Enhancing cinnamon essential oil activity by nanoparticle encapsulation to control seed pathogens

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Abstract

Natural biocides, such as cinnamon (*Cinnamomum zeylanicum*) bark essential oil, have enormous potential as antimicrobials but are limited by their volatility and rapid degradation. To counteract this and to prolong the efficacy of the biocide, cinnamaldehyde (CNAD, the main bioactive compound of cinnamon essential oil) was encapsulated into mesoporous silica nanoparticles (MSNPs). The synthesised CNAD-MSNPs can be used to tackle the issue of global crop loss; every year, more than 40% of global food production (estimated at $500 billion USD) is lost to diseases. This is despite the annual use of over two million tonnes of pesticides. To address seed borne diseases, CNAD-MSNPs were incorporated into a sodium alginate seed coating. As a proof of concept, this system was tested against *Pseudomonas syringae* pv. *pisi*, the causative agent of pea bacterial blight, and demonstrated to increase the number of symptomless plants by 143.58% twenty days after sowing. Additionally, the concentration of CNAD present in the alginate coating was estimated to be <0.0000034% (v/v); up to 90,000-fold lower than concentrations of free cinnamon oil previously reported to control some bacterial diseases. Furthermore, alginate-treated seeds germinated faster than control plants, and were physiologically similar, demonstrating the dual benefit of this treatment. To the best of our knowledge, this is the first study to exploit the combined properties of essential oils, alginate and MSNPs as a seed treatment to control bacterial phytopathogens. Moreover, this study proved that the antimicrobial activity of plant products can be significantly enhanced by MSNP encapsulation, allowing volatile biocides,

such as essential oils, to be used effectively at very low concentrations to treat and prevent microbial diseases in crops.

Keywords

Essential oils, Cinnamaldehyde, Alginate, Mesoporous Silica Nanoparticles, Phytopathogens, Crop protection

1. Introduction

Essential oils (EOs) are naturally occurring, highly antimicrobial and biologically active compounds produced by secondary metabolism in aromatic plants. Many EOs are widely used in several industries, have GRAS (Generally Regarded as Safe) status and appear in the EAFUS (Everything Added to Food in the US) list. These compounds are safe, have low toxicity, are generally accepted by the public and have strong antimicrobial properties, making them promising candidates to substitute chemicals and antibiotics currently used in agriculture (Bajpai et al., 2011). However, their use as free compounds can be hindered by their inherent characteristics such as high volatility, or predisposition to premature degradation and poor miscibility in aqueous substances. The encapsulation of EOs into nanoparticles such as mesoporous silica nanoparticles (MSNPs), has been shown to prevent volatilisation of the oils while improving their stability, immiscibility in aqueous solutions and long-term antimicrobial effects (Bernardos et al. 2014; Bravo Cadena et al*.,* 2018*;* Chan et al. 2017).

MSNPs are composed of silicon dioxide (SiO_2), also known as silica, a material of growing interest because of its properties including chemical stability, versatility and

58 biocompatibility (Slowing et al., 2008). Also, it decomposes in the natural environment
59 into relatively harmless silicic acid by-products (Diaconu et al., 2010). Silica nanoparticles
60 can be easily functionalised and are more stable due to their Si-O bonds (Liong et al.,
61 2008). In addition, they have tuneable pore size and porosity (Trewyn et al., 2007), with
62 simple and low-cost synthesis methods (Kwon et al., 2013). Furthermore, the size of
63 MSNPs can be adjusted to control or prevent internalisation into plant systems; plants
64 walls typically have pores of 3-8 nm (Carpita and Gibeaut, 1993) so only MSNPs smaller
65 than the largest pores are able to penetrate the cell wall into the plasma membrane. Silica
66 also plays an important physiological role in plants (Solanki et al., 2015) and its
67 characteristics and natural presence in the environment and plant systems make it a
68 promising material for biocide delivery in agriculture.

69 MSNPs have been reported to have mainly beneficial effects on plants (Haghighi et al.,
70 2012; Lin et al., 2004a, 2004b; Lu et al., 2002; Nair et al., 2011; Shah and Belozerova, 2008;
71 Siddiqui et al., 2014; Siddiqui and Al-Whaibi, 2014; Sun et al., 2014; Suriyaprabha et al.,
72 2012), improving tolerance to abiotic stress, enhancing plant growth, development and
73 yields (DeRosa et al., 2010). Their application in agriculture consequently presents
74 comparatively low risks with respect to toxicity and safety to the environment and food
75 chain introduction. The present work utilises cinnamaldehyde (CNAD, the main bioactive
76 compound of cinnamon essential oil) loaded MSNPs, which we previously demonstrated
77 can eliminate up to 99.99% of bacterial growth of five different bacterial strains with
78 MSNPs encapsulation highly increasing its antimicrobial activity *in vitro* by 10-fold
79 compared to free cinnamon EO (Bravo Cadena et al., 2018).

In this study, an alginate seed coating was developed to incorporate CNAD-loaded MSNPS. Alginate, or alginic acid, is a natural polysaccharide widely present in brown algae, consisting of β- 1,4-linked D-mannuronic acid (M) and α- 1,4-linked L-guluronic acid (G) residues arranged in homopolymeric blocks (MM and GG) together with alternating sequence blocks (MG) (Joint FAO/WHO Expert Committee on Food Additives, 1997; Smidsrød, 1974). An alginate solution can form a hydrogel in the presence of divalent cations such as Ca^{+2} due to selective binding of the ions to the GG blocks (Simpson et al., 2004; Smidsrød, 1974). 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 (Grant et al., 1973).

Alginate has been widely used in many industries, including agriculture, where it has been employed as fertilizer (Guo et al., 2006), biofertilizer (Ivanova et al., 2005), and soil conditioner (Abd El-Rehim, 2006), to produce artificial or synthetic seeds (Asmah et al., 2011; Khor et al., 1998; Pintos et al., 2008; Redenbaugh, 1983; Sakhanokho et al., 2013; Sharifeh et al., 2011; Slade et al., 1989) or to encapsulate biocontrol agents (Fravel et al., 1985). It is safe to use, biodegradable, and has GRAS status. Additionally, alginate and other natural polymers have been shown to promote plant activities such as germination, root and shoot elongation and growth (Abd El-Rehim, 2006; Luan et al., 2002), and a degraded product of alginate has been shown to act as a growth promoter and an enhancer of enzymatic activity (González et al., 2013; Idrees et al., 2011; Le et al., 2003; Naeem et al., 2011; Sarfaraz et al., 2011).

As a proof of concept, the CNAD-MSNPs alginate coating was tested on common peas (*Pisum sativum*) against bacterial blight. This disease is caused by *Pseudomonas syringae* pv. *pisi*, which is a seed-borne and seed-transmitted pathogen. It has been reported in most pea growing areas worldwide and can cause devastating effects, significantly reducing yield and seed quality (Roberts, 1993).

Pea bacterial blight is usually controlled by crop rotation and hygiene measures (disinfecting and cleaning equipment and machinery), sowing time, and employing disease-free seeds and resistant cultivars. *P. syringae* pv. *pisi* can remain viable in seeds for at least three years (Lawyer and Chun, 2001) and infected crops may result from sowing infected seeds and bacteria spreading between plants through contact or water splashing (Grondeau et al., 1991). In the UK, bacterial blight is mainly controlled through a seed certification scheme (The Plant Health (Great Britain) SI 1987/1758, 1987); however, a negative seed test result does not necessarily guarantee a seed batch is free of the pathogen and only one infected seed in 10,000 (0.01%) is enough to initiate an epidemic and destroy a crop under favourable conditions (Hollaway et al., 2007; Taylor and Dye, 1976). Therefore, it is important to develop effective treatment measures that will not present potential risks such as bacterial resistance or human and environmental hazards. Hence, essential oils are promising candidates for preventing bacterial diseases in agriculture.

The objectives of this study were: (i) to enhance the antimicrobial activity of cinnamon essential oil by nanoparticle encapsulation, and (ii) to develop an alginate seed coating capable of incorporating CNAD-loaded MSNPs to be used as a seed treatments against

plant pathogens. The results from this work demonstrated the nanoparticle-enhanced activity of cinnamon essential oil and the feasibility of this system as a potential seed treatment to prevent and control the incidence of microbial infections in crops.

2. Materials and Methods

2.1 Loading (immobilisation) of CNAD onto MSNPs

Mesoporous silica nanoparticles, previously synthesised and characterised by our research group (see (Huang et al., 2014) for full characterisation details), were used throughout this study. Synthesised MSNPs were imaged *via* transmission electron microscope (TEM) using a JEOL JEM 2100 at 200 kV. Samples were prepared by dry deposition onto holey carbon-coated TEM copper grids (Agar Scientific, UK), and air-dried overnight.

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 3-aminopropyltriethoxysilane (2.3 ml) were dissolved in 20 ml dichloromethane (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 and stirred for 5.5 hours at room temperature. The solution was centrifuged and washed with acetonitrile and water. The resulting loaded nanoparticles were dried at room temperature under vacuum for 36 hours and collected. For more information about the loading and unloading of CNAD onto MSNPs, see our previous study (Bravo Cadena, et al*.,* 2018).

2.2 Development of an Alginate Seed Coating

143 Pea seeds ("Kelvedon Wonder" cultivar) were sterilised by immersing seeds in 70%
144 ethanol (Sigma Aldrich, UK) in a sterile flask for 1 minute before washing seeds with sterile
145 water for an additional minute. Seeds were then immersed in a 2% sodium hypochlorite
146 (Sigma Aldrich, UK) solution for 5 minutes and washed 10 times with sterile water (1
147 minute per wash). They were then drained and placed in a sterile petri dish and air dried.
148 Only seeds that remained wrinkled after sterilisation were used.

149 Sterile, dry seeds were coated by immersing peas in a 1% or 3% (w/v) stirred sodium
150 alginate (Sigma Aldrich, UK) solution and swirled for 2-3 minutes to remove any bubbles
151 on the seeds' surface. They were transferred into a 0.1 M or 2 M $CaCl_2 \cdot 2H2O$ solution
152 (≥99.0% Sigma Aldrich, UK) at room temperature and left for 30 minutes, swirling
153 occasionally, to allow the coat to form around the seeds. After coating, seeds were placed
154 in a sterile petri dish and allowed to dry overnight.

155 Fifteen pea seeds were coated with each of the four resulting combinations (1% or 3%
156 alginate with 0.1M or 2M $CaCl_2$) and with two additional formulations containing 25
157 mg/ml MSNPs. To evaluate the effects of the seed coating; germination and plant height
158 were monitored and measured two weeks after sowing. Shoot and root emergence, root
159 length and number of secondary roots, leaves and nodes were also measured (data not
160 shown).

161 For the infection experiments, a seed coating using 1% sodium alginate and 0.1 M $CaCl_2$
162 was selected and 2 mg/ml CNAD-loaded MSNPs were dissolved in the sodium alginate
163 solution before the seed coating protocol. Alginate-MSNPs-coated seeds were used as the

test group while alginate-coated seeds without the addition of MSNPs were used as a control group. The experiment was performed in triplicate with 26 seeds per treatment in 3 experimental batches.

2.2.1 Atomic Force Microscope (AFM)

Alginate layers (1% and 0.1 M; or 3% and 2M sodium alginate with $CaCl_2$) 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).

2.3 Effect of Alginate Seed Coating containing CNAD-MSNPs against Pea Bacterial Blight *in planta*

2.3.1 Microorganism 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