DRUG DELIVERY NANOSYSTEMS AS PLANT "VACCINES": FABRICATION AND ASSESSMENT OF THEIR USE FOR PLANT PROTECTION AGAINST BROAD HOST-RANGE NECROTROPHIC PATHOGENS

by

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This doctoral dissertation is dedicated to all members of my personal learning network, who have shaped and nurtured me, intellectually and spiritually along this challenging journey, by direct interactions, or indirect example. I feel fortunate to be here, thankful for having you in my life, and inspired to try to move the world forward.

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ABSTRACT

Drug-delivery nano-systems enhances the potency of bioactive molecules due to its increase membrane permeability, as a result of their sub-cellular size. The concept of engineered nano-carriers may be a promising route to address confounding challenges in agriculture that could lead to an increase in crop production while reducing the environmental impact associated with crop protection and food production. A key motivation of this work is to evaluate the potential use of drug delivery nanosystems in agriculture, especially in the area of disease control. To this end, identifying the most suitable materials to serve as carrier and cargo is imperative. Understanding their bioactive properties and their physical-chemical characteristics is critical because these influences not only their biological effects on plants and environmental impact, but also, the fabrication process and potential scaling-up, enabling practical and relevant field applications in the future.

In this work, chitosan was selected as nano-carrier material because of its biological and chemical properties. The chemical structure of chitosan allows spontaneous assemble of core-shell like nanostructures via ionic gelation, has enabled it to be used as nano-carrier biomaterial intended for delivery of bioactive cargo. In agriculture, the use of chitosan is of special interest due to its immune-modulatory activity elicited in plants. However, due to its inherent molecular heterogenicity, the formulation and fabrication of stable and low inter-batch variability chitosan nanocarriers via ionic gelation is difficult and time consuming.

A myriad of different bioactive molecules has been tested as payload, encapsulated into chitosan-based delivery nano-systems for a range of purposes ranging from biomedicine, pharmaceutical, food and agriculture. In this work plant derived essential oils were selected as bioactive payload. Essential oils are at the core of the plant communication process with their phytobiome, including plant pathogens. Molecules from essential oils can carry an air-borne message serving as a plant-to-plant communication system (a phenomenon known as allelopathy) that activate the plant defense mechanisms. Encapsulation of essential oils into chitosan nanocarriers is only possible by forming nano-emulsions.

Despite the potential benefits from the use of chitosan and essential oils in agriculture, its use at a large scale has been hindered by the overwhelming inconsistencies in the current literature, regarding their formulation and fabrication. This work addresses these problems and presents evidence that support the feasibility of producing highly chitosan nanocarriers loaded with essential oils, in a facile and rapid way, using FDA-grade materials only, without the need of expensive or specialized instrumentation.

The plant-pathogen compatible interaction between *A. thaliana* and *B. cinerea* was used as biological model to test the hypothesis that chitosan nano-carriers and essential oil nano-emulsions can enhance the quantitative disease resistance of plants against broad host-range necrotrophic pathogens. We found that these treatments display a dose-dependent response in plants triggering a systemic immune response. Image-based phenotyping analysis showed that chitosan nanoparticles alone, as well as loaded with d-limonene, significantly enhanced the disease resistance of *A. thaliana* against *B. cinerea*. Nano-emulsions using essential oils from cinnamon, clove, coriander and red thyme also produced similar effects on the defense response in the pathosystem under study. Functional analysis of the differentially expressed genes from treated plants revealed that these treatments up-regulated the biological process involved in “stress management”, while down-regulated the biological process required for normal growth and development during ideal, non-stressful conditions.

1. INTRODUCTION

The concept of nanoencapsulation of bioactive molecules for controlled release and targeted delivery purposes, combines elements brought from diverse fields of knowledge. From physical-chemistry, to material science applied in the formulation of engineered nano-carriers, to molecular biology applied in the assessment of their effect on biological models for biomedical or agricultural uses. This work evaluates the potential of drug delivery technologies, traditionally developed for pharma, and biomedical purposes, to be translated into scalable, stable, and cost-effective solutions for agricultural applications.

From the lessons learned from the biomedical field, chapter two provides a comprehensive literature review on the advantages and disadvantages of different strategies for drug nanoencapsulation, and their prospective benefits if transferred from the biomedical field into the agriculture domain. We also present a critical analysis of such approaches and identify the main challenges that have delayed the effective transfer across fields, preventing, therefore, further adoption.

Chapter three presents a strategy to leverage on the natural ability of chitosan and d-limonene to elicit plant immune response, hence the term plant "vaccine". This chapter introduces a chitosan-based nanocarrier loaded with d-limonene and evaluates its ability to enhance the quantitative disease resistance against broad host necrotrophs, using Arabidopsis thaliana-botrytis cinerea as a model pathosystem. Following a careful analysis, we unveil the physical-chemical drivers governing the formation of stable chitosan-based nanocarriers loaded with d-limonene nanoemulsion, offering a new avenue for high-scale fabrication of stable nanocarriers with low batch-batch variation. Furthermore, we provide evidence of the biological processes and metabolic pathways induced by the proposed nanocarrier and related to enhanced immune resistance.

In chapter four, further evaluation is presented, assessing the potential effect of a selection of natural essential oils, as elicitors of the plant immune response. The chapter navigates the physical-chemical drivers governing the formation of stable oil-in-water (O/W) nano emulsions, which allows for a rational approach for fabrication of scalable, repeatable, and cost-effective nano emulsions. The work presented here demonstrates the opportunity for a controlled high-scale

fabrication of nanoemulsion that can be customized on-demand for virtually any bioactive essential oil. In addition, we explored the hermetic induced effect from a set of nano emulsified essential oils on the plant defense response; while we shed light onto the biological processes and metabolic pathways involved in the enhanced defense resistance in the pathosystem here under study, using cinnamon nanoemulsion as active ingredient model.

Finally, summary of the work here contained and recommendations for future work are presented in chapter five.

2. NANOSCALE DRUG DELIVERY SYSTEMS: FROM MEDICINE TO AGRICULTURE

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Abstract

The main challenges in drug delivery systems are to protect, transport and release biologically active compounds at the right time in a safe and reproducible manner, usually at a specific target site. In the past, drug nano-carriers have contributed to the development of precision medicine and to a lesser extent have focused on its inroads in agriculture. The concept of engineered nano-carriers may be a promising route to address confounding challenges in agriculture that could perhaps lead to an increase in crop production while reducing the environmental impact associated with crop protection and food production. The main objective of this review is to contrast the advantages and disadvantages of different types of nanoparticles and nano-carriers currently used in the biomedical field along with their fabrication methods to discuss the potential use of these technologies at a larger scale in agriculture. Here we explain what is the problem that nano-delivery systems intent to solve as a technological platform and describe the benefits this technology has brought to medicine. Also here we highlight the potential drawbacks that this technology may face during its translation to agricultural applications, based on the lessons learned so far from its use for biomedical purposes. We discuss not only the characteristics of an ideal nano-delivery system, but also the potential constraints regarding the fabrication including technical, environmental and legal aspects. A key motivation is to evaluate the potential use of these systems in agriculture, especially in the area of plant breeding, growth promotion, disease control, and post-harvest quality control. Further, we highlight the importance of a rational design of nano-carriers and

identify current research gaps to enable scale-up relevant to applications in the treatment of plant diseases, controlled release of fertilizers, and plant breeding.

Keywords: *Drug delivery systems, Nanotechnology, Agriculture, Encapsulation, Phytonanotechnology*.

2.1 Introduction

The potency and efficacy of an exogenously administrated bioactive molecule heavily depend on the extent of its prolonged availability in the intended final site of action. In turn, its availability depends on the intrinsic factors related to the nature of the molecule itself, such as its solubility (Savjani et al., 2012), pKa (Manallack, 2007), affinity for the receptor (Rang, 2006), molecular weight (Lajiness et al., 2004), among others. These characteristics largely influence the membrane permeability of the molecules and therefore, its capability to ingress to the target cell and produce its biological activity in it. On the other hand, some extrinsic factors such as the physiological stage of the receptor organism, enzymatic machinery and external pH in the surrounding environment, make the drug prone to inactivation or degradation. Moreover, some other substances encountered throughout the organisms during the distribution process may interact with the drug in different ways resulting in either inactivation by the formation of molecular complexes, or either synergistic or antagonistic interactions (Foucquier and Guedj, 2015) which may modulate the potency of the drug or generate unexpected responses (FDA, 2012). After its administration, the processes of absorption, distribution throughout the circulatory system and subsequent metabolism may lead to physicochemical modifications due to the dynamic interaction with its new surrounding environment.

In order to successfully execute its therapeutic effect, a bioactive molecule must overcome every unfavorable physiological condition to reach its target in such a way that, a proper amount of active compound (i.e. adjusted within its therapeutic window) enters the target cell at a proper time. The challenge of drug delivery is to accomplish the release of the drug agents at the right time in a safe and reproducible manner, usually to a specific target site.

Drug delivery systems are engineered devices used to transport a pharmaceutical compound throughout the body in order to release its therapeutic cargo in a controlled manner (NIH, 2016).

By encapsulating the molecules within a protective shell-like structure, potential physical-chemical or enzymatic disruptions of the active compound are diminished. In turn, not only the bioavailability of the active compound is increased but also undesirable side effects resulting from unspecific systemic distribution are reduced (Felice et al., 2014). Nano-encapsulation of bioactive compounds helps to reduce the frequency of dosing needed during treatment and also may confer physical protection to the drug during storage prior to its use for controlled release of cargo (Choudhury et al., 2017).

One of the most notable advantages offered by nano-delivery systems for drug therapy is the controlled drug release not only at a specific location level but in a time-dependent manner via passive or active targeting. Passive targeting drug nano-carrier is designed based on pathophysiological features from the targeted tissue that allow the accumulation of the nano-sized delivery system on it. On the other hand, active targeting refers to the coupling or assembly of surface-active ligands onto the surface of the drug delivery systems, which are able to recognize and interact with a receptor in the target cell. As a result of the interaction between ligands and receptors, the drug delivery specificity and nanoparticle up-take is enhanced (Felice et al., 2014b). Different types of ligands have been successfully tested in vitro such as engineered antibodies, growth factors (Lee et al., 2010), vitamins (Chen et al., 2010), and aptamers (Colombo et al., 2015). Describing the complete pathway which had to take the controlled drug delivery systems from their very origins to their current state is not within the scope of this review. However, a highly detailed review describing the evolution of controlled drug delivery systems from their non-biodegradable macro-scaled state, up to the more updated biocompatible nano-carriers used in therapeutics is available. (Hoffman, 2008).

The challenge of drug delivery is to accomplish the release of the drug agents at the right time in a safe and reproducible manner, usually to a specific target site. In this sense, medicine and agriculture share similar challenges and final goals. Similarly, nano delivery systems that have contributed to the development of precision medicine by delivering therapeutic molecules in a controlled manner have potential applications in agriculture. For instance, the use of encapsulated agrochemicals into nano-carriers to deliver pesticides to the desired crop to provide a focused delivery of the required dose (i.e. diminished application dosages), time-controlled release, and less eco-toxicity is not only an expanding area of research but a potential growth market (Slattery

et al., 2019). Other areas within agriculture that could benefit from nano-encapsulation approaches include plant breeding (Kim et al., 2015), plant nutrition (Rai et al., 2015), growth promotion (Siddiqui and Al-Whaibi et al, 2015), disease control (Nuruzzaman et al., 2016), and post-harvest quality control (Yadollahi et al., 2010) to name a few. Conversely, agricultural materials such as cellulose (Bhandari et al., 2018, 2017) and chitosan (Cai and Lapitsky, 2019) have been used as base materials to develop drug delivery systems.

Nano-carriers intended for drug delivery can be prepared from a variety of materials such as proteins, polysaccharides, synthetic polymers and inorganic metallic salts (Panchapakesan et al., 2011; Wang et al., 2012). The selection of matrix materials depends on many factors such as the size of nanoparticles required; the physical properties of the drug (e.g., aqueous solubility and stability); the surface characteristics such as charge and permeability; the degree of biodegradability, biocompatibility and toxicity; drug release characteristics of the final product; and challenges involved in regulatory approvals. Scalability and approval from regulatory governmental entities are two other major concerns when the intention is to release a product to the market, which are closely related to the formulation and fabrication. The main objective of this review is to contrast the advantages and disadvantages of different types of nanoparticles and nano-carriers currently used in the biomedical field along with their fabrication methods to discuss the potential use of these technologies at a larger scale in agriculture. We also aim to highlight and discuss the applications of nano-encapsulation technology in agriculture and its potential drawbacks. Specifically, we address the use of nano-delivery systems as a non-viral vector for gene delivery in plant cells, and for the delivery of nutrients during plant growth promotion and crop protection.

2.2 Nano-Drug delivery systems from the engineering perspective

Ideally, nano delivery systems should fulfill certain technical and economical requirements. Table 1 presents a summary of the characteristics of an ideal nano-carrier for biomedical and agricultural purposes. First, the materials used as carriers should not trigger any adverse response in the recipient organism. Also, not only the matrix material should be biocompatible, but its degradation products. Second, the mechanical properties of the polymer must provide prolonged protection to its cargo allowing chemical stability over time. Third, the scalability of the fabrication process

should be technologically feasible and economically viable. Accordingly, the processes employed for the elaboration of nano-carriers should yield consistent results in a batch to batch basis, in terms of size, polydispersity, encapsulation efficiency and stability. Finally, the materials to act as nano-carriers should be carefully selected since they not only must meet the technical criteria to address mandatory regulations prior to being commercialized (Tinkle et al., 2014), but they also must display good performance in terms of cost/benefit and eco-compatibility.

Table 2.1: Characteristics of an ideal nano-carrier for agricultural purposes

Fabrication conditions	Encapsulation properties	Release profile
✓ Mild conditions ✓ Scalable ✓ Low-cost ✓ Reproducible ✓ Low batch-to-batch variability	✓ Stable ✓ No early cargo release/leakage ✓ Non-toxic ✓ Biodegradable ✓ Eco-compatible ✓ Water soluble	✓ Controlled ✓ Targeted ✓ Stimuli sensitive (pH, light, temperature)

Table 2 presents a summary of the advantages and disadvantages of drug delivery nano-carriers with potential use in agriculture. In general, drug nano-encapsulation depends on the physicochemical nature of the encapsulation matrix, the cargo, and the method to carry out the process. However, regardless of the encapsulation matrix and cargo nature; or the method used to fabricate the drug-loaded nano-carriers, a plethora of reports confirm that some processes to elaborate them have the potential to be standardized since their reproducibility is fairly consistent.

Table 2.2: Summary of advantages and disadvantages of drug delivery nano-carriers with potential use in agriculture

Type of nano-carrier	Advantages	Disadvantages
Mesoporous silicon-based materials	Stable structure. Tunable and uniform pore size. Controlled release of cargo.	Inorganic. Non-biodegradable. Potential cell lysis caused by silanol groups interacting with membrane lipids.

Table 2.2 continued

Solid lipidic nanoparticles (SLNs)	Improves solubility in water of hydrophobic cargo. Hydrophilic cargo possible. Relatively inexpensive production. Biocompatible/biodegradable. Feasible production scaling-up.	Low load capacity Low Encapsulation efficiency High water content in dispersions (70%-99.9%). Premature cargo release during storage.
Nano-emulsions	-Highly stable to gravitational separation and aggregation. - Improves solubility in water of hydrophobic cargo. - Biocompatible/biodegradable. - Relatively inexpensive production. - Suitable for incorporating lipophilic cargo. - Increase efficacy of antimicrobial agents.	- High amounts of surfactant needed to achieve oil droplets of nanometric sizes. -
Dendrimers	Functionalization of peripheral groups determines solubilization and enables targeted delivery of cargo. Suitable for incorporating lipophilic or lipophobic cargo. PAMAM dendrimers are reported to be relatively resistant to hydrolysis.	Cytotoxicity reported on cationic dendrimers. Toxicity correlated with the number of surface amine groups. Pharmacokinetics, biodistribution, biodegradation, and chronic toxicity of PAMAM dendrimers are not yet clearly understood.
Nanocrystals	Carrier-free (i.e. they are almost 100% drug). Improves bioavailability of water-insoluble compounds. Improves drug adhesiveness to surface cell membranes. Enhances particle stability in suspension Increase drug dissolution velocity.	Difficult to control morphology and crystallinity of final product. Highly time/money/energy demanding. Need for large amounts of organic solvents (Bottom-up approach). Residual presence of surfactants, solvents or stabilizers (top-down approach). Specialized equipment is needed.
Hydrogels	Complete bio and eco-compatibility. Relatively inexpensive production. Easy to fabricate.	Batch to batch variation due to the heterogeneity of the polymer. Fine tuning formulation required to achieve stable particles.

2.3 Nanoparticles and nano-carriers for agriculture: advantages and disadvantages

2.3.1 Metallic nanoparticles

Due to their chemical nature, metallic nanoparticles such as gold and silver display enhanced physicochemical properties when presented as nanometric particles. Taking advantage of these properties, major efforts on research has focused on the development of devices, predominantly in the biomedical field, for detection and treatment. Chemical sensors are one of the most prominent biomedical applications of metallic nanoparticles (Guo and Irudayaraj, 2011). For instance, gold nanoparticles conjugated with specific oligonucleotides can sense complementary deoxyribonucleic acid (DNA) strands, detectable by color changes (Kouassi and Irudayaraj, 2006)). Furthermore, gold nanoparticles can be readily functionalized with antibodies and oligonucleotides (Yu and Irudayaraj, 2007) (Orendorff et al., 2006) (Wang et al., 2010); (Wang and Irudayaraj, 2013), (Sun and Irudayaraj, 2009a, 2009b), enzymes (Majouga et al., 2015). These hybrid nanostructures are also active elements of a number of biosensor assays to detect gene products in plants (Kadam et al., 2014, 2017), drug and gene delivery systems (Ding et al., 2014). Although metallic nanoparticles are widely used in detection, these have limited applications as delivery systems.

2.3.2 Mesoporous silicon-based nano-carriers (mpsnps)

Silicon-based mesoporous materials belong to the group of inorganic nano-carriers widely used as drug delivery systems. This approach takes advantage of the highly stable porous surface of silicon mesoporous materials to fill with bioactive cargo. Ideally, loaded pores are capped and the cargo is released intracellularly (Xu et al., 2019). One of the main advantages of MPSNPs is their stability, which confers the ability to cope with physical stress such as temperature and pH variations in their surrounding environment. Moreover, their tunable and uniform pore size (3-50 nm) allows them not only to load relatively high amounts of drug cargo due to their high surface area and large pore volume but to selectively functionalize candidate molecules onto its surface (Perez et al., 2017) (See figure 1). Different synthesis protocols to obtain fine-tuned large-pore mesoporous nano-carriers and their suitability in the delivery of proteins, enzymes, antibodies, and nucleic acids were explored (Knezevic, 2015).

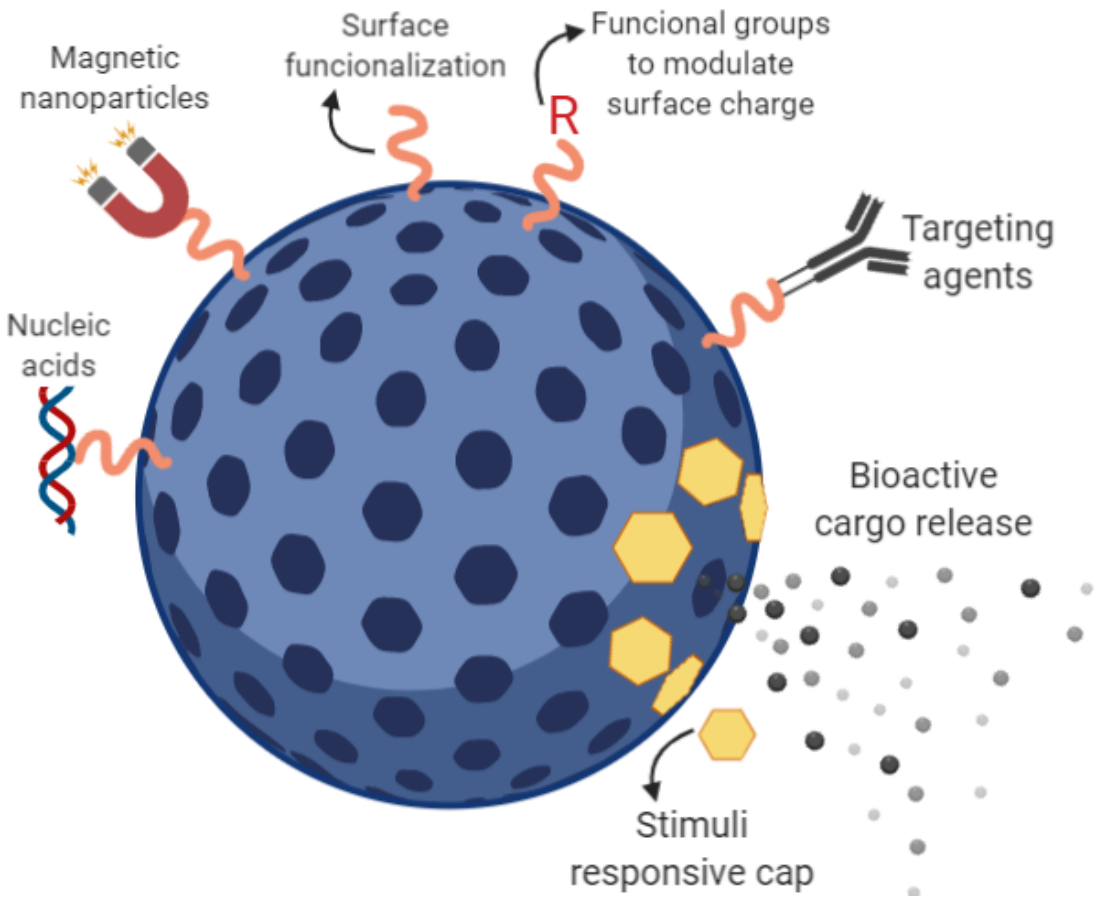

Fig. 2.1: Mesoporous silicon-based nano-carriers (MPSNPs). Mesoporous silicon-based nano-carriers (MPSNPs). Schematic representation of a mesoporous silicon-based nanocarrier. The bioactive cargo can be loaded into the porous spaces via passive adsorption or active anchoring. Stimuli responsive caps can be design to prevent early cargo release and detach from its pore allowing controlled release. Targeted cargo delivery can be performed by attachment of targeting agents onto previously functionalized particle surface.

The extended use of silicon-based mesoporous nano-carriers in clinical applications has been delayed due to the lack of pharmacokinetic-pharmacodynamic studies concerning biodistribution, clearance, therapeutic efficacy, and safety are important parameters that need further attention in the quest of providing competent porous nanoparticles (Shahbazi et al., 2012) For instance, it has been demonstrated that mesoporous silica nanoparticles is not completely hemo-compatible; such phenomena have been attributed to the surface density of silanol groups interacting with the surface of phospholipids or the red blood cell membranes resulting in hemolysis (Zhao et al., 2011). One of the potential drawbacks of the use of MPSNPs in agriculture is its non-biodegradability and lack of data on bioaccumulation to meet regulatory standards.

However, due to their intrinsic physico-chemical properties, the scope of use of MPSNPs include a wide range of applications such as: (i) water decontamination through adsorption of radioactive pollutants (Iqbal and Yun, 2018), separation of dyes (Shinde et al., 2017); (ii) catalysis (Munz et al., 2016; Verho et al., 2014); (iii) delivery of agrochemicals (Yi et al., 2015); (iv) chromatography (Ahmed et al., 2014) to mention a few.

2.3.3 Solid lipid nanoparticles (sln)

Solid lipid nanoparticles (SLN) (figure 2) are spherical nanoparticles, which makes these ideal candidates for the encapsulation of lipophilic bioactive compounds. The main advantage of SLN relies on their relatively low fabrication cost with the potential for scaling-up of production (Pallerla and Prabhakar, 2013). However, potential disadvantages for its use in agriculture include poor cargo loading capacity and early cargo expulsion after polymorphic transition during storage (Singhal et al., 2011) (Pardeshi et al., 2012).

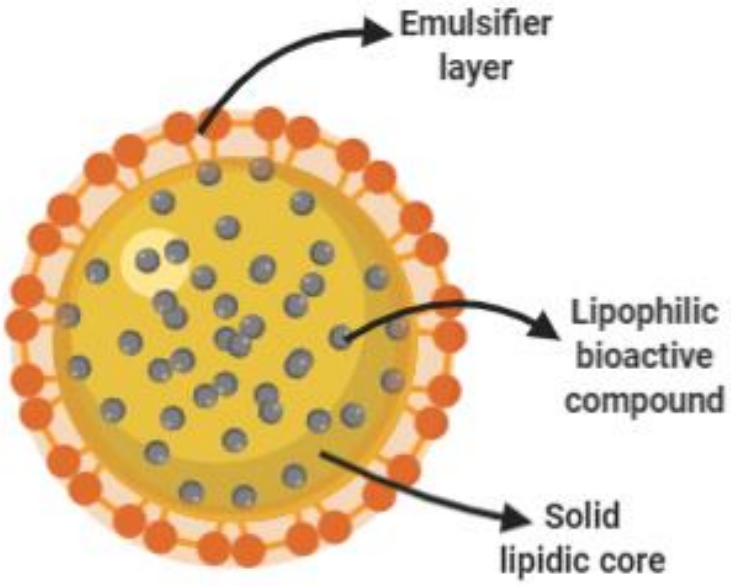

Fig. 2.2: Solid lipid nanoparticles (SLN). Schematic representation of a solid lipidic nanoparticle. During SLN fabrication, a lipophilic bioactive cargo is dissolved in a liquid hot lipid matrix. Under proper formulation and operational conditions, nanoparticles are formed assisted by an emulsifier as the lipidic core solidifies at room temperature.

SLN have been successfully implemented in a wide range of applications. In the biomedical field, for instance, it has been used to increase both the solubility of several poorly soluble drugs, (Padhye and Nagarsenker, 2013), (Patel et al., 2012) just to mention some. In the cosmetic industry, they have been used to encapsulate UV blockers such as 3,4,5-trimethoxybenzoylchitin (TMBC), 2-hydroxy-4-methoxybenzophenone and vitamin E for use as sunscreen (Wissing and Müller, 2001) (Song and Liu, 2005). In the food industry, SLNs have been used to encapsulate antioxidant molecules such as ferulic acid and tocopherol (Oehlke et al., 2017), natural

antimicrobial compounds (Piran et al., 2017), and hydrophobic flavoring agents (Eltayeb et al., 2013).

2.3.4 Nano-capsules

Nano-capsules are nano-vesicular systems in which drugs are enclosed in an inner cavity created by a unique polymeric membrane (see figure 3). Nano-encapsulation enhances drug delivery and efficacy, but the different methods used for the preparation of nano-capsules frequently produce dispersions with low drug loading. This is a serious disadvantage when the aim is to obtain therapeutic concentrations (Mora-Huertas et al., 2010). Similar to NLPs, the application of nano-capsules also extends from the pharmaceutical sector for the encapsulation and delivery of drugs, to the food industry and agriculture, as well as application in cosmetics and personal care in the form of cosmeceuticals.

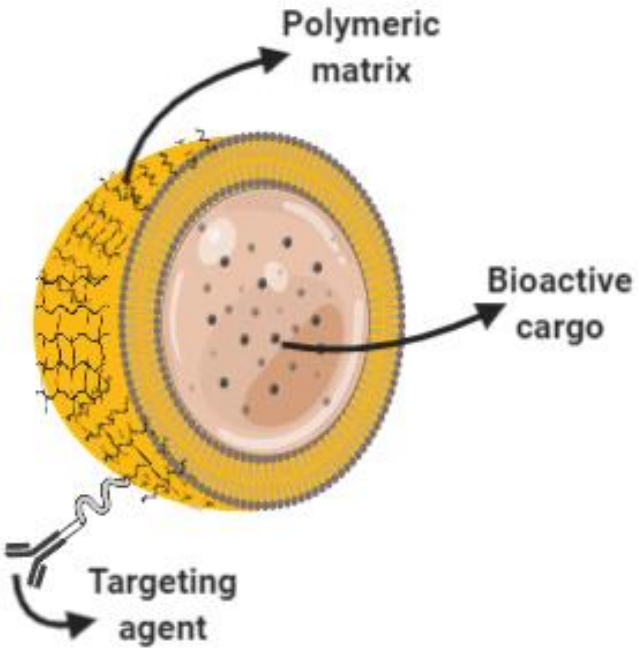

Fig. 2.3: Core shell nano-capsules for drug delivery. Schematic representation of a nanocapsule. Bioactive cargo is encapsulated into a core-shell polymeric matrix. Polymer surface can be functionalized and decorated with targeting agents enabling targeted delivery.

Drug loaded nano-capsules are especially useful for skincare and dermatological treatments because of their enhanced bioavailability in dermal cells. Ebselen (Eb) is an example of a repurposed drug with poor aqueous solubility which requires a sophisticated delivery system such as nano-encapsulation for topical application as a promising, safe and complementary alternative to the treatment of cutaneous candidiasis (Jaromin et al., 2018). Examples of commercially available cosmeceutical products are ‘Hydra flash bronzer’ a facial skin moisturizer, ‘Soleil soft-touch anti-wrinkle sunscreen’, “Soleil instant cooling sun’ and ‘primordiale optimum lip’ produced by Lancôme®. These products claim to contain nano-capsules of vitamin E and antioxidant agents as active ingredient. A more comprehensive list of readily

available cosmeceuticals products containing nano-capsules and SLN is available in the literature (Lohani et al., 2014).

The food industry is taking advantage of the benefits of nano-encapsulating essential oils to enhance their antimicrobial activity against food borne-pathogens to increase their solubility when loaded into polymeric nano-capsules (Granata et al., 2018).

In agriculture, nano-encapsulation technology has been used for the delivery of currently available pesticide molecules (Yin et al.,2012). However, the increased water solubility, which is desirable for pesticide efficiency, brings environmental and in turn, regulatory concerns. By studying a commercially available insecticide with an encapsulated active ingredient, Slattery et.al. demonstrated that by encapsulating the in nano-sized carriers, the active ingredient's water solubility increases. Enhanced water solubility disrupts foundational assumptions on its chemical behavior of the pesticide, such as its hydrophobicity (K_{OW}) and soil sorption (K_d). The hydrophobicity (K_{OW}) and soil sorption (K_d) values are numerical descriptors used to predict the environmental fate of a molecule (pesticide) and its toxicity. By encapsulating the pesticide molecules into nano-sized carriers, these indexes may not adjust to the prediction models once built based on their free un-encapsulated forms. Thus, complicating the use of hydrophobicity metrics to predict their fate and toxicity. Determining how carrier size influences the hydrophobicity (K_{OW}) and soil sorption (K_d) of a given pesticide, and thus its mobility through soil and water, is important to our understanding of whether the current pesticide's toxicity risk assessments are sufficient to protect against products that incorporate nano-encapsulation technology (Meredith et al., 2016) (Slattery et al., 2019).

2.3.5 micelles, liposomes, and nano-emulsions

Micelles are spontaneously self-arranged spherical aggregates made of surfactant molecules. Liposomes are spherical vesicles with at least one lipid bilayer, and nano-emulsions are surfactant-assisted homogeneous suspensions of nano-sized droplets of a dispersed phase in a continuous phase. They all display spherical shape (Pavlic et al., 2009) and facilitated a controlled release of cargo (Godfroy, 2009) (Joo et al., 2013). Besides their inherent biocompatibility, their surface can be modified and functionalized for conjugation with targeting moieties which enable targeting to specific sites, improving efficacy and potency (Vabbilisetty and Sun, 2014).

In general, liposomes (figure 4b) are used to encapsulate water-soluble compounds because they are comprised of a lipid bilayer separating an aqueous internal compartment from the bulk aqueous phase. Whereas oil in water (O/W) nano-emulsions are used to encapsulate hydrophobic compounds. In contrast, polymeric micelles (Figure 4a) are used to encapsulate both hydrophobic and hydrophilic compounds depending on the design. Block copolymers have a hydrophilic and a lipophilic block. Block-copolymers can easily reach NP size higher than 20 nm and close the liposomes.

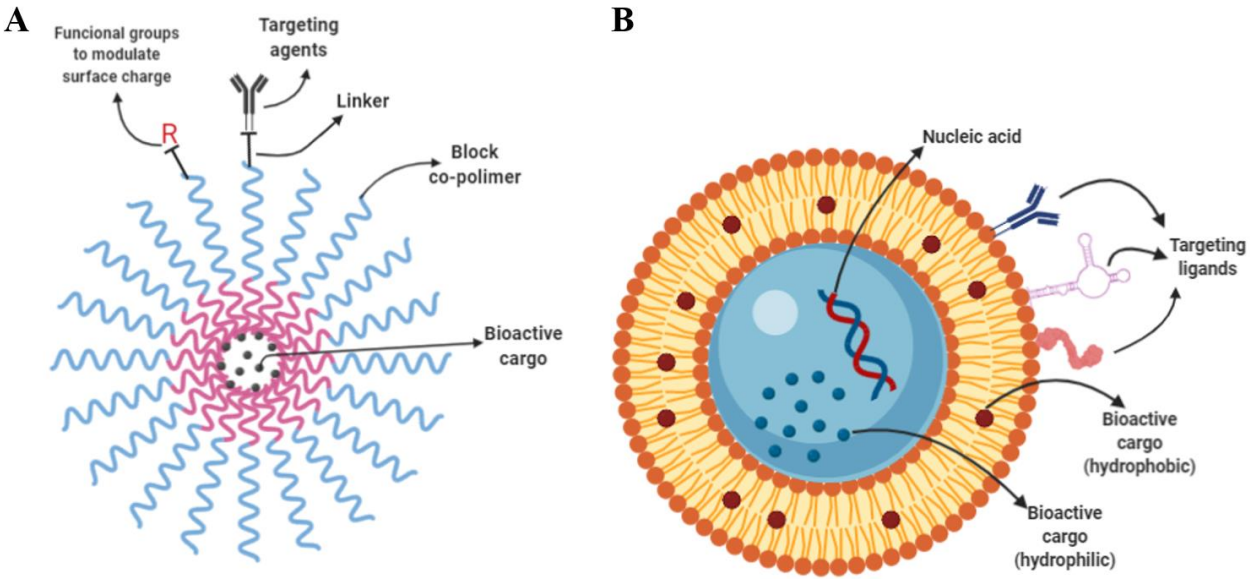

Fig. 2.4: Polymeric micelles and liposomes. A) Schematic representation of a polymeric micelles composed by a co-block polymer (red and blue wavy lines). The core shell is formed encapsulating the bioactive cargo inside. The surface can be functionalized with linker molecules and further decorated with targeting ligands to enable targeted delivery. B) Depiction of a liposome containing hydrophilic cargo in its core a hydrophobic cargo allocated in the bilayer. Surface functionalization can be achieved by anchoring of targeting ligands such as antibodies, proteins and aptamers.

The applications of micelles, liposomes, and nano-emulsions include the encapsulation of poorly water-soluble bioactive molecules to be further incorporated into aqueous products. For instance, for biomedical purposes, a plethora of different types of drug-loaded nano-emulsions is available including oral, topical, intranasal and ocular administration (Yukuyama et al., 2017). In the food industry, several types of different nano-emulsions have also been used as carriers of natural occurring, but poorly soluble flavors, colors, preservatives and antioxidant agents (Donsi, 2018). Increased attention has been focused on the nano-emulsification of essential oils because it has been proven that when presented on a nanometric scale, their antimicrobial activity is enhanced.

Moreover, its long-term stability is also enhanced (fig.5). In a recent work D-limonene was used to prevent the formation of biofilms on *E.coli* O157:H7 at sub-lethal doses, by blocking the quorum sensing mediated autoinducer-2 (AI-2) communication and curli-related gene expression (Wang et al., 2018).

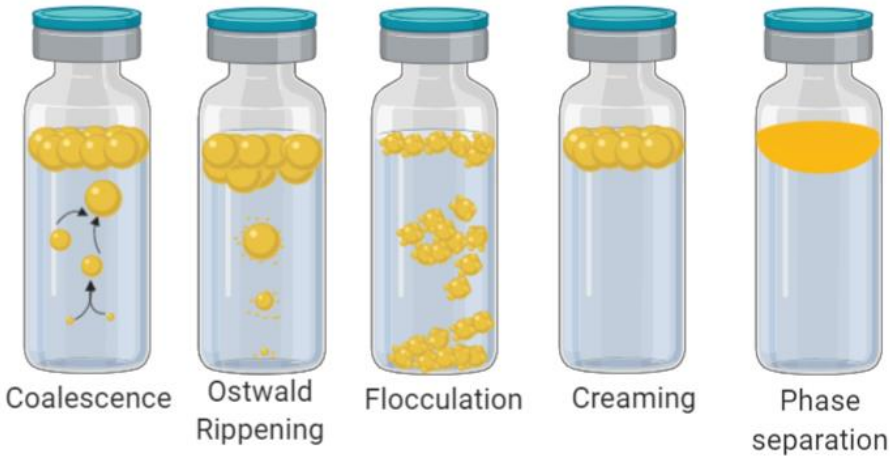

Fig. 2.5: Types of emulsion destabilization. Schematic depiction of how emulsions naturally tend to separate its phases. **i) Coalescence** occur when two separate oil droplets merge into a single larger oil droplet because surfactant monolayers fuse together**. ii) Ostwald ripening** is the most common way of nano-emulsion failure. Larger oil droplets become larger at expense of smaller oil droplets driven by the pressure difference between to oil droplets of different diameters. The process accelerates as the diameter difference increases. **iii) Flocculation** occurs when oil droplets collide, but instead of coalescence, they remain as independent droplets. Co-joined droplets form clusters that precipitate. with enough time, the before mentioned processes produce **iv) creaming** and later on they lead to complete **v) phase separation.**

2.3.6 Dendrimers

Dendrimer structures are comprised of three components (figure 6): a focal core, dendrons, and cavities formed between dendrons (Safari and Zarnegar, 2014). Some of the desirable characteristics of dendrimers are their uniform molecular weight and their three-dimensional structure with peripheral groups that determine solubility, making them relatively easy to design

upon specific demands. Further, their smaller hydrodynamic volume and lower molecular volume compared with linear polymers of similar molecular weight (Markowicz-Piasecka and Mikiciuk-Olasik, 2016). Exposed terminal groups in dendrimeric particles mostly control their chemical interactions with the molecular environment. Their properties such as nanometer size range, ease of preparation and functionalization, also their multiple copies of surface groups displaying stability, make them an attractive system for drug delivery. However, despite their initial popularity in drug delivery, at present, serious concerns exist on the cytotoxicity of cationic dendrimers, which has led to further investigation of alternatives to overcome this issue. The toxicity of dendrimers mainly comes from the high cationic charge density in the periphery, where charges interact with the biological cell membrane and then result in membrane disruption (Tsai and Imae, 2011).

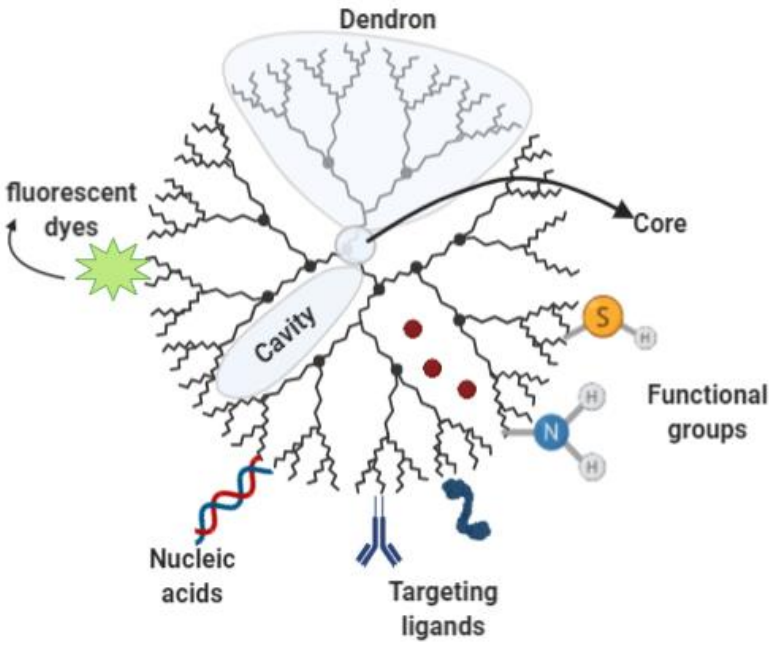

Fig. 2.6: Dendrimer structure and functionalization Schematic representation of a Dendron comprising Dendron units branching out of a focal core interspaced by cavities. Bioactive cargo can be encapsulated into cavities. Dendron ends can be functionalized allowing targeting ligand attachment, fluorophore molecules, nucleic acids among other molecules of interest.

Dendrimer-based non-viral vectors for gene delivery have gained traction over the past two decades, especially in the field of biomedicine for cancer treatment. In plants, the use of cationic polyamidoamine (PAMAM) vector assisted by ultrasound has been used for DNA delivery. Amani et al, demonstrated in alfalfa cells, that single and double-stranded DNA transfection efficiency can be significantly improved when PAMAM dendrimers are used assisted by sonication (Amani et al., 2018). Production of dendrimers can be approached in two different ways: convergent approach and divergent approach, each with its own limitations (Gupta and Nayak, 2015). Although the main applications of dendrimers are in drug/gene delivery for biomedical applications (Mendes et al., 2017), several other applications exist (Abbasi et al., 2014).

Dendrimers are also useful for agricultural purposes. They may improve the delivery of agrochemicals intended to either promote growth or discourage diseases. For instance, in 2016, a crop protection company (Adama) licensed Starpharma's (ASX:SPL) Priostar dendrimer technology for the development of an enhanced 2,4-D herbicide for the US market. According to the manufacturer, some of the potential benefits from the use of dendrimer technology in crop protection include improved efficacy, more concentrated formulations to reduce transport costs, reduction in solvent requirements and increased adhesion. Stapharma's lysine dendrimer based Vivagel® managed to achieve clinical approval (Moura et al., 2019) which indicates the technological feasibility of mass-production. However, the technical details regarding up-scaled production are not publicly available.

The use of dendrimers for crop protection faces the challenge impose by mass-production. The main challenge relies on preserving their purity and monodispersity upon up-scaled manufacture. Technical details with respect to improved reaction conditions and purification of half- and full-generation PAMAM dendrimers to overcome the critical limitations for upscaling this class of polymers are available elsewhere (Ficker et al, 2017).

2.3.7 Nanocrystals

Nanocrystals are another nanotechnological approach to deliver poorly soluble drugs. In contrast to the prior mentioned drug delivery system platforms, nanocrystals have several unique traits. Drug delivery nanocrystals are carrier-free colloidal delivery systems (i.e. they are almost 100% drug). Thus, drug nanocrystals possess the merits of improving the oral bioavailability of water-insoluble compounds, reducing administered dose, avoiding abnormal absorption thus minimizing utilization of large excipients, increasing dissolution velocity, increasing adhesiveness to surface cell membranes, and increasing particle stability (Wang et al., 2011). Conventionally, drug nanocrystals can be produced whether from a top-down or a bottom-up approach. The demand for energy, time and money is high for top-down approaches such as milling or high-pressure homogenization. For instance, high-pressure methods require specialized equipment able to deliver up to 1700 bar for over one hundred homogenization cycles, and the milling method requires hours if not days to achieve the desired particle size, depending on the drug properties (Lu et al., 2016). Moreover, the grinding process may contaminate or denature labile drugs which may

lead to unexpected side effects on the recipient patient. Further concerns exist on the potential loss of bioactivity and molecular integrity due to severe thermogenesis derived from the milling process. Other disadvantages for the top-down methods are: The lack of complete control of the morphology and crystallinity of the final product; particle aggregation/agglomeration issues; losses of the product due to drug adherence to equipment surfaces and residual presence of surfactants, solvents or stabilizers (Padrela et al., 2018).

In comparison, bottom-up processes are achieved through nucleation and subsequent crystallization. One way to achieve nucleation is by mixing the drug with an antisolvent by simple stirring. Another way is to remove the solvent via spray and freeze-drying. Subsequent crystallization does require high energy methods such as sonication or intense micro-stirring (Lu et al., 2016). Another approach to producing drug nanocrystals is based on supercritical carbon dioxide ($ssCO_2$). The details on the roles of $ssCO_2$ as solvent, co-solvent and as an additive for the production of drug nanocrystals are comprehensively reviewed elsewhere (Padrela et al., 2018). Amongst the main disadvantages of the bottom-up methods to produce drug nano-crystals are: i) the difficulty to control the particle size, nucleation, and growth of crystals that may lead to both, undesired morphologies or amorphous crystallinities and subsequent particle agglomeration; ii) the need for large amounts of organic solvents; iii) fine-tuning solvent/antisolvent formulation is time-consuming; iv) need for solvent removal; v) labile drugs may denature during heating solvent removal; vi) need for specialized equipment for $ssCO_2$-based nanocrystals (Padrela et al., 2018).

The use of nanocrystals in agriculture has enormous potential for sustained and efficient nutrient delivery into crops. For instance, nitrogen can be applied in the form of Urea-Hydroxyapatite nanohybrids. When tested in rice fields, urea-hydroxyapatite nanohybrids significantly enhanced nitrogen bioavailability, resulting in higher crop yields, while reducing the nitrogen input up to 50%, when compared to granular urea (Kottegoda et al., 2017). The efficacy of hydroxyapatite nanoparticles as Phosphorus fertilizer has also been studied in andisols and oxisols. Montalvo et al., showed that the effect of phosphorus in the form of hydroxyapatite nanoparticles, in the wheat dry matter production significantly depends on the type of soil these particles are applied on. Nano-Hydroxyapatite in strongly phosphorous sorbing soils had more effect on shoot dry matter production and plant phosphorous uptake than bulk-HAP but less than the water-soluble triple superphosphate. This is maybe due to the propensity of nano-hydroxyapatite to aggregate, thus

reducing both the mobility and the dissolution rate of the particles (Montalvo et al., 2015). Since nano-nutrient/soil particle interaction is strongly affected by the intrinsic heterogeneity of the soil, it is reasonable to study alternative nano-nutrient up-take pathways in plants. In a recent study, Avellan et. al., analyzed how nano-crystals move throughout the plant, from the leaves to the roots, using gold nanoparticles as a model in wheat. They found that "regardless of their coating and sizes, the majority of the transported AuNPs accumulated in younger shoots (10–30%) and in roots (10–25%), and 5–15% of the NPs <50 nm were exuded into the rhizosphere soil. A greater fraction of larger sizes AuNPs (presenting lower ζ potentials) was transported to the roots" (Avellan et al., 2019).

Accounting for these disadvantages, scaling up of its production has been a challenge. It is also worth noting that there is a lack of cytotoxicity studies, and the details of the intracellular fate of the nanocrystals are not well understood (Junyaprasert and Morakul, 2015).

2.3.8 Nanogels

Nanogels are hydrophilic cross-linked networks forming polymer chains that absorb substantial amounts of aqueous solutions. Due to their conformational tridimensional structure, hydrogels are capable of imbibing bioactive molecules solubilized in water or aqueous fluids. The presence of chemical crosslinks (tie-points or junctions) or physical crosslinks, such as entanglements or crystallites, are responsible for their characteristic conformational structure and size (Himi and Maurya, 2013), which can be fine-tuned via chemical control of the formulation and the process to obtain the hydrogel nanoparticles. The main advantages of hydrogels, when used as drug delivery systems, is their complete biocompatibility due to their high content of water (Caló and Khutoryanskiy, 2015). On the other hand, one of the major drawbacks of these types of particles is the batch-to-batch variation due to the heterogeneity of the polymer itself, such as the case for chitosan-based drug delivery systems.

Coacervation or ionic gelation method is one of the most common processes carried out to produce this type of nanoparticles because it is easy to implement and requires un-expensive materials. In general, the process involves the mixture of two aqueous phases, where one of which is the polymer and the other is the dissolved cross-linker. It is common to use an oil/water emulsion as one of the aqueous phases containing the bioactive hydrophobic molecule or drug of interest to be

encapsulated within the forming capsule. The method is relatively easy to perform since it does not require sophisticated equipment, which is imperative for the scaling up. However, the final characteristics of the produced nanoparticles, such as size, polydispersity, and stability, are highly sensitive to changes in the fabrication conditions, such as pH, ionic strength, stirring speed, addition rate and type and concentration of polymers and cross-linkers. Chitosan-based nano-carriers are of special interest in agriculture. However, the literature regarding both, nanoparticle formation via ionic gelation and cargo release profile, is overwhelmingly inconsistent (Huang et al., 2015) (Cai and Lapitsky, 2019). An example is illustrated in figure 7 showing factors that influence chitosan nanoparticles' formation and stability.

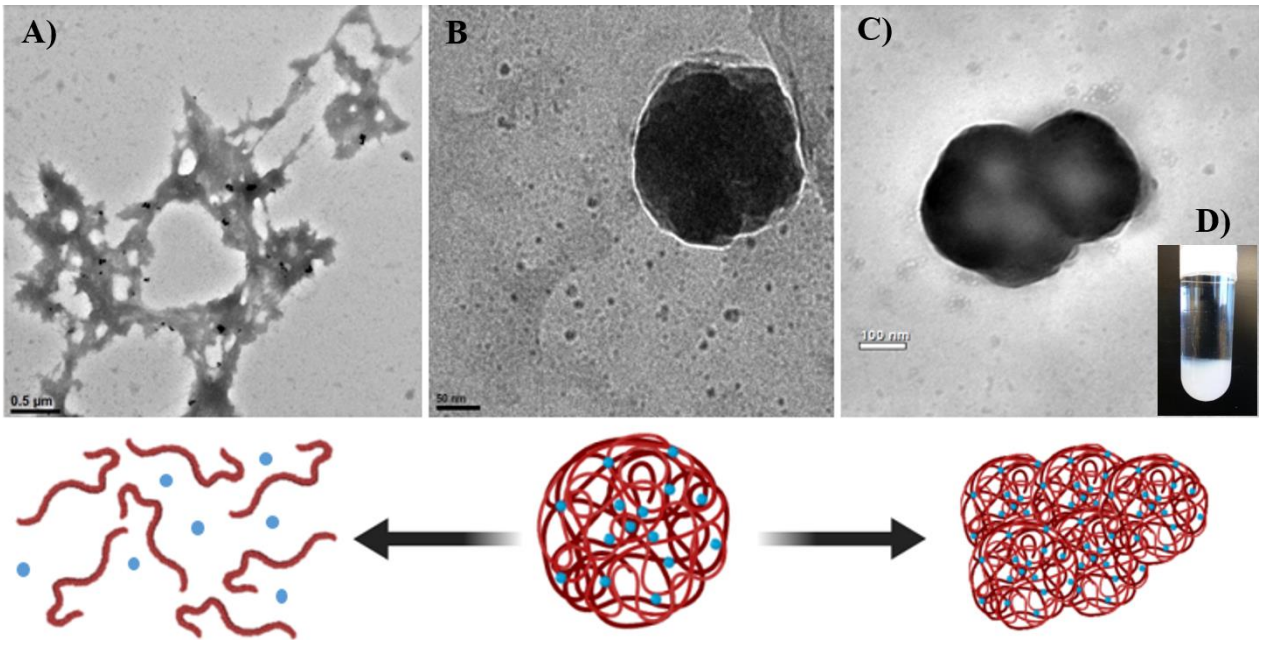

Fig. 2.7: Factors influencing formation and stability of chitosan-based nanoparticles mediated by the cross-linker tripolyphosphate (TPP). Formation and stability of chitosan-based nanoparticles are sensitive to formulation and preparation conditions. A) When the amount (per mole) of cross-linker (TPP) is insufficient relative to the amount (per mole of NH_3^+ from chitosan), chitosan particles (b) rapidly dissolve at pH levels below its pKa. When the pH of the solution is not acid enough, amino groups from chitosan deprotonate preventing chitosan to dissolve and then failing to form electrostatic interactions with the crosslinker, resulting in particle dissolution and ulterior precipitation. B) Excess of crosslinker in the solution result in particle aggregation and d) further precipitation

Typical applications of hydrogels revolve around the biomedical field, including drug encapsulation, transport and delivery; tissue engineering for wound-healing treatment; and 3-D cell culture. Nonetheless, hydrogels can also be used as antimicrobial agents. Chitosan, for instance, is a polymer commonly used to fabricate nano-carriers, naturally displays antimicrobial

activity. Metal ions, such as Ti3+, Fe3+, Ag+, Cu2+, and Zn2 can also be incorporated into non-antimicrobial hydrogels in order to confer antimicrobial properties. Incorporation of some metallic ions can also confer catalytic, photo-responsive, photochemical, redox and conductive properties to hydrogels (Wahid et al., 2017). In agriculture, the use of chitosan nanoparticles are of special interest due to its immune-modulatory activity elicited in plants. Chitin is a pathogen-associated molecular pattern (PAMP), detected by a transmembrane chitin receptor (LysM/CERK1) in plant cells. Sensing chitosan triggers an intracellular defense immune response (i.e. PTI – Pathogen triggered immunity) involving the activation of kinases and up-regulation of defense-related genes, such as plant defensin PDF1.2, resulting in jasmonic acid and ethylene accumulation associated with immunity to necrotrophs (Malerba and Cerana, 2016) (Mengiste, 2012).

2.4 Drug delivery systems in the agriculture

According to the United Nations, the estimated world population projected for 2050 will be 9.7 billion people. The increasing world population brings challenges that may imbalance the food production chain at various levels such as social, economic, technologic and environmental. Efforts to find new strategies that will allow improving the quantity and quality of food supply under a scheme of sustainability are imperative to meet the demands of the incoming population. The application of engineered nano-carrier devices, intended for the delivery of encapsulated molecules, could be a promising alternative to meet the future agriculture needs of increased productivity. Phytonanotechnology (i.e. the application of nanotechnology in plants) may improve the way we grow crops. Nano-delivery systems enable the controlled release of agrochemicals (e.g., fertilizers, pesticides, and herbicides) and target-specific delivery of biomolecules (e.g., nucleotides, proteins, and activators) (Wang et al., 2016) (see Figure 8 and Table No.3).

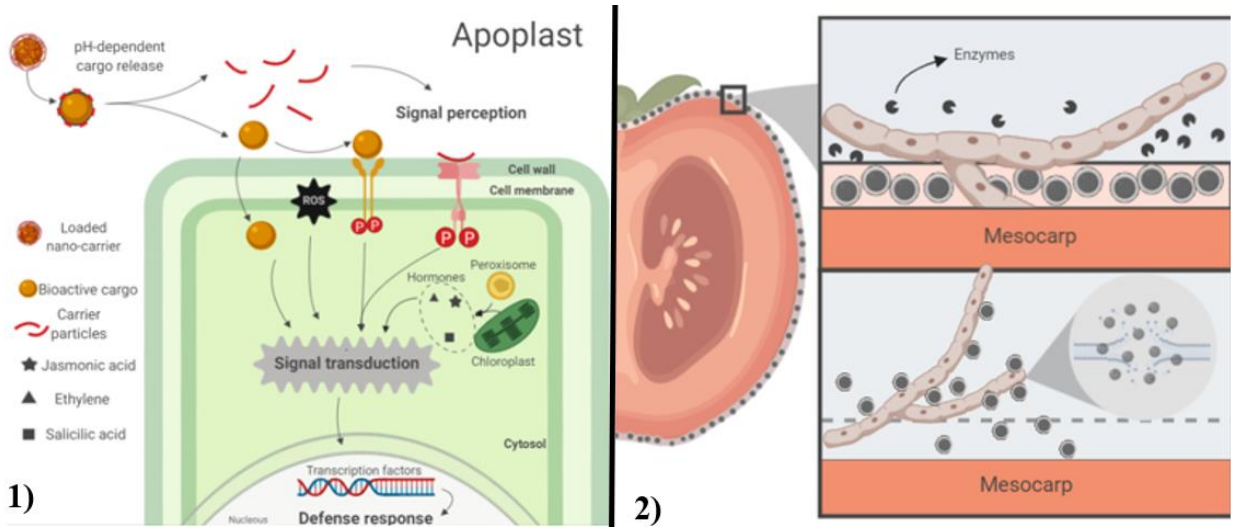

Fig. 2.8: Schematic representation of the potential mode of action of drug-nanocarriers applied in the agriculture: 1) Example of a potential mode of action of drug nano-carriers for systemic protection of plants: A pH-sensitive polymeric loaded nano-carrier enters the plant apoplast and releases its cargo. The bioactive payload may enter to the plant cell cytosol or b) Activate a signaling cascade upon recognition by a transmembrane receptor triggering the plant defense immune response. Carrier molecules (e.g. chitosan) can also elicit an immune response in plants upon recognition by receptors. 2. Example of a potential mode of action of drug nano-carriers for post-harvest produce protection: **2a:** Fungal extracellular enzymes degrade an edible coating with embedded nano-carriers**. 2b:** Drug nano-carriers loaded with antimicrobial compounds are released from the coating. Fungal membrane is disrupted by direct contact with antimicrobial compounds**.**

Table 2.3: **Some commercial product of nanofertilizers.**

Commercial product	Content	Company
Nano-Gro™	Plant growth regulator and immunity enhancer	Agro Nanotechnology Corp., FL, United States
Nano Green	Extracts of corn, grain, soybeans, potatoes, coconut, and palm	Nano Green Sciences, Inc., India
Nano-Ag Answer®	Microorganism, sea kelp, and mineral electrolyte	Urth Agriculture, CA, United States
Biozar Nano-Fertilizer	Combination of organic materials, micronutrients, and macromolecules	Fanavar Nano-Pazhoohesh Markazi Company, Iran

Table 2.3 continued

Nano Max NPK Fertilizer	Multiple organic acids chelated with major nutrients, amino acids, organic carbon, organic micro nutrients/trace elements, vitamins, and probiotic	JU Agri Sciences Pvt. Ltd, Janakpuri, New Delhi, India
Master Nano Chitosan Organic Fertilizer	Water soluble liquid chitosan, organic acid and salicylic acids, phenolic compounds	Pannaraj Intertrade, Thailand
TAG NANO (NPK, PhoS, Zinc, Cal, etc.) fertilizers	Proteino-lacto-gluconate chelated with micronutrients, vitamins, probiotics, seaweed extracts, humic acid	Tropical Agrosystem India (P) Ltd, India

Source: Ram Prasad, Atanu Bhattacharyya et al.Frontiers in Microbiology, 8, JUN, 6 2017 (creativecommons.org/licences/by/4.0)

Nano-encapsulated pesticides offer enhanced controlled release of cargo and enhanced efficacy. Regarding the development of nano-pesticides is worth noting that a common practice in this industry is to focus on the modification of already registered existing molecules, rather than discovering new molecules. This is due to the costs associated with the development and further registration which is a process often measured in years. A commercially available capsule suspension insecticide (Environmental Protection Agency (EPA) Reg. No. 67760-104-53883) with 5.9% γ-cyhalothrin; and an EPA registered capsule suspension insecticide with 22.8% λ-cyhalothrin (EPA Reg. Number 100-1295, Greensboro, NC, USA) are two examples of nano-pesticides currently available in the market under this reformulation scheme (Slattery et al., 2019) (Meredith et al., 2016).

2.4.1 Nano-carriers as a non-viral vector for gene delivery in plant cells

In order to obtain higher crop production yields, it is necessary to develop new plant varieties by introducing traits that ideally enables them to better resist different environmental-derived abiotic stresses or pathogen-mediated diseases along with the generation of higher biomass under limited resources. The transfer of genes to the target plant cells is challenging due to the rigid plant cell

wall which prevents the exogenous particle movement from the outside to the cytoplasm (Abd-Elsalamet.al., 2014). There is evidence that the plant's nano-particle uptake is strongly dependent on the cell wall pore diameter (i.e. exclusion size limit), which may vary amongst different tissues and organs. In general, the plant cell wall's exclusion size limit is up to 50nm (Cunningham et al., 2018). Due to its small size, nanoparticle-enabled gene delivery into plant cells pose a promising option for genetic engineering for agriculture. The first reported example of this was done by Torney et al., who managed to develop a 3-nanometer pore mesoporous nanoparticle (MSN) able to transport DNA and chemicals into isolated plant cells that interact with leaves. MSN were designed in such way that gold nanoparticles capped the pores in order to avoid cargo leakage and release the content in the intended target to trigger gene expression under controlled-release conditions. (Torney et al., 2007).

For plant genetic recombination purposes, exogenous gene delivery into plant cells is required. In animal models, nanoparticle penetration into cells is often reported to be improved when mediated with ultrasound. Ultrasound-assisted gene delivery is in use for plants because of its easier operation, lower cost and no plant specificity constraints among others (Liu et al., 2005). Nevertheless, the main disadvantage of ultrasound-mediated technique is that naked DNA is highly sensitive to external high energy sources and as a result, it may suffer damage, especially when increasing ultrasonic strength and time to achieve high transfection efficiency; so the ultrasound-mediated transgenic method has been largely restricted in practice (Yu-qin et al., 2012). Interestingly, DNA-nanoparticle complexes can protect DNA from ultrasound damage as well as from enzymatic degradation (Liu et al., 2005). DNA-nanoparticle complexes that have been studied before included Zinc and Calcium phosphate (Yu-qin et al., 2012) (Naqvi et al., 2012).

Foreign particle uptake in plants can naturally occur either via endocytosis or by direct penetration. In plants, different engineered nanomaterials can be used for nanoparticle-mediated DNA transfer using gene-nanoparticle (NP) anchoring using zinc, calcium phosphate, silica, gold, magnetite, strontium phosphate, magnesium phosphate and manganese phosphate (Sokolova and Epple, 2008) (Rai et al., 2012) and carbon-based materials such as starch (Sun et al., 2009) fullerenes, single-walled carbon nanohorns (SWCNHs), single-walled carbon nanotubes (SWCNTs), multi-walled carbon nanotubes (MWCNTs) (Burlaka et al., 2015) and dendrimers. However, it has been reported that nanoparticle uptake by plant cells undergoes faster when positively charged

nanoparticles are used rather than negatively charged nanoparticles, perhaps due to the preference of the negatively charged cell wall for cations (Cunningham et al., 2018).

Chitosan-based nano-carriers are a promising platform for cargo delivery into plant cells because it is positively charged, amongst other advantages it has. A recent study demonstrated organelle-targeted delivery and transient expression of genetic material via chitosan-complexed single-walled carbon nanotube carriers. Successful transformation of chloroplasts was achieved in mature *Eruca sativa, Nasturtium officinale, Nicotiana tabacum,* and *Spinacia oleracea* plants and in isolated *Arabidopsis thaliana* mesophyll protoplasts.

Since the plastid genome is maternally inherited in most plants, organelle-specific gene delivery is important because it can prevent the potential proliferation of genes to weedy relatives (Kwak et al., 2019). In this specific study, the authors showed that chitosan-complexed single-walled carbon nanotubes (SWNTs) uptake mechanism was described by the lipid exchange envelope penetration (LEEP) model, whereby the ability of nanoparticles to penetrate the cell membrane and the chloroplast envelope is governed primarily by the nanoparticle size and surface charge (Kwak et al., 2019)

In conclusion, nanoparticle assisted gene delivery systems initially developed for medical purposes has been shown to display the same delivery function in plant cells. It is worth noting that the individual performance of DNA delivery into plant cells must be evaluated on a case to case basis since the results presented in literature has several inconsistencies related to the transformation efficiencies achieved by different materials on different plant models. However, the evidence suggests that the concept of non-viral gene delivery into plant cells is promising. The specific design of nanoparticles should respond to the specific demands of the plant model/gene to be transferred, therefore no universal or generic delivery system for gene delivery into plants has been developed.

2.4.2 Nano-delivery systems for Nutrition and growth promotion in plants

Commercial fertilizers play a critical role in improving crop yields, however inherent inefficiencies derived from the nature of the soil, plant health, environmental conditions, or the fertilization method among other factors, can lead to dire negative economic and environmental consequences

that may endure in the long term. Not all the nutrient ions in fertilizer applied to a field soil are uptaken by the growing crop. At least three things can happen to the remaining residues from chemical fertilization: They may persist in the soil or, washed away by water leaching through the soil either downwards or throughout the surface or, lost to the atmosphere by volatilization.

In particular, higher than optimum nitrogen, phosphorus and potassium levels can lead to excessive plant and algal growth in waterways that can degrade potable water, fisheries, and recreational areas; leach nitrates into underground or sea waters and release nitrogen-oxides into the atmosphere. Phosphorous losses are also a major environmental concern derived from excessive fertilization in agriculture. It is estimated that the overall efficiency of applied phosphorus to the soil is less than 20% (Balemi and Negisho, 2012). Nutrient depletion leads to a variety of plant symptoms which affects the overall yield of a crop. Similarly, over-fertilization leads to an ecological imbalance which is hard to restore. Excessive soluble salts from fertilizers alter soil salinity, which in turn alters the soil pH; lower pH values diminish the availability of nutrients to plants by causing an imbalance in the soil native microbial ecology, responsible for nutrient solubilization.

Excessive fertilization is common due to soil nutrient heterogeneity. Overfertilization releases to the environment nutrients that cause, for instance, eutrophication of water bodies. Estimated losses of nitrogen, phosphorus and potassium are around 40–70 %, 80–90 %, and 50–90 % respectively. In a practical scenario, very less concentration (much below to minimum desired concentration) reaches the targeted site due to leaching of chemicals, drift, runoff, evaporation, hydrolysis by soil moisture, and photolytic and microbial degradation losses (. Thus, nano-delivery systems for controlled release emerge as a highly valuable technology with the potential to strengthen the responsive capabilities of a sustainable food chain supply.

The application of nanotechnology for fertilizer delivery is encouraging. Patent applications related to nano-fertilizers are growing consistently according to the world intellectual property organization database. a 10% increment in patent filings related to nano-fertilizers from China, in a period of fewer than 3 years (01/2014 -11/2016). This is consistent with data reported by Mastronardi et al who noted a 10x (c.a) increase in patent results (Ref. SciFinder) over a 10-year period from 2002 to 2012 (Mastronardi et. al., 2015). Current applications of nanotechnology in

fertilization and plant protection can be divided into three different categories: 1) Nanoscale fertilizer inputs, which describes examples of nano-sized reformulation of fertilizer input in such a way that the size of the fertilizer or supplement is reduced down to nano-scale. 2) Nanoscale additives, which include the additives presented as nanoparticles and added to bulk materials, and 3) nanoscale coatings or host materials for fertilizers, which includes nano-thin films or nanoporous materials used to encapsulate fertilizers for the controlled release of nutrients in crops. (Mastronardi et al., 2015).

Current applications of nanotechnology in fertilization and plant protection can be divided into three different categories 1. Nanoscale fertilizer inputs, which describe examples of nano-sized reformulation of fertilizer input in such a way that the size of the fertilizer or supplement is reduced down to the nano-scale. 2. Nanoscale additives, which include the additives presented as nanoparticles and added to bull materials. And 3, nanoscale coatings or host materials for fertilizers, which include nano-thin films or nanoporous materials used to encapsulate fertilizers for the controlled release of nutrients in crops (E.Mastronardi et al. 2015). Gao et.al., working with spinach, have shown an enhancement of plant growth when titanium dioxide nanoparticles (TiO2-NPs) were administered to the seeds or when they were sprayed onto the leaves. TiO2-NPs were shown to increase the activity of several enzymes and promote the adsorption of nitrate, which accelerated the transformation of inorganic nitrogen into organic nitrogen (Gao et al., 2008). The current understanding of the mechanisms involved in nanoparticle uptake and translocation from leaves to roots were discussed earlier in this document (see section 3.7).

2.4.3 The importance of nano-delivery systems for disease and pests control in crops

In 1985, Pimentel and Levitan reported that approximately 500 million kilograms of pesticides were applied to plants in the United States (U.S) each year, but only 0.1% of this reach its desired target to effectively eliminate pests (Pimentel and Levitan, 1986). Over twenty-five years later, in 2011, Pimentel and Burgess reinforced this statement, stating that 545 million kilograms of pesticides were applied to crops in the United States each year, and several applications show that less than 0.1% of these pesticides reach their target (Pimentel and Burgess, 2012). The use of pesticides including herbicides, insecticides, and fungicides is consistently increasing worldwide, but nowadays, we do not know exactly by how much. According to the United States Department

of Agriculture (USDA), the total pesticide expenditures in U.S. agriculture reached close to $12 billion in 2008, a 5-fold increase in real terms (adjusted for inflation) since 1960, but well below the $15.4-billion peak reached in 1998 (Fernandez-Cornejo et al., 2014). The most recent report about pesticide usage dates to 2017 covering data from 2008 to 2012. According to the report, by 2012 over an estimated 380.000 tons were used in the US, from which 282.000 tons were herbicides, with a total expenditure over 9 billion $US (Atwood and Paisley-Jones, 2017). The lack of up-dated data reports in this regard makes it difficult to enable an informed pesticide policy debate, as well as sway science-based decisions in the right direction.

It is conceivable that improving the targeting and accuracy of pesticides could substantially reduce the amount of toxic chemicals that are applied to crops and improve the yield and safety of agriculture. Ideally, a pesticide should be able to remain active regardless of the environmental conditions in order to perform its intended biocide action. Correspondingly, it should also overcome the defense mechanisms from the pest it must target, it should also be harmless to the surrounding flora and fauna, and be engineered in such a way that it can be mass-produced at the lowest possible cost in order to guarantee economic returns to farmers. Current pesticides fail to completely fulfill these requirements, which results in more frequent and higher doses application schemes, and therefore, higher economic and environmental costs. Nanomaterials used as a pesticide or as a carrier material have exhibited functional properties such as stiffness, permeability, crystallinity, thermal stability, and biodegradability over commonly used pesticides (Bordes et al., 2009).

Increasing wealth of knowledge in the literature regarding the development and use of pesticide-loaded nano-carriers intended for crop protection supports the importance of this technology towards sustainable agriculture by increasing the potency and bioavailability of pesticides, thus reducing the total amount of agrochemicals released in the environment. Pesticides such as β-cypermethrin (an insecticide) (Lijuan et. al., 2007), tebuconazole (a fungicide) (Díaz-Blancas et al., 2016), and atrazine (a herbicide) (Oliveira et al., 2015) presented as nano-encapsulated formulations are some examples of the potential use of nano-carriers to enhance the biological activity of active ingredients and also increase their stability over time. Zhao et al, (2017) demonstrated that it is feasible to develop a nano-emulsified pesticide displaying not only high stability over time (90 days) but also stronger absorption on negatively charged surfaces, which

are desirable characteristics for spray-based foliar applications of pesticides in crops (Zhao et al., 2017)

2.5 Prospects of nano-delivery system technology in agriculture

Based on the data collected from the literature, we expect at least two main positive impacts of the extended, prolonged and improved use of nano-delivery technology translocated into the food production chain. The first is related to the technical aspects of pesticide usage. Similar to the role they play in the medical field, nano-delivery systems can increase the controlled-release properties of the pesticide, increased solubility of active ingredients, protection against premature degradation and increase the stability of active ingredients. Another advantage is that non-target surrounding or distant flora and fauna will be less affected as a result of reducing exposure to toxic chemical compounds. In addition, the technical constraints concerning the massive production of nano-carriers for use in agriculture should be correlated with the economical boundaries which limit the production costs and configures the potential revenues for producers. Additional studies are required to assess, not only the fate of nano-encapsulation materials and payloads, and the resulting physical-chemical and biological performance, but the long-term environmental risks and economic viability.

2.6 Conclusions

It is clear that there is an immense need to develop methods or technologies that allow us to cope with the contrasting challenges of the food supply chain. For instance, the toxicity threshold of materials used in the delivery system is species-dependent and responses to these are driven by a series of factors including not only the nanomaterial itself but the environmental and physiological conditions on which they are applied. Another noteworthy factor is the broader impact of the delivery system to the environment, while in the medical systems, it is localized to the individual receiving the treatment. Impacts on plant growth, and therefore on product yield and food quality, have been reported. However, several gaps exist in understanding the dynamics of interactions between plants and engineered nanomaterials (ENMs). Given the lack of experimental standardization and the divergent responses, even within similar plant species, it is challenging to foresee the challenges on the use of ENMs in plants. (Zuverza-Mena et al., 2016). Finally, there is

an imperative need to standardize and validate protocols to assess the positive and negative impact of nano-carriers in an experimental setting, and scale-up of testing can yet be another challenge. Most of the currently available information stems from experiments under controlled conditions, making it difficult to predict the real potential of functional prototypes. Research efforts could focus on controlled release, particle stability, and environmental fate and toxicity to make this a fully-embedded technology.

2.7 Conflict of Interest

The submitted work was not carried out in the presence of any personal, professional or financial relationships that could potentially be construed as a conflict of interest.

Author Contributions

The initial framework and idea were conceptualized by Vega and Irudayaraj. All authors contributed towards the writing of this article.

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2.8 References

Abbasi, E., Aval, S. F., Akbarzadeh, A., Milani, M., Nasrabadi, H. T., Joo, S. W., et al. (2014). Dendrimers: synthesis, applications, and properties. *Nanoscale Res. Lett.* 9, 247. doi:10.1186/1556-276X-9-247.

Abd-Elsalam, K. A., and Alghuthaymi, M. A. (2015). Nanobiofungicides: Are they the Next Generation of Fungicides? *J. Nanotechnol. Mater. Sci.* 2. doi:10.15436/2377-1372.15.0.

Ahmed, A., Myers, P., and Zhang, H. (2014). Synthesis of Nanospheres-on-Microsphere Silica with Tunable Shell Morphology and Mesoporosity for Improved HPLC. doi:10.1021/la503015x.

Amani, A., Zare, N., Asadi, A., and Asghari-Zakaria, R. (2018). Ultrasound-enhanced gene delivery to alfalfa cells by hPAMAM dendrimer nanoparticles. *Turkish J. Biol.* doi:10.3906/biy-1706-6.

Atwood, D., and Paisley-Jones, C. (2017). Pesticides Industry Sales and Usage. *United States Environ. Prot. Agency*, 24. Available at: https://www.epa.gov/sites/production/files/2017-01/documents/pesticides-industry-sales-usage-2016_0.pdf.

Avellan, A., Yun, J., Zhang, Y., Spielman-Sun, E., Unrine, J. M., Thieme, J., et al. (2019). Nanoparticle Size and Coating Chemistry Control Foliar Uptake Pathways, Translocation, and Leaf-to-Rhizosphere Transport in Wheat. *ACS Nano* 13, 5291–5305. doi:10.1021/acsnano.8b09781.

Balemi, T., and Negisho, K. (2012). Management of soil phosphorus and plant adaptation mechanisms to phosphorus stress for sustainable crop production: A review. *J. Soil Sci. Plant Nutr.* 12, 547–561. doi:10.4067/S0718-95162012005000015.

Bordes, P., Pollet, E., and Avérous, L. (2009). Nano-biocomposites: Biodegradable polyester/nanoclay systems. *Prog. Polym. Sci.* 34, 125–155. doi:10.1016/j.progpolymsci.2008.10.002.

Burlaka, O. M., Pirko, Y. V., Yemets, A. I., and Blume, Y. B. (2015). Plant genetic transformation using carbon nanotubes for DNA delivery. *Cytol. Genet.* 49, 349–357. doi:10.3103/S009545271506002X.

Cai, Y., and Lapitsky, Y. (2019). Pitfalls in analyzing release from chitosan/tripolyphosphate micro- and nanoparticles. *Eur. J. Pharm. Biopharm.* 142, 204–215. doi:10.1016/J.EJPB.2019.06.020.

Caló, E., and Khutoryanskiy, V. V. (2015). Biomedical applications of hydrogels: A review of patents and commercial products. *Eur. Polym. J.* 65, 252–267. doi:10.1016/j.eurpolymj.2014.11.024.

Chen, S., Zhao, X., Chen, J., Chen, J., Kuznetsova, L., Wong, S. S., et al. (2010). Mechanism-based tumor-targeting drug delivery system. Validation of efficient vitamin receptor-mediated endocytosis and drug release. *Bioconjug. Chem.* 21, 979–87. doi:10.1021/bc9005656.

Choudhury, Samrat Roy; Ordaz, Josue; Lo, Chiao Ling; Damayanti, Nur P; Zhou, Feng; Irudayaraj, J. (2017). Zinc oxide nanoparticles-induced reactive oxygen species promotes multimodal cyto- and epigenetic toxicity. *Toxicol. Sci.* 156, 261–274. doi:10.1093/toxsci/kfw252.

Colombo, M., Mizzotti, C., Masiero, S., Kater, M. M., and Pesaresi, P. (2015). Peptide aptamers: The versatile role of specific protein function inhibitors in plant biotechnology. *J. Integr. Plant Biol.* 57, 892–901. doi:10.1111/jipb.12368.

Cunningham, F. J., Goh, N. S., Demirer, G. S., Matos, J. L., and Landry, M. P. (2018). Nanoparticle-Mediated Delivery towards Advancing Plant Genetic Engineering. *Trends Biotechnol.* 36, 882–897. doi:10.1016/j.tibtech.2018.03.009.

Díaz-Blancas, V., Medina, D., Padilla-Ortega, E., Bortolini-Zavala, R., Olvera-Romero, M., Luna-Bárcenas, G., et al. (2016). Nanoemulsion Formulations of Fungicide Tebuconazole for Agricultural Applications. *Molecules* 21, 1271. doi:10.3390/molecules21101271.

Ding, Y., Jiang, Z., Saha, K., Kim, C. S., Kim, S. T., Landis, R. F., et al. (2014). Gold nanoparticles for nucleic acid delivery. *Mol. Ther.* 22, 1075–83. doi:10.1038/mt.2014.30.

Donsì, F. (2018). "Applications of Nanoemulsions in Foods," in *Nanoemulsions: Formulation, Applications, and Characterization*, 349–377. doi:10.1016/B978-0-12-811838-2.00011-4.

Eltayeb, M., Bakhshi, P. K., Stride, E., and Edirisinghe, M. (2013). Preparation of solid lipid nanoparticles containing active compound by electrohydrodynamic spraying. *Food Res. Int.* 53, 88–95. doi:10.1016/J.FOODRES.2013.03.047.

FDA (2012). Guidance for industry. Drug interaction studies study design, data analysis, implications for dosing, and labeling recommendations. *Guid. Doc.*, 79.

Felice, B., Prabhakaran, M. P., Rodríguez, A. P., and Ramakrishna, S. (2014a). Drug delivery vehicles on a nano-engineering perspective. *Mater. Sci. Eng. C* 41, 178–195. doi:10.1016/j.msec.2014.04.049.

Felice, B., Prabhakaran, M. P., Rodríguez, A. P., and Ramakrishna, S. (2014b). Drug delivery vehicles on a nano-engineering perspective. *Mater. Sci. Eng. C*. doi:10.1016/j.msec.2014.04.049.

Fernandez-Cornejo, J., Nehring, R., Osteen, C., Wechsler, S., Martin, A., and Vialou, A. (2014). Pesticide Use in US Agriculture: 21 Selected Crops, 1960-2008, EIB-124. *U.S. Dep. Agric. Econ. Res. Serv*. May, 1960–2008.

Ficker, M., Paolucci, V., and Christensen, J. B. Improved large-scale synthesis and characterization of small and medium generation PAMAM dendrimers. doi:10.1139/cjc-2017-0108.

Foucquier, J., and Guedj, M. (2015). Analysis of drug combinations: current methodological landscape. *Pharmacol. Res. Perspect.* 3, e00149. doi:10.1002/prp2.149.

Godfroy, I. (2009). Polymeric Micelles – The Future of Oral Drug Delivery. *J. Biomater. Appl. Rev.* 3, 216–232. doi:10.1351/pac200476071321.

Granata, G., Stracquadanio, S., Leonardi, M., Napoli, E., Consoli, G. M. L., Cafiso, V., et al. (2018). Essential oils encapsulated in polymer-based nanocapsules as potential candidates for application in food preservation. *Food Chem.* 269, 286–292. doi:10.1016/j.foodchem.2018.06.140.

Guo, C., and Irudayaraj, J. (2011). Fluorescent Ag Clusters via a Protein-Directed Approach as a Hg(II) Ion Sensor. *Anal. Chem.* 83, 2883–2889. doi:10.1021/ac1032403.

Gupta, V., and Nayak, S. K. (2015). Dendrimers: a Review on Synthetic Approaches. *J. Appl. Pharm. Sci.* 5, 117–122. doi:10.7324/JAPS.2015.50321.

Himi, M., and Maurya, S. D. (2013). Review Article Preparation and Evaluation of Stomach Specific Ipn Hydrogels for Oral Drug Delivery : a Review. 3, 131–140.

Hoffman, A. S. (2008). The origins and evolution of “controlled” drug delivery systems. *J. Control. Release* 132, 153–163. doi:10.1016/j.jconrel.2008.08.012.

Huang, Y., Cai, Y., and Lapitsky, Y. (2015). Factors affecting the stability of chitosan/tripolyphosphate micro- and nanogels: Resolving the opposing findings. *J. Mater. Chem. B* 3. doi:10.1039/C5TB00431D.

Iqbal, S., and Yun, J.-I. (2018). Decontamination of radionuclides by functionalized mesoporous silica under gamma irradiation. *RSC Adv.* 8, 32211–32220. doi:10.1039/C8RA05939J.

Jaromin, A., Zarnowski, R., Pi, M., Etka-Ottlik, ˛, Andes, D. R., and Gubernator, J. (2018). Topical delivery of ebselen encapsulated in biopolymeric nanocapsules: drug repurposing enhanced antifungal activity. *Nanomedicine*. doi:10.2217/nnm-2017-0337.

Joo, K. Il, Xiao, L., Liu, S., Liu, Y., Lee, C. L., Conti, P. S., et al. (2013). Crosslinked multilamellar liposomes for controlled delivery of anticancer drugs. *Biomaterials* 34, 3098–3109. doi:10.1016/j.biomaterials.2013.01.039.

Junyaprasert, V. B., and Morakul, B. (2015). Nanocrystals for enhancement of oral bioavailability of poorly water-soluble drugs. *Asian J. Pharm. Sci.* 10, 13–23. doi:10.1016/j.ajps.2014.08.005.

Kadam, U., Moeller, C. A., Irudayaraj, J., and Schulz, B. (2014). Effect of T-DNA insertions on mRNA transcript copy numbers upstream and downstream of the insertion site in arabidopsis thaliana explored by surface enhanced raman spectroscopy. *Plant Biotechnol. J.* 12, 568–577. doi:10.1111/pbi.12161.

Kadam, U. S., Schulz, B., and Irudayaraj, J. M. K. (2017). Multiplex single-cell quantification of rare RNA transcripts from protoplasts in a model plant system. *Plant J.* 90, 1187–1195. doi:10.1111/tpj.13537.

Kim, H., Kim, S.-T., Kim, S.-G., and Kim, J.-S. (2015). Targeted Genome Editing for Crop Improvement. *Plant Breed. Biotechnol.* 3, 283–290. doi:10.9787/PBB.2015.3.4.283.

Knezevic, N. (2015). Large Pore Mesoporous Silica Nanomaterials for Application in Delivery of Biomolecules. *Nanoscale* 7, 2199–2209. doi:10.1039/C4NR06114D.

Kole, C., Kumar, D. S., and Editors, M. V. K. (2016). *Plant Nanotechnology Principles and Practices*.

Kottegoda, N., Sandaruwan, C., Priyadarshana, G., Siriwardhana, A., Rathnayake, U. A., Berugoda Arachchige, D. M., et al. (2017). Urea-Hydroxyapatite Nanohybrids for Slow Release of Nitrogen. *ACS Nano* 11, 1214–1221. doi:10.1021/acsnano.6b07781.

Kouassi, G. K., and Irudayaraj, J. (2006). Magnetic and Gold-Coated Magnetic Nanoparticles as a DNA Sensor. *Anal. Chem.* 78, 3234–3241. doi:10.1021/ac051621j.

Kwak, S. Y., Lew, T. T. S., Sweeney, C. J., Koman, V. B., Wong, M. H., Bohmert-Tatarev, K., et al. (2019). Chloroplast-selective gene delivery and expression in planta using chitosan-complexed single-walled carbon nanotube carriers. *Nat. Nanotechnol.* 14, 447–455. doi:10.1038/s41565-019-0375-4.

Lajiness, M. S., Vieth, M., and Erickson, J. (2004). Molecular properties that influence oral drug-like behavior. *Curr. Opin. Drug Discov. Devel.* 7, 470–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15338956.

Lee, K., Silva, E. A., and Mooney, D. J. (2010). Growth factor delivery-based tissue engineering: general approaches and a review of recent developments. *J. R. Soc. Interface* 8.

Liu, Y., Yang, H., and Sakanishi, A. (2005). Ultrasound: Mechanical gene transfer into plant cells by sonoporation. *Biotechnol. Adv.* 24, 1–16. doi:10.1016/j.biotechadv.2005.04.002.

Lohani, A., Verma, A., Joshi, H., Yadav, N., and Karki, N. (2014). Nanotechnology-Based Cosmeceuticals. *ISRN Dermatol.* 2014, 1–14. doi:10.1155/2014/843687.

Lu, Y., Li, Y., and Wu, W. (2016). Injected nanocrystals for targeted drug delivery. *Acta Pharm. Sin. B* 6, 106–113. doi:10.1016/J.APSB.2015.11.005.

Mahendra Rai, Shivaji Deshmukh, A. G. and K.-E. (2012). strategic nanoparticles mediated gene transfer in plants and animals. *Curr. Nanosci.*, 170–179.

Majouga, A., Sokolsky-Papkov, M., Kuznetsov, A., Lebedev, D., Efremova, M., Beloglazkina, E., et al. (2015). Enzyme-functionalized gold-coated magnetite nanoparticles as novel hybrid nanomaterials: Synthesis, purification and control of enzyme function by low-frequency magnetic field. *Colloids Surfaces B Biointerfaces* 125, 104–109. doi:10.1016/j.colsurfb.2014.11.012.

Malerba, M., and Cerana, R. (2016). Chitosan Effects on Plant Systems. *Int. J. Mol. Sci.* 17. doi:10.3390/ijms17070996.

Manallack, D. T. (2007). The pKa Distribution of Drugs: Application to Drug Discovery. *Perspect. Medicin. Chem.* 1, 25–38. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2754920&tool=pmcentrez&rendertype=abstract.

Markowicz-Piasecka, M., and Mikiciuk-Olasik, E. (2016). *Dendrimers in drug delivery*. doi:10.1016/B978-0-323-42866-8.00002-2.

Mengiste, T. (2012). Plant Immunity to Necrotrophs. *Annu. Rev. Phytopathol.* 50, 267–294. doi:10.1146/annurev-phyto-081211-172955.

Meredith, A. N., Harper, B., and Harper, S. L. (2016). The influence of size on the toxicity of an encapsulated pesticide: A comparison of micron- and nano-sized capsules. *Environ. Int.* doi:10.1016/j.envint.2015.10.012.

Montalvo, D., McLaughlin, M. J., and Degryse, F. (2015). Efficacy of hydroxyapatite nanoparticles as phosphorus fertilizer in andisols and oxisols. *Soil Sci. Soc. Am. J.* 79, 551–558. doi:10.2136/sssaj2014.09.0373.

Mora-Huertas, C. E., Fessi, H., and Elaissari, A. (2010). Polymer-based nanocapsules for drug delivery. *Int. J. Pharm.* 385, 113–142. doi:10.1016/j.ijpharm.2009.10.018.

Moura, L. I. F., Malfanti, A., Peres, C., Matos, A. I., Guegain, E., Sainz, V., et al. (2019). Functionalized branched polymers: Promising immunomodulatory tools for the treatment of cancer and immune disorders. *Mater. Horizons* 6, 1956–1973. doi:10.1039/c9mh00628a.

Munz, D., Wang, D., Moyer, M. M., Webster-Gardiner, M. S., Kunal, P., Watts, D., et al. (2016). Aerobic Epoxidation of Olefin by Platinum Catalysts Supported on Mesoporous Silica Nanoparticles. doi:10.1021/acscatal.6b01532.

Naqvi, S., Maitra, a. N., Abdin, M. Z., Akmal, M., Arora, I., and Samim, M. (2012). Calcium phosphate nanoparticle mediated genetic transformation in plants. *J. Mater. Chem.* 22, 3500–3507. doi:10.1039/c2jm11739h.

NIH (2016). Drug Delivery Systems: Getting Drugs to Their Targets in a Controlled Manner | National Institute of Biomedical Imaging and Bioengineering. *Sci. Educ.* Available at: https://www.nibib.nih.gov/science-education/science-topics/drug-delivery-systems-getting-drugs-their-targets-controlled-manner.

Nuruzzaman, M., Rahman, M. M., Liu, Y., and Naidu, R. (2016). Nanoencapsulation, Nano-guard for Pesticides: A New Window for Safe Application. *J. Agric. Food Chem.* 64, 1447–1483. doi:10.1021/acs.jafc.5b05214.

Oehlke, K., Behsnilian, D., Mayer-Miebach, E., Weidler, P. G., and Greiner, R. (2017). Edible solid lipid nanoparticles (SLN) as carrier system for antioxidants of different lipophilicity. doi:10.1371/journal.pone.0171662.

Oliveira, H. C., Stolf-Moreira, R., Martinez, C. B. R., Grillo, R., de Jesus, M. B., and Fraceto, L. F. (2015). Nanoencapsulation Enhances the Post-Emergence Herbicidal Activity of Atrazine against Mustard Plants. *PLoS One* 10, e0132971. doi:10.1371/journal.pone.0132971.

Orendorff, C. J., Gearheart, L., Jana, N. R., and Murphy, C. J. (2006). Aspect ratio dependence on surface enhanced Raman scattering using silver and gold nanorod substrates. *Phys. Chem. Chem. Phys.* 8, 165–170. doi:10.1039/b512573a.

Padhye, S. G., and Nagarsenker, M. S. (2013). Simvastatin Solid Lipid Nanoparticles for Oral Delivery: Formulation Development and In vivo Evaluation. *Indian J. Pharm. Sci.* 75, 591–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24403661 [Accessed December 4, 2018].

Padrela, L., Rodrigues, M. A., Duarte, A., Dias, A. M. A., Braga, M. E. M., and de Sousa, H. C. (2018). Supercritical carbon dioxide-based technologies for the production of drug nanoparticles/nanocrystals – A comprehensive review. *Adv. Drug Deliv. Rev.* 131, 22–78. doi:10.1016/j.addr.2018.07.010.

Pallerla, S. M., and Prabhakar, B. (2013). A Review on Solid Lipid Nanoparticles. *Int. J. Pharm. Sci. Rev. Res.* 20, 196–206.

Palmerston Mendes, L., Pan, J., and Torchilin, V. P. (2017). Dendrimers as Nanocarriers for Nucleic Acid and Drug Delivery in Cancer Therapy. *Molecules* 22. doi:10.3390/molecules22091401.

Panchapakesan, B., Book-Newell, B., Sethu, P., Rao, M., and Irudayaraj, J. (2011). Gold nanoprobes for theranostics. *Nanomedicine* 6, 1787–1811. doi:10.2217/nnm.11.155.

Pardeshi, C., Rajput, P., Belgamwar, V., Tekade, A., Patil, G., Chaudhary, K., et al. (2012). Solid lipid based nanocarriers: An overview. *Acta Pharm.* 62, 433–472. doi:10.2478/v10007-012-0040-z.

Patel, K., Padhye, S., and Nagarsenker, M. (2012). Duloxetine HCl Lipid Nanoparticles: Preparation, Characterization, and Dosage Form Design. *AAPS PharmSciTech* 13, 125–133. doi:10.1208/s12249-011-9727-6.

Pavlic, J. I., Mares, T., Bester, J., Jansa, V., Daniel, M., and Iglic, a (2009). Encapsulation of small spherical liposome into larger flaccid liposome induced by human plasma proteins. *Comput. Methods Biomech. Biomed. Engin.* 12, 147–50. doi:10.1080/10255840903081180.

Perez, R. A., Singh, R. K., Kim, H., and Kim, T. (2017). Silica-based multifunctional nanodelivery systems toward regenerative medicine. 772–799. doi:10.1039/c7mh00017k.

Pimentel, D., and Burgess, M. (2012). Small amounts of pesticides reaching target insects. *Environ. Dev. Sustain.* 14, 1–2. doi:10.1007/s10668-011-9325-5.

Pimentel, D., and Levitan, L. (1986). Pesticides: Amounts Applied and Amounts Reaching Pests. *Bioscience* 36, 86–91. doi:10.2307/1310108.

Piran, P., Kafil, H. S., Ghanbarzadeh, S., Safdari, R., and Hamishehkar, H. (2017). Formulation of Menthol-Loaded Nanostructured Lipid Carriers to Enhance Its Antimicrobial Activity for Food Preservation. *Adv. Pharm. Bull.* 7, 261–268. doi:10.15171/apb.2017.031.

Rai, M., Ribeiro, C., Mattoso, L., and Duran, N. (2015). Nanotechnologies in food and agriculture. *Nanotechnologies Food Agric.*, 1–347. doi:10.1007/978-3-319-14024-7.

Rang, H. P. (2006). The receptor concept: pharmacology's big idea. *Br. J. Pharmacol.* 147 Suppl, S9-16. doi:10.1038/sj.bjp.0706457.

Safari, J., and Zarnegar, Z. (2014). Advanced drug delivery systems: Nanotechnology of health design A review. *J. Saudi Chem. Soc.* doi:10.1016/j.jscs.2012.12.009.

Savjani, K. T., Gajjar, A. K., and Savjani, J. K. (2012). Drug solubility: importance and enhancement techniques. *ISRN Pharm.* 2012, 195727. doi:10.5402/2012/195727.

Shahbazi, M.-A., Herranz, B., and Santos, H. A. (2012). Nanostructured porous Si-based nanoparticles for targeted drug delivery. *Biomatter* 2, 296–312. doi:10.4161/biom.22347.

Shinde, P., Sayam, A., Gupta, S., Singh, B., Polshettiwar, V., and Prasad, B. L. V (2017). Amphi-functional mesoporous silica nanoparticles for dye separation. doi:10.1039/c7ta03904b.

Siddiqui Mohamed H Al-Whaibi Firoz Mohammad Editors, M. H. (2015). *Nanotechnology and Plant Sciences Nanoparticles and Their Impact on Plants*.

Singhal, G. B., Patel, R. P., Prajapati, B. G., and Patel, N. A. (2011). Solid Lipid Nanoparticles and Nanolipid Carriers: as novel solid lipid based drug carrier. *Int. Res. J. Pharm.* 2, 40–52.

Slattery, M., Harper, B., and Harper, S. (2019). Pesticide encapsulation at the nanoscale drives changes to the hydrophobic partitioning and toxicity of an active ingredient. *Nanomaterials* 9. doi:10.3390/nano9010081.

Sokolova, V., and Epple, M. (2008). Inorganic nanoparticles as carriers of nucleic acids into cells. *Angew. Chem. Int. Ed. Engl.* 47, 1382–95. doi:10.1002/anie.200703039.

Song, C., and Liu, S. (2005). A new healthy sunscreen system for human: Solid lipid nannoparticles as carrier for 3,4,5-trimethoxybenzoylchitin and the improvement by adding Vitamin E. *Int. J. Biol. Macromol.* 36, 116–119. doi:10.1016/j.ijbiomac.2005.05.003.

Sun, L., and Irudayaraj, J. (2009a). PCR-free quantification of multiple splice variants in a cancer gene by surface-enhanced Raman spectroscopy. *J. Phys. Chem. B* 113, 14021–5. doi:10.1021/jp908225f.

Sun, L., and Irudayaraj, J. (2009b). Quantitative surface-enhanced Raman for gene expression estimation. *Biophys. J.* 96, 4709–16. doi:10.1016/j.bpj.2009.03.021.

Sun, L., Zhao, Q., Xiang, J., Shi, J., Wang, L., Hu, S., et al. (2009). Adsorption of NO and NH3 over CuO/γ-Al2O3 catalyst by DRIFTS. *Huagong Xuebao/CIESC J.* 60, 444–449. doi:10.1007/s11771.

Tinkle, S., Mcneil, S. E., Mühlebach, S., Bawa, R., Borchard, G., Barenholz, Y. C., et al. (2014). Nanomedicines: Addressing the scientific and regulatory gap. *Ann. N. Y. Acad. Sci.* 1313, 35–56. doi:10.1111/nyas.12403.

Torney, F., Trewyn, B. G., Lin, V. S.-Y., and Wang, K. (2007). Mesoporous silica nanoparticles deliver DNA and chemicals into plants. *Nat. Nanotechnol.* 2, 295–300. doi:10.1038/nnano.2007.108.

Tsai, H. C., and Imae, T. (2011). *Fabrication of dendrimers toward biological application*. 1st ed. Elsevier Inc. doi:10.1016/B978-0-12-416020-0.00003-6.

Vabbilisetty, P., and Sun, X.-L. (2014). Liposome surface functionalization based on different anchoring lipids via Staudinger ligation. *Org. Biomol. Chem.* 12, 1237. doi:10.1039/c3ob41721b.

Verho, O., Gao, F., Johnston, E. V, Wan, W., Nagendiran, A., Zheng, H., et al. (2014). Mesoporous silica nanoparticles applied as a support for Pd and Au nanocatalysts in cycloisomerization reactions. *APL Mater.* 2, 113316. doi:10.1063/1.4901293.

Wahid, F., Zhong, C., Wang, H. S., Hu, X. H., and Chu, L. Q. (2017). Recent advances in antimicrobial hydrogels containing metal ions and metals/metal oxide nanoparticles. *Polymers (Basel).* 9. doi:10.3390/polym9120636.

Wang, L., Li, X., Zhang, G., Dong, J., and Eastoe, J. (2007). Oil-in-water nanoemulsions for pesticide formulations. *J. Colloid Interface Sci.* 314, 230–235. doi:10.1016/j.jcis.2007.04.079.

Wang, P., Lombi, E., Zhao, F.-J., Kopittke, P. M., Nel, A., al., et, et al. (2016). Nanotechnology: A New Opportunity in Plant Sciences. *Trends Plant Sci.* 21, 699–712. doi:10.1016/j.tplants.2016.04.005.

Wang, R., Vega, P., Xu, Y., Chen, C.-Y., and Irudayaraj, J. (2018). Exploring the anti-quorum sensing activity of a D-limonene nanoemulsion for Escherichia coli O157:H7. doi:10.1002/jbm.a.36404.

Wang, Y., Chen, J., and Irudayaraj, J. (2011). Nuclear Targeting Dynamics of Gold Nanoclusters for Enhanced Therapy of HER2 $^{+}$ Breast Cancer. *ACS Nano* 5, 9718–9725. doi:10.1021/nn2032177.

Wang, Y., and Irudayaraj, J. (2013). Surface-enhanced Raman spectroscopy at single-molecule scale and its implications in biology. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 368, 20120026. doi:10.1098/rstb.2012.0026.

Wang, Y., Lee, K., and Irudayaraj, J. (2010). SERS aptasensor from nanorod–nanoparticle junction for protein detection. *Chem. Commun.* 46, 613–615. doi:10.1039/B919607B.

Wang, Y., Newell, B. B., and Irudayaraj, J. (2012). Folic Acid Protected Silver Nanocarriers for Targeted Drug Delivery. *J. Biomed. Nanotechnol.* 8, 751–759. doi:10.1166/jbn.2012.1437.

Wissing, S. A., and Müller, R. H. (2001). Solid lipid nanoparticles (SLN)--a novel carrier for UV blockers. *Pharmazie* 56, 783–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11683123 [Accessed December 4, 2018].

Xu, C., Lei, C., and Yu, C. (2019). Mesoporous silica nanoparticles for protein protection and delivery. *Front. Chem.* 7. doi:10.3389/fchem.2019.00290.

Yadollahi, A., Arzani, K., and Khoshghalb, H. (2010). The role of nanotechnology in horticultural crops postharvest management. *Acta Hortic.* 875, 49–56. doi:10.17660/ActaHortic.2010.875.4.

Yi, Z., Hussain, H. I., Feng, C., Sun, D., She, F., Rookes, J. E., et al. (2015). Functionalized Mesoporous Silica Nanoparticles with Redox-Responsive Short-Chain Gatekeepers for Agrochemical Delivery. doi:10.1021/acsami.5b02131.

YIN, Y., GUO, Q., HAN, Y., WANG, L., and WAN, S. (2012). Preparation, Characterization and Nematicidal Activity of Lansiumamide B Nano-Capsules. *J. Integr. Agric.* 11, 1151–1158. doi:10.1016/S2095-3119(12)60109-9.

Yu-qin, F. U., Lu-hua, L. I., Pi-wu, W., Jing, Q. U., Yong-ping, F. U., and Hui, W. (2012). Delivering DNA into Plant Cell by Gene Carriers of ZnS Nanoparticles. 28, 672–676.

Yu, C and Irudayaraj, J. (2007). Multiplex Biosensor Using Gold Nanorods. doi:10.1021/AC061730D.

Yukuyama, M. N., Tomiko, E., Kato, M., Löbenberg, R., and Araci Bou-Chacra, N. (2017). Challenges and future prospects of nanoemulsion as drug delivery system. *Curr. Pharm. Des.* 23, 495–508. doi:10.2174/1381612822666161027l.

Zhao, X., Zhu, Y., Zhang, C., Lei, J., Ma, Y., and Du, F. (2017). Positive charge pesticide nanoemulsions prepared by the phase inversion composition method with ionic liquids. *RSC Adv.* 7, 48586–48596. doi:10.1039/C7RA08653A.

Zhao, Y., Sun, X., Zhang, G., Trewyn, B. G., Slowing, I. I., and Lin, V. S. Y. (2011). Interaction of mesoporous silica nanoparticles with human red blood cell membranes: Size and surface effects. *ACS Nano* 5, 1366–1375. doi:10.1021/nn103077k.

Zuverza-Mena, N., Martínez-Fernández, D., Du, W., Hernandez-Viezcas, J. A., Bonilla-Bird, N., López-Moreno, M. L., et al. (2016). Exposure of engineered nanomaterials to plants: Insights into the physiological and biochemical responses-A review. *Plant Physiol. Biochem.* doi:10.1016/j.plaphy.2016.05.037.

3. HORMESIS INDUCING ESSENTIAL OIL NANO-DELIVERY SYSTEM

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Abstract

Botrytis cinerea a broad host-range necrotrophic phytopathogen, establishes compatible interactions with hosts by deploying multi-gene dependent infection strategies, rendering simply inherited resistance ineffective to fight off this pathogen. To date, synthetic fungicide application remains the most common method to control the ensuing disease. Since essential oils serve as intermediators during phytobiome communication, we hypothesize that they have the potential to enhance the quantitative disease resistance against BHN by eliciting the adaptive stress response (hormesis) in plants. Using basic engineering principles of nano-carrier design, we demonstrate the facile development of stable and scalable essential oil (EO) nano-emulsions for controlling *Botrytis cinerea* in a model plant, *Arabidopsis thaliana*. We show that these facile nano-emulsions significantly enhanced the disease resistance of the plant system by reducing the necrotic area by up to 50% compared to untreated plants. RNA-seq analysis indicated that successful treatments upregulated autophagy, ROS scavenging and activation of jasmonic acid signaling pathway.

3.1 Introduction

The effect of stress on biological systems can be debilitating or restorative, depending on the dosage. High dose or continuous exposure to stress factors result in cellular failure. In contrast, low dose, or intermittent exposure to stressors, activate a complex signal/receptor mechanism that counter balance the noxious effects restoring homeostasis. This biphasic physiological resilience phenomenon is known as hormesis [1,2]. Hormetins are chemical elicitors of the hormetic response.

In plants, the secondary metabolites are termed as hormetins[3], which acts as intermediators in a feedback loop system. Secondary metabolites are produced in plants by the immune system upon perception of environmental cues, including secondary metabolites.

The plant immune system comprises of a highly sensitive network of cellular receptors that transduce signals from the surrounding environment, into and between cells, via biochemical messages [4,5]. Upon perception, these biochemical signals trigger a cascade of biological response at the cellular level that escalates to a systemic level, shifting the overall physiological status of the plant, from a basal growing status, to a secondary metabolism [6,7]. The onset of the secondary metabolism displayed by stressed plants to overcome sub-optimal growing conditions, results in enhanced biosynthesis and accumulation of a chemically diverse and biologically versatile group of small and volatile compounds that are collectively known as essential oils (EOs)[8,9]. These compounds serve as an extra defense barrier protecting plants against predators and disease, as well as to endure environmental challenges.

Due to the volatile nature of these molecules, EO are at the core of the plant communication process with their phytobiome[10] including plant pathogens[11]. Molecules from EO can carry an air-borne message that protects plants against predators by attracting larger animals that predate on them[12], or by repelling them directly[10]. These molecules also serve as a plant-to-plant communication system (a phenomenon known as allelopathy) whether to help each other by alerting the surrounding plants on the presence of a pathogen, or to harm each other by preventing the growth of competing plants by disrupting their photosynthetic process[13]. Moreover, there is evidence that EO activates the plant defense mechanism known as Pathogen/Damage Associated Molecular Pattern (PAMP/DAMP) which in turn, results in enhanced production of secondary metabolites stored in the form of EO[14,15].

Chemically, the composition of EO includes terpenes, terpenoids and, phenylpropanoids (non-terpenoid compounds) [16]. Terpenes are perhaps the most abundant type of molecules in EO and encompasses a myriad of monoterpenes and sesquiterpenes, including their oxygenated derivatives such as monoterpene and sesquiterpene alcohols, aldehydes, ketones, esters, peroxides and phenols [17]. Biologically, the role of these molecules in the plant's defense response is broad. For instance, when stored and accumulated in the leaves, some molecules in EO absorb UV light and protects

the plant against detrimental levels of UV radiation[18] and prevents water loss. Some flavonoids present in EO frequently found to be associated with sugar molecules[19], confer protection to the cell membranes form water crystals under freezing conditions[20,21] and serve as a chelating agent protecting plants from toxic heavy metal ions[22–24]. Some molecules that form EO are cytotoxic to insects, which deters an attack by predators, and are antimicrobial, which help to prevent pathogen invasion and colonization.

The chemical heterogeneity and biological versatility of EOs have attracted much attention in the industry due to their potential utility outside its natural context. The main challenge in using EOs may stem from the fact that they are poorly soluble in water. Surfactant assisted emulsification is a common process carried-out to produce homogeneous blends of oil and water assisted by a surface-active agent (i.e. surfactant). However, the diameter of oil droplets in traditional emulsions are micrometric, therefore their bioactivity is low. Nano-emulsions, in contrast, are more stable than traditional emulsion [25] and more potent due to their enhanced bioavailability [26] because of their increased membrane permeation.

Proper formulation and fabrication of nano-emulsions can be costly, difficult and time consuming. Submicrometric oil droplets can be formed by *i)* high-energy or *ii)* low energy methods. In general, high energy methods emphasize on brute force steps to achieve droplet deformation, such as high shear forces used in colloidal mills, or high pressure in membrane-based homogenizers. Low energy methods, in contrast, rely on chemical "persuasion" to achieve droplet deformation during the emulsification process. This is achieved by fine-tuning the formulation. Large scale production of nano-emulsions via high energy could render them prohibitively expensive for its use in agricultural. Given that past work on nano-emulsification via low energy methods is overwhelmingly inconsistent stalling this field. We hypothesize that a rationalized approach to fabrication and application will help to design new formulations for drug-delivery systems that can be actually be used in practice with significant benefit to the society.

The incongruences in the literature appear to stem from one fundamental factor: the overreliance on the hydrophilic-lipophilic balance (HLB) for formulation purposes. The fundamental problem with the HLB is that it only describes the behavior of a given surfactant in water while ignoring all other components of the system (i.e. oil, salinity, and temperature), thereby overlooking the

theoretical foundations to properly describe the emulsification process. Salager et. al., proposed a different model called the Hydrophilic-lipophilic difference (HLD)[27,28] where the the formulation variables (i.e. nature of both the surfactant and the oil, salinity, and temperature) are accounted for into a single variable that is more useful for comparing nano-emulsion formations in the literature. In the current literature, this inconsistency hinders the development of more impactful insights [29]. It is worth noting that the ultimate droplet size of an emulsion is determined by a balance between the two opposing processes: Droplet break-up, and re-coalescence [30]. Droplet break-up is described by the capillary number which depends directly on the inertial forces (i.e. flow of velocity U in a fluid of viscosity η) and, inversely on the surface tension of the fluid [29]. The relative viscosity (ηD/ηC) is a crucial aspect of nano-emulsion preparation because it can influence the final droplet size of the emulsion [31]. The large variation of nano-emulsion composition, formation, and fabrication conditions impose an extra layer of complexity in the analysis of biological effects of EOs in cellular models. Confounding effects may be caused by either by loss of bioactivity of molecules present in EOs because of high energy emulsification methods (e.g. thermal denaturation), or addition of potentially toxic components in the formulation (e.g. alcohols)[26].

Our approach will lay the foundation to develop EONEs for crop protection by taking advantage of the enhanced bioactive properties, more specifically, against brad host necrotrophs (BHN). BHN, are a group of highly invasive plant pathogenic microorganisms that do not rely on a single strategy to successfully infect, colonize, and suppress the host immune defense response. Instead, these processes are mediated by several strategies including the production of cell wall degrading enzymes, non-host specific toxins (botrydial), high levels of ROS, necrosis-inducing factors, and an array of secondary metabolites. Since no true plant resistance can be achieved against broad host necrotrophic pathogens via plant breading, to date, synthetic fungicide application still remains the most common method to control the disease it causes, although it is a practice that is becoming increasingly restricted, not only due to concerns on sustainability, but also due to the increasing resistance to synthetic chemical fungicides.

Botrytis cinerea is a broad host-range fungal necrotrophic pathogen that infects many economically relevant fruit bearing trees, shrubs and vegetables. Due to its economic importance, *B. cinerea* has not only been classified as the second most important plant pathogen [33] but it has also been considered the most common pathogen responsible for the post-harvest decay of fruits

and vegetables. The development of novel alternatives to treat the grey rot from *B. cinerea* is imperative since resistance to chemical fungicides by *B. cinerea* has been already detected [32–34]. Moreover, fungicides are environmentally costly since they are persistent, and may have negative effects on human health. The economic losses in crops worldwide caused by this pathogen are estimated to be up to $100 billion per year [35]. About a decade ago, the average cost of chemical treatment of grey mold disease for all crops around the world was c.a €40/ha [36]. By the beginning of the new millennium the estimated market of fungicides specifically targeted against Botrytis ('botryticides') cost €540 million (2001), representing 10% of the world fungicide market [36]. By 2005, the total fungicide market was estimated at US$ 8,916 [37] and the world market for Botrytis control products was estimated at US$15-25 million [38]. The vineyard segment represents 50% of the value of the total market for botryticides [36]. This is consistent with the facts that, on one hand, the disease caused by *B, cinerea* in grape clusters (known as Botrytis bunch rot) is the most important disease of grape crops in the world [39] causing negative impacts on the yield and quality of harvest.

Both the impossibility of relying on plant breeding approaches to combat broad host necrotrophs, and the high risks posed by synthetic fungicides on the environment and on the health of humans and off-target ecosystem, brings the challenge of finding eco-compatible solutions to combat broad host necrotrophs. However, natural products usually require high doses for disease control in crops and the beneficial effects are often mild to moderate, such as the case for biocontrol products. Since it is well documented that the biological roles of the compounds present in the EOs are associated with the plant's defense response, either by direct antimicrobial activity against pathogens, or repelling off insects, we hypothesized that exposure of plants to nano-emulsified EOs may result in the activation of the JA hormone signaling pathway that is triggered upon insect or herbivore attack, or necrotrophic pathogen infection. Some of the molecules present in EOs also serve as intermediators in a plant-to-plant cross-talk communication process (allelopathy) [40] that triggers this specific defense response in plants situated at close proximity from a plant facing an on-going infection or insect attack [13]. Using the biological plant-pathogen model system model *A. thaliana* (Col-0) challenged with the necrotrophic fungi *Botrytis cinerea*, we predict that the QDR of treated plants with nano-emulsified EO will be enhanced, and therefore the extent of the lesion produced by the pathogen will be significantly lower than in untreated plants.

The main objective of our work is two-fold. First, to develop a rational formulation for the production of concentrated, translucent, essential oil-based nano-emulsions under low energy conditions using a range of essential oils such as cinnamon, clove, coriander, geranium, oregano, peppermint and red thyme. Second, to explore the systemic hormetic effect of the developed nano-emulsions on the quantitative disease resistance in *A. thaliana* (Col-0) infected with the necrotrophic fungal pathogen *B. cinerea.*

3.2 Materials and methods

3.2.1 Materials

Commercially available 100% pure EO from Cinnamon (*Cinnamomum cassia*) and Peppermint (*Mentha piperita*) (Now Foods, Bloomingdale, IL, USA), clove bud, oregano, coriander seed, geranium, and red thyme (Aura Acacia, Urbana, IA,USA) were used for the preparation of nano-emulsions. Commercially available soybean oil (The J.M Smucker Co., Orrville, OH, USA) was used to modulate the viscosity of the organic phase of the nano-emulsion (NE). Propylene glycol (Ward's science, West Henrietta, NY, USA) and polyethylene glycol (PEG) 4000 (ref 95904 Fluka, Thermo Fisher Scientific; Waltham, MA, USA) were used to modulate the viscosity of the aqueous phase of NE. Tween80® (P4780, Sigma-Aldrich, St. Louis, MO, USA) was used as a surfactant agent during the emulsification process. All materials/reagents were used as received.

Viscosity measurements

Dynamic viscosity was measured from 5 different blends of Essential oil to soybean oil containing 95:5, 90:10, 80:20, 75:25, 50:50 and, 5:95 (percentage weight ratios) respectively. PEG4000 and propylene glycol were used as models of aqueous phase viscosity modulators. The dynamic viscosity was measured from 4 different concentrations of each of the polymers: 75%, 50%, 25% and, 10% (w/w). Viscosity measurements were performed in triplicate in a stabinger viscometer SVM3001 Anton Paar set at 25°C. The results are expressed as the average value.

Surface tension measurements

50g of PEG400 and propylene glycol in dH_2O were prepared at 75%, 50%, 25% and 10% (w/w). For measuring the surface tension, the wilhelmy method was performed with a platinum plate

using force tensiometer K100MKII goniometer K100 (Krüss Hamburg, Germany). The surface tension measurements from each sample were conducted at room temperature for 60 seconds recording data every 10 seconds. Experiments were performed in triplicate per sample. Results are expressed as the average between replicas (n=3). The aluminum plate was torched-cleaned before each new measurement.

Nano-emulsion formation

The spontaneous emulsification method explained by Komaiko and McClements was performed [92]. In this work the organic phase consisted of 5% (w/w) of a 95:5 (%w/w) blend of Essential oil:SBO thoroughly mixed with Tween80® at a weight ratio of 3:1. The surfactant and the oil phase were mixed under magnetic stirring at room temperature for 2-3 min at room temperature. The freshly formed organic phase was then titrated at a rate of 0.5mL/min into 95% (w/w) of aqueous phase containing either propylene glycol or PEG4000 at one of the previously mentioned concentrations (i.e. 75, 50, 25 and, 10 (%w/w). The aqueous phase was kept at a constant magnetic stirring speed of 500 rpm at room temperature. The formed nano-emulsion was kept under magnetic stirring for 2-3 minutes after the organic phase was fully added into the aqueous phase.

3.2.2 Nano-emulsion particle size characterization

Dynamic light scattering (DLS)

Only translucent emulsions were selected for particle size distribution analysis. Nano-emulsion particle size distribution was measured with dynamic light scattering (Nano ZS, Malvern, Worcestershire, UK), at a scattering angle of 173° using a 633 nm laser with each measurement at an average of 11 runs, each of 10 sec duration. Undiluted freshly prepared samples were measured at 25°C and results are reported as an average of 2 measurements. The intensity average emulsion droplet size diameter, and polydispersity of each sample was obtained from the cumulant analysis of each sample's correlation function.

Transmission electron microscopy

Formvar carbon film 400 mesh copper TEM grids were negatively charged using a PELCO easiGlow™ discharge flow at 10 mA for one minute in order to allow aqueous solutions to spread

easily on the grid's surface. After glow discharge, 1.5µL of translucent, freshly prepared undiluted nano-emulsion sample was placed onto the TEM grid surface along with 1.5 µL of 1% uranyle acetate 1N in water in order increase image contrast. The TEM grid containing the nano-emulsion sample and the negative stain was let to rest for 1 minute. TEM images were acquired using a Tecnai T12 (120KV) transmission electron microscope.

3.2.3 Dynamic morpho-physiological assessment of the systemic effect of nano-emulsions on the plants' defense immune system of wild type *A. thaliana* (Col-0)

Plant material and growing conditions

In order to assess the systemic effect of essential oil nano-emulsions (EONEs) on the innate immune mechanism of plants, a system to grow plants hydroponically was per Conn, et.al. 2013 [93] using *Arabidopsis thaliana* wild type (Col-0) as a biological model. By this means, access to the radicular system of the plant is enabled without compromising the integrity of plants.

Briefly, 2 to 3 individual seeds were carefully placed on a seed holder (pierced black microcentrifuge lid) containing germination medium agar. Seed holders loaded with seeds were placed in germination boxes containing germination nutrient solution (suppl. Info) and then seeds were stratified at 4 ºC for 48h in darkness. After the stratification period, germination boxes were incubated under a 12h:12h day/night photoperiod with an irradiance of 150 µmol photons m-2.s-1 at the plant level, at 24 ºC and 55% atmospheric relative humidity. On day 4 post germination, extra seedlings were carefully removed from seed holders to guarantee one plant per holder only. After 7 days of germination, the germination solution was gradually replaced with basal nutrient solution (BNS) (Suppl. Info) daily for 3 days, replacing 33%, 50% and 100%, respectively. A week later, 14-day old seedlings were individually transferred to 50 mL plant holders containing BNS and further incubated for 2 more weeks. 4-week-old plants were used for further experimentation.

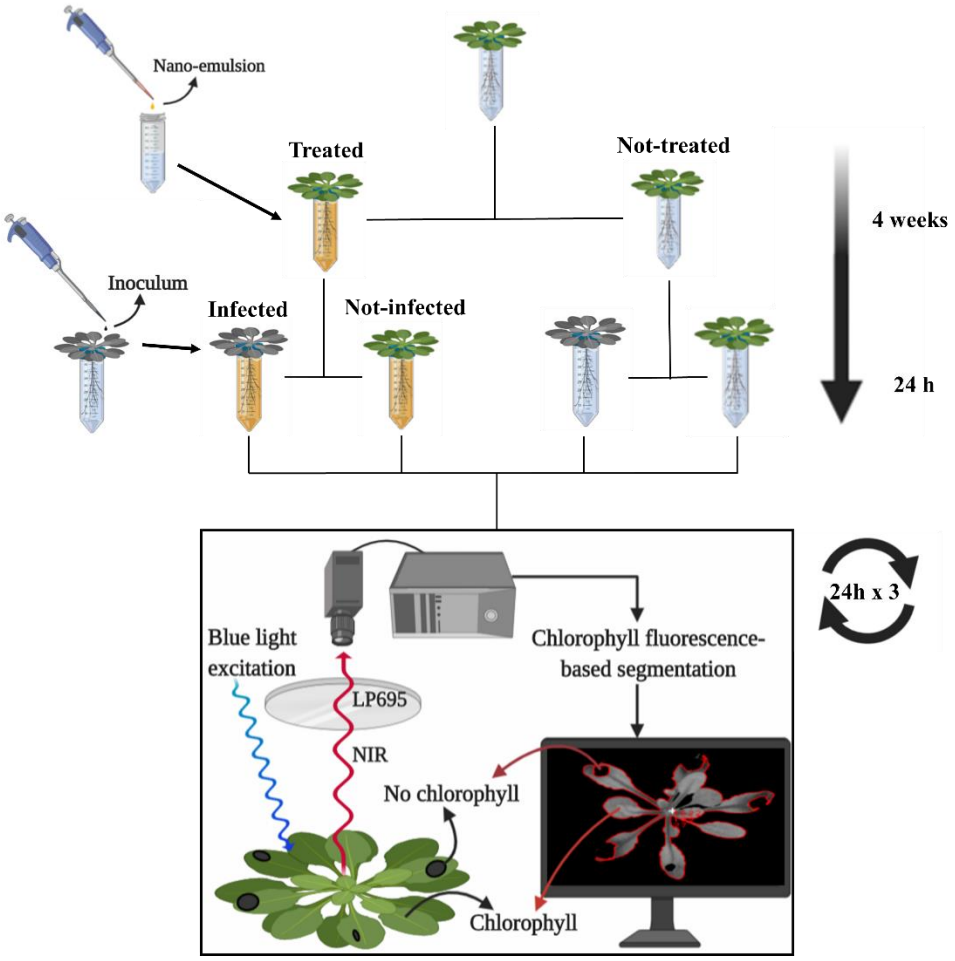
Multispectral Image-based phenotyping assessment of treated plants

Roots from 4-week-old plants were treated with freshly prepared EONEs at 2 different concentrations (50 and 500 µg/mL) under hydroponic conditions. Basal nutrient solution (BNS) was leveled up to 50 mL after dissolution of each EONEs treatment. A set of 9 individual plants

were used for each of the experimental conditions. Image-based phenotyping analysis was performed using an ARIS B.V automatic top-view imaging machine vision system equipped with a multispectral light source (Aris, Eindhoven, Netherlands). The chlorophyll-based masking segmentation was used to measure the area of the plant rosette immediately after treatment (t=0), and then every 24 hours for 3 consecutive days. The treated plants were kept under controlled conditions, previously described. On day 3, plants were flash-frozen with liquid nitrogen and stored at -80 ^{o}C. A two-way ANOVA was performed to assess the interaction of each of the concentration levels under investigation every 24 hours, over a 72 hour time period, for each treatment.

Assessment of the systemic effect of treatments on the quantitative disease resistance (QDR) against B. cinerea via image-based phenotyping

Fungal disease was evaluated as previously described [94]. Briefly, for the *Botrytis cinerea* disease assay, 4 leaves from pretreated 4-week-old plants were drop inoculated with a conidial suspension (2.5 x 105 spores/mL) of *B. cinerea* strain B05.10 in 1% Sabouraud Maltose Broth and maintained under a transparent cover at high humidity for 72 hours. The lesion area size per leaf was measured via Image-based phenotyping analysis was performed using an ARIS B.V automatic top-view imaging machine vision system equipped with a multispectral light source (Aris, Eindhoven, Netherlands). The chlorophyll-based masking segmentation was used to distinguish necrotic tissue in leaves. The statistical analysis was carried out by conducting a balanced nested ANOVA on the necrotic area on the infected leaves per plant for each of the treatments (n=20).



Scheme 1: Workflow of the evaluation of EONEs via quantitative image-based phenotyping. Scheme no.1 is a representation of the multispectral image-based analysis system to assess the hormetic effect of EONEs on the quantitative disease resistance in the model plant-pathogen model system A. thaliana-B. cinerea. Roots of 4-week-old A. thaliana (Col-0) are treated by dissolving the tested EONE in the basal nutrient solution, and the leaves are infected with the inoculum 24 hours after treatment. Images are collected daily for 3 days. During image acquisition, the automated system shines light of different wavelengths onto the plants rosettes. Chlorophyll absorb short wavelength light (blue) and the longer (NIR) wavelength reflected light is filtered through a LP696 filter that blocks lights ultraviolet (UV) and visible (VIS). Collected images are automatically segmented based on their chlorophyll fluorescence. Lack of fluorescence from necrotic tissue is not displayed in the processed image.

Physiological assessment of treated plants via RNA-seq

Plant tissue was grounded with pestle and mortar with liquid nitrogen and total RNA was extracted with Trizol reagent according to the manufacturer's instructions (Sigma-Aldrich). Three independent biological replicates per condition were used to quantitatively assess the differential gene expression between treated plants against untreated plants via RNA-seq analysis. Illumina sequencing was carried out by Novogene Bioinformatics Technology Co., Ltd., in Beijing, China.

3.2.4 Statistical analysis

Multispectral Image-based phenotyping assessment of treated plants

For the statistical analysis, an orthogonal model was used with nine independent plants for each of the concentrations of EONEs under study. A two-way ANOVA was conducted to evaluate the effect of each of the different levels of tested concentrations on the relative rosette area growth over three time points, per treatment. A pair-wise comparison using Tukey HSD test was conducted to compare the differences between the LSmeans for each of the levels on the model. An alpha value was set at 0.01 to establish statistical significance.

Phenotypical and physiological assessment of the systemic effect of EONEs on the quantitative disease resistance of A. thaliana (Col-0) against B. cinerea via image-based phenotyping

Phenotyping assessment of the effect of EONEs was quantitatively assessed by measuring the resulting necrotic area 72 hours post infection, from four different leaves per plant. Five independent plants were used as biological replicates per treatment. A nested ANOVA model was carried out to assess the effect of treatments on the quantitative disease resistance in the plant-pathogen model system under study. Statistical significance was set at alpha (α) = 0.01.

Differential gene expression analysis

The gene expression levels between two different experimental conditions (treatment Vs Control) were compared. Both experimental groups consisted of three biological replicates. The differential expression analysis between the two experimental conditions was performed using the DESeq2 R package according to Anders, 2010[95]. The DESeq2 R package provides statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. Therefore, if the read count of the *i*-th gene in *j*-th sample is Kij, there is: Kij $\sim$ NB(μij,σij2) and the resulting P values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate.

$$Kij \sim NB(\mu ij, \sigma ij2)$$

Functional analysis

Gene Ontology (GO) and KEGG enrichment analysis were conducted with a adjusted p-value < 0.05 to assess statistical significance. Significantly enriched metabolic or signal transduction pathways associated with differentially expressed genes, compared to the whole genome background from the KEEG analysis was conducted as described by Kanehisa, 2000[96].

$$P = 1 - \sum_{i=0}^{m-1} \frac{\binom{M}{i}\binom{N-M}{n-i}}{\binom{N}{n}}$$

Where, N is the number of all the genes with a KEGG annotation, n is the number of differentially expressed genes in N, M is number of all genes annotated to specific pathways and m is the number of differentially expressed genes in M.

3.3 Results and discussion

Since the infection mechanisms from broad host necrotrophic pathogens (BHN) are complex and depend on a plethora of different genes, true immunity from plants against BHN cannot be achieved through simply inherited resistance. However, resistance to BHN can be quantitatively enhanced. To date, synthetic fungicide application remains the most common method to control the diseases caused by BHNs, although it is a practice that is becoming increasingly restricted. The motivation of this work is to investigate the potential of drug-delivery nano-systems conventionally used in the biomedical field, comprising of different plant derived EO as an eco-compatible and safe alternative to synthetic fungicides to control BHN. Factoring in the limitations of using EO in a large scale, such as poor solubility in water and related low potency, we also investigated the main drivers of isothermal formation of nano-emulsions under low energy conditions, with the purpose of simplifying the formulation and fabrication process. We further investigated the hermetic effect of these essential oil-based nano-emulsions in the QDR of plants against BHN in the *A. thaliana-B. cinerea* pathosystem model and the molecular mechanisms behind it.

3.3.1 Essential oil nano-emulsion formulation, production, and characterization:

For essential oil-based nano-emulsions, low energy methods are preferred since the molecules present in the EO from plants have high vapor pressure and are thermolabile. Therefore, in order

to prevent losses due to evaporation and/or denaturation and consequential loss of biological function, it is imperative to not subject EOs to harsh conditions during the nano-emulsification process. Low energy methods for the formulation of nano-emulsions at room temperature are available in the literature [41–44]**.** However, these methods often require extensive, time demanding trial-and-error experimentation. This approach is usually based on investigating the effect of systematically changing the formulation variables and fabrication conditions of the droplet size. In general, the formulation variables include the oil and surfactant, as well as the salinity and temperature of the system. Further, the composition, as well as production configuration such as order and rate of individual component addition, and agitation speed amongst others. Reported results in the literature are often difficult to reproduce when the exact components and conditions are not replicated.

Here, we report on a rational approach to rapidly formulate, and simply produce, highly stable and translucent EONEs at room temperature via spontaneous emulsification (a low energy method) by using the HLD (Eq.1), and the modulation of the capillary number (eq.2), instead of focusing on the HLB as commonly reported in the literature. In this work, we investigated the effect of modulating the viscosities (η) and surface tensions (γ) of the continuous phase using polyethylene glycol (PEG) and propylene glycol (PG) as co-solvent models. Soybean oil (SBO) was used as the viscosity modulator model for the organic (dispersed) phases from 7 different types of EOs.

Eq. 1: Hydrophilic-lipophilic difference*:*

$$HLD = Cc - k * EACN - \alpha * \Delta T + f(S)$$

Where: *Cc is the Characteristic value or critical curvature of the surfactant(s),*
K, is the scaling factor for the oil fraction described for EACN (generally taken to be 0.17),
EACN is the Effective Alkane Carbon Number of the oil(s),
(α) is a constant. For typical anionics α=0.01 and for typical ethoxylates α=-0.06. For typical sugar surfactants α=0.
ΔT is the difference in temperature from the standard, 25°C.
S is the salinity of the system expressed in g NaCl /100 ml (aqueous phase). The salinity term is 0.13S for non-ionics and ln(S) for ionics.

Since the droplet break-up during the emulsion formation depends on the capillary number (Eq.1) [29], which in turn depends on the fluid viscosity and surface tension, we characterized the co-solvents under investigation in order to assess their potential effect during the emulsification

process. Fig. 1a-b show that when tested under identical conditions, the effect of propylene glycol (PG) on the viscosity of the aqueous phase is not as strong as that from polyethylene glycol (PEG). However, PG reduces the surface tension of the aqueous phase significantly more than PEG at concentrations over 50% (w/w). Fig. 1c shows how a gradual increment in the soybean oil proportion in the organic phase blend increases its viscosity. We believe that the addition of SBO into the nano-emulsion formulation has a dual purpose: It may contribute to the nano-emulsification process by adjusting the viscosity differential, but may also serve as a biological active ingredient, in the defense response of the plant-pathogen model system under study.

Eq. 2: Capillary number:

$$Ca = \frac{U\eta}{\gamma}$$

Where: (U) is the flow of velocity,
(η) is the fluid of viscosity and
(γ) is the surface tension.

Further formulation iterations were conducted at a fixed HLD by systematically altering the capillary number of the system, by modifying the viscosities of both, the aqueous and organic phases. The viscosity of the aqueous phase was modified by changing the concentration and type of co-polymer in the formulation. The viscosity of the organic phase was modified by blending soybean oil (SBO) with the EO at different proportions (w/w).We fixed the formulation to a blend of EO:SBO %(w/w) 95:5 for the organic phase, since the change in viscosity for this blend is minimum (Fig. 1c), but allows the incorporation of SBO. The HLD value was fixed to negative five with Tween80®, using an effective alkane carbon number (EACN) of seven at 25°C.

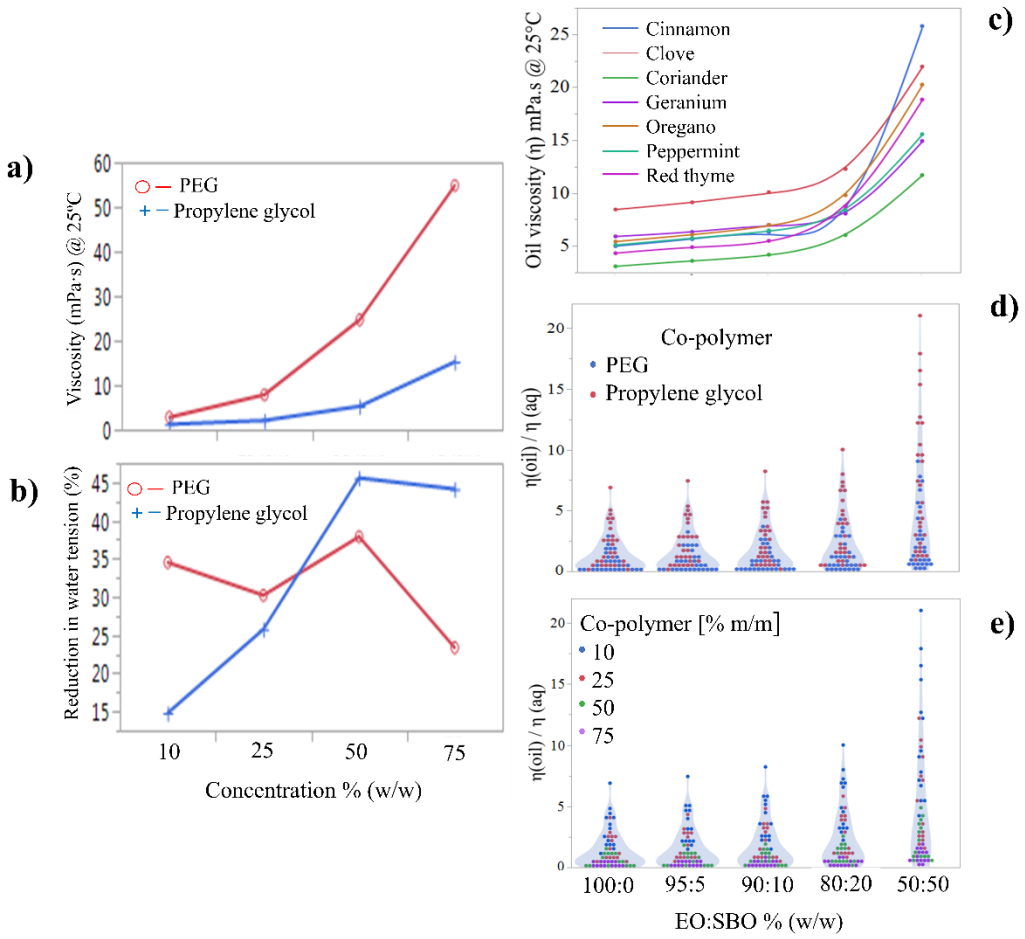


Fig. 3.1: Comparative characterization of the rheologic and viscometric properties of the modified aqueous and organic phases. 1a-b, shows the effect of polyethylene glycol (PEG) and propylene glycol (PG) on the viscosity and surface tension of water at 4 different concentrations. c) Shows the effect of incremental addition of soybean oil (SBO) on the viscosity of essential oils. (d-e) Violin plots show the distribution of the viscosity differential for different concentrations of PEG or PG on the blends of organic phase containing varying levels of cinnamon oil as a model system.

Our results suggest that investigating the viscosity differential (ηD/ηC) between the aqueous (continuous) and organic phase (dispersed), rather than the HLB, is more important for fabricating EONEs. HLB fails to explain the behavior of surfactant, temperature, and salt in the system, but the ηD/ηC influences the capillary number of the system, a phenomenon that governs droplet formation during the emulsification process. When the oil is much less viscous than the aqueous phase, oil droplet break-up is difficult but possible under higher flow velocity. On the other hand, when the organic (dispersed) phase is much more viscous than the aqueous (continuous) phase, oil droplet break-up is impossible since the oil droplets absorb the inertial energy, and they spin instead of split [29]. When the differential in viscosities lays within an optimal range, the capillarity

number in the system is greater than the critical capillary number, allowing droplet breakup. The optimal range of ηD/ηC where droplet disruption during emulsion formation is most efficient[30] is between 0.1 and 5.0. The ηD/ηC can be modulated by the addition of polymers in the aqueous phase and viscous oils in the organic phase. By following this approach, we were able to produce concentrated translucent nano-emulsions with each of the seven different EOs tested using only a few iterations on the ηD/ηC via changes on the propylene glycol concentration, instead of using a multifactorial experimental design normally reported in the literature. The ηD/ηC for cinnamon, clove, coriander, geranium, peppermint, red thyme, and oregano that yielded translucent nano-emulsion were 0.37, 3.5, 1.3, 1.2, 1.1, 0.32 and, 0.4 respectively.

We set a constant HLD value (-5) with Tween80® comprising of a blend of EO:SBO (95:5) % w/w in the organic phase for all the formulations, and modified the viscosity of the water with PEG or PG. Table 1 shows that using PG in the aqueous phase, resulted in a translucent o/w nano-emulsions under the operational conditions previously described (i.e. 25°C, 500rpm, SOR=3, dripping at 0.5 mL/min). In contrast, none of the emulsions containing PEG resulted in translucent nano-emulsions and therefore were not subject to further analysis. It is possible that the inability of PEG to produce translucent EONEs could be explained by *i)* the increased viscosity it yields (Fig. 1a) combined with its inability to reduce the surface tension in the aqueous phase (Fig. 1b). In contrast, propylene glycol reduces the surface tension of the water significantly more ($p<0.001$) than PEG, specially at concentrations above 50% (w/w) (Fig. 1b); and *ii)* the viscosity of propylene glycol is significantly lower ($p<0.0001$) than that from PEG at all of the concentrations evaluated (Fig. 1b). Due to the relative low viscosity of EOs, the incorporation of PEG impairs the viscosity differential between the two phases of the system (Fig. d-e), preventing nano-emulsification of EO under low energy conditions. PEG has been successfully used before to produce small, translucent nanoemulsions[50], but it requires the assistance of high shear homogenization devices.

Table 3.1: Main characteristics of some undiluted essential oil nano-emulsions obtained using propylene glycol and soybean oil as viscosity modulators.

Essential oil	Viscosity (mPa·s)	ηD/ηC ratio	Nano-emulsion mean droplet diameter (nm)	Polydispersity	Appearance of undiluted nano-emulsion
Cinnamon	4.991	0.3686	155.6	0.252	Translucent
Clove	8.456	1.66	74.77	0.346	Translucent
Coriander	3.082	4.21	49.3	0.25	Translucent
Geranium	5.912	1.19	59.61	0.25	Translucent
Peppermint	5.096	2.64	155	0.285	Translucent
Oregano	5.422	0.39	337.1	0.304	Translucent
Red thyme	4.322	0.31	256.9	0.19	Translucent

All of the EONEs described contain a SBO:EO weight (%) ratio of 5:95. The proportion of propylene glycol in each formulation varies. DLS characterization was conducted on freshly prepared EONEs without further dilution or filtration.

DLS analysis was performed on translucent emulsion only, without further filtration or dilution. Droplet characterization on previously diluted emulsions are common in the literature [44–46]. However, misinterpretations of particle size distributions may result due to artifacts in the droplet size distribution caused by dilution of the NEs (Supplementary information – SI.1).

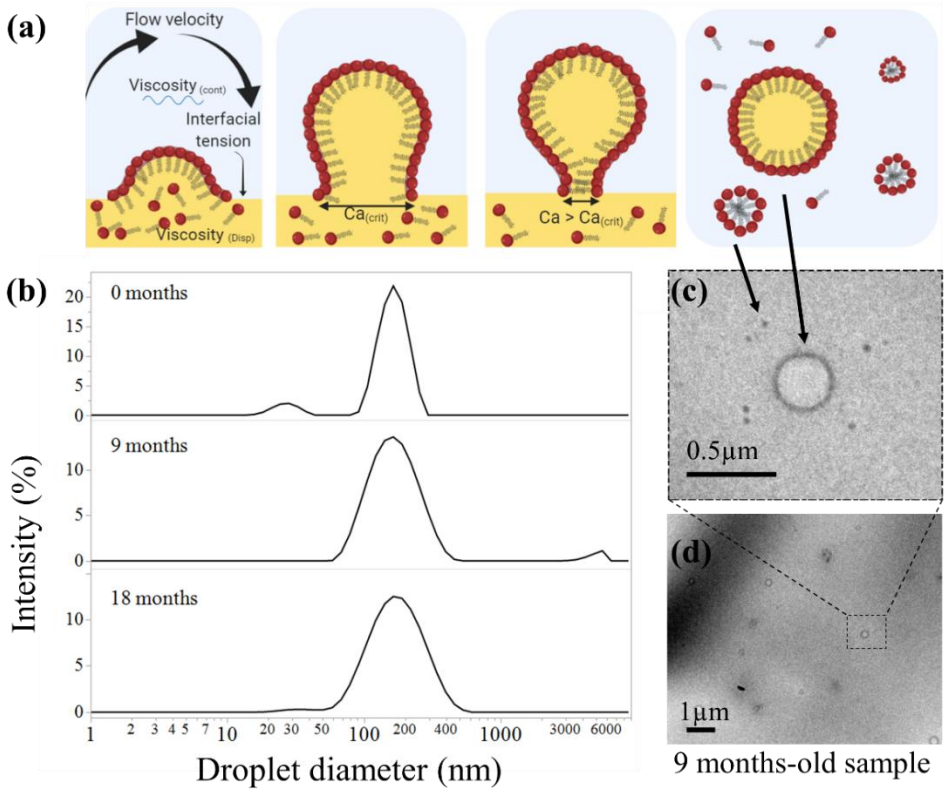


Fig. 3.2: Formation and characterization of stable EONEs under low energy conditions by modulation of the viscosity differential with propylene glycol and soybean oil. .Fig. 2a is a schematic representation of the nano-emulsion formation process via spontaneous emulsification driven by the modulation of the viscosity in continuous phase under mild conditions. First, upon contact with the aqueous phase (containing a viscosity modulator), the organic phase (added dropwise) experienced a deformation driven by the flow velocity of the agitation process and enabled by the viscosity differential (ηD/ηC) between the continuous and dispersed phases. Fig. 2b. presents the droplet size distribution of three independent cinnamon oil nano-emulsions at three different times, obtained via dynamic light scattering. Fig.2 (c,d) shows the transmission electron micrographs (TEM) from 9 old-month cinnamon nanoemulsion demonstrating stability and conforms to the size distribution from the DLS analysis.

Free surfactant molecules in excess may interact with each other forming free micelles. An optimized micelle-free finely-tuned formulation may be possible using the Net Average Curvature (NAC) theory first described by Sabatini et al.[47], and further developed upon by Acosta [48,49]. NAC uses the geometrical characteristic, ξ of a surfactant which depends on its head area and tail length, driving its adopted configuration based on its interaction with oil and water to predict the direction of the (average) curvature. In theory, it is possible to estimate the necessary amount of certain surfactant (or blend of surfactants) needed per gram of oil for a desired droplet size by combining the HLD-NAC approaches, but this is beyond the scope of this work.

3.3.2 Assessment of the hormetic dose response of EONEs in the plant-pathogen model system *A. thaliana (Col-0)-B. cinerea.* via image-based phenotyping

Although the mechanisms involved in volatile mediated plant-plant interactions have yet to be fully elucidated, evidence suggests that these molecules not only prime the immune system upon perception by eliciting the expression of defense related genes [14], but also induce their biosynthesis *de novo*, which comes with a metabolic costs [50]. Under adverse conditions, plants face a trade-off between growth and defense. Stressed plants shift their physiological status by diverting energy and resources that are used to sustain growth and development under otherwise normal conditions, to favor the defense mechanisms in order to endure adversity to trigger survival responses. Therefore, a phenotypical hallmark displayed by plants under such stressful circumstances, signals the halt of growth [51].

Fig.3 shows the effect over time of EONEs from cinnamon, clove, coriander, geranium, peppermint, oregano and red thyme on the mean growth rate of *A. thaliana* rosette upon exposure of the root to EONEs at two different concentrations (50 and 500 μg/mL). The growth behavior from untreated plants under the same incubation conditions are presented for each of the two concentrations for reference. We found that although the experimental conditions were kept identical, the control group (untreated plants) showed a slight difference ($p=0.0243$) between the two groups over time. Specifically, the mean relative grow on day 3, where the difference between the two groups was the strongest ($p=0.0014$). This indicates that there is some variation that could not be explained by the experimental conditions. Due to this, a lower of alpha(α) = 0.01 was used to test the statistical significance of the interaction between the two concentration levels over time in the forthcoming treatments. However, the relative growth of *A. thaliana* seedlings, measured as the relative difference in the area of the rosette compared to its initial size, was significantly different for both groups after day 2, indicating that the image-based phenotyping approach using a chlorophyll-derived fluorescence for image treatment and analysis, is sensitive enough to discern statistical noise from biological signal.

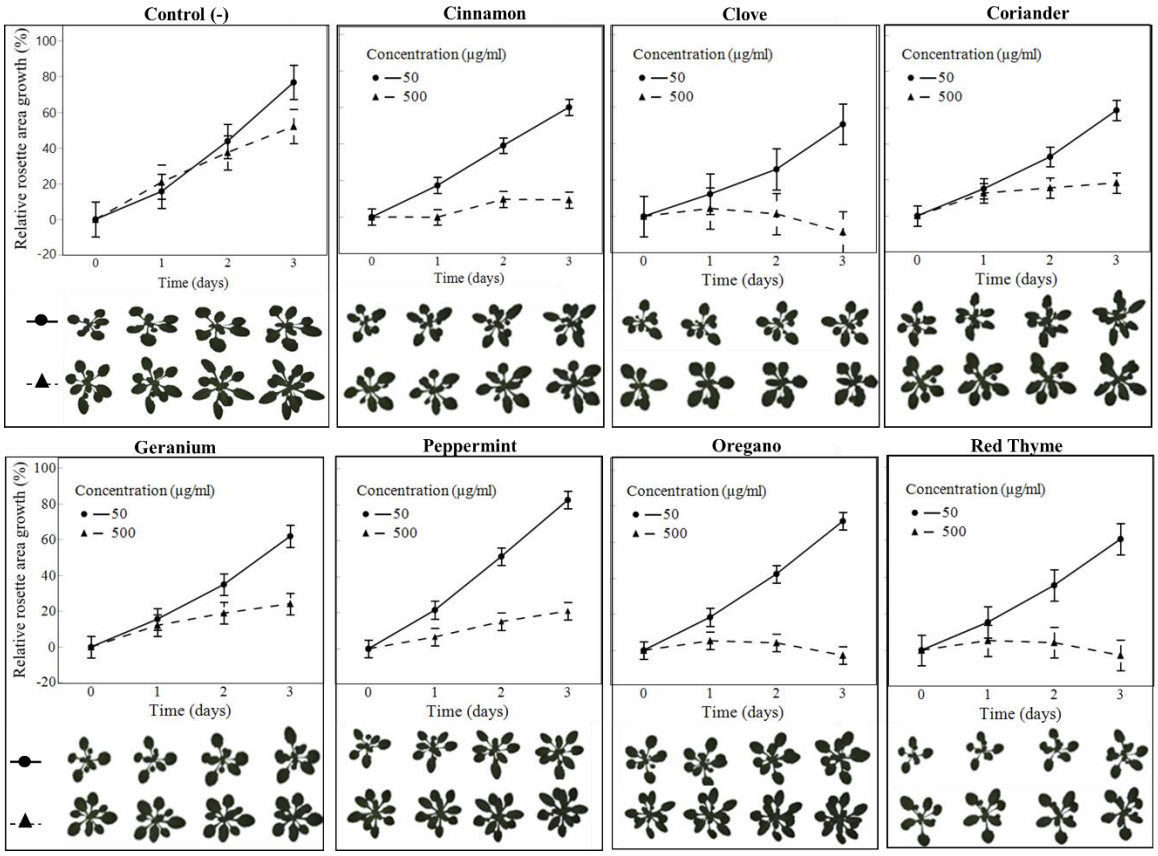


Fig. 3.3: Assessment of the hormetic dose-dependent response from A. thaliana to various essential oil nano-emulsions. Fig.3: The least square mean plots from the two-way ANOVA conducted for each of the concentrations under study, represent the mean relative growth of the rosettes from a set of 9 independently grown seedlings under identical hydroponic conditions. Error bars represent the 95% confidence interval of the LS means. To test the differences between LS means, the pairwise comparison Tukey HSD test was employed at alpha (α) = 0.01 to determine the statistical significance. Levels not connected by the same letter are significantly different.

The image-based phenotyping analysis conducted on the EONEs previously mentioned demonstrates a concentration dependent response on the growth of *A. thaliana* plants under investigation. Fig. 3 show the interaction plots from the full set of EONEs at the two tested concentrations. In general, none of the EOs used in the nano-emulsions negatively altered the relative growth of the *A. thaliana* over time when treating its roots with 50 µg/mL. However, upon increasing the concentration to 500 µg/mL, the mean growth of the rosettes from the *A. thaliana* seedlings was significantly less. This was true for all of the EONEs tested, although to a different extent as expected. For instance, nano-emulsion formulations with cinnamon, clove, oregano and red thyme completely halted the growth of treated plants in less than 24 h, whereas nano-emulsions from coriander, geranium and peppermint, although equally effective, were not as efficient. On an average, none of the treated plants with 500 µg/mL EONEs grew over 20% relative as noted from

the measured rosette area. Since we exposed the roots to the EONEs, we measured the effects on the rosette (see fig. 4), our nano-emulsified EO triggered a *systemic* immune response.

A key element in the transition between growing status to a *systemic* defense response status is the plant hormone jasmonic acid (JA) which counterbalances the salicylic acid (SA) hormone signaling system that prevails under optimal growing conditions [4]. JA carries the "alarm message" to distal parts of the plant triggering the cellular defense response at a systemic level. The biosynthesis and role of jasmonates in the plant immune response has been extensively described in the literature [52–55]. Briefly, (JA) is synthesized from polyunsaturated fatty acids (PUFAs), such as α-linolenic acid (α-LeA) of membranes through the octadecanoid pathway that involves the translocation of lipid intermediates from the chloroplast membranes to the cytoplasm and later on into peroxisomes. There is evidence that VOCs, the main constituents of EO, triggers the oxylipin pathway[56] which is critical for JA formation [57]. Given that the nano-emulsion formulation comprise of a blend of soybean oil (rich in PUFAs) and EO (rich in VOCs), it is feasible that the change in growth upon treatment with EONEs, is a consequence of activation of the JA signaling system.

Intracellularly, JA turns into methyl jasmonate (MeJa). MeJa induces metabolic reprogramming that contributes to the regulation of the trade-off between defense mode and plant growth by inhibiting cell proliferation and halting cell expansion while enhancing the defense response [58,59]. MeJa is also responsible for the induction of biosynthesis and subsequent accumulation of secondary metabolites (such as volatile organic compounds in EO) [60]. It is well established in the literature that Ethylene (ET) and JA are the main contributors to the defense response of plants against necroptrophs, whereas salicylic acid (SA) primarily regulates the defense resistance to biotrophic and hemibiotrophic pathogens [61].

3.3.3 Phenotypical and physiological assessment of the systemic effect of EONEs on the quantitative disease resistance of *A. thaliana* (Col-0) against *B. cinerea* via image-based phenotyping

Due to their necrotrophic lifestyle, the restriction of the HR from plants is a paramount strategy that plants can employ to prevent disease progression once infected. Although information on the genetic control of HR is scarce, there are a number of potential mechanisms that plant cells may

undertake to achieve it. A growing body of evidence suggests that plants can deter BHN infection by preventing the accumulation of ROS intracellularly, by upregulating the autophagocytic process and by enhancing JA production. We hypothesized that exposure of plant roots to EONEs may trigger these biological processes, resulting in enhanced quantitative disease resistance against BHN. In order to test this, we conducted RNA-seq analysis from plants treated with cinnamon NE, because this treatment resulted in the most enhanced quantitative defense response compared to untreated plants. The RNA-seq analysis showed that a total of 1405 differentially expressed genes (DEGs) were uniquely expressed by the treated plants, whereas 1073 genes were expressed by untreated plants only. Most of the DEGs in the treated phenotype participated in the activation of secondary metabolism, transmembrane transport, JA-mediated metabolism, and response to stress. (Supplementary information-SI 2) In contrast, DEGs from untreated plants were associated with the process of growth and development, such as cell cycle phase transition, cellular responses to brassinosteroid and cytokinin stimulus, as well as responses to gibeberellin , amongst others. (Supplementary information-SI 2). Fig. 6b shows the gene ontologies for differentially expressed biological functions from treated vs. untreated plants.

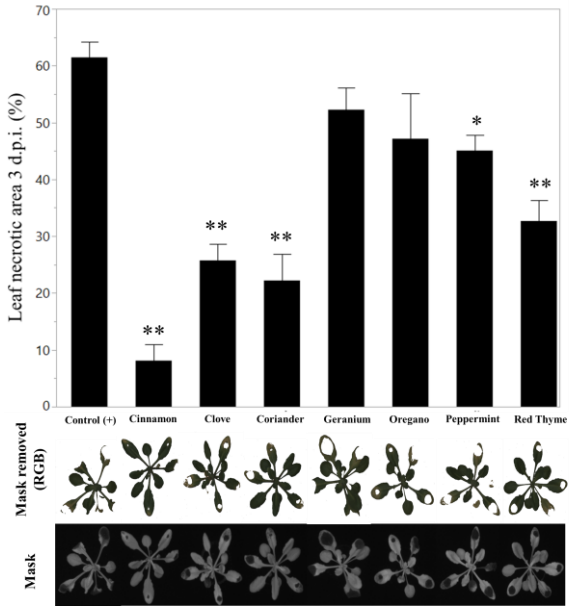


Fig. 3.4: Effect of essential oil nano-emulsions (EONEs) on the quantitative disease resistance (QDR) in the plant-pathogen model system A. thaliana – B-cinerea assessed by automated phenotyping using chlorophyll fluorescence-based segmentation. Bars represent the mean and standard error of a nested model measuring necrotic areas from four leaves per plant and 5 plants per treatment. Statistical differences were evaluated per the nested ANOVA followed by a pair-wise comparison with a Dunnett's adjustment relative to the control group. Asterix on top of the bars indicate a significant difference between the treatment and the control group (*$p<0.05$ and **$p<0.001$). Images in the bottom depict an example of each of the treatments. Mask and removed mask (RGB) shows the image segmentation based on fluorescence emited by the chlorophyll upon excitation with blue light. Gaps in the leaves indicate areas no fluorescence (i.e. necrotitic areas).

As shown in Fig. 5 nano-emulsions from cinnamon, clove, coriander, and red thyme showed a significant effect on the QDR of *A. thaliana* against *B. cinerea*. The strongest effect was produced by cinnamon nano-emulsion, with a mean necrotic area of 10%, which is c.a. 50% less than the untreated plants. The necrotic area observed in plants treated with coriander, clove, and red thyme EONEs were, on an average, between a third to a half of the necrotic area observed in the control group. The EO extracted from cinnamon bark contains mainly cinnamaldehyde (65.00 to 80.00%) and Eugenol (5.00 to 10.00%)[62]. Eugenol is also present in the essential oil from clove buds, where is its main constituent (70-95%) along with eugenol citrate (up to 20%) and β-caryophyllene (12–17 %) [63]. The relative composition of the EO extracted from coriander seeds is mainly linalool (72.7%) followed by λ-terpinene (8.8%), α-pinene (5.5%), camphor (3.7%), limonene (2.3%), geranyl acetate (1.9%) and p-cymene (1.5%), although the oil composition may change depending on the maturity of the seed [64]. The major components in the EOs from red thyme have been identidied as thymol (48%), γ-terpinene (31%), and p-cymene (8%) [65].

The role of cinnamaldehyde [66], eugenol [67], thymol [68] and linalool [69] and terpinene [70] in the defense response of plants have been studied. In general VOC from EO induce the JA-defense response [69] For instance, there is evidence that cinnamaldehyde elicit a defense response in plants that result in significatively less accumulation of salicilic acid, which indicates up-regulation of the JA/ET pathway[71]. The ability to transduce infection signals from focal points into a systemic response in a coordinated and timely manner is imperative to effective plant defense. The intrinsicacies of the transcription dynamics related to plant immunity are available in the literature [72] as well as a description of the major transcription regulators during the plant immune response against necrotrophic pathogens [73.]

As mentioned before, the mechanisms involved in the cross-communication mediated by VOCs is not completely elucidated but evidence suggests that some of the mechanisms in the translocation of these molecules from its source to where they accumulate may involve simple diffusion, vesicle-mediated transport and, transporter-mediated membrane transport [74]. Several genes have been identified for JA-responsive transporters involved in the membrane transport of various secondary metabolites including the families: ABC (ATP-binding cassette) transporter, NRT (Nitrate-peptide transporter), MATE (multidrug and toxic compound extrusion) and PUP (purine permease) [75]. In *A. thaliana*, there are around 120 genes for ABC proteins, 53 for NRT, 56 for MATE and , 21 PUP

genes [74]. GSEA-GO[76] showed that exposure of roots to cinnamon NE resulted in enhanced transporter activity (see table 2). Specifically, nucleotide transmembrane transporter activity (GO:0000295, GO:0051503, GO:0015215, GO:0005347). Consistent with the fact that these are JA-dependent transporters, the gene set enrichment analysis (GSEA) also revealed that the biosynthetic process of JA (GO:0009695 and GO:0009694) were significantly enriched in the treated plants, as well as the toxin biosynthetic process (GO:0009403) which are part of the secondary metabolite metabolic synthesis. Finally, the GSEA analysis showed that by treating the plants with cinnamon nano-emulsion at the tested concentration, significantly enriched the gene set involved in the positive regulation of transcription from RNA polymerase II promoter in response to stress (GO:0061408 and GO:0036003).

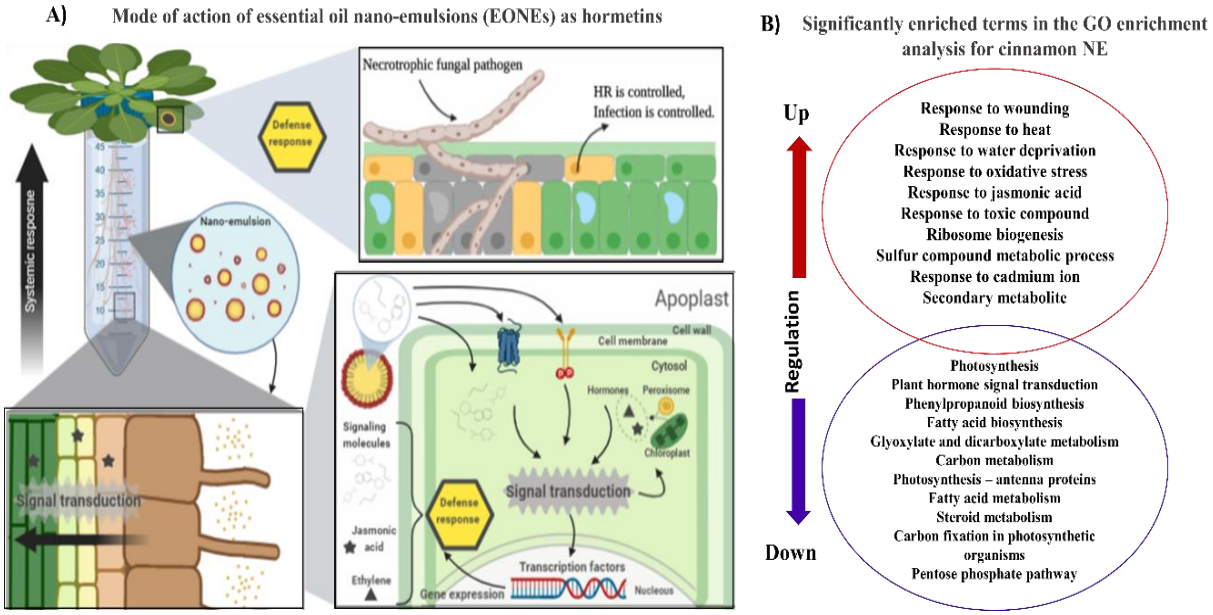


Fig. 3.5: Mode of action of essential oil nano-emulsions (EONEs) as hormetins. Fig. 5a is a schematic representation of the mode of action of cinnamon essential oil nano-emulsions (EONEs) in the activation of hormesis leading to enhanced quantitative disease resistance against broad host necrotrophs (BHN) in the plant-pathogen system A. thaliana-B. cinerea. b) List of biological targets up regulated and down regulated upon exposure to cinnamon oil nano-emulsion.

The transcriptome from *A. thaliana* plants treated with cinnamon oil NE were analyzed to elucidate the biological mechanisms involved in the enhanced QDR caused by this treatment in our model systemplant-pathogen model system. The gene ontology (GO) functional analysis on the differentially expressed genes (DEGs) in table 3 revealed that, after 3 days post treatment, the set of genes that were significantly up-regulated compared to untreated plants were those involved in

the biological functions controlling the major defense responses, including the responses to heat, water deprivation, oxidative stress and toxic compounds. Further, the genes that control the activation of secondary metabolism were upregulated, including those responsible for the biosynthesis of sulfur-compounds. VOCs from the EO are rich in such type of compounds [77,78]. In contrast, the GO annotations from genes controlling major biological functions related to growth and development were down-regulated.

The JA-mediated defense is vital for the plants to enhance resistance to *B. cinerea* [79]. As discussed before, activation of the JA-mediated defense response shifts the physiological status of the plant, halting growth and activating defense-associated mechanism. Ontologies associated with JA-based physiology were shown to be significantly enriched in plants treated with cinnamon EONE, including biomarkers such as FAMT, PDF1.2, VSP2 and WRKY, amongst others [80–82] . These findings suggest that the enhanced systemic QDR, observed in the phenotyping assays is most likely caused by molecules from the cinnamon NE.

KEGG functional annotations on the DEGs from plants treated with cinnamon NE indicate that amongst the main up-regulated metabolic pathways were the ribosome and the proteasome biogenesis (see table 4). This is most likely due to an attempt from cells to maintain homeostasis since, especially under conditions of cellular stress, ribosomes, ER and proteasomes are substrates for selective degradation (i.e. selective autophagy) through complex mechanisms that are only recently beginning to emerge [83]. GO terms for biomarkers of autophagy were significantly upregulated in plants treated with cinnamon NE, including gene members of the families ATG, BAG, RAPTOR, VTI, RING and ATI (suppl. info). For instance, atBAG4 gene that appears to inhibit apoptotic-like plant cell death (i.e. HR) and functions in stress tolerance in plants; as well as inhibitors of cell death domains such as BIR1 (a.k.a BAK1) and BAG6, which is thought to be implicated in endoplasmic reticulum stress-induced cell death via regulation of apoptosis-inducing factor (AIF) [84]. We also found that KIN2 gene was significantly enriched in plants treated with cinnamon oil NE. KIN genes promote phosphorylation of ATG genes and the TOR complex subunit RAPTOR which inhibits TOR activity and initiate autophagy [85]. There is evidence that BOS1 (BOTRYTIS SUSCEPTIBLE 1) and BOI (BOTRYTIS SUSCEPTIBLE INTERACTOR) seem to restrict pathogen induced necrosis[73]. Our GSEA results show that BOI gene is overrepresented in plants treated with cinnamon NE. This gene Has E3 ubiquitin ligase activity

and interacts with and ubiquitinates BOS1 (Botrytis Susceptible 1) preventing caspase activation resulting in attenuation of cell death [86].

Gene biomarker VTI11, encoding a member of SNARE gene family was also significantly enriched (Soluble N-ethylmaleimide-sensitive-factor Attachment protein Receptor). Components of the SNARE machinery are required for the fusion process and autophagosome membrane expansion [87]. This may explain the reason why KEGG annotations for the GSEA of DEGs indicates that the snare interaction in vesicular transport (ath04130) was significantly enriched (supplementary information). Fig.6b indicates that the in general, biological processes involved in carbon and energy metabolism were significantly downregulated. This is consistent with the fact that tight linkages between autophagy and energy metabolism, particularly sugar signaling, exist [84]. One of the overrepresented GO terms in the plants treated with cinnamon NE was the ATI2 gene. This gene encodes an Atg8-interacting protein. This interaction contributes to the selective autophagy, targeting mitochondria, protein aggregates, chloroplasts and invading pathogens that is critical during stress tolerance [84].

Table 3.2: Comparison of significantly enriched gene ontology terms from plants treated with cinnamon EONE

Cinnamon NE treated group	Control group
Adenine nucleotide transmembrane transporter activity.	At DNA binding
Adenine nucleotide transport.	Carbonate dehydratase activity
Alkali metal ion binding.	Cell cycle phase transition
ATP transmembrane transporter activity	Cellular response to brassinosteroid stimulus
ATP transport	Cellular response to cytokinin stimulus
Energy reserve metabolic process	Condensed chromosome centromeric region
Glycogen metabolic process.	Condensed chromosome kinetochore
Jasmonic acid biosynthetic process	Cytokinin activated signaling pathway
Jasmonic acid metabolic process	Fluid transport
Nucleotide transmembrane transporter activity	Meiosis II
Nucleotide transport.	Meiosis II cell cycle process
Positive regulation of transcription from RNA polymerase II promoter in response to heat stress.	Prenyltransferase activity
Positive regulation of transcription from RNA polymerase II promoter in response to stress.	Purine nucleoside biosynthetic process
Potassium ion binding	Purine ribonucleoside biosynthetic process
Purine nucleotide transmembrane transporter activity	Response to cytokinin
Purine nucleotide transport.	Response to gibberellin
Pyruvate kinase activity	Voltage gated cation channel activity
Toxin biosynthetic process.	Water transmembrane transporter activity
	Water transport

KEGG notations from the GSEA, indicates that the most significantly enriched pathway from the treated plants correspond to the control of the alpha linoleic acid metabolism. As discussed before, linolenic acid is a precursor for the biosynthesis of JA, via β-oxidation. A key element in the formulation of EONEs is the addition of soybean oil as a viscosity modulator. Hence, the function of SBO in the EONEs is dual: facilitates the formation of EO nano-sized droplets and promotes the JA response in plants, resulting in enhanced QDR. Gene ontologies related to the biosynthesis of jamsonic acid (see table 3) supports this conclusion.

Table 3.3: Comparison of significantly enriched KEGG annotations from plants treated with cinnamon EONE

Cinnamon NE treated group	Control group
Alpha linoleic acid metabolism	Aminoacyl tRNA biosynthesis
Arachidonic acid metabolism	Butanoate metabolism
Ascorbate and aldatate metabolism	Carbon fixation in photosynthetic organisms
Biosynthesis of amino acids	Carbon metabolism
Circadian rhythm – Plant	DNA replication
Cysteine and methionine metabolism	Fructose and mannose metabolism
Fatty acid degradation	Glyoxylate and dicarboxylate metabolism
Folate biosynthesis	Nicotinate and nicotinamide metabolism
Glutathione metabolism	Nitrogen metabolism
Glycine, serine and threonine metabolism	One carbon pool by folate
Histidine metabolism	Other glycan degradation
Lysine degradation	Pentose and glucuronate interconversions
Peroxisome	Pentose phosphate pathway
Phenylalanine, tyrosine and tryptophan biosynthesis	Phagosome
Protein processing in endoplasmic reticulum	Photosynthesis
Purine metabolism	Photosynthesis antenna proteins
RNA degradation	Plant hormone signal transduction
Spliceosome	Steroid biosynthesis
Sulfur metabolism	Terpenoid backbone biosynthesis
Valine, leucine and isoleucine degradation	Various types of N-glycan biosynthesis

Finally, amongst the major metabolic pathways that were significantly enriched in the treated plants were the glutathione metabolism, and the cysteine and methionine metabolism (see table 4). In plants, glutathione in a paramount hallmark of stress related response, involved in the cell detoxification of ROS [88,89]. Cysteine is the metabolic precursor of glutathione and other key components involved in the signal transduction in plants under stress [90].

The nascent field of phytobiome research attempts to overcome the challenge of reconciling the learnings in the fields of agriculture and ecology while paving the way to engineer strategies for

better crop and ecosystem management [10]. Similarly, the concepts of "hormesis" and "biphasic mechanism" are generally considered as two independent forms of biological response, ergo, the research efforts and literature describing them were historically diverted into the fields of toxicology and pharmacology. And lastly, we have attempted to merge the concepts of drug delivery, traditionally restricted to biomedical applications, into the realm of agriculture [91].

The aqueous-to-oil phase viscosity differential can be modulated using soybean oil and food-grade polymers to facilitate the oil-droplet disruption leading to the formation of nano-sized oil droplets. By fixing a unique HLD value to c.a. -5, we have simplified the nano-emulsion formulation process. We were able to formulate and produce translucent nano-emulsions under low energy conditions at room temperature by developing a rational approach using the droplet capillarity and emulsion hydrophilic-lipophilic differences (HLD) as numerical indicators, rather than extensive, time consuming HLB analysis for formulation purposes. Eight different essential oils were used to produce such nano-emulsions and characterized for particle size, shape, and distribution.

Here, we demonstrate that EONEs from cinnamon, clove, coriander and red thyme significantly enhanced the quantitative disease resistance in the model plant-pathogen system, *A. thaliana-B. cinerea* under controlled experimental conditions. Comparative, quantitative RNA-seq analyses with cinnamon NE-treated plants and untreated plants showed that the main physiological processes enabling the enhancement of QDR are related to the up-regulation of autophagy, JA-dependent cell-to-cell communication and ROS scavenging detoxification. These processes combined, allowed the tight control of hypersensitive response, hindering disease progression.

Given the vast heterogeneity of EO, and the intrinsic technical difficulty of working with them due to their poor water solubility, specific molecules that trigger the host defense response and the ensuing mechanism is still not clear, suggesting opportunities for further studies.

Author contributions

PV-V and JI conceived the idea. PV-V designed and carried out the experiments, analyzed the data and wrote the initial manuscript. NM and JI supervised the findings of this work, discussed experiments and results. All authors contributed to writing the manuscript.

3.4 References

1. Mattson, M. P. Hormesis defined. *Ageing Research Reviews* **7**, 1–7 (2008).
2. Calabrese, E. J. & Mattson, M. P. How does hormesis impact biology, toxicology, and medicine? *npj Aging Mech. Dis.* **3**, 1–8 (2017).
3. Colla, G. *et al.* Plant Hormesis Management with Biostimulants of Biotic Origin in Agriculture. *Front. Plant Sci* **8**, 1762 (2017).
4. Jones, J. D. G. & Dangl, J. L. The plant immune system. *Nature* **444**, 323–329 (2006).
5. Scheres, B. & Van Der Putten, W. H. The plant perceptron connects environment to development. *Nature* **543**, 337–345 (2017).
6. Austen, N., Walker, H. J., Lake, J. A., Phoenix, G. K. & Cameron, D. D. The Regulation of Plant Secondary Metabolism in Response to Abiotic Stress: Interactions Between Heat Shock and Elevated CO2. *Front. Plant Sci.* **10**, 1–12 (2019).
7. Singh, A. K., Dhanapal, S. & Yadav, B. S. The dynamic responses of plant physiology and metabolism during environmental stress progression. *Molecular Biology Reports* **47**, 1459–1470 (2020).
8. Isah, T. Stress and defense responses in plant secondary metabolites production. *Isah Biol Res* **52**, 39 (2019).
9. Dhifi, W., Bellili, S., Jazi, S., Bahloul, N. & Mnif, W. Essential Oils' Chemical Characterization and Investigation of Some Biological Activities: A Critical Review. *Medicines* **3**, 25 (2016).
10. Leach, J. E., Triplett, L. R., Argueso, C. T. & Trivedi, P. Communication in the Phytobiome. *Cell* **169**, 587–596 (2017).
11. Venturi, V. & Fuqua, C. Chemical signaling between plants and plant-pathogenic bacteria. *Annu. Rev. Phytopathol.* **51**, 17–37 (2013).
12. Capinera, J. L. *et al.* Natural Enemy Attraction to Plant Volatiles. in *Encyclopedia of Entomology* 2567–2570 (Springer Netherlands, 2008). doi:10.1007/978-1-4020-6359-6_2150
13. Cheng, F. & Cheng, Z. Research progress on the use of plant allelopathy in agriculture and the physiological and ecological mechanisms of allelopathy. *Frontiers in Plant Science* **6**, 1020 (2015).
14. Holopainen, J. K. & Blande, J. D. Where do herbivore-induced plant volatiles go? *Frontiers in Plant Science* **4**, 185 (2013).
15. Jones, J. D. G., Vance, R. E. & Dangl, J. L. Intracellular innate immune surveillance devices in plants and animals. *Science (80-.).* **354**, (2016).
16. Zuzarte, M. & Salgueiro, L. Essential oils chemistry. in *Bioactive Essential Oils and Cancer* 19–61 (Springer International Publishing, 2015). doi:10.1007/978-3-319-19144-7_2

17. Dhifi, W., Bellili, S., Jazi, S., Bahloul, N. & Mnif, W. Essential Oils' Chemical Characterization and Investigation of Some Biological Activities: A Critical Review. *Medicines* **3**, 25 (2016).

18. Hikosaka, S., Ito, K. & Goto, E. Effects of Ultraviolet Light on Growth, Essential Oil Concentration, and Total Antioxidant Capacity of Japanese Mint. *Environ. Control Biol.* **48**, 185–190 (2010).

19. Yang, Y. *et al.* Chemical Mapping of Essential Oils, Flavonoids and Carotenoids in Citrus Peels by Raman Microscopy. *J. Food Sci.* **82**, 2840–2846 (2017).

20. Fürtauer, L., Weiszmann, J., Weckwerth, W. & Nägele, T. Dynamics of plant metabolism during cold acclimation. *International Journal of Molecular Sciences* **20**, (2019).

21. Maruyama, K. *et al.* Metabolic pathways involved in cold acclimation identified by integrated analysis of metabolites and transcripts regulated by DREB1A and DREB2A. *Plant Physiol.* **150**, 1972–1980 (2009).

22. Ramakrishna, A. & Ravishankar, G. A. Influence of abiotic stress signals on secondary metabolites in plants. *Plant Signaling and Behavior* **6**, 1720–1731 (2011).

23. Koutsaviti, A., Ioannou, E. & Roussis, V. Bioactive seaweed substances. in *Bioactive Seaweeds for Food Applications: Natural Ingredients for Healthy Diets* 25–52 (Elsevier, 2018). doi:10.1016/B978-0-12-813312-5.00002-9

24. Cherrak, S. A. *et al.* In Vitro Antioxidant versus Metal Ion Chelating Properties of Flavonoids: A Structure-Activity Investigation. *PLoS One* **11**, e0165575 (2016).

25. Jansen, K. M. B., Agterof, W. G. M. & Mellema, J. Droplet breakup in concentrated emulsions. *J. Rheol. (N. Y. N. Y).* **45**, 227–236 (2001).

26. Wooster, T. J. *et al.* Biological fate of food nanoemulsions and the nutrients they carry – internalisation, transport and cytotoxicity of edible nanoemulsions in Caco-2 intestinal cells. *RSC Adv.* **7**, 40053–40066 (2017).

27. Salager, J. Guidelines to handle the formulation , composition and stirring to attain emulsion properties on design (type , drop size , viscosity and stability). (1996).

28. Salager, J.-L., Bullón, J., Pizzino, A., Rondón-González, M. & Tolosa, L. Emulsion Formulation Engineering for the Practitioner. *Encycl. Surf. Colloid Sci.* (2007). doi:DOI: 10.1081/E-ESCS-120045970

29. Abbott, S. Surfactant Science : Principles and Practice. *Surfactant Sci. Princ. Pract.* 1–249 (2015).

30. Wooster, T. J., Golding, M. & Sanguansri, P. Ripening Stability. 12758–12765 (2008).

31. Gupta, A., Eral, H. B., Hatton, T. A. & Doyle, P. S. Nanoemulsions: Formation, properties and applications. *Soft Matter* **12**, 2826–2841 (2016).

32. Grabke, A., Li, X., Schnabel, G. & Fern, D. Independent Emergence of Resistance to Seven Chemical Classes of Fungicides in Botrytis cinerea. *Phytopathology* **17**, 424–432 (2014).

33. Leroux, P. Chemical control of Botrytis and its resistance to chemical fungicides. in *Botrytis: Biology, Pathology and Control,* 195–222 (Springer, 2007).

34. Hu, M., Cox, K. D. & Schnabel, G. Resistance to Increasing Chemical Classes of Fungicides by Virtue of " Selection by Association " in Botrytis cinerea. **106**, 1513–1520 (2016).

35. Hua, L. *et al.* Pathogenic mechanisms and control strategies of Botrytis cinerea causing post-harvest decay in fruits and vegetables. *Food Qual. Saf.* **3**, 111–119 (2018).

36. Dean, R. *et al.* The Top 10 fungal pathogens in molecular plant pathology. **13**, 414–430 (2012).

37. Morton, V. & Staub, T. A Short History of Fungicides A Short History of Fungicides. *Am. Phytopathol. Soc.* 1–12 (2008). doi:10.1094/APSnetFeature-2008-0308.A

38. Elad, Y. & Stewart, A. Microbial control of botrytis spp. in *Botrytis: Biology, Pathology and Control* 223–241 (Springer Netherlands, 2007). doi:10.1007/978-1-4020-2626-3_13

39. Smith, D. Botrytis Bunch Rot (Gray Mold) of Grapes. *Oklahoma State University. Cooperative extension. United States Department of Agriculture (USDA). National Institute of Food and Agriculture.* (2019). Available at: https://grapes.extension.org/botrytis-bunch-rot-gray-mold-of-grapes/. (Accessed: 5th May 2020)

40. Sakai, A. & Yoshimura, H. Monoterpenes of Salvia leucophylla. *Curr. Bioact. Compd.* **8**, 90–100 (2012).

41. Forgiarini, A., Esquena, J., González, C. & Solans, C. Formation of nano-emulsions by low-energy emulsification methods at constant temperature. *Langmuir* **17**, 2076–2083 (2001).

42. Komaiko, J. & McClements, D. J. Low-energy formation of edible nanoemulsions by spontaneous emulsification: Factors influencing particle size. *J. Food Eng.* (2015). doi:10.1016/j.jfoodeng.2014.09.003

43. Chang, Y. & Mcclements, D. J. Optimization of Orange Oil Nanoemulsion Formation by Isothermal Low-Energy Methods: Influence of the Oil Phase, Surfactant, and Temperature. (2014). doi:10.1021/jf500160y

44. Gupta, A., Badruddoza, A. Z. M. & Doyle, P. S. A General Route for Nanoemulsion Synthesis Using Low-Energy Methods at Constant Temperature. *Langmuir* **33**, 7118–7123 (2017).

45. Zhong, J., Liu, X., Wang, Y., Qin, X. & Li, Z. γ-Oryzanol nanoemulsions produced by a low-energy emulsification method: An evaluation of process parameters and physicochemical stability. *Food Funct.* **8**, 2202–2211 (2017).

46. Hashemnejad, S. M., Badruddoza, A. Z. M., Zarket, B., Ricardo Castaneda, C. & Doyle, P. S. Thermoresponsive nanoemulsion-based gel synthesized through a low-energy process. *Nat. Commun.* **10**, 1–10 (2019).

47. Acosta, E., Szekeres, E., Sabatini, D. A. & Harwell, J. H. Net-average curvature model for solubilization and supersolubilization in surfactant microemulsions. *Langmuir* **19**, 186–195 (2003).

48. Acosta, E. J. The HLD-NAC equation of state for microemulsions formulated with nonionic alcohol ethoxylate and alkylphenol ethoxylate surfactants. *Colloids Surfaces A Physicochem. Eng. Asp.* **320**, 193–204 (2008).

49. Kiran, S. K. & Acosta, E. J. Predicting the morphology and viscosity of microemulsions using the HLD-NAC model. *Ind. Eng. Chem. Res.* **49**, 3424–3432 (2010).

50. Sharifi-Rad, J. *et al.* Biological activities of essential oils: From plant chemoecology to traditional healing systems. *Molecules* **22**, 70 (2017).

51. Huang, H., Liu, B., Liu, L. & Song, S. Jasmonate action in plant growth and development. (2017). doi:10.1093/jxb/erw495

52. Wasternack, C. & Hause, B. The missing link in jasmonic acid biosynthesis. *Nat. Plants* **5**, 776–777 (2019).

53. Wasternack, C. & Strnad, M. Jasmonates: News on occurrence, biosynthesis, metabolism and action of an ancient group of signaling compounds. *International Journal of Molecular Sciences* **19**, (2018).

54. Ruan, J. *et al.* Jasmonic acid signaling pathway in plants. *International Journal of Molecular Sciences* **20**, (2019).

55. Ahmad, P. *et al.* Jasmonates: Multifunctional roles in stress tolerance. *Frontiers in Plant Science* **7**, 813 (2016).

56. Song, G. C. & Ryu, C. Two Volatile Organic Compounds Trigger Plant Self-Defense against a Bacterial Pathogen and a Sucking Insect in Cucumber under Open Field Conditions. 9803–9819 (2013). doi:10.3390/ijms14059803

57. Dave, A. & Graham, I. A. Oxylipin signaling: A distinct role for the jasmonic acid precursor cis-(+)-12-oxo-phytodienoic acid (cis-OPDA). *Front. Plant Sci.* **3**, (2012).

58. Bömer, M., Brien, J. A. O., Pérez-salamó, I. & Krasauskas, J. and cell wall protein composition in arabidopsis COI1-dependent jasmonate signalling affects growth , metabolite production and cell wall protein composition in arabidopsis. (2018). doi:10.1093/aob/mcy109

59. Huot, B., Yao, J., Montgomery, B. L. & He, S. Y. Growth-defense tradeoffs in plants: A balancing act to optimize fitness. *Mol. Plant* **7**, 1267–1287 (2014).

60. Ho, T. T., Murthy, H. N. & Park, S. Y. Methyl jasmonate induced oxidative stress and accumulation of secondary metabolites in plant cell and organ cultures. *International Journal of Molecular Sciences* **21**, (2020).

61. Glazebrook, J. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu. Rev. Phytopathol.* **43**, 205–227 (2005).

62. Rao, P. V. & Gan, S. H. Cinnamon: A multifaceted medicinal plant. *Evidence-based Complement. Altern. Med.* **2014**, (2014).

63. Nurdjannah, N. & Bermawie, N. Cloves. in *Handbook of Herbs and Spices: Second Edition* **1**, 197–215 (Elsevier Inc., 2012).

64. Mandal, S. & Mandal, M. Coriander (Coriandrum sativum L.) essential oil: Chemistry and biological activity. *Asian Pac. J. Trop. Biomed.* **5**, 421–428 (2015).

65. Borugă, O. *et al.* Thymus vulgaris essential oil: chemical composition and antimicrobial activity. *J. Med. Life* **7 . 3**, 56–60 (2014).

66. Yoon, M. Y., Cha, B. & Kim, J. C. Recent trends in studies on botanical fungicides in agriculture. *Plant Pathol. J.* **29**, 1–9 (2013).

67. Wang, C. & Fan, Y. Eugenol enhances the resistance of tomato against tomato yellow leaf curl virus. *J. Sci. Food Agric.* **94**, 677–682 (2014).

68. Banani, H. *et al.* Thyme and savory essential oil efficacy and induction of resistance against botrytis cinerea through priming of defense responses in apple. *Foods* **7**, (2018).

69. Tanaka, K. *et al.* Multiple roles of plant volatiles in jasmonate-induced defense response in rice. *Plant Signal. Behav.* **9**, (2014).

70. Rienth, M., Crovadore, J., Ghaffari, S. & Lefort, F. Oregano essential oil vapour prevents Plasmopara viticola infection in grapevine (Vitis Vinifera) and primes plant immunity mechanisms. *PLoS One* **14**, (2019).

71. Alvarez, A., Montesano, M., Schmelz, E. & Ponce de León, I. Activation of shikimate, phenylpropanoid, oxylipins, and auxin pathways in Pectobacterium carotovorum elicitors-treated moss. *Front. Plant Sci.* **7**, 328 (2016).

72. Moore, J. W., Loake, G. J. & Spoel, S. H. Transcription Dynamics in Plant Immunity. *Plant Cell* **23**, 2809–2820 (2011).

73. Mengiste, T. Plant Immunity to Necrotrophs. *Annu. Rev. Phytopathol.* **50**, 267–294 (2012).

74. Shitan, N. Secondary metabolites in plants: Transport and self-tolerance mechanisms. *Bioscience, Biotechnology and Biochemistry* **80**, 1283–1293 (2016).

75. Shitan, N., Sugiyama, A. & Yazaki, K. Functional analysis of jasmonic acid-responsive secondary metabolite transporters. *Methods Mol. Biol.* **1011**, 241–250 (2013).

76. Subramanian, A. *et al.* Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *Proc. Natl. Acad. Sci.* **102**, 15545–15550 (2005).

77. Ikram, R., Low, K. H., Hashim, N. B., Ahmad, W. & Nasharuddin, M. N. A. Characterization of Sulfur-Compounds as Chemotaxonomic Markers in the Essential Oils of Allium Species by Solvent-Free Microwave Extraction and Gas Chromatography–Mass Spectrometry. *Anal. Lett.* **52**, 563–574 (2019).

78. Kasaian, J., Asili, J. & Iranshahi, M. Sulphur-containing compounds in the essential oil of Ferula alliacea roots and their mass spectral fragmentation patterns. *Pharm. Biol.* **54**, 2264–2268 (2016).

79. Zhang, N., Zhou, S., Yang, D. & Fan, Z. Revealing Shared and Distinct Genes Responding to JA and SA Signaling in Arabidopsis by Meta-Analysis. *Front. Plant Sci.* **11**, 908 (2020).

80. Article, H. *et al.* Jasmonic acid carboxyl methyltransferase regulates development and herbivory-induced defense response in rice. (2011). doi:10.1111/jipb.12436

81. Mengiste, T. Plant Immunity to Necrotrophs. *Annu. Rev. Phytopathol.* **50**, 267–294 (2012).

82. Lai, Z., Wang, F., Zheng, Z., Fan, B. & Chen, Z. A critical role of autophagy in plant resistance to necrotrophic fungal pathogens. *Plant J.* **66**, 953–968 (2011).

83. Beese, C. J., Brynjólfsdóttir, S. H. & Frankel, L. B. Selective Autophagy of the Protein Homeostasis Machinery: Ribophagy, Proteaphagy and ER-Phagy. *Frontiers in Cell and Developmental Biology* **7**, 373 (2020).

84. Kabbage, M., Kessens, R., Bartholomay, L. C. & Williams, B. The Life and Death of a Plant Cell. *Annu. Rev. Plant Biol.* **68**, 375–404 (2017).

85. Su, T. *et al.* Autophagy: An Intracellular Degradation Pathway Regulating Plant Survival and Stress Response. *Frontiers in Plant Science* **11**, 164 (2020).

86. Luo, H. *et al.* The arabidopsis botrytis susceptible1 interactor defines a subclass of RING E3 ligases that regulate pathogen and stress responses. *Plant Physiol.* **154**, 1766–1782 (2010).

87. Liu, Y. & Bassham, D. C. Autophagy: Pathways for Self-Eating in Plant Cells. *Annu. Rev. Plant Biol.* **63**, 215–237 (2012).

88. Hasanuzzaman, M., Nahar, K., Anee, T. I. & Fujita, M. Glutathione in plants: biosynthesis and physiological role in environmental stress tolerance. *Physiology and Molecular Biology of Plants* **23**, 249–268 (2017).

89. Hameed, A., Sharma, I., Kumar, A., Azooz, M. M. & Ahmad, H. *Glutathione Metabolism in Plants under Environmental Stress*. *Oxidative Damage to Plants* (Elsevier Inc., 2014). doi:10.1016/B978-0-12-799963-0.00006-X

90. Romero, L. C. *et al.* Cysteine and cysteine-related signaling pathways in arabidopsis thaliana. *Molecular Plant* **7**, 264–276 (2014).

91. Vega-Vásquez, P., Mosier, N. S. & Irudayaraj, J. Nanoscale Drug Delivery Systems: From Medicine to Agriculture. *Frontiers in Bioengineering and Biotechnology* **8**, 79 (2020).

92. Komaiko, J. S. & Mcclements, D. J. Formation of Food-Grade Nanoemulsions Using Low-Energy Preparation Methods: A Review of Available Methods. *Compr. Rev. Food Sci. Food Saf.* **15**, 331–352 (2016).

93. Conn, S. J. *et al.* Protocol : optimising hydroponic growth systems for nutritional and physiological analysis of Arabidopsis thaliana and other plants. 1–11 (2013).

94. Liao, C. J., Lai, Z., Lee, S., Yun, D. J. & Mengiste, T. Arabidopsis HOOKLESS1 regulates responses to pathogens and abscisic acid through interaction with MED18 and acetylation of WRKY33 and ABI5 chromatin. *Plant Cell* **28**, 1662–1681 (2016).

95. Anders, S. & Huber, W. Differential expression analysis for sequence count data. *Genome Biol.* **11**, R106 (2010).

96. Kanehisa, M. & Goto, S. KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Research* **28**, 27–30 (2000).

4. A NANO-VACCINE FOR PLANT PROTECTION: CHITOSAN NANOCARRIERS LOADED WITH D-LIMONENE PROTECTS *A. THALIANA* AGAINST *BOTRYTIS CINEREA*

Abstract

Botrytis cinerea is a broad host-range necrotrophic (BHN) pathogen that employs a complex infectious mechanism that require multiple genes for its onset; therefore, true resistance to this pathogen cannot be conferred through simply inherited resistance. The disease it causes in crops is devastating and results in vast economic losses worldwide. Synthetic fungicide application remains the most common method to control the pathogen, but this comes' with a significant environmental cost. Drug delivery systems from naturally occurring biomaterials can yield a potential solution to combat this disease. We hypothesized that engineered chitosan-based nanocarriers loaded with D-limonene can trigger an on-demand systemic defense response in plants that enhances its quantitative disease resistance against BHN by restricting the extent of the hypersensitive response as a result of up-regulation of anti-oxidant activity, autophagy and jasmonic acid biosynthesis. We fabricated Chitosan nano-carriers encapsulated with d-limonene as cargo through a rational formulation via ionic gelation and spontaneous emulsification method, respectively to produce Chitosan d-limonene nano particles (CdlNPs). The therapeutic effect of CdlNPs on the plants' defense response against necrotrophic fungal pathogens was evaluated by monitoring the dynamic morpho-physiological changes in plants treated with CdlNPs via multispectral image-based phenotyping and quantitative gene set enrichment analysis, respectively in a *A.thaliana* (Col-0) – *Botrytis cinereal* model system. *We found that the effect of the treatments followed a dose-dependent response. CSNPs, D-limonene nano-emulsions (dlNE), and CdlNPs significantly enhanced the quantitative disease resistance (QDR) of A. thaliana to B. cinerea*. Functional analysis of the differentially expressed genes revealed that CNPs halted plant growth, but up-regulated the main process controlling response to stress.

4.1 Introduction

Botrytis cinerea is a broad host fungal necrotrophic pathogen able to infect over 500 different plant species including many economically relevant fruit bearing trees, shrubs and vegetables. The

economic losses in crops worldwide caused by this pathogen are estimated to be up to $100 billion per year[1] . Due to its economic importance, *B. cinerea* has not only been classified as the second most important plant pathogen[2] but has also been considered the most common pathogen responsible for post-harvest decay of fruits and vegetables. Due to its natural ability to infect multiple hosts, grey mold disease caused by *B.cinerea* is difficult to control. This pathogen does not rely on a single strategy to successfully infect, colonize, and suppress the host immune defense response. Instead, these processes are mediated by several strategies including the production of cell wall degrading enzymes, non-host specific toxins (botrydial), high levels of ROS, necrosis-inducing factors, and an array of secondary metabolites[2]. Moreover, *B. cinerea* may activate specific virulence arsenals depending on host species and tissue type contributing to its success as a widespread pathogen of many plants[3]. Since no true plant resistance can be achieved against *B.cinerea* via plant breading, to date, synthetic fungicide application still remains the most common method to control the disease, although it is a practice that is becoming increasingly restricted, not only due to the sustainability considerations, but also due to the increasing resistance to chemical fungicides by *B.cinerea* already noted[10–12]. Furthermore, fungicides are costly and may have negative effects on human health.

Phytopathogens live at the expense of their hosts by deriving the nutrients they require for growth and reproduction from plant tissue. Depending on their lifestyles, three different types of plant pathogens exist: biotrophs, necrotrophs and hemibiotrophs. In general, biotrophic pathogens highjack the plant's innate immune system using specialized infection structures (hyphae or haustoria) within living plant cells from which nutrients are uptaken[4] and specific effectors that allow infection to progress unnoticed [5]. In contrast, necrotrophic pathogens actively kill plant cellsby producing and releasing degrading enzymes and toxic proteins[3]. The hemibiotrophic pathogenic lifestyle is displayed by pathogens that can shift from a biotrophic lifestyle at early infection stages, to a necrotrophic lifestyle as the infection progresses to its later stages[5]. The molecular mechanisms and the infection strategies mounted by pathogens displaying the different types of lifestyles are reviewed elsewhere in greater detail[3,6].

IN the context of plant immunity, the ability to detect and recognize elicitors is defined as perception [7]. The defense system based on the perception of molecular signatures from pathogens or "cellular debris" resulting from pathogen attack, known as pathogen/damage associated

molecular pattern (PAMP/DAMP), comprise the first line of defense that plants rely on to protect themselves. The effector triggered immunity (ETI), driven by a gene-to-gene basis within the plant-pathogen specific interaction, comprises of the second layer of protection from plants. Effectors are the molecular "weaponry" that pathogens use to invade a host plant. The intricacies of the plant immune system are reviewed elsewhere[8] . Overall, the PAMP/ETI defense mechanism is effective against biotrophic pathogens, but not very effective against the necrotrophic pathogens for two main reasons. First, the PAMP/ETI response is driven by gene-by-gene interaction and second, the PAMP/ETI triggers the hypersensitive response (HR), a controlled cellular death process carried out to sacrifice cells around the pathogen, in order to enclose the pathogen within the necrotic tissue. Since necrotrophic pathogens strive on necrotic tissue, triggering the HR eases the diseases progression. Moreover, the infection mechanisms from most of the necrotrophic pathogens, specially the broad host necrotrophic, do not rely on a gene-to-gene interaction, but deploy a wide range of molecular strategies that cannot be counteracted by a single gene from the plant defense genetic repertoire.

Simply inherited resistance traits confer protection to plants against biotrophs and host-specific necrotrophs (HSNs), but not to broad host-range necrotrophs (BHNs); in contrast, plant resistance to broad host necrotrophs (BHNs) is complex as it involves multiple immune response mechanisms [6,7]. Generally, resistance to host specific necrotrophs is conditioned by single genes conferring complete immunity whereas resistance to broad-host necrotrophs is quantitative, requiring many genes for full resistance[3]. However, even though true resistance against BHN cannot be achieved, quantitative resistance can be enhanced (*i.e* decelerate infection rate and limit the extent of infection).

For enhancement of the quantitative disease resistance against necrotrophic pathogens, chitosan-based nano-carriers are a promising platform for "drug" delivery to plant cells and plant derived essential oils are a promising cargo[9]. The function of chitosan is dual: It serves as a delivery vehicle of essential oils, and also acts as an elicitor of the plants immune system. Amongst the molecular events that are triggered by the exposure of plants to chitosan include callose apposition, increase in cytosolic CA2+, activation of MAP kinases, up-regulation of genes coding for ROS scavenging and antimicrobial enzymes, chromatin alterations, synthesis of alkaloids, and activation of the JA/ET pathway involved in the plant defense response mounted against necrotrophs[10,11]. The role

of essential oils is also dual: They can activate the plant's defense response mounted when under herbivore attack, the same that needs to be triggered upon necrotrophic infection, to actively kill fungal pathogens because of their strong antimicrobial properties. However, the droplet size, polydispersity and stability of both chitosan-based nanocarriers and nano-emulsions are highly dependent on formulation variations and fabrication methods; therefore, making it challenging to mass-produce for agricultural purposes.

We believe that exposure of plant roots to d-limonene nano-emulsion and d-limonene encapsulated in chitosan carriers (CdlNps), triggers a systemic defense response in plants that activates the jasmonic acid hormone signaling route resulting in halted plant growth. We predict that these treatments enhance the quantitative disease resistance in leaves against broad host necrotrophs using *A. thaliana* (Col-0) - *B.cinerea* as a model system, by restricting the extent of the hypersensitive response in the infected tissue. The objectives of this work are: *i)* to fabricate chitosan-based nano-carriers following a rational approach via the ionic gelation method using both low and medium molecular weight chitosan; *ii)* Fabricate a nano-emulsion using -d-limonene as essential oil model following a rational approach with FDA-approved (G.R.A.S) materials under low-energy conditions at room temperature and; *iii)* quantify the defense response from treated plants, by measuring the infected area via multispectral image-based phenotyping assessment, using chlorophyll-based masking segmentation, and *iv)* quantitatively compare the effect of treatments on the impaired or enhanced biological mechanisms of treated plants and untreated plants via RNA-seq analysis of differentially expressed genes.

4.2 Materials and methods

4.2.1 Functional characterization of chitosan

Commercially available samples from low and medium molecular weight chitosan were analyzed as received from the vendors without further modification.

Degree of deacetylation (DD%) estimation via proton nuclear magnetic resonance (^{1}H-NMR):

The estimation of the degree of deacetylation was performed [50] using a Bruker AV-III-HD-400 equipped with a 5mm BBFO probe. 10 mg of chitosan powder was dissolved in 2 mL of solvent (0.04 mL of deuterium chloride 20% in 1.96 mL D_2O) using a vortex. 1 mL of dissolved chitosan

was transferred to an NMR tube and placed into the magnet. Temperature was adjusted at 70°C and the sample was introduced in the instrument for 10 min before conducting the experiment. The ^{1}H NMR experiment was conducted by a single pulse sequence with pre-saturation of the solvent. The delay before the application of the pulse was 6 sec and the acquisition time was 2 sec for a total relaxation time (recycle time) of 8 sec between each transient. The degree of deacetylation (%) was calculated for low and medium molecular weight chitosan using the integrals obtained from the peak of proton H^1 of deacetylated monomer (H1-D) and the peak of the three protons of acetyl group (H-Ac) as described in Eq.1:

$$\text{Eq.1: } DD(\%) = \left(\frac{1HD}{H1D+\left(\frac{HAc}{3}\right)}\right) \times 100$$

Determination of available NH_3^+ groups from chitosan via potentiometric titration:

The determination of the amount of available amino groups from low and medium molecular weight chitosan samples was conducted according to Lapitsky. et al. [51] using Eq. 2. In this method, 30 mg of chitosan powder was first dissolved in 20 mL of acidified dH_20 (circa pH 2.0) with 30 µL of HCl (12M). Potentiometric titrations of aqueous chitosan solutions were prepared at room temperature using a pH meter equipped with a pH/ATC probe. The solutions were titrated with 20 mM NaOH solution for up to a pH of 9 when full deprotonation of pH-sensitive NH_3^+ charges occur. The amount in moles of freely available amino groups NH_3^+ per gram of chitosan, Np, was calculated for both low and medium molecular weight chitosan samples as received from the manufacturer as follows:

$$\text{Eq.2: } V^{add} = \frac{\left(N_p\left(\frac{-10^{-pH}}{K_a+10^{-pH}}\right)+N_{HCl}^{in}-V^{in}\left(10^{-pH}-\frac{K_w}{10^{-pH}}\right)\right.}{C_{NaOH}^{stock}+10^{-pH}-(K_w/10^{-pH})}$$

4.3 Fabrication of loaded chitosan-based nanocarriers:

4.3.1 Fabrication of chitosan nanocarriers

Low and medium molecular weight chitosan-based nanocarriers were fabricated at room temperature via the ionic gelation method using sodium tripolyphosphate (Na-TPP) as cross-linker. An aqueous solution of chitosan was prepared by dissolving 1 mg/mL of chitosan in acidified dH20 (acetic acid 1%v/v, pH 4.5 adjusted with NaOH) under magnetic stirring at room temperature. Once homogenized, an aqueous Na-TPP solution was added dropwise (0.5 mL/min)

into the chitosan solution at a volumetric ratio of 1:10 (chitosan:Na-TPP) under constant agitation at room temperature. Different concentrations of Na-TPP aqueous solution were prepared in order to identify an optimal NH_3^+: $P_3O_{10}^-$ molar ratio that enabled stable nanoparticle formation. The assessed NH_3^+: $P_3O_{10}^-$ molar ratios were 1:0.1; 1:0.5, 1:1, 1:2, respectively. All experiments were performed in triplicate. Particle formation and stability were assessed via TEM image analysis and dynamic light scattering (DLS) particle size distribution analysis. Once identified the optimal NH_3^+: $P_3O_{10}^-$ molar ratio for chitosan nanoparticle formation, both low and medium molecular weight chitosan nanoparticles were fabricated by dissolving chitosan in ascorbic acid (0.1M, pH 2.5) instead of acetic acid. Particle formation and stability were assessed as previously mentioned in triplicate.

Isothermal fabrication of d-limonene nano-emulsion

d-limonene nano-emulsion was obtained under low energy conditions at room temperature via spontaneous emulsification. Briefly, a (circa) 5% wt d-limonene was added dropwise under constant magnetic stirring at room temperature into an aqueous solution containing propane-1,2-diol (0.04M). Polyethylene glycol sorbitan monooleate was used as a surfactant at a weight ratio of 3:1 with respect to the organic phase. Droplet formation and stability were assessed via TEM image analysis and dynamic light scattering (DLS) particle size distribution analysis. All experiments were conducted in triplicate.

Encapsulation of d-limonene nanoemulsion into chitosan-based nanocarriers

d-limonene Nano-Emulsion loaded into low and medium molecular weight Chitosan NanoCarriers (CdlNPs) was performed as follows. First, 1 mg/mL of chitosan was dissolved at room temperature in aqueous acetic acid 1%v/v (pH 4.5. adjusted with NaOH) under constant magnetic stirring. Then, an aliquot of freshly prepared d-limonene nano-emulsion, equivalent to a 10% v/v of the final volume was added to the chitosan solution under constant agitation. Finally, the cross-linking step was carried out as previously described using Na-TPP at a NH_3^+: O_3^- molar ratio of 1:0.5. Morphology and size distribution of formed NE@CNSPs was assessed via TEM image analysis and dynamic light scattering (DLS) analysis. Confirmation of d-limonene nanoemulsion encapsulation, and encapsulation efficiency were assessed via ATR-FTIR and gas chromatography,

respectively. All of the experiments were conducted in triplicate. CdlNPs were also prepared in ascorbic acid (0.1M, pH 2.5) using low molecular weight chitosan only, as previously described.

4.4 Characterization of chitosan nanocarriers (CSNPs), d-limonene nanoemulsion (NE) and d-limonene loaded chitosan nanocarriers (CdlNPs)

The particle size distribution was characterized by DLS, using a Zetasizer Nano ZS (Malvern, UK) dynamic and electrophoretic light scattering instrument with a backscatter measurement angle set at 173° with automatic attenuator selection. The particle size distribution and polydispersity analysis were performed by fitting the intensity autocorrelation functions using the cumulant analysis. Experiments were performed with 3 independently freshly prepared samples without adjusting the dispersion pH, ionic strength or concentrations, and each sample was measured three times.

In addition to measuring the size distributions, the nanoparticle size and morphologies were probed by scanning TEM using a Tecnai T12 120KV transmission electron microscope (TEM). Samples were loaded onto formvar carbon film 400 copper mesh substrates pretreated with a PELCO easiGlow™ Glow Discharge chamber for 1 min in order to negatively charge its surface and thus, facilitate particle attachment. Then, 3 µL of freshly prepared sample (i.e. CSNPs, limonene nano-emulsion (NE) or CdlNPs) were loaded onto the previously treated substrate and was left to stand for 5 min. Samples were then subjected to negative staining with 1% uranyle acetate (1N) in water for 1 min before image acquisition.

CdlNPs samples were subjected to further experimentation to confirm the encapsulation of d-limonene nano-emulsion and estimate the encapsulation efficiency. Confirmation of encapsulation was assessed via ATR-FTIR spectroscopy using a Spectrum 100 FTIR Spectrometer (Perkin Elmer,Waltham, MA). In this test, FTIR spectra from both low (LMw) and medium (MMw) molecular weight chitosan powder and from pure d-limonene were compared to the spectra from LMw and MMw nanocarriers loaded with d-limonene nano-emulsion. CdlNPs samples for ATR-FTIR experiments were obtained by centrifugation of 5 mL of sample at 13200 x g at 4ºC for 10 min. The pellet was collected and further used for analysis.

Finally, the efficiency of LMw and MMw chitosan-based nano-carriers to encapsulate d-limonene nano-emulsion was assessed via gas chromatography. For this test, 5 mL of freshly prepared CdlNPs from both LMw and MMw chitosan were centrifuged at 13200x g for 10 min at 4ºC and the supernatant was transferred to a 15 mL tube. An equal volume of hexane was then added to the supernatant and thoroughly mixed with a vortex for 3 min. Unencapsulated d-limonene dissolved in hexane was recovered from the most superficial layer on the supernatant and further injected in a gas chromatograph (Agilent 7820A) using a Stabilwax-DB column (Restek). The temperature gradient was set from 60ºC to 250ºC at a rate of 10ºC/min, using a split injection ratio of 80. The concentration of recovered d-limonene was calculated from a previously constructed calibration curve (supplementary data), and the encapsulation efficiency was estimated as:

$$Eq.3: Encapsulation\ efficiency\ (\%) = \left(\frac{[Limonene]_{initial} - [limonene]_{recovered}}{[Limonene]_{initial}}\right) \times\ 100$$

4.5 Dynamic morpho-physiological assessment of the systemic effect of chitosan nanoparticles (CSNPs), d-limonene nano-emulsion (NE) and d-limonene loaded chitosan-based nano-carriers, on the plants' defense immune system of wild type *A.thaliana* (Col-0).

4.5.1 Plant material and growing conditions

In order to assess the systemic effect of CSNPS, NE and CDLNPS on the innate immune mechanism of plants, a system to grow plants hydroponically was adopted [52] using *Arabidopsis thaliana* wild type (Col-0) as the biological model. By this means, access to the radicular system of the plant is enabled without compromising the integrity of the plants.

Briefly, 2 to 3 individual seeds were carefully placed on a seed holder (pierced black microcentrifuge lid) containing germination medium agar. Seed holders loaded with seeds were placed on germination boxes containing germination nutrient solution and then seeds were stratified at 4 ºC for 48h in darkness. After the stratification period, germination boxes were incubated under a 12h:12h day/night photoperiod with an irradiance of 150 µmol photons/ m^2s^1 at the plant level, at 24 ºC and 55% atmospheric relative humidity. On day 4 post-germination, extra seedlings were carefully removed from seed holders to guarantee one plant per holder only. After 7 days of germination, the germination solution was gradually replaced with basal nutrient solution daily for 3 days, replacing 33%, 50% and 100%, respectively. A week later, 14-day old seedlings

were individually transferred to 50 mL plant holders containing BNS and further incubated for two more weeks. Four-week old plants were used for further experimentation.

Multispectral Image-based phenotyping assessment of treated plants

4-week-old *A. thaliana* (Col-0) plants, independently grown under hydroponic conditions in 50 mL modified tubes with basal nutrient solution (BNS) as previously described, were treated by exposing their roots to 4 different types of treatments freshly prepared, namely, LMw chitosan solution in ascorbic acid (0.1M, pH 2.5), LMw-CSNPS, d-limonene NE and CdlNPs. Each treatment was tested at 4 different concentrations (0.01, 0.1, 0.5 and 1% v/v). A set of nine individual plants were used per each of the experimental conditions. Image-based phenotyping analysis was performed using an ARIS B.V automatic top-view imaging machine vision system equipped with a multispectral light source (Aris, Eindhoven, Netherlands). The chlorophyll-based masking segmentation was used to measure the area of the plant's rosette immediately after treatment (t=0), and then every 24 hours for 3 consecutive days. The relative growth of plants was calculated according to Eq. 4. The treated plants were kept under controlled growing conditions, as previously described. On day 3, each plant was flash-frozen with liquid nitrogen and stored at -80 ^{o}C for further molecular analysis. A two-way ANOVA was performed to describe the interaction effect between each of the concentrations under investigation over time, per type of treatment, on the relative growth of plants under study.

$$\text{Eq. 4: } Rel.growth\ (\%) = \left(\frac{Rosette\ Area_{final} - Rosete\ area_{initial}}{Rosette\ Area_{initial}}\right) \times 100$$

Assessment of the systemic effect of treatments on the quantitative disease resistance against B. cinereal via image-based phenotyping

Fungal disease assays were conducted as previously described [53]. In brief, for the *Botrytis cinerea* disease assay, 4 leaves from pretreated 4-week-old plants, grown under hydroponic conditions as previously described, were drop inoculated with a conidial suspension (2.5 x 105 spores/mL) of *B. cinerea* strain B05.10 in 1% Sabouraud Maltose Broth and maintained under a transparent cover at high humidity for 72 hours. The lesion area size per leaf was measured via Image-based phenotyping analysis was performed using an ARIS B.V automatic top-view imaging machine vision system equipped with a multispectral light source (Aris, Eindhoven, Netherlands). The

chlorophyll-based masking segmentation was used to distinguish necrotic tissue in leaves. The necrotic area from individual leaves was measured using imageJ software. The relative necrotic area was calculated according to Eq. 5. The statistical analysis was carried out by conducting a balanced nested ANOVA on the necrotic area on the infected leaves per plant for each of the treatments (n=20).

$$Eq.5: Necrotic\ area\ (\%) = \left(\frac{Necrotic\ area}{Total\ area}\right) \times 100$$

4.5.2 4.4. Physiological assessment of treated plants via RNA sequencing and transcriptome data analysis.

Sample collection and preparation:

RNA extraction, quantification and qualification:

Frozen, pre-treated plant tissue stored at -80 ^{o}C, was grounded with pestle and mortar with liquid nitrogen and total RNA was extracted with Trizol reagent according to the manufacturer's instructions (Sigma-Aldrich). Illumina sequencing was carried out at Novogene Bioinformatics Technology Co, Ltd. RNA degradation and contamination was monitored on 1% agarose gels. RNA purity was checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA) and RNA integrity and quantitation were assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

Library preparation for transcriptome sequencing

Library preparation for transcriptome sequencing was carried out using a total amount of 1 µg of RNA per sample as input material. Sequencing libraries were generated using NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5X). First strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase (RNase H-). Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. Remaining overhangs were

converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3' ends of DNA fragments, NEBNext Adaptor with hairpin loop structure were ligated to prepare for hybridization. In order to select cDNA fragments of preferentially 150~200 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA). Then 3 μl USER Enzyme (NEB, USA) was used with size-selected, adaptor ligated cDNA at 37 °C for 15 min followed by 5 min at 95 °C before PCR. Then PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. At last, PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system. The clustering and sequencing of the index-coded samples was performed on a cBot Cluster Generation System using PE Cluster Kit cBot-HS (Illumina) according to manufacturer's instructions. After cluster generation, the library preparations were sequenced on a Illumina platform and paired-end reads were generated.

Data analysis

Quality control

Raw data (raw reads) of FASTQ format were firstly processed through fastp. In this step, clean data (clean reads) were obtained by removing reads containing adapter and poly-N sequences and reads with low quality from raw data. At the same time, Q20, Q30 and GC content of the clean data were calculated. All of the downstream analyses were based on the clean data with high quality.

Mapping to reference genome

Reference genome and gene model annotation files were downloaded from genome website browser (NCBI/UCSC/Ensembl) directly. Paired-end clean reads were mapped to the reference genome using HISAT2 software. HISAT2 uses a large set of small GFM indexes that collectively covers the whole genome. These small indexes (called local indexes), combined with several alignment strategies, enable rapid and accurate alignment of sequencing reads.

Quantification

Feature counts was used to count the read numbers mapped of each gene, including known and novel genes. And then RPKM of each gene was calculated based on the length of the gene and reads count mapped to this gene. RPKM, Reads Per Kilobase of exon model per Million mapped reads, considers the effect of sequencing depth and gene length for the read count at the same time, and is currently the most commonly used method for estimating gene expression levels.

Differential expression analysis

Differential expression analysis between two conditions (three biological replicates per condition) was performed using DESeq2 R package. DESeq2 provides statistical analysis for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting P values were adjusted using the Benjamini and Hochberg's approach for controlling the False Discovery Rate (FDR). Genes with an adjusted P value < 0.05 found by DESeq2 were assigned as differentially expressed.

Enrichment analysis

A common way for searching shared functions among genes is to incorporate biological knowledge provided by biological ontologies. Gene Ontology (GO) annotates genes to biological processes, molecular functions, and cellular components in a directed acyclic graph structure, and Kyoto Encyclopedia of Genes and genomes (KEGG) annotates genes to pathway. Gene Ontology (GO) enrichment analysis of differentially expressed genes was implemented by the clusterProfiler R package, in which gene length bias was corrected. GO terms with corrected P-value less than 0.05 were considered significantly enriched by differential expressed genes. For KEGG pathway enrichment analysis, clusterProfiler R package to test the statistical enrichment of differential expression genes in KEGG pathways was used.

4.6 Results and discussion

4.6.1 Functional characterization of chitosan

Chitosan formulated nanocarriers have the potential to be used in agriculture for plant and crop protection due to their immune-stimulant bioactivity, however, their particle size, polydispersion and stability are highly dependent on formulation variations and fabrication methods; therefore, making it challenging to mass-produce[9]. This is almost certainly due to the intrinsic heterogenous molecular weights and surface charges of chitosan, produced by deacetylation of natural chitin from shellfish or other organisms, that makes it challenging to fine-tune the formulation procedures and ensure reproducible processing when using the ionic gelation method. The literature regarding both, nanoparticle formation via ionic gelation and cargo release profile, is overwhelmingly inconsistent[12,13].A common thread from the literature is that chitosan-based nanoparticle formation is highly sensitive to changes in either the formulation or experimental conditions such as pH, ionic strength, reactant concentrations, volumetric ratios, injection and stirring rate among other.

We predicted that fabrication of chitosan-based nano-carriers can be optimized and simplified by following a rational approach based on the characterization of chitosan rather than following a multivariate experimental approach. By estimating the amount of available amino (NH_3^+) groups and the percentage of deacetylation, the formulation process for fabrication of chitosan-based nano-carriers via ionic gelation can be simplified using both low and medium molecular weight chitosan.

Ionic gelation (IG) is the method of choice to fabricate chitosan-based nanoparticles (CSNPs) because it requires no harsh conditions or specialized equipment. Since the formation of chitosan–based nano-carriers via ionic gelation depends on the electrostatic interaction between positively charged amino groups from chitosan, and negatively charged groups from the cross-linker, we hypothesized that the fabrication of chitosan nano-carriers can be optimized and simplified. Although the ionic gelation method is simple to perform, the literature regarding both, nanoparticle formulation and its cargo release profile assessment, is overwhelmingly inconsistent[12,13].

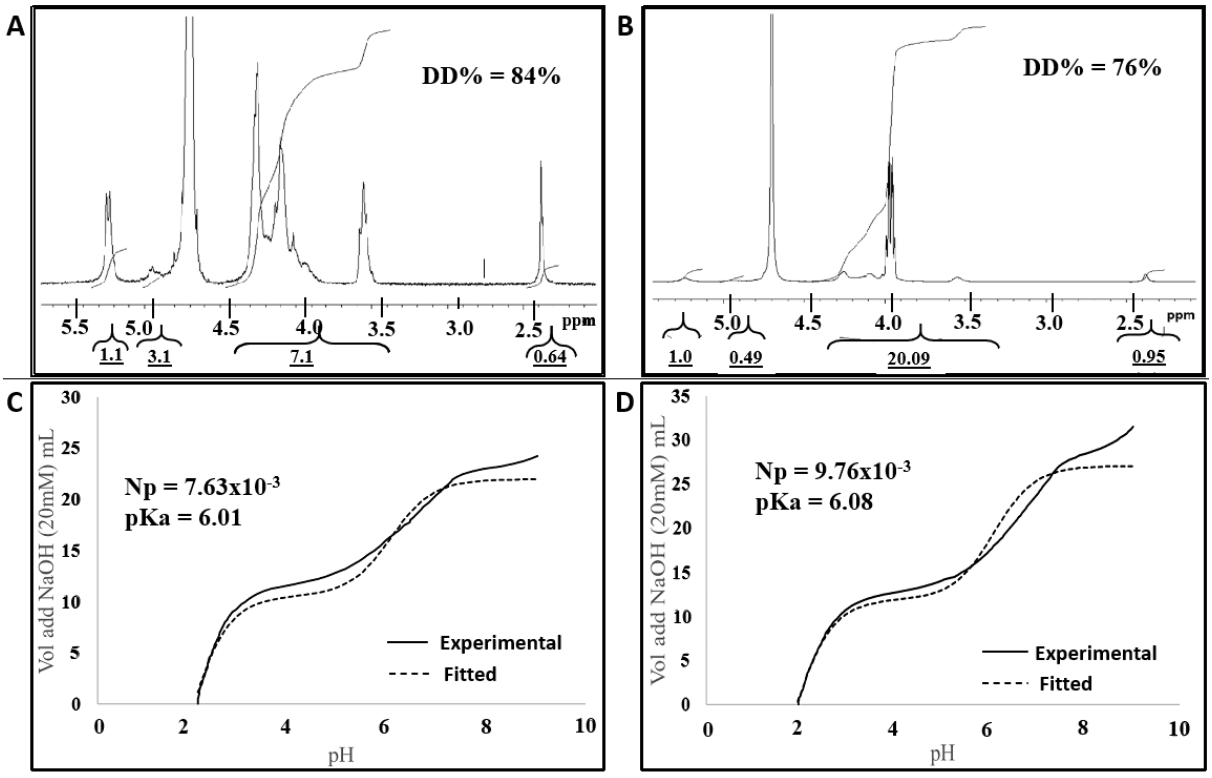


Fig. 4.1: Functional characterization of low and medium molecular weight chitosan. Estimation of the degree of deacetylation (DD) and Np forms a low and medium molecular weight chitosan using eq. 1 and eq. 2 respectively.

The Np values shown in Fig.1C-D were calculated per previous methods[19]. The model describes the protonation response of the free amino groups from chitosan in aqueous solution as a function of pH. When the pH is low (i.e. circa pH 2), the amino groups are fully protonated, which allows for the solubilization of chitosan in water. Rapid amino group deprotonation starts at pH levels close to 3 and continues until the pH in the solution is closer to its pKa value, where 50% of amino groups are deprotonated, causing a shift in the rate of deprotonation. Above pKa values, deprotonation continues until the solution acidity is neutral. Under alkaline conditions, amino groups from chitosan are fully deprotonated, rendering the polymer water insoluble. It is worth noting that the behavior described from the model is similar in both the low and medium molecular weight chitosan. This indicates that the estimation of the Np value, is more informative and useful for further formulation purposes than the degree of deacetylation. This is important because the estimation of the Np value requires no specialized instrumentation, whereas the estimation of the degree of deacetylation does require instrumentation.

Since the pH determines the protonation of the amino groups in chitosan, which in turn enables the cross-linking process in cooperation with negatively charged ion from the cross-linker. Tripolyphosphate is alkaline in solution, therefore, changes in the volumetric ratios between TPP and chitosan solution are also critical for the cross-linking to take place. Higher ratios of TPP solution may increase the pH of the solution above the pKa of chitosan leading to its deprotonation and consequent precipitation. The amount of both available amino groups from the chitosan (Np) and the amount of negatively charged groups from the cross-linker which are the main drivers of the reaction, are key factors affecting the formation of chitosan-based nanoparticles via ionic gelation. Fig.1. also shows that the amount of available amino groups in chitosan (Np) depends on the degree of deacetylation of the parental chitin polymer and molecular weight. Failing to balance the amount of available amino groups and cross-linker molecules leads to particle destabilization[9,12]. Excess of cross-linker molecules results in particle aggregation and further precipitation and insufficient amount of cross-linker molecules will result in solubilization of chitosan nano-carriers and premature cargo release.

4.6.2 Formulation and Fabrication of chitosan-based nanocarriers loaded with essential oil nano-emulsions: A rational approach

Fabrication of chitosan nanocarriers

One of the major drawbacks in the scale-up of the fabrication of chitosan-based nanoparticles for agricultural purposes is the batch-to-batch variation due to the heterogeneity of the polymer itself [9]. The fabrication of chitosan-based nano-carriers can be optimized and simplified following a rational approach based on the characterization of chitosan, rather than following a multivariate experimental approach, by estimating the amount of available amino (NH_3^+) groups and its percentage of deacetylation, both low and medium molecular weight chitosan.

We found that the optimal NH_3^+: $P_3O_{10}^-$ molar ratio in the nanoparticle formation using both low and medium molecular weight chitosan via ionic gelation was identified to be 1:0.5. Fig. 2. Shows that chitosan nanoparticles were successfully fabricated by dissolving chitosan in ascorbic acid (0.1M, pH 2.5) instead of acetic acid (1%v/v, pH 4.5.). Using acetic acid as the protonation agent during chitosan nanoparticle formation for its intended use in agriculture will completely defeat its purpose, due to its toxicity. Acetic acid affects the cell membranes of plants, causing rapid

breakdown/desiccation of foliage tissue upon contact[14]. In contrast, the well-known antioxidant activity from ascorbic acid, may be beneficial to the overall plant health, counteracting the deleterious effects of reactive oxygen accumulation in tissues from plants undergoing pathogen attack or other abiotic stressors[15–17].

The literature on the fabrication of chitosan nanocarriers, emphasizes the effect of systematic variation of individual component factors (e.g. concentration, molecular weight, degree of deacetylation), as well as the operation conditions under which the ionic gelation is performed (pH, temperature, agitation speed, injection rate) on the final size distribution and particle stability. Here we confirmed that, for formulation purposes, estimating the amount of NH3+ available groups per gram of chitosan) is the most relevant characteristic of chitosan. Regardless of its degree of deacetylation and molecular weight, the number if experimental iterations during the formulation are greatly reduced.

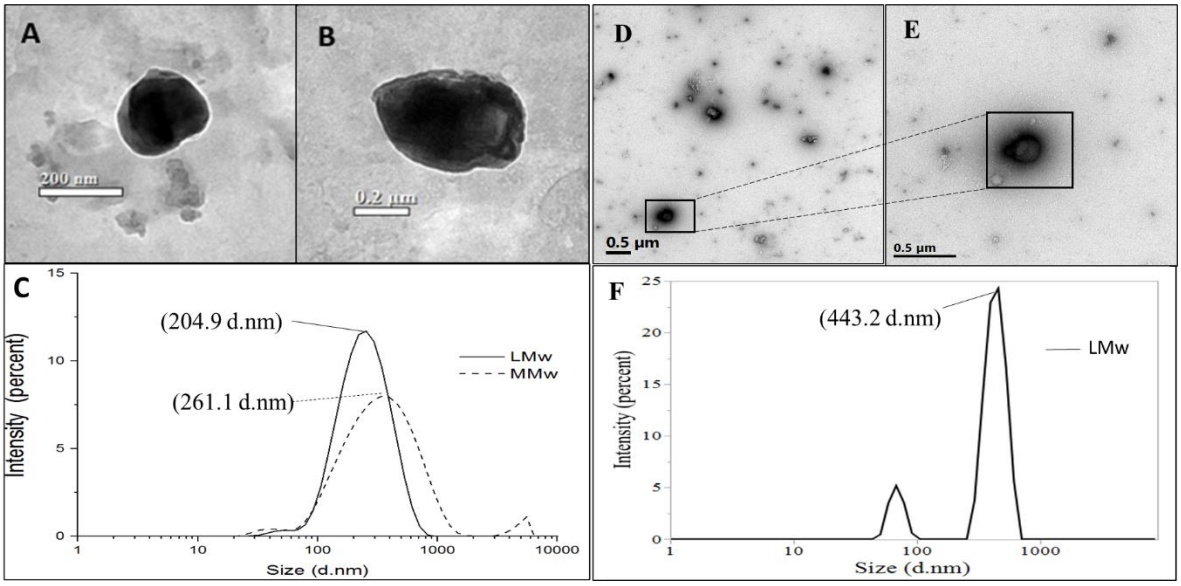


Fig. 4.2. Characterization of chitosan-based nanoparticles. Chitosan nanoparticles were obtained from low and medium molecular weight chitosan via ionic gelation using sodium tripolyphosphate (TPP) as cross-linker at a NH_3^+ : O_3 molar ratio = 1:0.5. (A-C) shows the size and shape characterization of nanoparticles obtained by dissolving chitosan in acetic acid (1% v/v; pH 4.6). Transmission electron micrographs of (A) low molecular weight (LMw) and (B) medium molecular weight (MMw) chitosan. (C) Dynamic light scattering analysis (DLS) of particle size distribution of both low and medium molecular weight chitosan. (D-F) shows the size distribution and shape characterization of nanoparticles formed when chitosan is dissolved in ascorbic acid (0.1M; pH 2.5). Mean particle diameter in (F) are 68.1 nm and 459 nm, respectively. Mean particle diameter of low and medium molecular weight chitosan-based particles are 204.9 and 261.1 d.nm, with PDI values of 0.237 and 0.419 respectively.

Fabrication of d-limonene nanoemulsion under low energy conditions via spontaneous emulsification.

The fabrication process of nano-emulsions traditionally involves a time-demanding trial-and-error approach by systematically changing formulation, composition and/or procedural variables such as the ratio of organic to aqueous phases, the surfactant type and ratio, and blends thereof[18]. Moreover, high energy inputs can be implemented, for instance, high temperature, high pressure, high shear, high frequency sonication, among others. Here we describe a method to rationally formulate nano-emulsions, comprising vegetable oils and water soluble polymers to act as viscosity modulators for the organic and aqueous phases respectively to facilitate the spontaneous formation of highly stable, translucent and water soluble emulsions containing sub-micrometric (i.e. nano-metric) under mild conditions at room temperature (i.e. low energy method). We hypothesized that the formulation of d-limonene nano-emulsion can be simplified and optimized following a rational approach based on the characterization and modulation of the dispersed (oil) and continuous (water) phases using FDA-approved (G.R.A.S) materials only under low-energy conditions at room temperature.

For producing essential oil-based nano-emulsions we set the formulation using the hydrophilic-lipophilic difference approach (i.e. $HLD = Cc - k * EACN - \alpha * \Delta T + f(S)$) as described by [18–20]. The organic phase composed of a blend of D-limonene with soybean oil and tween80® as surfactant. (Tween80 Cc = -3.7; d-limonene and soybean oil EACN values are 7 and 18 respectively). The resulting HLD value for the formulation described is c.a.-5, when using a 95:5 % w/w d-limonene:soybean oil blend as organic phase. D-limonene:Soybean oil (SBO) 95:5 %wt at HLD -5 produced translucent nano-emulsions (1b) when using propylene glycol (33.3% w/w) as co-solvent. We found that at the specific conditions investigated, in general, PEG yielded much larger capillary numbers than those obtained with propylene glycol across the different tested concentrations (75, 50 and 25% (w/w)) (Supplementary information – Fig.1). We found that propylene glycol enables nano-emulsification of d-limonene, whereas PEG hinders it, when using a blend of d-limonene:Soybean oil of 95:5. Incremental addition of soybean oil increases the viscosity of the organic phase (Supplementary information – Fig.2). All other variables with potential impact on the emulsification process, such as temperature, water salinity, agitation speed, dripping rate and surfactant to oil ratio (SOR) were kept constant. The differences in viscosities between PEG and propylene glycol may explain the different results during nano-emulsification,

when using these two polymers as co-solvents, since the viscosity influences the capillary number, and therefore, droplet deformation, during the emulsion formation.

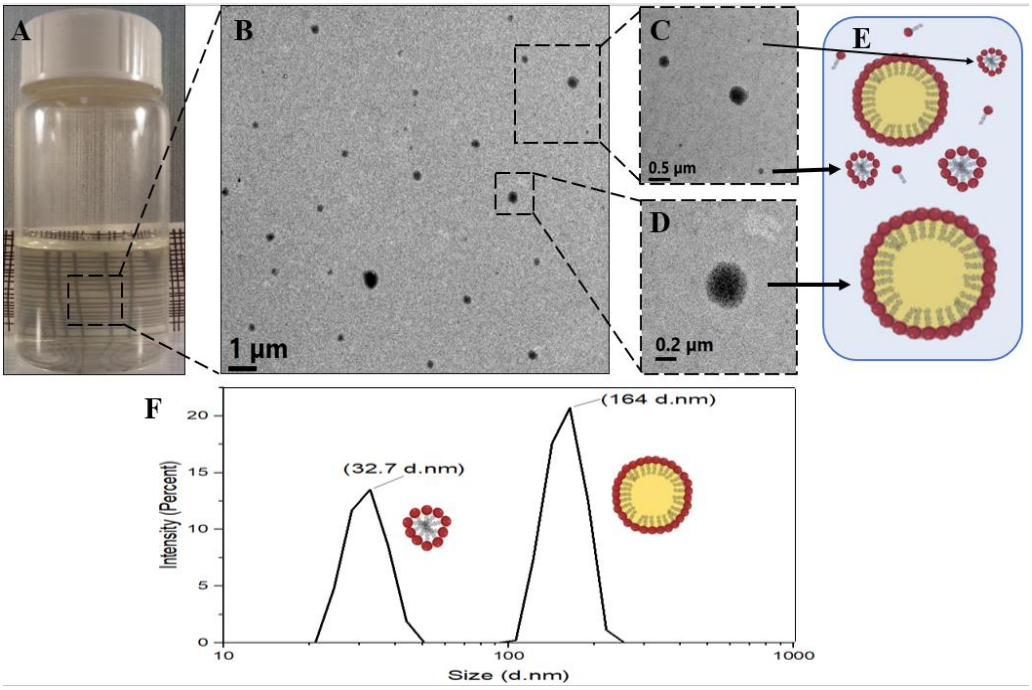


Fig. 4.3. Characterization of d-limonene nano-emulsion produced under low energy conditions via spontaneous emulsification. (A) Undiluted, translucent d-limonene nano-emulsion obtained via spontaneous emulsification at 25 ^{o}C. (B-D) shows the nanosize d-limonene confirmed via transmission electron microscopy and dynamic light scattering (F) techniques. (E) is a schematic representation of d-limonene droplets and surfactant micelles presented in figures (B-D).

Here, we demonstrated the feasibility to consistently encapsulate essential nano-emulsions in chitosan nanocarriers using low energy methods, regardless of the Degree of deacetylation or molecular weight of the chitosan. We also demonstrated that translucent nano-emulsions can be easily prepared using food grade components only without. the need for harsh conditions (e.g. changes in temperature or solvents/alcohols) nor specialized instrumentation (i.e. high energy methods). An optimized micelle-free finely-tuned formulation may be possible using the Net Average Curvature (NAC) theory firstly described by Sabatini et al.[21]., and further developed upon by Acosta et al.[19,22,23] but that objective is not within the scope of this work.

Encapsulation and characterization of d-limonene nanoemulsion into chitosan-based nanocarriers:

Essential oils as cargo with chitosan capping have been investigated [24–28]. Despite the wealth of knowledge over the last past decade on the potential benefits of chitosan-based nanocarriers loaded with essential oils, this technology still faces numerous challenges in terms of consistency and scalability posed by the intrinsic heterogeneity of the constitutive materials. In this work we have developed a rational formulation and fabrication of both, chitosan nanocarriers and nano-emulsification of essential oils that has robust applications. Fig. 4 shows the successful encapsulation of essential oils into chitosan nanocarriers as confirmed through independent multiple experimental analysis.

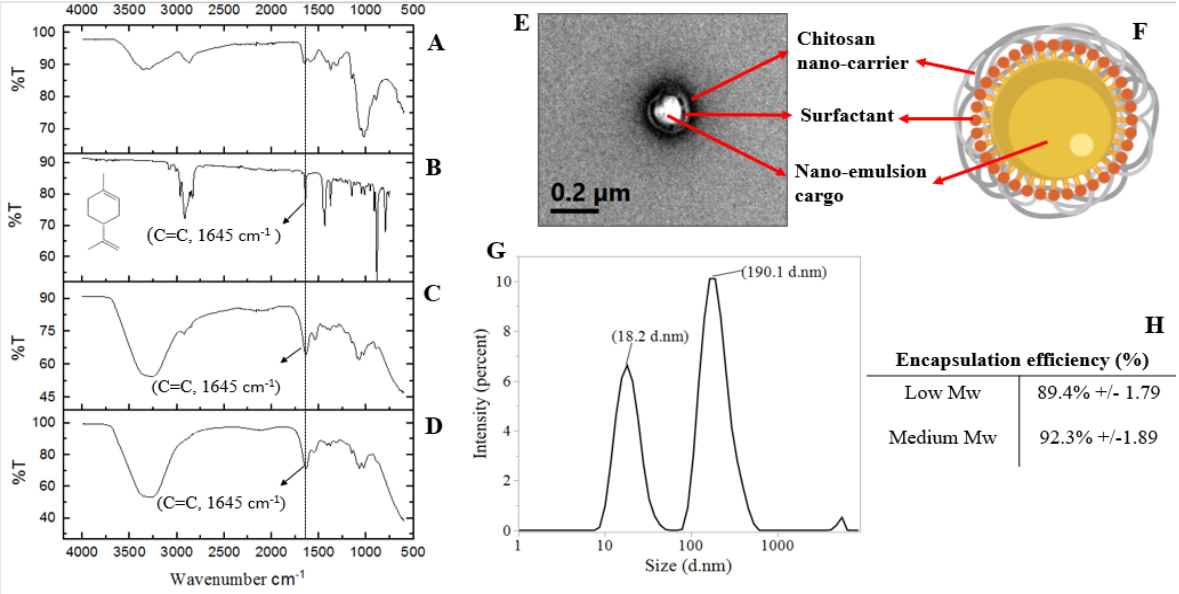


Fig. 4.4: Is a comprehensive characterization of chitosan nano-carriers loaded with d-limonene nano-emulsion. It shows a comparative characterization of low and medium molecular weight chitosan nano-carriers loaded with d-limonene nano-emulsion. (A-D) correspond to the ATR-FTIR spectral analysis of chitosan powder (A), D-limonene (B), Low and medium molecular weight chitosan nano-carriers loaded with d-limonene nano-emulsion (C) and (D), respectively. (E). Shows a transmission electron micrograph of a low molecular weight chitosan-based nano-carrier loaded with d-limonene nano-emulsion. (F) is a schematic representation of (E). (G). Shows the particle size distribution measured via dynamic light scattering. (H). presents the encapsulation efficiency (%) of low and medium molecular weight chitosan nanocarriers (n=3). A one-sided t-test shows that the effect of chitosan type is not significant ($p>0.05$) on the encapsulation efficiency (%) of d-limonene nano-emulsion.

Assessment of the systemic effect of treatments on the quantitative disease resistance against B. cinerea via image-based phenotyping

Chitosan displays several natural properties that, in combination, make of it a highly promising candidate to be considered for the development of an alternative treatment to control broad host necrotrophic pathogens such as *B.cinerea*. Perhaps the most relevant feature of chitosan might be its ability to elicit the plant defense immune system response. There is strong evidence that chitosan elicits the production of plant hormones, especially jasmonic acid [29]

In *A.thaliana*, methyl jasmonate (MeJa) induces a cellular metabolic reprogramming in order to adapt to stress conditions regulating the trade-off between defense mode and plant growth, by inhibiting cell proliferation and therefore halting cell expansion while enhancing the plant defense response [30]. Fig. 5. shows the effect of exposing the roots from *A. thaliana* (Col-0) to different treatments tested at 4 different concentrations overtime on the growth of the plants' rosette. At the lowest concentration, none of the treatments significantly affected the growth and development of *A.thaliana* under controlled conditions. In contrast, at the highest tested concentration (i.e. Chitosan 500 µg/mL and d-limonene 10mg/mL), the (alive) mean rosette area rapidly decreased when the roots of *A. thaliana* were treated with d-limonene nano-emulsion encapsulated in chitosan based nano-carriers (CdlNPs). This may indicate that there is a concentration threshold for both chitosan and d-limonene when exceeded, becomes toxic for plant cells. We demonstrated that at the same volumetric concentration (1% v/v, 0.1M, pH 2.5), the ascorbic acid in the background of the chitosan treatments did not significantly affect the growth of *A. thaliana* rosette area under the same experimental conditions (Supplementary information). Interestingly, treating roots with d-limonene nano-emulsion effectively slows down the growth of the *A. thaliana* 3 days after treatment, when using only 100 µg /mL. However, the effect on the mean rosette growth significantly increased with concentration by a factor of 10, effectively halting the mean rosette growth after 1 day of treatment. Similar results were observed for CSNPs and CdlNPs at a concentration of 0.5% (v/v). Interestingly, the effect of d-limonene nano-emulsion seems to be masked when encapsulated in a chitosan-based delivery system.

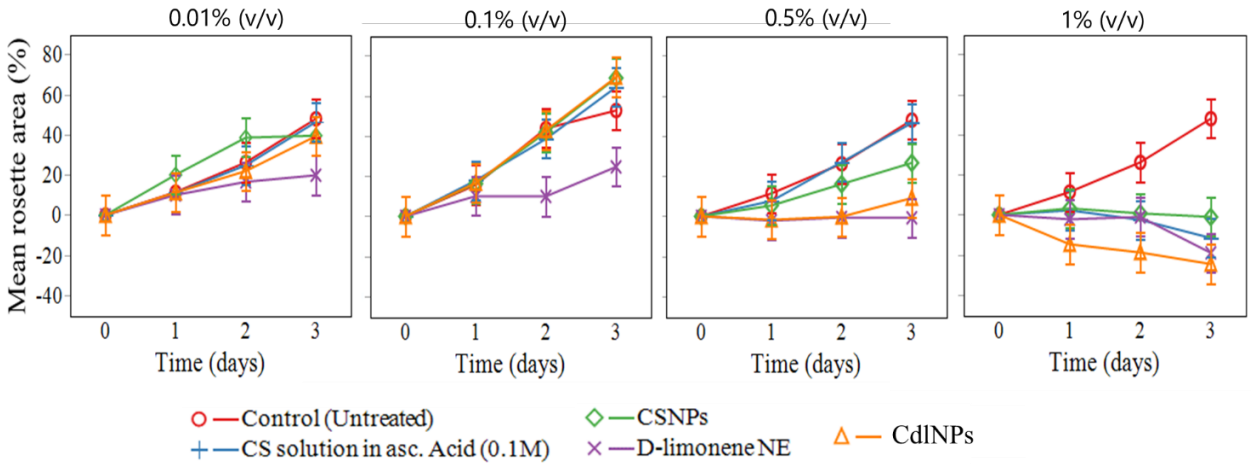


Fig. 4.5: Identification of the optimal hormetic dose response of chitosan nanoparticles (CSNPs), d-limonene nano-emulsion and chitosan-based nano-carriers loaded with d-limonene (CdlNPs) on the growth of A. thaliana (Col-0) under controlled conditions via Image-based phenotyping. Interaction plots from a balanced design containing n=9 independent plants/treatment/concentration. Bars represent a 95% confidence interval. Treatment*Time (days)*Concentration (% v/v) Leverage, P<.0001.

Although the mode of action of chitosan as plant immune-modulator is not completely understood, there is evidence that the elicitation of the plant immune system upon exposure to chitosan can be mediated by the direct interaction with the chromatin and by its recognition by receptors (Pattern triggered immunity – PTI) [31]. In *A. thaliana* there are at least two different mechanisms involved in the chitosan perception. The membrane protein chitin elicitor receptor kinase 1 (CERK1) is the major chitin receptor in Arabidopsis and rice and it is required for the activation of downstream signaling for gene regulation [32]. Besides CERK1, lysin motif receptor-like kinase4 also recognizes chitin and it is important for full induction of chitin derived signaling [33].

Regarding the effect of d-limonene on the mean rosette area of *A. thaliana*, there is evidence suggesting that d-limonene up-regulates the expression of the marker gene pdf1.2, leading to the activation of jasmonate pathway in *A. thaliana*, which halts plant growth. Fujioka et al., demonstrated that d-limonene enhances the resistance of *A. thaliana* against the *hemibiotroph Colletotrichum* higginsianum [34]. There is also evidence that terpenes activate the autophagocitic pathway in mammalian cells [35] including neuroblastoma cells [36,37] and lung cancer cells [38]. Since many of the molecular markers involved in the hypersensitive response in plants and apoptosis in mammals are conserved [39,40], it is plausible that in plants, such response might also be similar to that mounted by mammalian cells.

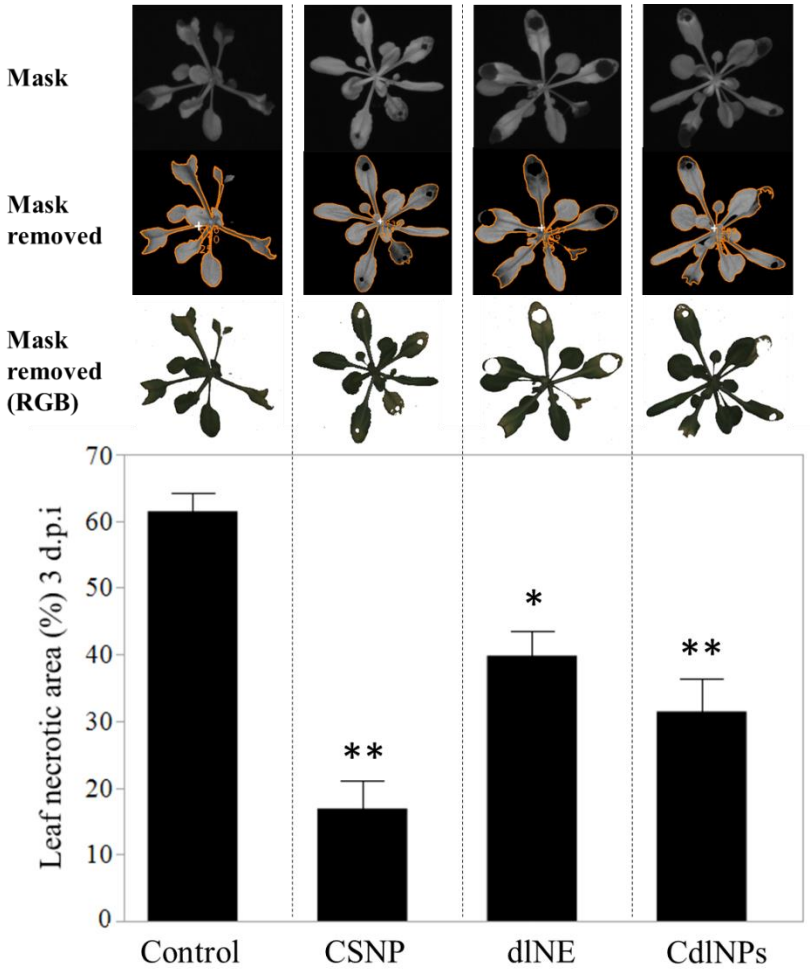


Fig. 4.6: Image-based phenotype assessment of the systemic effect of chitosan nanoparticles (CSNP), d-limonene nano-emulsion (dlNE) and chitosan-based nano-carriers loaded with d-limonene (CdlNPs) on the quantitative disease resistance of A. thaliana (Col-0) to B. cinerea. Bars represent the mean and standard error of a nested model measuring necrotic areas from four leaves per plant and 5 plants per treatment. Statistical differences were evaluated according to a nested ANOVA followed by a pair-wise comparison with a Dunnett's adjustment relative to the control group. Asterix on top of the bars indicate a significant difference between the treatment and the control group (*$p<0.05$ and **$p<0.001$). Images in the bottom depict an example of each of the treatments. Mask and removed mask (RGB) shows the image segmentation based on the fluorescence emited by chlorophyll upon excitation with blue light. Gaps in the leaves indicate areas with no fluorescence (i.e. necrotitic areas).

As shown in Fig. 6, all of the tested treatment significantly reduced the necrotic area in infected leaves. The strongest effect on the quantitative disease resistance of *A. thaliana* against *B. cinerea* was produced from CSNPs and CdlNPs, decreasing the relative necrotic area in the leaves 3- fold and 2-fold respectively, compared to the control. In order to better understand the biological mechanisms involved in the enhanced quantitative disease resistance triggered by CSNPs, RNAseq-based quantitative analysis of differentially expressed genes were compared between treated and untreated plants. Fig. 7 shows that in general, the biological processes involved in the

defense mechanisms against stressors were activated, whereas the biological process involved in the growth and development were downregulated. These findings are consistent with the image-based phenotyping analysis, indicating that perception of low doses of CSNPs in the roots, activates a systemic defense response, in the distal parts of the plant, such as infected leaves (see fig. 6).

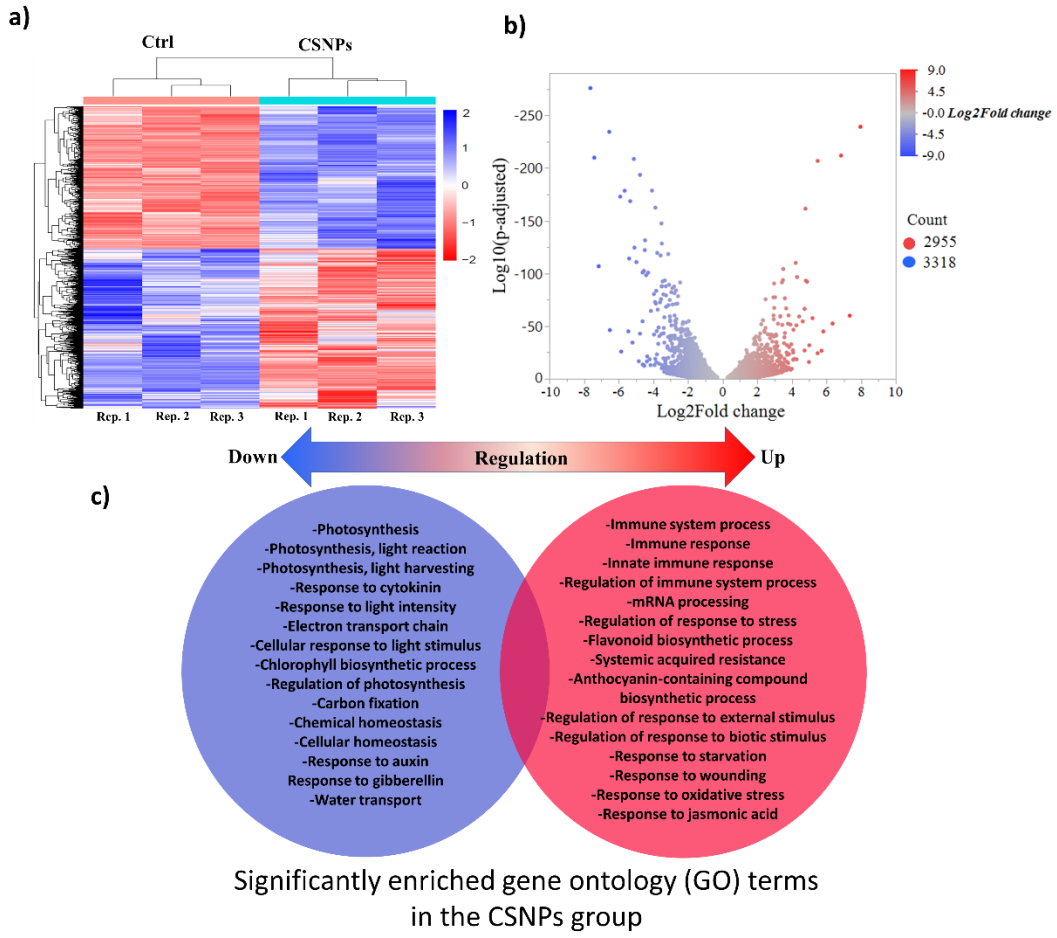


Fig. 4.7: Quantitative analysis of differentially expressed genes: CSNPs Vs Control. (A) Hierarchical Clustering Heatmap: Overall results of FPKM cluster analysis, clustered using the log2(FPKM+1) value. Red color indicates genes with high expression levels, and blue color indicates genes with low expression levels. (B) Volcano plot: Horizontal axis for the fold change of genes in different samples. Vertical axis for statistically significant degree of changes in gene expression levels. The points represent genes, red dots indicate upregulated differentially expression genes, blue dots indicate downregulated differential gene expression. (C): Functional analysis of differentially expressed genes from plants treated with CSNPs showing some of the gene ontology terms (GO) for the significantly enriched biological functions.

It is not clear how the perception of chitosan by plant cells via transmembrane receptors, activates the downstream events that lead to the control of the genes involved in the defense response of the plant, but it has been demonstrated that chitosan perception is an important pathway for plant responses to fungal necrotrophs[7]. Perception of chitin is linked to the activation of the immune response regulated by the JA/ET signaling pathway [41] and it is also linked to the expression of the chitinases and glucanases, which in turn are linked to JA- and ET- dependent pathways associated with immunity to necrotrophs[7]. We found that the biomarker genes associated with the activation of the JA-dependent defense response, such as, plant defensin genes *PDF2.1*, *PDF2.2* and *PDF2.4*, as well as vegetative storage proteins *VSP1* and *VSP2*, were up regulated in the treated plant group. Ethylene response transcription factors such as *ERF106, ERF071, ERF098, ERF094, ETR2, ERF015. ABR1*, were also upregulated. These findings suggest that PAMP-triggered immunity (PTI) by CSNPs perception may up-regulate the activation of the JA/ET pathway via up regulation of the ethylene response factor (erf) implicated in the ET signaling. Both hormones are hallmarks in the defense against fungal necrotrophic pathogens. Our results also showed that expression of Wax inducer 1 (*WIN1*) gene was also up regulated by exposure to CSNPs. *WIN1* triggers wax synthesis during cuticle production, which is instrumental to protecting the plant against various forms of biotic and abiotic stress[42].

It has been demonstrated that JA/ET pathways are botrytis-induced kinase 1 (bik1) dependent, in a response phenomenon known as triple response. A recent report suggest that chitin perception requires BIK1 and CERK1 which interacts with BIK1[43]. In this sense, BIK1 might play an integrating role between the pattern recognition receptor (PRR) sensing chitosan from the extracellular environment, to the nucleus where the gene expression is regulated.

WRKY transcription factors are one of the largest families of transcriptional regulators found exclusively in plants. In Arabidopsis *WRKY18*, *WRKY40* and *WRKY60* have partially redundant roles in response to the hemibiotrophic bacterial pathogen *Pseudomonas syringae* and the necrotrophic fungal pathogen *Botrytis cinerea*, with *WRKY18* to play a more important role than the other two. Our result shows an increased expression of *WRKY18* and *WRKY40* but not *WRKY60* in plants treated with CSNPs. Co-expression of *WRKY40* and *WRKY 60* have been linked with increased susceptibility of *A. thaliana* to *B. cinerea*. *WRKY40* may play a role in activation of the JA-dependent defense response via JA-isoleucine biosynthesis activation, resulting from the

complex formed with the coronatine insensitive 1 (COI1) and JAZ repressors, that leads to activation of *WRKY18* gene[44].

Finally, chitin perception by plants may have a positive role in the activation of autophagy, which is a cellular strategy to restrict the extent of the cellular death caused during the hypersensitive response. At the epigenetic level, a major signature of the autophagy activation is the overexpression of histone deacetylases [45] also involved in the activation of jasmonic acid and ethylene-sensitive defense mechanisms[46] . Our results showed an increased upregulation of histone deacetylases *HDA2, HDA14 and HDA18* in plants treated with CSNPs. *HDA2* and *HDA18* belongs to the class II and *HDA14* to the class III histone deacetylases[47]. Class II histone deacetylases interacts with a small number of protein complexes mainly involved in energy metabolism and metabolite transport, halting growth and altered levels in sugars, amino acids, and ADP contents [48]. Since *HDA14* exerts control functions on the RuBisCO activase [49] its overexpression results in decreased carbon fixation. These results are consistent with the observed phenotype from treated plants with CSNPs displaying halted growth.

In conclusion, we demonstrate the feasibility of rational fabrication of chitosan-based nanocarrier loaded with d-limonene nano-emulsion and its potential application in agriculture for plant protection purposes against broad host necrotrophic pathogens. The literature reports the use of the degree of acetylation and molecular weight, as the main characteristics from chitosan to account for formulation purposes. However, our results indicate that the amount of available amino groups (NH_3^+) per gram of chitosan is more relevant than the degree of deacetylation or molecular weight for nanocarrier fabrication via ionic gelation. Similarly, our results also indicate that the viscosity differential between the aqueous and organic phases in the emulsion, paired with the use of HLD, is more relevant than the HLB. By following this approach, we were able to rapidly and inexpensively develop a reliable process to produce highly stable chitosan nanocarriers loaded with d-limonene nanoemulsion.

Further, we quantitatively demonstrate the efficacy of these particles in enhancing the defense response in plants against the broad host necrotrophic pathogens in the *A. thaliana*, *B. cinerea* compatible model system. The biological systemic effect of chitosan and d-limonene on the

activation of the plant immune response followed a dose-response pattern and is enhanced when presented as nanoparticles.

In summary, our results showed that perception of CSNP, d-limonene nano-emulsion (NE) and encapsulated d-limonene nano-emulsion in chitosan nanocarriers (CdlNPs) by the plant's surveillance system, triggered major immune responses leading to an increase in quantitative resistance against necrotrophs: ROS scavenging (i.e. antioxidant environment), activation of autophagy and induction of jasmonate/ethylene and ABA hormone signaling. In combination, all these strategies together could contribute to the restriction of the hypersensitive response therefore, necrotrohic invasion by *B. cinerea* was significantly hindered in treated plants compared to the untreated ones.

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4.7 References

(1) Hua, L.; Yong, C.; Zhanquan, Z.; Boqiang, L.; Guozheng, Q.; Shiping, T. Pathogenic Mechanisms and Control Strategies of Botrytis Cinerea Causing Post-Harvest Decay in Fruits and Vegetables. *Food Qual. Saf.* **2018**, *3* (July), 111–119. https://doi.org/10.1093/fqsafe/fyy016.

(2) Dean, R.; Kan, J. A. N. A. L. V. A. N.; Pretorius, Z. A.; Hammond-kosack, K. I. M. E.; Pietro, A. D. I.; Spanu, P. D.; Rudd, J. J.; Dickman, M.; Kahmann, R.; Ellis, J.; Foster, G. D. The Top 10 Fungal Pathogens in Molecular Plant Pathology. *Mol. Plant Pathol.* **2012**, *13* (4), 414–430. https://doi.org/10.1111/J.1364-3703.2011.00783.X.

(3) Laluk, K.; Mengiste, T. Necrotroph Attacks on Plants: Wanton Destruction or Covert Extortion? *Arabidopsis Book* **2010**, *8*, e0136. https://doi.org/10.1199/tab.0136.

(4) Mendgen, K.; Hahn, M. Plant Infection and the Establishment of Fungal Biotrophy. *Trends in Plant Science*. Trends Plant Sci August 1, 2002, pp 352–356. https://doi.org/10.1016/S1360-1385(02)02297-5.

(5) Koeck, M.; Hardham, A. R.; Dodds, P. N. The Role of Effectors of Biotrophic and Hemibiotrophic Fungi in Infection. *Cellular Microbiology*. NIH Public Access December 2011, pp 1849–1857. https://doi.org/10.1111/j.1462-5822.2011.01665.x.

(6) Lai, Z.; Mengiste, T. Genetic and Cellular Mechanisms Regulating Plant Responses to Necrotrophic Pathogens. *Current Opinion in Plant Biology*. 2013, pp 505–512. https://doi.org/10.1016/j.pbi.2013.06.014.

(7) Mengiste, T. Plant Immunity to Necrotrophs. *Annu. Rev. Phytopathol.* **2012**, *50* (1), 267–294. https://doi.org/10.1146/annurev-phyto-081211-172955.

(8) Jones, J. D. G.; Dangl, J. L. The Plant Immune System. *Nature* **2006**, *444* (7117), 323–329. https://doi.org/10.1038/nature05286.

(9) Vega-Vásquez, P.; Mosier, N. S.; Irudayaraj, J. Nanoscale Drug Delivery Systems: From Medicine to Agriculture. *Frontiers in Bioengineering and Biotechnology*. Frontiers Media S.A. February 18, 2020, p 79. https://doi.org/10.3389/fbioe.2020.00079.

(10) Malerba, M.; Cerana, R. Chitosan Effects on Plant Systems. *Int. J. Mol. Sci.* **2016**, *17* (7), 1–15. https://doi.org/10.3390/ijms17070996.

(11) Chandra, S.; Chakraborty, N.; Dasgupta, A.; Sarkar, J.; Panda, K.; Acharya, K. Chitosan Nanoparticles: A Positive Modulator of Innate Immune Responses in Plants. *Sci. Rep.* **2015**, *5*, 15195. https://doi.org/10.1038/srep15195.

(12) Huang, Y.; Cai, Y.; Lapitsky, Y. Factors Affecting the Stability of Chitosan/Tripolyphosphate Micro- and Nanogels: Resolving the Opposing Findings. *J. Mater. Chem. B* **2015**, *3* (29). https://doi.org/10.1039/C5TB00431D.

(13) Cai, Y.; Lapitsky, Y. Pitfalls in Analyzing Release from Chitosan/Tripolyphosphate Micro- and Nanoparticles. *Eur. J. Pharm. Biopharm.* **2019**, *142*, 204–215. https://doi.org/10.1016/J.EJPB.2019.06.020.

(14) Smith-Fiola, D.; Gill, S. Vinegar: An Alternative To Glyphosate? **2017**. https://doi.org/10.1016/S0022-0248(99)00711-3.

(15) Das, K.; Roychoudhury, A. Reactive Oxygen Species (ROS) and Response of Antioxidants as ROS-Scavengers during Environmental Stress in Plants. *Front. Environ. Sci.* **2014**, *2*, 53. https://doi.org/10.3389/fenvs.2014.00053.

(16) Muckenschnabel, I.; Goodman, B. A.; Williamson, B.; Lyon, G. D.; Deighton, N. Infection of Leaves of Arabidopsis Thaliana by Botrytis Cinerea: Changes in Ascorbic Acid, Free Radicals and Lipid Peroxidation Products. *J. Exp. Bot.* **2002**, *53* (367), 207–214.

(17) Akram, N. A.; Shafiq, F.; Ashraf, M. Ascorbic Acid-a Potential Oxidant Scavenger and Its Role in Plant Development and Abiotic Stress Tolerance. *Frontiers in Plant Science*. Frontiers Research Foundation April 26, 2017, p 613. https://doi.org/10.3389/fpls.2017.00613.

(18) Salager, J.-L.; Bullón, J.; Pizzino, A.; Rondón-González, M.; Tolosa, L. Emulsion Formulation Engineering for the Practitioner. *Encycl. Surf. Colloid Sci.* **2007**, No. 1: 1, 1 — 16. https://doi.org/DOI: 10.1081/E-ESCS-120045970.

(19) Acosta, E. J. The HLD-NAC Equation of State for Microemulsions Formulated with Nonionic Alcohol Ethoxylate and Alkylphenol Ethoxylate Surfactants. *Colloids Surfaces A Physicochem. Eng. Asp.* **2008**, *320* (1–3), 193–204. https://doi.org/10.1016/j.colsurfa.2008.01.049.

(20) Abbott, S. Surfactant Science : Principles and Practice. *Surfactant Sci. Princ. Pract.* **2015**, 1–249.

(21) Sabatini, D. A.; Acosta, E.; Harwell, J. H. Linker Molecules in Surfactant Mixtures. *Curr. Opin. Colloid Interface Sci.* **2003**, *8* (4–5), 316–326. https://doi.org/10.1016/S1359-0294(03)00082-7.

(22) Acosta, E.; Szekeres, E.; Sabatini, D. A.; Harwell, J. H. Net-Average Curvature Model for Solubilization and Supersolubilization in Surfactant Microemulsions. *Langmuir* **2003**, *19* (1), 186–195. https://doi.org/10.1021/la026168a.

(23) Kiran, S. K.; Acosta, E. J. Predicting the Morphology and Viscosity of Microemulsions Using the HLD-NAC Model. *Ind. Eng. Chem. Res.* **2010**, *49* (7), 3424–3432. https://doi.org/10.1021/ie9013106.

(24) Jamil, B.; Abbasi, R.; Abbasi, S.; Imran, M.; Khan, S. U.; Ihsan, A.; Javed, S.; Bokhari, H.; Imran, M. Encapsulation of Cardamom Essential Oil in Chitosan Nano-Composites: In-Vitro Efficacy on Antibiotic-Resistant Bacterial Pathogens and Cytotoxicity Studies. *Front. Microbiol.* **2016**, *7*, 1580. https://doi.org/10.3389/fmicb.2016.01580.

(25) Wu, C.; Wang, L.; Hu, Y.; Chen, S.; Liu, D.; Ye, X. Edible Coating from Citrus Essential Oil-Loaded Nanoemulsions: Physicochemical Characterization and Preservation Performance. *RSC Adv.* **2016**, *6* (25), 20892–20900. https://doi.org/10.1039/C6RA00757K.

(26) Fakhreddin, S.; Zandi, M.; Rezaei, M.; Farahmandghavi, F. Two-Step Method for Encapsulation of Oregano Essential Oil in Chitosan Nanoparticles : Preparation , Characterization and in Vitro Release Study. *Carbohydr. Polym.* **2013**, *95* (1), 50–56. https://doi.org/10.1016/j.carbpol.2013.02.031.

(27) Kalagatur, N. K.; Ghosh, O. S. N.; Sundararaj, N. Antifungal Activity of Chitosan Nanoparticles Encapsulated With Cymbopogon Martinii Essential Oil on Plant Pathogenic Fungi Fusarium Graminearum. **2018**, *9* (June), 1–13. https://doi.org/10.3389/fphar.2018.00610.

(28) Mishra, N.; Rai, V. K.; Yadav, K. S.; Sinha, P.; Kanaujia, A.; Chanda, D.; Jakhmola, A.; Saikia, D.; Yadav, N. P. Encapsulation of Mentha Oil in Chitosan Polymer Matrix Alleviates Skin Irritation. **2016**, *17* (2), 482–492. https://doi.org/10.1208/s12249-015-0378-x.

(29) Jia, X.; Meng, Q.; Zeng, H.; Wang, W.; Yin, H. Chitosan Oligosaccharide Induces Resistance to Tobacco Mosaic Virus in Arabidopsis via the Salicylic Acid-Mediated Signalling Pathway. *Nat. Publ. Gr.* **2016**, No. April, 1–12. https://doi.org/10.1038/srep26144.

(30) Bömer, M.; Brien, J. A. O.; Pérez-salamó, I.; Krasauskas, J. And Cell Wall Protein Composition in Arabidopsis COI1-Dependent Jasmonate Signalling Affects Growth , Metabolite Production and Cell Wall Protein Composition in Arabidopsis. **2018**, No. June. https://doi.org/10.1093/aob/mcy109.

(31) Igarashi, D.; Bethke, G.; Xu, Y.; Tsuda, K.; Glazebrook, J.; Katagiri, F.; Pattern-Triggered Immunity Suppresses Programmed Cell Death Triggered by Fumonisin B1. *PLoS One* **2013**, *8* (4), e60769. https://doi.org/10.1371/journal.pone.0060769.

(32) Miya, A.; Albert, P.; Shinya, T.; Desaki, Y.; Ichimura, K.; Shirasu, K.; Narusaka, Y.; Kawakami, N.; Kaku, H.; Shibuya, N. CERK1, a LysM Receptor Kinase, Is Essential for Chitin Elicitor Signaling in Arabidopsis. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104* (49), 19613–19618. https://doi.org/10.1073/pnas.0705147104.

(33) Wan, J.; Tanaka, K.; Zhang, X. C.; Son, G. H.; Brechenmacher, L.; Nguyen, T. H. N.; Stacey, G. LYK4, a Lysin Motif Receptor-like Kinase, Is Important for Chitin Signaling and Plant Innate Immunity in Arabidopsis. *Plant Physiol.* **2012**, *160* (1), 396–406. https://doi.org/10.1104/pp.112.201699.

(34) Fujioka, K.; Gotoh, H.; Noumi, T.; Yoshida, A.; Noutoshi, Y.; Inagaki, Y.; Yamamoto, M.; Ichinose, Y.; Shiraishi, T.; Toyoda, K. Protection Induced by Volatile Limonene against Anthracnose Disease in Arabidopsis Thaliana. *J. Gen. Plant Pathol.* **2015**, *81* (6), 415–419. https://doi.org/10.1007/s10327-015-0621-z.

(35) Kim, T.; Song, B.; Cho, K. S.; Lee, I. S. Therapeutic Potential of Volatile Terpenes and Terpenoids from Forests for Inflammatory Diseases. *Int. J. Mol. Sci.* **2020**, *21* (6). https://doi.org/10.3390/ijms21062187.

(36) Berliocchi, L.; Chiappini, C.; Adornetto, A.; Gentile, D.; Cerri, S.; Russo, R.; Bagetta, G.; Corasaniti, M. T. Early LC3 Lipidation Induced by D-Limonene Does Not Rely on MTOR Inhibition, ERK Activation and ROS Production and It Is Associated with Reduced Clonogenic Capacity of SH-SY5Y Neuroblastoma Cells. *Phytomedicine* **2018**, *40*, 98–105. https://doi.org/10.1016/j.phymed.2018.01.005.

(37) Russo, R.; Cassiano, M. G. V.; Ciociaro, A.; Adornetto, A.; Varano, G. P.; Chiappini, C.; Berliocchi, L.; Tassorelli, C.; Bagetta, G.; Corasaniti, M. T. Role of D-Limonene in Autophagy Induced by Bergamot Essential Oil in SH-SY5Y Neuroblastoma Cells. *PLoS One* **2014**, *9* (11), 1–19. https://doi.org/10.1371/journal.pone.0113682.

(38) Yu, X.; Lin, H.; Wang, Y.; Lv, W.; Zhang, S.; Qian, Y.; Deng, X.; Feng, N.; Yu, H.; Qian, B. D-Limonene Exhibits Antitumor Activity by Inducing Autophagy and Apoptosis in Lung Cancer. *Onco. Targets. Ther.* **2018**, *11*, 1833–1847. https://doi.org/10.2147/OTT.S155716.

(39) Olvera-Carrillo, Y.; Van Bel, M.; Van Hautegem, T.; Fendrych, M.; Huysmans, M.; Simaskova, M.; van Durme, M.; Buscaill, P.; Rivas, S.; Coll, N. S.; Coppens, F.; Maere, S.; Nowack, M. K. A Conserved Core of Programmed Cell Death Indicator Genes Discriminates Developmentally and Environmentally Induced Programmed Cell Death in Plants. *Plant Physiol.* **2015**, *169* (4), 2684–2699. https://doi.org/10.1104/pp.15.00769.

(40) Mur, L. A. J.; Kenton, P.; Lloyd, A. J.; Ougham, H.; Prats, E. The Hypersensitive Response; The Centenary Is upon Us but How Much Do We Know? *J. Exp. Bot.* **2008**, *59* (3), 501–520. https://doi.org/10.1093/jxb/erm239.

(41) Yin, H.; Li, S.; Zhao, X.; Du, Y.; Ma, X. CDNA Microarray Analysis of Gene Expression in Brassica Napus Treated with Oligochitosan Elicitor. *Plant Physiol. Biochem.* **2006**, *44* (11–12), 910–916. https://doi.org/10.1016/j.plaphy.2006.10.002.

(42) Kannangara, R.; Branigan, C.; Liu, Y.; Penfield, T.; Rao, V.; Mouille, G.; Höfte, H.; Pauly, M.; Riechmann, J. L.; Broun, P. The Transcription Factor WIN1/SHN1 Regulates Cutin Biosynthesis in Arabidopsis Thaliana. *Plant Cell* **2007**, *19* (4), 1278–1294. https://doi.org/10.1105/tpc.106.047076.

(43) AbuQamar, S.; Moustafa, K.; Tran, L.-S. P. Mechanisms and Strategies of Plant Defense against *Botrytis Cinerea*. *Crit. Rev. Biotechnol.* **2017**, *37* (2), 262–274. https://doi.org/10.1080/07388551.2016.1271767.

(44) Rushton, P. J.; Somssich, I. E.; Ringler, P.; Shen, Q. J. WRKY Transcription Factors. *Trends Plant Sci.* **2010**, *15* (5), 247–258. https://doi.org/10.1016/j.tplants.2010.02.006.

(45) Luo, M.; Cheng, K.; Xu, Y.; Yang, S.; Wu, K. Plant Responses to Abiotic Stress Regulated by Histone Deacetylases. *Frontiers in Plant Science*. Frontiers Media S.A. December 15, 2017, p 2147. https://doi.org/10.3389/fpls.2017.02147.

(46) Zhou, C.; Zhang, L.; Duan, J.; Miki, B.; Wu, K. Histone Deacetylase19 Is Involved in Jasmonic Acid and Ethylene Signaling of Pathogen Response in Arabidopsis. *Plant Cell* **2005**, *17* (4), 1196–1204. https://doi.org/10.1105/tpc.104.028514.

(47) Liu, X.; Yang, S.; Zhao, M.; Luo, M.; Yu, C. W.; Chen, C. Y.; Tai, R.; Wu, K. Transcriptional Repression by Histone Deacetylases in Plants. *Mol. Plant* **2014**, *7* (5), 764–772. https://doi.org/10.1093/mp/ssu033.

(48) König, A. C.; Hartl, M.; Pham, P. A.; Laxa, M.; Boersema, P. J.; Orwat, A.; Kalitventseva, I.; Plöchinger, M.; Braun, H. P.; Leister, D.; Mann, M.; Wachter, A.; Fernie, A. R.; Finkemeier, I. The Arabidopsis Class II Sirtuin Is a Lysine Deacetylase and Interacts with Mitochondrial Energy Metabolism. *Plant Physiol.* **2014**, *164* (3), 1401–1414. https://doi.org/10.1104/pp.113.232496.

(49) Hartl, M.; Füßl, M.; Boersema, P. J.; Jost, J.; Kramer, K.; Bakirbas, A.; Sindlinger, J.; Plöchinger, M.; Leister, D.; Uhrig, G.; Moorhead, G. B.; Cox, J.; Salvucci, M. E.; Schwarzer, D.; Mann, M.; Finkemeier, I. Lysine Acetylome Profiling Uncovers Novel Histone Deacetylase Substrate Proteins in Arabidopsis . *Mol. Syst. Biol.* **2017**, *13* (10), 949. https://doi.org/10.15252/msb.20177819.

(50) Lavertu, M.; Xia, Z.; Serreqi, A. N.; Berrada, M.; Rodrigues, A.; Wang, D.; Buschmann, M. D.; Gupta, A. A Validated 1H NMR Method for the Determination of the Degree of Deacetylation of Chitosan. *J. Pharm. Biomed. Anal.* **2003**, *32* (6), 1149–1158. https://doi.org/10.1016/S0731-7085(03)00155-9.

(51) Lapitsky, Y.; Zahir, T.; Shoichet, M. S. Modular Biodegradable Biomaterials from Surfactant and Polyelectrolyte Mixtures. *Biomacromolecules* **2008**, No. 9, 166–174. https://doi.org/10.1021/bm7009416.

(52) Conn, S. J.; Hocking, B.; Dayod, M.; Xu, B.; Athman, A.; Henderson, S.; Aukett, L.; Conn, V.; Shearer, M. K.; Fuentes, S.; Tyerman, S. D.; Gilliham, M. Protocol : Optimising Hydroponic Growth Systems for Nutritional and Physiological Analysis of Arabidopsis Thaliana and Other Plants. **2013**, 1–11.

(53) Liao, C.-J.; Lai, Z.; Lee, S.; Yun, D. J.; Mengiste, T. Arabidopsis HOOKLESS1 Regulates Responses to Pathogens and Abscisic Acid through Interaction with MED18 and Acetylation of WRKY33 and ABI5 Chromatin. *Plant Cell* **2016**, *28* (July), tpc.00105.2016. https://doi.org/10.1105/tpc.16.00105.

5. SUMMARY AND RECOMMENDATIONS

The overall purpose of the present work was to evaluate the potential of drug delivery nanotechnologies, traditionally developed for pharma, and biomedical purposes, to be translated into scalable, stable, and cost-effective solutions for agricultural applications. Specifically, evaluating their ability to enhance the quantitative disease resistance against broad host necrotrophs, using *Arabidopsis thaliana-Botrytis cinerea* as a model pathosystem. The methodological approach employed in this work can be divided in two main sections: The first part consisted in understanding the physical-chemical drivers governing the formation of loaded nano-carriers, enabling the rationalization of the fabrication methods aiming to reduce the batch-to-batch variability, while increasing their stability. Thus, paving the way into potential fabrication at scale for agricultural purposes.

There are certain caveats, however, that must be factored in. First is the scale-up of production which should not only be technically feasible, but also economically viable in the future. For instance, the biological mechanisms for up-take, distribution inside the plant, and potential accumulation in tissues must be investigated. This is important because it poses a risk for humans and animals fed with nano-particle containing plants. If the materials used for nano-carrier fabrication are not biodegradable, these can be accumulated along the trophic chain, potentially reaching toxic levels at the upper levels of the chain. Therefore, the use of natural materials and food grade materials are preferred for nano-carrier fabrication. Chitosan and essential oils were used in this work because they meet these criteria.

Another important criterion to be accounted for is the effective treatment dosage needed to trigger the desired response in plants. This is important because it directly correlates with the economic viability of the treatment. Too high or too frequent a dose may dramatically increase the cost per treated hectare. If the cost of treatment per hectare exceeds the cost caused by the disease affecting the untreated crops, then this technology would be prohibitively expensive for agricultural purposes. Hence additional characterization in adverse environmental conditions for stability, release, and degradation mechanism needs to be performed. In this work evidence on how the potency of the treatments is increased by reducing the particle size of chitosan carriers and essential

oil emulsions is presented. Increasing the potency of the treatment may present an opportunity to decrease the cost while increasing the effectivity of this technology for crop protection purposes.

Lastly, a critical component is mass production given the vast nature of agricultural systems, unlike drug delivery for human health. In this work, we have presented a rational approach for cost-effective fabrication of chitosan nano-carriers loaded with essential oil nano-emulsions for agricultural purposes. In general, the first part of this work argued about why chitosan and essential oils are of interest for the development and use of drug-delivery systems for crop protection purposes and presented compelling evidence on how chitosan nano-carriers loaded with essential oil nano-emulsions can be cost-effectively fabricated and customized.

The second part consisted in quantifying how effectively such treatments enhanced the plants defense response against broad host necrotrophic pathogens, and second, understanding which are the biological mechanisms involved in such response from plants upon exposure to the treatments. Based on the findings derived from this research, the following recommendations for future work are formulated:

Using the Np (i.e. the amount of NH_3^+ available groups per gram of chitosan) instead of deacetylation degree or molecular weight as the main characteristic of chitosan during formulation purposes is recommended when using the ionic gelation method to fabricate chitosan nanocarriers. By following this approach, the experimental design to determine the conditions required for nanoparticle formation can be drastically reduced. This approach identifies a narrow range of conditions that are likely to be successful rather than the traditional approach, where various levels of multiple parameters are systematically tested to find the conditions needed for nanoparticle formation. This approach yields similar results regardless of the molecular weight of chitosan and its deacetylation degree, which vary significantly in this natural material and are the main two characteristics frequently discussed in the literature.

Using the viscosity differential (η_c/η_d) between the continuous (c) and dispersed (d) phases and the HLD (hydrophilic-lipophilic difference), instead of the HLB (hydrophilic-lipophilic balance) during the formulation of essential oil nano-emulsions. By following this rational approach, the experimental design during nano-emulsion formulation can be drastically reduced, where a few iterations are needed, rather than a large number of experiments that are usually required during

full factorial experimental design. Moreover, this approach enables nano-emulsion formation under low energy conditions, thus, preventing potential denaturation of bioactive molecules. Finally, when using dynamic light scattering analysis for droplet characterization purposes, the use of undiluted samples is recommended. Diluting nano-emulsions may modify the droplet size and polydispersity and thus, inadvertently result in potential misleading conclusions when investigating different formulations or emulsification conditions.

The nano-emulsification process described in this work facilitates the production and customization of nano-emulsions using virtually any essential oil available, without compromising its chemical or biological properties. Thus, an opportunity is presented here to explore the biological effects of different essential oils on different biological models, that may result in interesting discoveries and useful industrial applications beyond agriculture.

Here the effectiveness of chitosan-based nanocarriers loaded with d-limonene nano-emulsions, and other essential oil nano-emulsions in enhancing the resistance of plants against broad host necrotrophs was demonstrated. It is feasible that these treatments may also be effective in enhancing the defense response in plants against other type of plant phytopathogens such as oomycetes. The hydroponic setup for plant growth employed and described in this work may serve as experimental platform to easily test the effect of these treatments against oomycetes such as *Phytophtora spp.*