during the first week after sowing. Silica nanoparticles have been shown to improve germination, provide nutrient availability, enhance plant dry weight and levels of proteins, chlorophyll and phenols; improve seedlings growth and quality, and increase plant stress resistance (Siddiqui & Al-Whaibi 2014; Suriyaprabha *et al.,* 2012; Lin *et al.,* 2004b; Haghighi *et al.,* 2012; Siddiqui *et al.,* 2014; Lin *et al.,* 2004a; Shah & Belozerova 2008; Janmohammadi & Sabaghnia 2015). Silicon, present as SiO_2 in soil, has a potentially important physiological role in plants; it is deposited as hydrated amorphous silica in the endoplasmic reticulum, cell wall and intercellular spaces and its deficiency increases the plant's susceptibility to lodging and infections (Solanki *et al.,* 2015). Its natural role in plant systems make silica a low-risk candidate to be used in agriculture to deliver antimicrobial cargo. MSNPs have previously been used to deliver DNA and chemicals into plant cells (Torney *et al.,* 2007) or to encapsulate pesticides such as validamycin (Liu *et al.,* 2006) and 1-naphthylacetic acid (Ao *et al.,* 2013) and the insecticide chlorfenapyr (Song *et al.,* 2012). In addition to providing efficient delivery and cargo stability, MSNPs have been shown to be safe on plants as well as on humans (Shin *et al.,* 2007). For these reasons, MSNPs are considered to be suitable delivery vehicles for volatile biocides such as EOs.

Other studies have previously evaluated EOs seed treatments, usually by soaking the seeds in a high concentration solution of EOs. Goggi *et al.* (2008) treated infected corn seeds with up to 1.6% (v/v) EOs, demonstrating the effectivity *in vitro* but not *in vivo* nor in field experiments due to volatilisation and loss of the biocides (Goggi *et al.,* 2008). This supports the need of a delivery vehicle such as MSNPs to protect the EO and enhance its antimicrobial activity *in planta*.

Eucalyptus, rosemary and niaouli EOs at 2% concentration have been shown to reduce the incidence and severity of bacterial leaf spot disease caused by *Xanthomonas perforans* in tomato 21 days after sowing (Mbega *et al.,* 2012). Moreover, eugenol (4 mg/ml) seed treatment of beans resulted in a highly significant reduction of *Xanthomonas campestris* pv. *phaseoli* var. *fuscans*, however, after 72 hours of incubation a significant reduction of germination was observed (Lo Cantore *et al.,* 2009). Cabbage seeds treated with thyme, oregano, cinnamon or clove oils had a reduced count of seed-associated bacteria, but relatively high concentrations (≥0.1%) were needed for a bactericidal effect. Additionally, concentrations of >1% cinnamon oil had a negative effect on germination while >3.3% resulted in significant seed damage (Van Der Wolf *et al.,* 2008). Likewise, high doses of clove, and oregano oils (5%, 10%) could inhibit fungal growth on seeds but also totally inhibited seed germination (Karaca *et al.,* 2017). Still, commercial CNAD products such as fungicide Cinnacure™ are applied at high CNAD doses of ~1% (v/v) (Environmental Protection Agency 2000b). The utilisation of MSNPs to deliver EOs such as CNAD allows the effective antimicrobial activity of the biocide at much lower doses that would not have a negative impact on the plant's germination or development.

The alginate solution utilised in this study contained 2 mg/ml MSNPs, which contain 0.0339 $\mu l_{CNAD}/ml_{alginate}$ (35.63 mg/L CNAD are released from 2 mg/ml loaded MSNPs as discussed in Chapter 6 (Bravo Cadena *et al.,* 2018)). This resulted in a CNAD concentration of <0.0000034% v/v present in the alginate coating, which is ~90,000-fold lower than concentrations of free cinnamon oil (0.3%) previously reported to control certain bacterial phytopathogens (Van Der Wolf *et al.,* 2008). The use of MSNPs was thus shown to permit much lower doses of biocide to be effective as antimicrobials. The CNAD-MSNP-alginate coating effectively decreased the percentage of plants infected by *P. syringae* pv. *pisi* from 50% of control plants to only 28.21% of treated plants displaying bacterial blight symptoms. Additionally, the treatment resulted in larger pods, greater wet and dry masses, and longer roots. It is important to mention that as the number of plants that died 20 days after infection was higher in the control; the difference between the treatments at day 50 was reduced, possibly underestimating its beneficial effect on the plants.

Future experiments using slightly higher MSNPs concentrations could be carried out to determine the optimal treatment to further reduce bacterial infection. Also, this treatment could be applied to different crops and several other essential oils could be used to treat and prevent a vast array of microbial diseases.

It is essential to mention that natural variation is an important source of divergence in biological experiments (Yarnes 2013) and that this intrinsic variability in terms of individual variation in germination, growth and rate of infection could explain the variance in Figure 7.7. Additionally, this study utilised an extreme condition where seeds were soaked in a highly concentrated bacterial inoculum which would be very unlikely to occur in the field. It has been demonstrated that the number of bacteria present on the seed at sowing affects the bacteria density at emergence as well as the symptoms developed (Grondeau, Poutier, *et al.,* 1992). Also, seeds were sown initially in plant culture media with a high water content, which has been demonstrated to increase the incidence of infection (Skoric 1927; Roberts 1992; Hollaway *et al.,* 1996; Roberts *et al.,* 1996). Despite this, the treatment of seeds with CNAD-MSNPs-alginate resulted in a statistically significant ($p<0.01$) protection of peas against the bacterial pathogen. In a more realistic scenario where seeds in a field are infected with lower levels of bacteria through water-splashing or contact with infected debris/plants; the level of protection would be expected to be even greater.

The treatment was particularly effective within the first week after sowing with efficacy decreasing over time, possibly due to the inevitable loss of the antimicrobial via evaporation or degradation. This treatment could therefore be highly effective as a first line of defence before germination. Moreover, the alginate-CNAD-MSNPs seed treatment not only provided protection from bacterial blight infection but also resulted in improved germination and plant development.

7.5. Conclusions

In conclusion, this is the first study to exploit the combined benefits of essential oils, MSNPs and alginate to enhance the antimicrobial activity of plant products. MSNPs protected the loaded EO from evaporation whilst enhancing its stability and enabling a controlled delivery of the biocide. Furthermore, MSNPs permitted the incorporation of hydrophobic and volatile antimicrobials such as EOs into a seed coating formulation and enabled a much lower dose of EO (<0.0000034% v/v CNAD) to be effective against the target pathogen, avoiding the use of higher doses that would otherwise negatively affect plant germination and development. The controlled slow-release of EOs from MSNPs resulted in a higher efficacy attained by increased solubility, stability and effectiveness, thereby showing a significant increase of symptomless plants protected from pea bacterial blight infection.

Chapter 8
Discussion and Conclusion

8.1. Discussion

The application of nanoparticles as delivery vehicles in agriculture has only recently started to gain attention. With the fast increase in population and the limited resources available, new approaches are being explored to fulfil the future generations' food consumption needs. Until recently, the main focus of nanotechnology has been for drug delivery in medicine and diagnostics. Its use in the food industry has also increased rapidly with applications in packaging and food preservation (Parisi *et al.,* 2015).

Different industries are currently investigating the potential use of nanotechnology, including medicine, food, energy, electronics, cosmetics, pharmaceuticals, and textiles (Gogos *et al.,* 2012; Nair *et al.,* 2010). In the case of agriculture, the application of nanotechnology has been investigated in the context of increasing yields and reducing losses through plant protection, nutrient management and growth stimulation (Gogos *et al.,* 2012).

In regard to plant protection, nanoparticles can be used to encapsulate and deliver biocides in a controlled manner while enhancing their effectivity and achieving a longer-lasting effect with much lower quantities of biocides needed. Mesoporous silica nanoparticles (MSNPs) are promising candidates for biocide delivery due to their biocompatibility, biodegradability, tuneable particle characteristics and low-cost synthesis (Trewyn *et al.,* 2007; Kwon *et al.,* 2013). They also have the potential to enable much more target delivery of biocide, reducing losses and environmental contamination since stealth delivery enables lowering of the quantities required to be effective.

The use of nanoparticles such as MSNPs to deliver cargo against pathogenic microorganisms has mainly focused on human pathogens (Qi *et al.,* 2013; Mosselhy *et al.,* 2016; Agnihotri *et al.,* 2015). In the case of agriculture, most reports are related to the control of fungal pathogens (Wanyika 2013; Janatova *et al.,* 2015; Bernardos *et al.,* 2014) or pests (Song *et al.,* 2012; Li-Xiong *et al.,* 2005; Ao *et al.,* 2013) and there is not much information about bacterial phytopathogens and their susceptibility against MSNPs or biocide-loaded MSNPs.

As discussed in Chapter 1 (Section 1.2.2), bacterial infections in agriculture are difficult to treat or control. Agricultural practices rely on hygienic measures to prevent the bacterial infection of crops. In some instances, copper compounds or antibiotics such as streptomycin and oxytetracycline are used; but these are only used for a few crops and are tightly regulated, besides causing bacterial resistance and negative environmental issues (Rodriguez *et al.,* 1997; Bajpai *et al.,* 2011). Therefore, innovative and effective ways to deal with bacterial diseases in agriculture are of extreme urgency.

The overall aim of this study was to evaluate MSNPs as delivery vehicles for natural biocides such as essential oils (EOs). Additionally, this thesis aimed to develop a seed treatment containing EO-loaded MSNPs to protect plants from seed-borne phytopathogens affecting agriculture.

8.1.1. Antimicrobial activity and species-specificity of essential oils

Natural products such as essential oils possess several advantages as antimicrobials. EOs have been produced and improved by plants throughout thousands of years to confer protection against potential pathogens, playing an important role in natural plant defence (Pradhanang *et al.,* 2003). As they are of natural origin, they are widely accepted and have GRAS status, and are commonly used in industries such as food and cosmetics. Furthermore, EOs are complex mixtures of antimicrobial components that are less likely to cause microbial resistance than conventional antibiotics (Yap *et al.,* 2014).

In this study, the antimicrobial activity of a variety of EOs against three bacterial species (*Pseudomonas syringae* pv. *pisi, Pseudomonas fluorescens* and *Pectobacterium carotovorum* subsp. *carotovorum*) was investigated (Chapter 2). More than 67% of EOs tested displayed specificity against phytopathogenic *P. syringae* pv. *pisi* over non-pathogenic *P. fluorescens* (Section 2.3.2.3). Mustard and cinnamon oils demonstrated the strongest antimicrobial activities with a minimum inhibitory concentration (MIC) of 0.016% after 24 hours of incubation (Section 2.3.3). As our research group had previously focused on the encapsulation of allyl isothiocyanate and cinnamaldehyde, the main components of mustard and cinnamon oil, respectively; these compounds were selected for MSNP loading. Also, a third EO, ajwain oil, was selected for encapsulation since it demonstrated strong antimicrobial activity (MIC 0.031%) against phytopathogen *P. syringae* pv. *pisi*.

P. syringae pv. *pisi* was chosen as a proof of principle for this investigation since it is a seed-borne pathogen, and this would enable the development of a seed treatment to control and prevent infection. Moreover, there are only a few studies that have reported on the use of EOs against *P. syringae* pv. *pisi* (Verma & Agrawal 2015; Iacobellis *et al.,* 2005; Lo Cantore *et al.,* 2004; Gormez *et al.,* 2015), but none include any of the oils selected for encapsulation.

8.1.2. MSNPs as delivery vehicles for agriculture

Despite the many advantages of EOs as antimicrobials, these compounds are prone to fast evaporation, easy degradation, and are poorly miscible in aqueous solutions. These inherent characteristics limit their use in fields such as agriculture.

Previous studies have demonstrated the effectiveness of EOs against microbial phytopathogens (Mbega *et al.,* 2012; Christian 2007; Božik *et al.,* 2017); nonetheless this effect is short-term and requires high concentrations of the antimicrobial to be effective besides having detrimental effects on germination and growth. In order to be able to successfully apply EOs to crops, a delivery system needs to be developed that can protect the active ingredients, while enhancing effectiveness and allowing a controlled or slow-release of the antimicrobial.

Silica nanoparticles enable the encapsulation of cargo, such as EOs, and its protection from degradation, resulting in an increased effectivity by facilitating a lower concentration to be delivered in a more concise and targeted manner. Furthermore, silica is the second most common element in soil (Ma *et al.,* 2011), plays an important role in natural plant defences and plant growth (Epstein 1999), is biocompatible (Slowing *et al.,* 2008), biologically inert and biodegrades into harmless silicic acid by-products (Diaconu *et al.,* 2010). MSNPs are therefore potential candidates for EO encapsulation due to their safe composition, porous structure that enables the incorporation of specific molecules, tuneable particle and pore size (Trewyn *et al.,* 2007), simple and low-cost synthesis (Kwon *et al.,* 2013), potential scale-up for industrial use, ease of surface functionalisation and ability to modify its surface properties such as charge or hydrophobicity (Wang *et al.,* 2016).

An additional advantage of MSNPs is that they can serve as a multipurpose treatment. While the cargo is protected and effectiveness is enhanced, the silica itself has a protective and beneficial effect on plants, as demonstrated in Chapter 4 (Section 4.3.2). Also, supplementary ingredients or molecules can be added to the NPs such as nutrients, growth stimulants or biocontrol agents (Lopez-Reyes *et al.,* 2015) that can enhance the effect of the treatment.

In this study, MSNPs were synthesised *via* the sol-gel method and spherical particles of approximately 100 nm in diameter were obtained (Chapter 3). The nanoparticle characterisation demonstrated that the synthesised MSNPs had characteristics comparable to those previously produced in our research group (Huang *et al.,* 2014). The as-synthesised MSNPs were loaded with EOs and their loading and unloading behaviours were assessed. Approximately 0.74 mg AIT, 0.0945 mg CNAD and 0.703 mg Ajwain EO were loaded per mg MSNPs (Section 3.3.3.1), which was comparable to other reports of encapsulated EOs (Bernardos *et al.,* 2014; Park & Pendleton 2012; Siahaan *et al.,* 2013). The release of the cargo from the MSNPs was evaluated during 24 h. Concentrations of 187.59 mg/L (AIT), 35.63 mg/L (CNAD), and 1405.88 mg/L (Ajwain EO) were detected in solution after being released from 10 mg MSNPs (Section 3.3.3.2). AIT- and CNAD-loaded MSNPs enabled a more controlled slow-release of the antimicrobial, while Ajwain oil was released much more quickly but allowing higher concentrations of biocide to be released into solution. After the encapsulation of EOs into MSNPs, the resulting EOs-loaded MSNPs could be incorporated into a seed treatment to protect plants against microbial phytopathogens.

8.1.3. Seed treatments for bacterial infections in crops

The successful encapsulation of EOs into MSNPs enables very low doses of biocides to be delivered precisely against target bacterial phytopathogens, enabling low doses to be more effective. In this study, an alginate seed coating was developed in order to incorporate the EO-MSNPs for biocide delivery *in planta* (Chapter 4). Two concentrations of sodium alginate and of $CaCl_2$ were studied to obtain the best formulation for the seed coat. All seeds treated with alginate germinated faster and developed better than the controls; however, a high $CaCl_2$ molarity (2 M) prevented germination by trapping the roots or shoots within the coat (Section 4.3.2). AFM analysis of alginate gels with different $CaCl_2$ concentrations demonstrated that a high molarity results in a softer and more flexible structure, while a lower molarity resulted in a harder and more brittle coat that could be penetrated by the roots and shoots (Section 4.3.3.2). Similar results using high $CaCl_2$ molarity have been obtained during alginate encapsulation of mammalian cells (Simpson *et al.,* 2004). The addition of MSNPs to the alginate gel altered its properties by reducing the formation of 'egg-box' dimers and making it more brittle, facilitating germination. Consequently, a low $CaCl_2$ molarity (0.1 M) was selected for the development of the seed treatment.

EO-loaded MSNPs were added to the alginate seed treatment previously developed and evaluated both *in vitro* and *in planta.* First, Ajwain-MSNPs were studied (Chapter 5) due to the higher concentration of biocide that could be released from the nanoparticles, compared to AIT- or CNAD-MSNPs. Ajwain-MSNPs were able to reduce *P. syringae* pv. *pisi* growth (optical density) by 47.87% and 41.42% after 24h and 48h, respectively, compared to untreated bacterial samples (Section 5.3.1). Cell viability was reduced >23% after Ajwain-MSNPs treatment, demonstrating the efficacy of this system *in vitro* (Section 5.3.1.1). However, the Ajwain-MSNP-alginate seed treatment *in planta* did not cause any statistically significant plant protection against the phytopathogen, despite an observable difference between treatments.

To determine if the capping of the MSNPs could enhance the biocide delivery and the effect of the EO-MSNPs system, a sugar cap was investigated (Chapter 6). The initial hypothesis was that a sugar cap that was readily degradable by the target pathogen would cause the delivery of the biocide and kill the cells in the immediate vicinity. Therefore, sugar assimilation studies were performed using ten different carbohydrates and three bacterial species (Section 6.3.1). It was found that lactose could only be degraded by one of the three bacterial species (*P. carotovorum* subsp. *carotovorum*) and thus, it was selected as a capping agent for AIT-, CNAD-, and Ajwain-loaded MSNPs. However, the *in vitro* studies in this approach did not result in the targeted delivery of the antimicrobials; possibly due to the consumption of the sugar cap triggering an increase in bacterial growth at a faster pace than the killing effect of the biocide being released. Interestingly, CNAD-MSNPs capped with lactose eliminated >99.8%, >99.9% and >95% of *P. fluorescens, P. syringae* pv. *pisi* and *P. carotovorum* subsp. *carotovorum*, respectively, compared to non-capped CNAD-MSNPs (Section 6.3.2). This system was also shown to eliminate >98% and >89% of *Escherichia coli* and *Pseudomonas aeruginosa,* respectively. This increased activity was attributed to the immobilisation of CNAD onto the surface of the MSNPs *via* the coupling agent APTES, which was confirmed through FT-IR analysis. Ruiz-Rico *et al.* (2017) have demonstrated the possibility to graft EO components onto silica NPs surfaces (Ruiz-Rico *et al.,* 2017).

The effective antimicrobial efficacy of the system was also achieved by grafting CNAD onto MSNPs using APTES as a coupling agent, without the addition of a sugar cap (Chapter 7). The addition of this system to the alginate seed treatment resulted in 71.79% symptomless plants after infection with *P. syringae* pv. *pisi*; compared to 50% of the control plants (Section 7.3.2.2). The seed treatment also improved germination and led to plants that developed better with greater wet and dry mass, longer roots, healthier leaves and nodes, and more chlorophyll a production (section 7.3.3). This confirmed the efficacy of this seed treatment to protect peas from bacterial blight infection and improve plant development.

8.1.4. Cost analysis

The system developed in this study presents a potential alternative to antibiotics and copper compounds currently used to control bacterial infections in agriculture. Other treatments such as the use of streptomycin sulphate salt and copper products are tightly regulated (Bajpai *et al.,* 2011; Commission Regulation 2002), present potential health and environmental risks and can cause antimicrobial resistance (Bajpai *et al.,* 2011; Rodriguez *et al.,* 1997). Furthermore, even though streptomycin products such as Agri-mycin 17® (Syngenta) have been shown to reduce bacterial blight infection up to 90%, they are toxic to seeds and reduce germination by up to 40.9% (Taylor & Dye 1976). Treating 1 kg of seeds with Agri-mycin 17® would cost £0.10. To compare the feasibility of the seed treatment developed throughout this thesis, a production cost analysis was estimated (Table 8.1). The costs per kilogram for the main compounds needed for the development of the aforementioned treatment were used to estimate the cost of 100 ml of treatment, which can be used to treat approximately 1 kg of seeds.

The alternative nanoparticle system which has been developed is safe, effective as a first line of defence and has a low-cost in terms of synthesis. Silica nanoparticles can be manufactured for £3 - £4.40 / kg; considering a production of 500-1000 kg per annum (*Personal communication*). To load 1 kg MSNPs, approximately 0.0945 kg CNAD would be needed, based on the loading efficiency of the MSNPs (Chapter 3). At a final concentration of 2 $mg_{MSNPs}/ml_{alginate}$ (Chapter 7), 100 ml of treatment could be used to coat approximately 1 kg of pea seeds. Taking this into consideration, this treatment would cost approximately £0.19 per kg of seed.

This estimated cost would need to be optimised for a large-scale production of the seed treatment as well as to include application costs and other minor components. Still, it can be a cost-effective system for seed protection against bacterial infections.

Table 8.1 Cost analysis. Total cost to produce 100 ml of the developed seed treatment (CNAD-MSNPs-alginate) to treat 1 kg of pea seeds.

Ingredient	Price per kg (lab-scale)	For 100 ml treatment	Cost per 100 ml treatment
MSNPs	£3 - £4.40 / kg	0.2 g	£0.0006
CNAD	£29 / kg	0.0189 g	£0.0005481
Sodium alginate	£87 / kg	1 g	£0.087
$CaCl_2$	£69.3 / kg	1.4701 g	£0.102
Total cost to treat 1 kg seeds			**£0.19**

8.1.5. Challenges of nanotechnology in agricultural applications

Only a very limited number of agriculturally applied nanotechnology products have so far reached the market. Two of the main issues delaying the application of nanotechnology to agriculture are public opinion and high production costs (Parisi *et al.,* 2015).

The public perception of nanomaterials applied to the agri-food sector can be acceptable if the benefits can outweigh the potential risks (Giles *et al.,* 2015). In the few last decades, there has been an increasing awareness of pollution and its consequences; including the negative effects of pesticides and their incorporation into the food chain, water bodies, and soil (Sachs 1993; Konuk 2016). This is becoming a public concern, and the use of certain nanomaterials could address this issue (Ranjeet *et al.,* 2014). So, a system that enables lower concentration of a killing agent to be delivered more precisely is likely to be seen to be a positive development.

Also, it has been shown that consumers have a favourable perception of agri-food nanotechnology, especially if compared to other approaches such as genetically modified crops (Giles *et al.,* 2015). In particular, the technology developed in this study utilises biocompatible and biodegradable silica nanoparticles, which have been shown to be mainly beneficial to plants (Alsaeedi *et al.,* 2017; Lin *et al.,* 2004a; Siddiqui *et al.,* 2014), as discussed in Chapter 1 (Section 1.3.1), and have been widely studied as carrier systems in medicine (Fangqiong *et al.,* 2012; Slowing *et al.,* 2008). The biocides (EOs) and coating material (alginate) utilised throughout this study are also of natural origin, which contributes to ensuring the public's positive perception of the seed treatment developed here.

In addition, nanotechnology can present a potential solution to enable a more effective delivery of pesticides. It has been estimated that pesticides cause damage of up to $100 billion USD annually due to their high toxicity and hazardous effects on health and the environment (Koul *et* *al.,* 2008). The aforementioned seed treatment could ensure the decreasing use of chemical pesticides that are tightly regulated and highly toxic (Bajpai *et al.,* 2011).

It has also been shown that consumers are not willing to pay more for products related to nanotechnology, despite the potential benefits this might have (Giles *et al.,* 2015). Hence, it is essential that nanomaterials can be applied in a cost-effective manner. As discussed in Section 8.1.4 of this chapter, the production costs of the seed treatment developed in this thesis is competitive compared to other current treatment methods such as streptomycin solutions against bacterial infections. This price could be even more competitive during large-scale production of the seed treatment. Additionally, the technology needed for nanomaterials applications on agriculture is more readily available since it is increasingly used in medicine and diagnostics (Jackson *et al.,* 2017; Nasimi & Haidari 2013).

8.2. Limitations

The research presented in this thesis had inevitable limitations that could have had potential impacts on the findings previously discussed as well as on the development of the project. Regarding project design, the most important limitation was time constraints. Each experiment (from the nanoparticle synthesis and loading and the coat development to the seed treatment and plant monitoring) was time-consuming, with a duration ranging from a couple of weeks to up to 2-3 months. This reduced the number of experiments that could be performed within the project's time limit.

Regulations and licensing was another important limitation regarding project design. The pathogen used throughout this thesis requires a specific license to be used in the UK, which necessitated the collaboration with another department of the University. This created an initial delay of the first experiments. Furthermore, other inter-departmental collaborations were necessary to conduct this research, which also resulted in experimental delays due to logistical issues.

Sample size, due to greenhouse space and material availability, was also a critical limitation to this project. Most plant experiments in Chapter 4 and Chapter 5 were carried out using 15 replicates per treatment. However, it was unclear if some differences between samples were statistically significant or not, due to the relatively small sample size. Therefore, experiments with a much larger sample size, using 78 plants per treatment, were carried out in Chapter 5 and Chapter 7. An even larger sample size would have been useful to obtain an improved statistical analysis of results.

Added to this, the initial experimental aims and objectives were broad; leading to necessary preliminary work with different pathogens, hosts, and biocides to select the most appropriate system. However, this led to the selection of a seed-borne pathogen that brought about the developed seed treatment. On the negative side, it was difficult to assess the level of bacterial infection since the plant host can still survive and grow after inoculation with *P. syringae* pv. *pisi*. Therefore, the selection of a more aggressive plant pathogen that would completely prevent plant development could demonstrate more clearly the efficiency of the treatment.

As described in Chapter 1, Section 1.4; the overall aim of this thesis was to develop a seed treatment containing EO-loaded MSNPs that can decrease the incidence of bacterial infections in agriculture. Despite the aforementioned limitations, the research presented in this thesis successfully resulted in such treatment, capable of reducing bacterial blight infection *in planta*.

8.3. Future Work

The research presented here suggests that nanotechnology holds enormous potential in terms of agriculture applications. To further develop the antimicrobial delivery system studied here, there are some areas where future work would enhance the activity of the encapsulated essential oils as well as the delivery and efficiency, while providing a more complete understanding of the potential environmental and health impacts.

As a proof of concept, this thesis used peas and *P. syringae* pv. *pisi* for the development of a treatment against bacterial diseases in agriculture. Future work with additional bacterial phytopathogens, and even fungal pathogens, could demonstrate the wide range of applications of this technology. Broadening the research to include more pathogens as well as different host plants would make this treatment more attractive for protection against multiple diseases.

Similarly, biocide studies could be complemented by encapsulating more essential oils to target specific pathogens. Also, other compounds could be added to the nanoparticles, such as chitosan, to trigger an improved immune response of the host and increase plant protection against diseases (El Hadrami *et al.,* 2010; Thakur & Sohal 2013). In fact, chitosan-capped MSNPs were initially developed but they were not further investigated due to time-constraints and to the efficacy achieved with CNAD-MSNPs. Other compounds can also be added to the system to achieve targeting of the MSNPs towards specific microorganisms.

Even though this work focused on the development of a seed treatment, the formulations could as well be implemented for foliar or soil applications in the near future. This could extend the efficacy period as it could function as a second treatment after the seed has germinated and the plant has started growing.

The fate of the loaded-MSNPs could also be studied in more detail with more internalisation studies that can confirm the absence of MSNPs from the plant's tissues. The internalisation studies in Chapter 4 suggest that the MSNPs were not internalised by the plant, but other confirmation experiments would be necessary in the future. Similarly, studies of the treated plant's progeny would be useful to determine if any MSNPs (in case of internalisation) could be passed onto the next generation of plants; and if this is the case, what would be the impact of the internalisation.

In terms of the fate of the nanoparticles, future long-term experiments could be carried out to see how long the nanoparticles take to degrade under different environmental conditions and assess the effect of the by-products on the plants. In the same way, the effect of loaded-MSNPs on the environment and other microorganisms, such as those found in soil, would need to be evaluated.

8.4. Conclusion

This is the first report of a seed treatment that combines the beneficial effects of alginate, essential oils and silica nanoparticles against bacterial diseases in crops. The research presented in this thesis successfully achieved the development of a seed treatment that has the potential to significantly decrease the incidence of bacterial infections in agriculture.

The encapsulation of volatile biocides such as essential oils into MSNPs was demonstrated to result in a slow controlled release of the antimicrobial. This enabled the efficient use of very low concentrations of antimicrobials, up to 90,000-fold lower than free oil concentrations previously reported to be effective against microbial pathogens.

The biocide-loaded nanoparticles incorporated into an alginate seed coating were able to decrease bacterial blight incidence by 143.58% compared to the control, validating the important use of NPs as delivery vehicles to protect and enhance the effect of the biocides. Furthermore, the seed treatment developed was multifunctional, not only protecting seeds as a first line of defence against bacterial infections but also positively affecting plant growth and development.

In conclusion, this study reports the successful design and development of a seed treatment to protect seeds against bacterial infections. It also demonstrates the practical application of MSNPs as delivery vehicles in agriculture. Moreover, it has the potential to be further optimised and expanded to treat other important diseases and to deliver a vast array of previously overlooked volatile biocides that can now be exploited and applied in agriculture.

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