Jasmonate-loaded nanosilicates for induction of plant defense responses



Morgan Wade

Hertford College

University of Oxford

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Personal

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Abstract

Diseases of seedlings cause massive pre-harvest losses, therefore seed treatments or coatings are used to combat such losses in agriculture. Plant immune systems can be prepared for later infection in a process called priming. Certain plant hormones, like jasmonates, contribute to this priming effect and can allow plant immune systems to respond stronger and more quickly to diseases.

Previous work has shown that jasmonate treatments can be effective immune priming agents in a variety of crop species. However, there is a trade-off between growth and immune function and seeds treated with too much hormone may not grow as well as untreated plants. If the dosage of the hormone treatment is too low the effect of the treatment is often short lived. The immune priming wears off putting young plants at risk to disease. Improving jasmonate seed treatments by including a biocompatible carrier compound could create longer-lasting immune priming without incurring costs to plant growth and development.

Mesoporous silica is used as both a biocompatible carrier for other compounds as well as a micronutrient supplementation in a variety of agricultural contexts. Zeolite clinoptilolite is a microporous aluminosilicate which is used both for binding fertilizers and pesticides and also as a soil additive to increase water retention. Both compounds have also shown promise as crop-improving supplements in the literature. Silica, while not considered an essential nutrient in plants, can improve a wide variety of metrics including improving tolerance to salinity, drought, and heat stress, strengthening cell walls and fibres, and inducing priming responses against pathogens.

This research tests the hypothesis that silicates like mesoporous silica nanoparticles and zeolite clinoplilolite can be used as carriers or in conjunction with plant hormones to activate plant immune systems and induced defense priming in crop plants. This research tested the efficacy of mesoporous silica nano-encapsulated jasmonic acid and methyl jasmonate and jasmonic acid and methyl jasmonate loaded zeolite clinoptilolite as immune priming compounds in pea (*Pisum sativum*) seedlings against the necrotrophic pathogen *Pythium ultimum*.

The results of this study show that jasmonic acid and methyl jasmonate can be successfully loaded into both mesoporous silica nanoparticles and ground zeolite clinoptilolite. The results showed that jasmonate-zeolite treatments are not effective

at reducing mortality and decreasing disease symptoms in a *Pisum sativum-Pythium ultimum* disease system. Gene expression results show that, despite this lack of efficacy, the zeolite-jasmonate treatments do seem to induce upregulation of JAR4 in peas, at a later time point than has been previously reported in the literature.

0.1 Extenuating Circumstances

0.1.1 Delays due to the COVID-19 Pandemic

My DPhil programm was a four year doctoral training program which began with one term of coursework and then two, three month long rotation projects. When the pandemic began in March 2020 I was just finishing up my first rotation in Gail Preston's laboratory. Due to covid-19 related closures, I was unable to complete my second rotation, planned to be in Helen Townley's laboratory. Labs across the university were closed from late March 2020 through July 2020. To compensate for this, I extended my first rotation and found an internship to meet my PIPS internship requirement for summer 2020.

My thesis project then started in September 2020. My supervisors were located in the Department of Plant Sciences (now the Department of Biology, South Parks Road) and at the Begbroke Science Park, north of the city of Oxford.

From the beginning of my project I faced significant disruptions due to the covid-19 pandemic. My initial access to the Begbroke labs was set back by four weeks due to delayed processing of safety paperwork. In addition, both departments were in low occupancy restrictions from the start of my project until the 6th of September 2021. We were only allowed four individuals in the Plant Sciences lab at any one time and three individuals in the labs at Begbroke. We were only allowed in the labs with the same people, in shifts. This caused delays not only for initial training in techniques but also in access to equipment and assistance when trouble shooting techniques and protocols, especially in work that requires a high degree of skills training, like using a TEM or GCMS.

Much of my early work, including generating nanoparticles and optimizing a protocol for detecting jasmonates required early use of a TEM and a GCMS, respectively. I spent months (Oct 2020 – July 2021) waiting on access and training for these pieces of equipment, including seeking access from other departments and other institutions. The GCMS in Plant Sciences required repairs several times during this period and the repairs were significantly delayed due to covid-19 restrictions imposed by the servicing companies as well as by the host department. TEM is necessary for characterizing the nanoparticles and GCMS is necessary for measuring and characterizing the jasmonates. Much of my project required having these initial characterizations of the nanoparticles and nanoparticle loading, and so lack of access to a GCMS and a TEM caused a severe bottle neck in my project progress.

I was able to somewhat compensate for these delays by working on the few parts of my project which did not require these techniques, like my pathogen assays and engaging with the literature, however I was limited in what I could

do. My project is primarily experimental and unfortunately could not be shifted to be a computational project.

Additionally, from October 2020 through August 2021 I was in a challenging housing situation and found it stressful and uncomfortable to be at home, which made working effectively from home difficult during that time.

All told, I estimate at least seven months of work time lost due to these cumulative delays; limited lab access and limited training and assistance for over the course of 12 months of shift rotas and limited occupancy, lack of reliable TEM access from Oct 2020 to July 2021, GCMS broken for a total of 6 weeks, etc.

This loss of time caused me to extend my DPhil by four months.

0.1.2 Delays due to ongoing medical concerns

During the course of my PhD I also experienced serious medical concerns, which required me to take one month out in January-February 2020 and three months out from January 2022 - March 2022. I received a two month medical leave extension for the time lost in 2022.

All told, I delayed my thesis submission by two terms or six months due to delays caused by covid-19 and my own medical concerns.

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List of Abbreviations

AOC . . . . . allene oxide cyclase

AOS . . . . . allene oxide synthase

ANOVA . . . . analysis of variance

CMA . . . . . corn meal agar

CTAB . . . . . cetyl trimethyl ammonium bromide

dHJ . . . . . dihydrojasmone

DMSO . . . . . dimethyl sufoxide

EDTA . . . . . ethylenediaminetetraacetic acid

FAO . . . . . Food and Agriculture Organization

GC-MS . . . . gas chromatography-mass spectrometry

HMSNP . . . hexagonal mesoporous silica nanoparticle

IPCC . . . . . Intergovernmental Panel on Climate Change

JA . . . . . jasmonic acid

JA-Ile . . . . . jasmonyl isoleucine

LC-MS/MS . liquid chromatography-mass spectrometry-mass spectrometry

[LOX] lipoxygenase

MCM41 . . . . Mobil composition of matter No. 41

MeJA . . . . . methyl jasmonate

MSNP . . . . . mesoporous silica nanoparticle

NP . . . . . nanoparticle

PDA . . . . . potato dextrose agar

PDB . . . . . potato dextrose broth

SEM . . . . . scanning electron microscopy

TAE . . . . . tris base, acetic acid and EDTA

TEM . . . . . transmission electron microscopy

TEOS . . . . . tetraethyl orthosilicate

WA . . . . . water agar

12-OPDA . . . 12-Oxo-phytodienoic acid

1 Introduction

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1.1 Motivation

Seedling diseases cause massive pre-harvest losses and seed treatments or coatings to combat such losses are commonplace in agriculture. However, most of these seed treatments incorporate pesticides, which can have negative environmental impact. This research aims to provide alternatives to pesticide use in agriculture. Plant

immune systems can be prepared for later infection in a process called priming. Certain plant hormones, like jasmonates, contribute to this priming effect and can allow plant immune systems to respond stronger and more quickly to diseases. Previous work has shown that exposing seeds to jasmonates prepares the growing plant's immune system to resist future infection. This is called immune-priming. However, there is a trade-off between growth and immune function, and seeds treated with too much hormone may not germinate or grow as well as untreated samples. Also, the effect of treatments is often short lived. Immune priming achieved through seed treatments can become attenuated over time, putting young plants at risk of disease. This project aims to overcome these issues by, (i) controlling the dose and exposure of the seed treatment and (ii) extending the time over which the seed treatment is delivered. In theory both aims can be accomplished by loading the hormones into biodegradable nanoparticles, which keep the hormones and seed treatment more stable for longer, as well as enabling more precise control over how much hormone is released to the seed.

This research proposes to use silicates, both silica (Si) nanoparticles and zeolite, to deliver jasmonate hormones as immune defense-promoting compounds to seeds via a seed treatment. Jasmonates are plant signaling hormones that have been shown to increase immune system defense responses and therefore may be a viable alternative to traditional pesticides. The silicate carriers will be used because they are both biocompatible and biodegradable. They also serve several additional functions in this proposed seed treatment: they allow precise application of jasmonates, they can prevent the jasmonates from evaporating, and can serve as a silica nutrient supplementation. This project compares the efficacy of the silicate-jasmonate seed treatment versus traditional seed-soaking treatments. It also attempts to examine the dose and rate of the release of the compounds.

Developing a plant-pathosystem to test the effectiveness of the treatments was an important part of the project. As jasmonate hormones control plant responses to necrotrophic pathogens and herbivores a necrotrophic pathogen was needed. Most necrotrophic pathogens produce aerosolized spores which make them difficult

to study and contain within a lab system. *Pythium ultimum* was chosen as the pathogen because it is an oomycete pathogen which does not produce aerosolized spores. The plant needed to be agronomically important and susceptible to seed and seedling infections. It was important that the plant-pathosystem could be set up in a mattter of days and not take weeks to produce results. For this reason common garden pea, *Pisum sativum* var. Kelvedon Wonder, was chosen for the plant species. Peas are key agricultural crops with quick germination which are susceptible to root rot diseases including *Pythium ultimum* and which are responsive to JA treatments (Wang et al. 2003, Babadoost and Malvick 2002, Farr and Rossman 2024).

1.2 Damping-Off Pathogens

The Food and Agriculture Organization (FAO) estimated in 2019 that every year plant disease costs the global economy $220 billion USD (Sarkozi 2019). This is in addition to the ramifications for food security and land use worldwide. As the population grows and climatic conditions change, novel approaches to sustainable agriculture become increasingly important. Climate change is rapidly accelerating disease incidence in both wild and agricultural pests. Global demands for food continually grow as populations increase (Burdon and Zhan 2020, Willis 2017, Antonelli et al. 2020), making plant diseases one of the biggest threats facing global food security (Savary et al. 2019, Strange and Scott 2005, FAO 2021, Mbow et al. 2019).

Plants get infected at all stages of their life cycle, but particularly crucial to food production systems are seedling and other pre-harvest diseases. These prevent or significantly reduce yield in crops and thus have a dramatic effect upon food systems (FAO 2021, Mbow et al. 2019, Gaur et al. 2020).

Plant pathogens include viruses, bacteria, fungi and fungi-like organisms, such as oomycetes. They can be broadly divided into two categories (i) those that obtain nutrients from living cells i.e. biotrophs, and (ii) those that kill plant cells and then obtain the majority of their nutrients from dead cells i.e. necrotrophs (Geeta and Mishra 2018, García-Guzmán and Heil 2014). Figure 1.1 shows diagrams

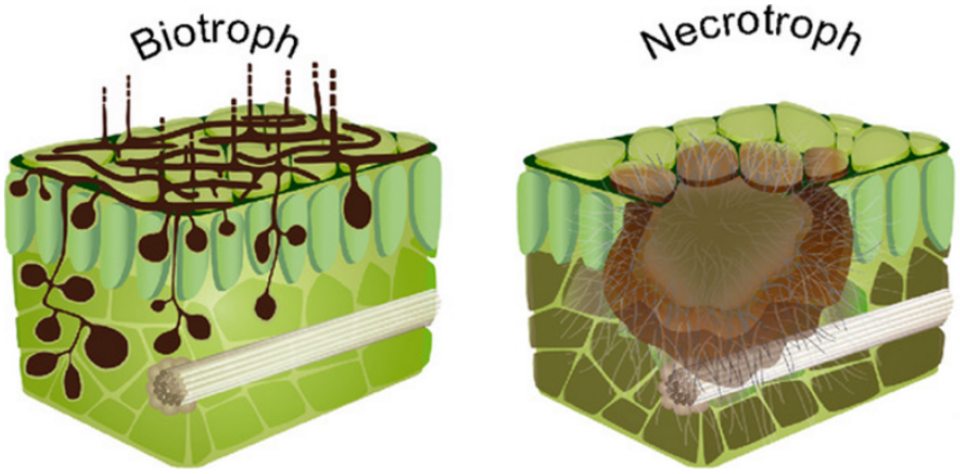


Figure 1.1: Biotrophic vs necrotrophic plant infection. Left: Diagram showing how biotrophic pathogens live within plant hosts: pathogen grows within and between cells and plant structures, siphoning nutrients from living cells and generally not causing active tissue necrosis. Right: Diagram showing how necrotrophic pathogens live within plant tissues: pathogen actively causes tissue necrosis in order to access nutrients and infect further tissue with mycelial structures. Adapted from García-Guzmán & Heil 2014 (García-Guzmán and Heil 2014)

of biotrophic and necrotrophic pathogen lifestyles within plants. However, a third category also exists – hemibiotrophs like *Magnaporthe oryzae*, *Pseudomonas syringae* and P*hytophthora infestans* which have distinct biotrophic and necrotrophic life phases (Lee and Rose 2010).

Furthermore, the line between biotrophic and necrotrophic lifestyles is not clear-cut. Traditionally, it has been thought that necrotrophic pathogens primarily employ 'break and enter' techniques like secreting toxins and cell wall degrading enzymes to gain access to plant tissues but that they do not otherwise try to modify the host environment or establish hyphal networks prior to killing the host cells (Rajarammohan 2021). However, there is increasing evidence that many pathogens, including those traditionally considered strict necrotrophs, will have both a biotrophic and necrotrophic infection phase (Lorang 2019). This 'silent' or 'cryptic' biotrophic phase is seen in the classic necrotrophic pathogens *Botrytis cinerea* and *Sclerotinia sclerotiorum* which have been shown to both dynamically modify the host cellular environment – outside of toxin and cell wall degrading enzyme secretion – and to have a biotrophic hyphal growth phase prior to killing

tissues for nutrients (Williams et al. 2011, Kabbage et al. 2015, Liang and Rollins 2018, Lorang 2019, Rajarammohan 2021).

Of particular interest in the case of seedling diseases, and thus pre-harvest losses, are the necrotrophic pathogens. Seeds and seedlings are small, nutrient-dense, and have under-developed defense systems, including relatively soft stem and root structures, meaning pathogens often do not need a long (or any) biotrophic phase in order to feed upon them (Mancini and Romanazzi 2014, Gaur et al. 2020). These include oomycetes and fungi which cause damping off diseases; a variety of diseases which result in seedling decay and pre-harvest plant death (Mancini and Romanazzi 2014, Lamichhane et al. 2017, Gaur et al. 2020).

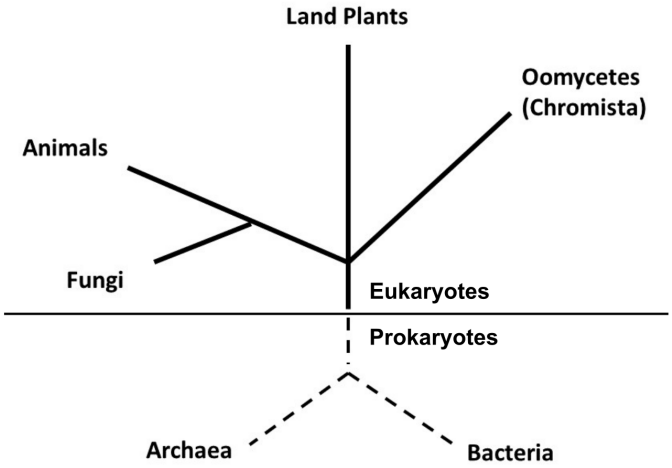


Figure 1.2: Schematic showing relationships between archaea bacteria, oomycetes, fungi, land plants, and animals. Adapted from Fry & Grünwald 2010 (Fry and Grünwald 2010)

Damping off diseases affect a wide variety of plant species. The only requirement is a humid seed environment, thus affecting both seeds started in greenhouses and those started in the field. A major cause of damping off diseases are oomycetes (Agrios 2005, Heffer et al. 2002, Fry and Grünwald 2010). Oomycetes are members of the proposed and polyphyletic Chromista kingdom, a diverse kingdom which, among others, contains oomycetes, brown algae, and diatoms, and which is evolutionarily distinct from fungi and other plant pathogens. Figure 1.2 shows a simplied phylogeny of the relationship between archaea, bacteria, oomycetes, fungi, land plants, and

animals. Oomycetes can reproduce both sexually and asexually, producing long-lasting oospores under stress conditions which can spread via water or soil and rapidly germinate when conditions suit as well as zoospores which germinate as cysts and can directly infect plant tissues(Martin and Loper 1999, Lévesque et al. 2010). One of the most common and virulent oomycetes is known as *Globisporangium ultimum* or *Pythium ultimum*.

Pythium ultimum was reclassified into *Globisporangium* in 2010 by Uzuhashi, Tojo, and Kakishima based upon phylogeny and morphology (Uzuhashi et al. 2010). However, *Globisporangium ultimum* is still commonly referred to as *Pythium ultimum* despite its renaming. The infection and disease caused by *Pythium ultimum* and *Globisporangium ultimum* are the same and *Pythium ultimum* as a name is in more common use than *Globisporangium ultimum* (Eggertson et al. 2023, Liu et al. 2023). Therefore, this author has chosen to proceed with the term *Pythium ultimum*.

Pythium/ (*Globisporangium*) species are common in most soil and water systems worldwide and can be difficult to treat or eradicate because of the number and types of spore they produce and the spores' durability in both water and soil substrates (Heffer et al. 2002, Fry and Grünwald 2010, Martin and Loper 1999). Figure 1.3 shows a simplified life cycle and infection cycle of oomycetes like *Pythium ultimum*.

1.2.1 *Pythium ultimum*

1.2.1.1 *Pythium ultimum* biology, host range, and disease

Pythium ultimum is a hardy pathogen and can survive a wide range of temperature and and moisture conditions. Perhaps as a result, *Pythium ultimum* has an incredibly broad host range, infecting well over 300 plant species and has been shown to have a primarily necrotrophic lifestyle ((Farr and Rossman 2024), Martin and Loper 1999). In fact, in contrast to many other necrotrophic pathogens, *Pythium ultimum* does not appear to have a cryptic or silent biotrophic phase (Lévesque et al. 2010). This loosely correlates with other more-necrotrophic-than-otherwise-pathogens – wide host ranges generally rely on brute-force techniques for gaining entry to plant tissues and thus nutrients while narrow host ranges often require more speciality

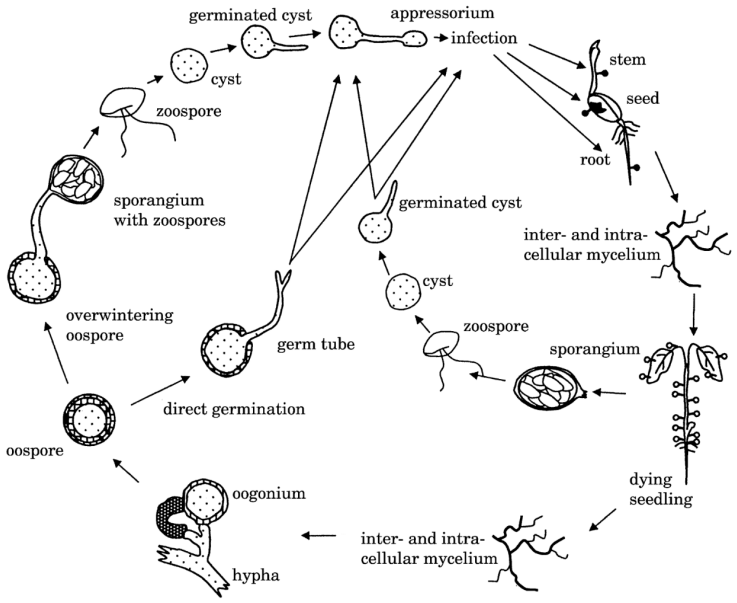


Figure 1.3: Schematic showing a simplified life cycle and host infection by oomycetes like *Pythium*. Oomycetes infect plant tiussue via exisitng openings like stomata and wounds and infilitrate the tissue using appressoria. *Pythium* can infect any part of the seedling, including seed, shoot, and root but primarily infects via root tissues. Once inside the plant, mycelia are formed both inter and intra cellularly, and cause tissue necrosis. Sporangia are produced along the mycelia and produce both sporangia, the structures which produce zoospores, motile asexual spores, and oogonia, the structures which produce oospores, thick-walled sexual spores. While zoospores form cysts which are stable for several days and once germinated can produce appressoria to infect new plant tissue. Oospores can germinate rapidly, forming a germ tube and infecting plant tissue right away or can stay dormant for years, surviving extreme temperatures and dehydration until either germinating themselves or producing sporangia and from there zoospores. Adapted from van West et al 2003 (van West et al. 2003)

and co-evolution (Mapuranga et al. 2022, Lévesque et al. 2010). While it is not clear if there are any true, fully necrotrophic pathogens which completely lack any biotrophic phase, *Pythium ultimum* appears to be the next closest thing.

Pythium ultimum produces both asexual and sexual spores. Its asexual spores, zoospores, are motile and can survive in water or moist soil for several days before forming cysts and germinating (Hendrix and Campbell 1973, Agrios 2005, van West et al. 2003). Meanwhile, the sexual spores, oospores, have a thick double wall and can survive for years in soil and water, withstanding extreme temperatures and aridity (Hendrix and Campbell 1973, Agrios 2005, van West et al. 2003). Oopsores

can then germinate either directly, producing a germ tube capable of infecting plant tissue via appressoria, the infection structures of oomycetes and fungi, or prodcuing a sporangia which in term creates zoospores, cysts, and appressoria. Figure 1.3 shows these alternate paths to infection.

The *Pythium* genus is diverse and contains over 300 plant pathogenic species, many of which have wide host ranges (Ho 2018, LéVesque and De Cock 2004). *Pythium* spp cause serious damage to crops worldwide. *Pythium ultimum* alone causes damping off and yield loss in economically important crops like rice, wheat, maize, sorghum, millet, cotton, soybean and sugarbeet as well as turfgrasses, beans, peas, chickpeas, squash, watermelon, peanuts, tomato, carrot, chilies, onions, celery, cauliflower, capsicum, coffee arabica, sweet potato and many others (Palmucci et al. 2011, Kraft et al. 1988, Abad 1994, Abreo et al. 2017, Martin and Loper 1999, Agarwal et al. 2021, Hyder et al. 2021, Alcala et al. 2016). The disease is very noticeable in seed, seedling, and transplant stages, particularly in nurseries, greenhouses and row cropping where seed and seedling density tends to be high (Hendrix and Campbell 1973). Symptoms develop quickly and kill large numbers of seedlings in both pre- and post-emergence stages. It becomes more difficult for *Pythium* to infect plants as they age and increase the thickness of their stem and primary root structures but *Pythium* can still infect mature plants through root hairs causing wilting, loss of vigour and reduced yields (Hendrix and Campbell 1973).

The losses caused by *Pythium* disease are therefore numerous and difficult to quantify, as they include losses of seeds and thus re-sowing costs, losses of mature plants, and the costs associated with sterilizing and removing *Pythium* from nursery and field soils. Lamichhane et al. estimate anywhere from 2-80% of crop losses due to *Pythium* infection, depending on agricultural setting with monoculture row cropping and nurseries as well as moist climates having the highest losses (Li et al 2022). An estimate for the United States and Ontario puts total crop losses due to *Pythium* in maize from 2016-2020 at over US$357 million and another for soybean across 28 US states estimates over US$779 million in losses in 2008 alone (Bickel and Koehler 2021, Navi et al. 2019). Because *Pythium* forms hardy

oospores and is transmitted primarily through water and drainage within soil it can be extremely difficult to remove *Pythium* from infected fields and nurseries meaning costs associated with *Pythium* diseases can compound from year to year. Additionally, *Pythium* transfers readily from plant to plant and oospores can remain dormant and attached to seeds for years, making *Pythium* difficult to eradicate from a system once it has entered (Golińska and Świecimska 2020).

1.2.1.2 *Pythium ultimum* control methods

Pythium control leans more towards integrated management strategies than complete eradication. Management strategies are varied but often rely on application of chemical fungicides, particularly metalaxyl, propamocarb, and fosetyl-Al. *Pythium* species vary in their natural resistance to fungicides and increasing use of fungicides like metalaxyl and propamocarb has lead to development of resistance in *Pythium* spp. (Golińska and Świecimska 2020, Alcala et al. 2016, Moorman and Kim 2004, Titone et al. 2009, Lookabaugh et al. 2018). Other management strategies include sanitation methods in nurseries and greenhouses, like sterilizing seeds, soils, irrigation equipment and tools. Plant breeding or genetic engineering for *Pythium* resistance is an important part of managing crop production under *Pythium* conditions (Golińska and Świecimska 2020, Lamichhane et al. 2017). An increasing area of interest in *Pythium* management is the use of biocontrol agents, like *Actinomycetes* spp., *Trichoderma* spp., and plant growth promoting rhizobacteria (El-Tarabily et al. 2009, Hyder et al. 2021, Tucci et al. 2011).

The primary method of preventing *Pythium* infection, damping off diseases, and other necrotrophic pathogen infections is by the application of pesticides. This can be either directly to the seeds prior to sowing, on the seedlings, or to soil or water systems after germination. Like all pesticides, this route of treatment poses its own problems; the risk to human health and the environment caused by accumulating concentrations of pesticide exposure, the emergence of pesticide-resistant pathogens, and off-target effects on insects and soil microbiota (Lamichhane et al. 2017, Nicolopoulou-Stamati et al. 2016, Sharma et al. 2019, Margni et al.

2002). However, fungicide treatments often fail to fully control *Pythium* diseases for several reasons. (i) *Pythium* symptoms appear early in a plant's life cycle, often at the seed stage, when signs of infection are harder to detect, (ii) *Pythium* spores are thick-walled and durable and difficult for pesticides to penetrate, (iii), *Pythium* spps host ranges are generally very broad and so *Pythium* can survive using surrounding plant species as a sink, (iv), spores can survive years in soil and water, meaning a farmer could spray and control *Pythium* one year only to have it return the next year or even years later (Agrios 2005, Hendrix and Campbell 1973, Martin and Loper 1999, Tsror 2023, Wu et al. 2020). As a result, research in this field is shifting to focus on other types of disease prevention and management (Gupta and Kumar 2020, Lamichhane et al. 2017, Salman and Abuamsha 2012).

Table 1.1: Table showing damping-off control methods in the literature. Fungicides were split into two categories, one category of fungicides which work against oomycete pathogens and one category of fungicides which work against fungal pathogens.

Method	**Pathogen**	**Effectiveness**	**Costs**	**Type**	**Source**
Fungicides: mefenoxam, metalaxyl, propamocarb, cyazofamid, fluopicolide, phosphites (potassium phosphite), fenamidone, mono- and di-potassium salts of phosphorous acid	Oomycetes: *Pythium* spp., *Phytophthora* spp.	Low to moderate	Using too often can cause pathogen resistance; toxic to aquatic organisms; acute toxicity to humans, birds, mammals, and insects when first applied	Soil drench, foliar spray, seed treatment	Wyenandt 2020, Koike and Tjosvold 2020, Wyenandt and van Vuuren 2022, Krieger 2010

Fungicides: Azoxystrobin, mono- and di-potassium salts of phosphorous acid, thiophanate methyl, iprodione, PCNB, fludioxonil	Fungi: *Rhizoctonia solani*, *Fusarium* spp.	Low to moderate	Using too often can cause pathogen resistance; toxic to aquatic organisms; acute toxicity to humans, birds, mammals, and insects when first applied	Soil drench, foliar spray, seed treatment	Wyenandt 2020, Koike and Tjosvold 2020, Wyenandt and van Vuuren 2022, Krieger 2010;
Sanitation: sanitation of trays, tables, and greenhouses prior to planting or starting seeds; good soil drainage; controlled irrigation; proper depth and spacing of seeds/seedlings; soil solarization	All	Moderate to high	Time consuming; resource consuming; only effective as long as it is maintained	Other	Wyenandt 2020, Koike and Tjosvold 2020, Wyenandt and van Vuuren 2022, Krieger 2010, Lamichhane et al. 2017

Biological control: *Bacillus subtillus* (Companion), *Streptomyces lydicus* (Actinovate), *Streptomyces griseoviridis* (Mycostop), *Trichoderma harzianum* (PlantShield, Rootshield), and *Trichoderma virens* (SoilGard)	Varies	Low to high, depending on pathogen, level of disease, growing conditions	More expensive than some fungicides; effectiveness varies more than fungicides	Soil drench, foliar spray, seed treatment	Wyenandt 2020, Koike and Tjosvold 2020, Wyenandt and van Vuuren 2022, Kevan and Shipp 2017
Disinfectants: hydrogen peroxide, peracetic acid, hydrogen dioxide	All	High	Kills all microorganisms, including normal root and soil microbiota	Soil drench, foliar spray, preplant dip, seed treatment	Wyenandt 2020, Wyenandt and van Vuuren 2022, Lamichhane et al. 2017

Soil fumigants: Hydrocyanide, acid gas, carbon disulphide, chloropicrin, ethylene dichloride, methyl bromide, paradichlorobenzene, methyl iodide, chloropicrin, methyl isothiocyanate, 1,3-dichloropropene, dimethyl disulfide, sulfuryl fluoride, dazomet, ethyl formate, ethanedinitrile, furfural or 2-propenal	All	High	High toxicity to all organisms; only practical for pre-planting soil sterilization; sale and use highly controlled	Soil fumigation	M. Wang et al. 2017, Wyenandt and van Vuuren 2022, M. Wang et al. 2017, Krieger 2010
Botanical fungicides: Milsana, jojoba oil, plant essential oils, cinnamaldehyde, plant phenolic compounds, plant alkaloid compounds	Varies	Low to High depending on compound and pathogen	Often expensive compared to fungicides; effectiveness depends heavily on compound and pathogen; degrade quickly	Seed treatment, soil drench, foliar treatment	El-Baky and Amara 2021, S. Sun et al. 2022, Pane et al. 2020

Metal nanoparticles (NPs): silver NPs, zinc oxide NPs, gold NPs, copper NPs	Varies	Low to High depending on compound and pathogen	Potential for accumulation in soil and plant biomass; off-target effects	Seed treatment, soil drench, foliar treatment	El-Baky and Amara 2021, Vanti et al. 2020
Inorganic salts: ammonium phosphate dibasic, potassium bicarbonate	*Sclerotium rolfsii* and *Pythium* sp	Moderate to high	Potential for accumulation in soil; off-target effects	Seed treatment,	Mansour et al. 2020
Clay minerals: Kaolin clay, bentonite	Varies	Low to high depending on pathogen	Needs to be re-applied; not effective against all pathogens; not suitable for all applications	Soil cover, foliar treatment	Mathre et al. 1999, Fabiyi 2023, EPA 1999

1.3 *Pisum sativum*

Legumes are considered a vital crop for achieving global food security under changing climatic conditions (Meena and Lal 2018, Foyer et al. 2016, Rawal and Navarro 2019). Peas (*Pisum sativum*) are high nutrient, cool-season legumes grown in temperate climates worldwide (Savage and Deo 1989). They are classified by end use into field or dry peas and green or garden peas. Field peas are grown for dry harvest as food or forage crop and green peas are grown for their immature seeds and pods as vegetables. According to the FAO 136 countries produce either field or green peas and annual global production exceeded 48 million tons in 2022.

Peas have a high protein (20-25%) and a low fat (<1%) content and are gaining

increased interest as a sustainable source of plant-based protein in the United States (Thavarajah et al. 2023) but are already grown at scale for human consumption and animal feed in the Russian Federation, China and India (Zhang et al. 2016). Additionally, peas, like other legumes, can establish nitrogen-fixing symbiosis with *Rhizobium* bacteria which helps maintain soil fertility and can decrease costs for growers (Faligowska et al. 2022, Knight 2012). Peas grow quickly and are a robust system for both commercial growth and lab stud

Peas are susceptible to a range of diseases, including powdery mildews, rusts and damping-off diseases like those caused by *Pythium* spp. (Khulbe and Sharma 2020, Singh et al. 2023, Hagedorn 2012). Peas also upregulate defense responses when exposed to jasmonates (Manzoor et al. 2012, Basu et al. 2021, Bera et al. 2020, Abbas et al. 2022) and have increased tolerance to drought and salinity when treated with silicates (Ismail et al. 2022, Orzoł et al. 2023, Sutulienė et al. 2022).

Pythium spp. have caused considerable losses in germination and crop yields in peas across North, Central and South America, Europe and Australia since the 1960s (Van Der Plaats-Niterink 1968, Kerr 1964, Harper 1964, Frezzi 1956, Escobar et al. 1967, Ewing 1959). Despite introduction of resistant varieties and and use of fungicide treatments *Pythium* disease continued to cause major crop losses through the 1990s and 2000s (Stasz et al. 1980, Hwang and Chang 1989, Hwang et al. 2000). Integrated management strategies including fungicide treatment, sanitation and biocontrol are common now and help control *Pythium* outbreaks but disease due to *Pythium* is still a major concern for private and commercial pea growers today (Hamid et al. 2012, Alcala et al. 2016, Kall et al. 2020, Gupta 2020, Gossen et al. 2024).

To the best knowledge of this author, peas have not previously been tested in a *Pythium ultimum* system with jasmonates and silicates. Taken together, these qualities make peas a suitable choice of study species for a testing a plant-defense promoting seed treatment.

1.4 Seed Treatments

Seed treatments are commonly used in agriculture to protect against crop diseases and as seed enhancers to improve crop yield. Seed treatments include disinfectants, pesticides, plant growth-promoting compounds, and fertilizer or micronutrients (Shakeel et al. 2019, Neergaard 1977, Rocha et al. 2019).

Crop enhancing seed treatments include chemicals or fertilizers to improve germination, treatments with beneficial bacteria to promote plant growth, and micronutrient dosing (Koch and Roberts 2014, Taylor et al. 1998, Kaufman 1991). As fungicide and pesticide resistance become more of a problem, researchers and growers are increasingly looking for alternative seed treatments. These include application of steam or drying, addition of microbiota such as rhizobacteria or other plant growth promoting bacteria, and the use of natural biocides derived from plant materials such as chitosan or essential oils (Koch and Roberts 2014, Bravo Cadena et al. 2018a, Bravo Cadena et al. 2018b, Bravo-Cadena 2018).

Both biocidal and crop enhancing seed treatments divide into roughly two categories: (i) those that are sprayed or soaked directly onto the seeds before planting, and (ii) those that are worked into seed coatings.

Seed coatings can both preserve the seed and the treatment until planting and also be carriers for additional nutrients or supplementation (Kaufman 1991, Neergaard 1977, Rocha et al. 2019, Bravo-Cadena 2018, Liu et al. 2006). Supplementation is the addition of nutrient amendments to soil or other growing substrate as a way to counteract existing nutrient deficiencies or to enhance growth. Supplementation is often used in conjunction with other properties, such as the addition of biocides, within a seed treatment.

1.5 Plant Immune Systems

Like all organisms affected by pests and diseases, plants need to be able to defend themselves in order to finsih their life cycles and produce seed for the next generation. Plants are sessile organisms and unable to move away from abiotic and biotic threats.

Plant immune systems have thus evolved to serve multiple functions in plants, with many processes coordinating defenses against both abiotic and biotic stressors under a complex system of interactions and cross-talks. The first line of defense a plant has is its outer tissues and stem structures. Plants can build a variety of physical defense structures creating thick, woody stems, tough bark, spines, thorns or fine hairs, trichomes, incorporating mineral deposits from the soil such a silica, creating waxy cuticles and leaf surfaces (Bigeard et al. 2015, Heil and Bostock 2002). Plants can also produce secondary metabolites to repel, poison, or irritate herbivores and pathogens (Bigeard et al. 2015, Heil and Bostock 2002, War et al. 2011). Some of these secondary metabolites are constitutively expressed, forming an additional baseline level of defense (Bigeard et al. 2015, War et al. 2011). Past the structural obstacles, plant immune systems are a combination of basal immunity in the form of pathogen-associated molecular pattern or PAMP-triggered immunity, known as PTI, and a more specific and stronger immune reaction called effector-triggered immunity or ETI (Doughari 2015, Jones and Dangl 2006). PTI occurs when the plant's transmembrane pattern recognition receptors (PRRs) recognize foreign proteins originating from bacteria, fungi, or oomycetes, called pathogen associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) (Jones and Dangl 2006). Once a pathogen or damage from a herbivore has been detected plants can induce a variety of defense responses. Stomatal closure, restriction of nutrient transfer, production of antimicrobial and anti-herbivore compounds like phytoalexins, phenolic compounds, flavonoids and tannins, production of defense response proteins and peptides, and generation of reactive oxygen species (ROS) are all potential consequences of PTI induction (Bigeard et al. 2015).

This basal immune response confers broad resistance against viruses, bacteria, fungal pathogens and herbivory meaning plants are generally immune to most pathogens (Bigeard et al. 2015). This is called non-host resistance. However, some pathogens have evolved to secrete effector molecules to overcome the plant's PTI defenses. Effector molecules either disable or confuse a plant's PTI responses, allowing the pathogen to become established in plant tissues before the plant can

mount an effective immune response (Jones and Dangl 2006). The plant can then evolve a defense response specific to the effectors, termed effector-triggered immunity or ETI. Figure 1.4 shows this canonical 'zig-zag' model of plant immunity.

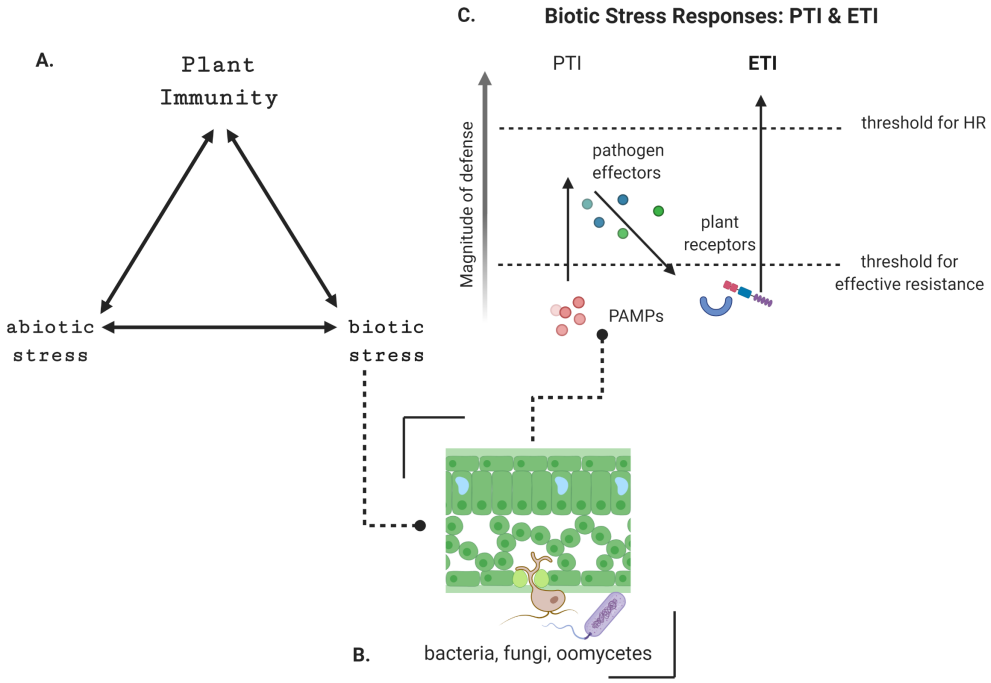


Figure 1.4: Overview of plant immunity. **(A)** shows the triangle of plant immunity where plant immune systems have to adapt to both abiotic and biotic factors and their interactions. **(B)** Infection of plant tissues by pathogens or damage by herbivores causes the release of pathogen associated molecular patterns (PAMPs) and/or damage associated molecular patterns (DAMPs). **(C)** A simplified 'zig-zag' model of plant immunity. PAMPs are detected by the plant causing an initial defense response, PAMP-triggered immunity or PTI. Some pathogens have evolved effector proteins to disable the plant PTI response and, in turn, plants have evolved effector-triggered immunity or ETI to reactive defense responses against these pathogens. Figure adapted from Jones and Dangl 2006 (Jones and Dangl 2006).

Downstream of both PTI and ETI the direction of plant immunity, and whether it is responding to mainly biotrophic or mainly necrotrophic pathogens, is controlled in part by hormone signalling networks (Pieterse et al. 2012, Denancé et al. 2013). Jasmonic acid (JA) and salicylic acid (SA) and their derivatives are the major plant defense hormones, broadly controlling responses to nectrophic and biotrophic pathogens respectively, though other plant signalling hormones like ethylene, gibberellins, auxins, cytokinins, brassinosteroids and nitric oxide also

interact with plant immune responses as well (Pieterse et al. 2012, Denancé et al. 2013). The cross over in pathogen lifestyles means there must be an interdependence and crosstalk also in SA and JA signalling. SA and JA are canonically treated as mutually exclusive and mutually inhibitory but more evidence is accumulating which suggests plant defense responses are mediated by a host of complex factors which utilize aspects of both signalling pathways (Li et a. 2019, Yang et al. 2019).

Plants have evolved two major pathways for responding to pathogens, one of which is regulated by the plant hormone salicylic acid and which is largely activated in response to biotrophic pathogens. The second is regulated by the jasmonates, plant hormones which are activated in response to necrotrophic pathogens. Induced resistance is thus often specific to either the jasmonic acid pathway or the salicylic acid pathway, though various elictors can activate these pathways, not just exogenous SA or JA (Mauch-Mani et al. 2017, Luyckx et al. 2017, Agrawal et al. 1999, Conrath et al. 2006).

1.6 Induced Resistance and Plant Defense Priming

Another interesting area for seed treatments is induced resistance. In induced resistance, seeds or seedlings are exposed to plant hormones, other signal molecules involved in plant defense responses, mechanical damage, pathogens or DAMPs/PAMPs, or abiotic stressors which induce immune response upregulation. After exposure to a resistance-inducing agent the plant upregulates its defense responses and, if exposed when it is still upregulating in response to the stimulus, often shows reduced disease symptoms and stronger immune responses when it next faces a pathogen, insect, or abiotic stressor (Van Bockhaven et al. 2013, Charng et al. 2023, Savvides et al. 2016, Liu et al. 2022, Agrawal et al. 1999). Figure 1.6 shows an overview of induced resistance in plants. Induced resistance can arise from exposure to both natural or synthetic compounds like plant hormones, plant defense response elicitors like flg 22, chitosan, or alginate, wounding from herbivores, a low-dose exposure to a pathogen, or introduction of microorganisms. These can be applied to foliar tissues,

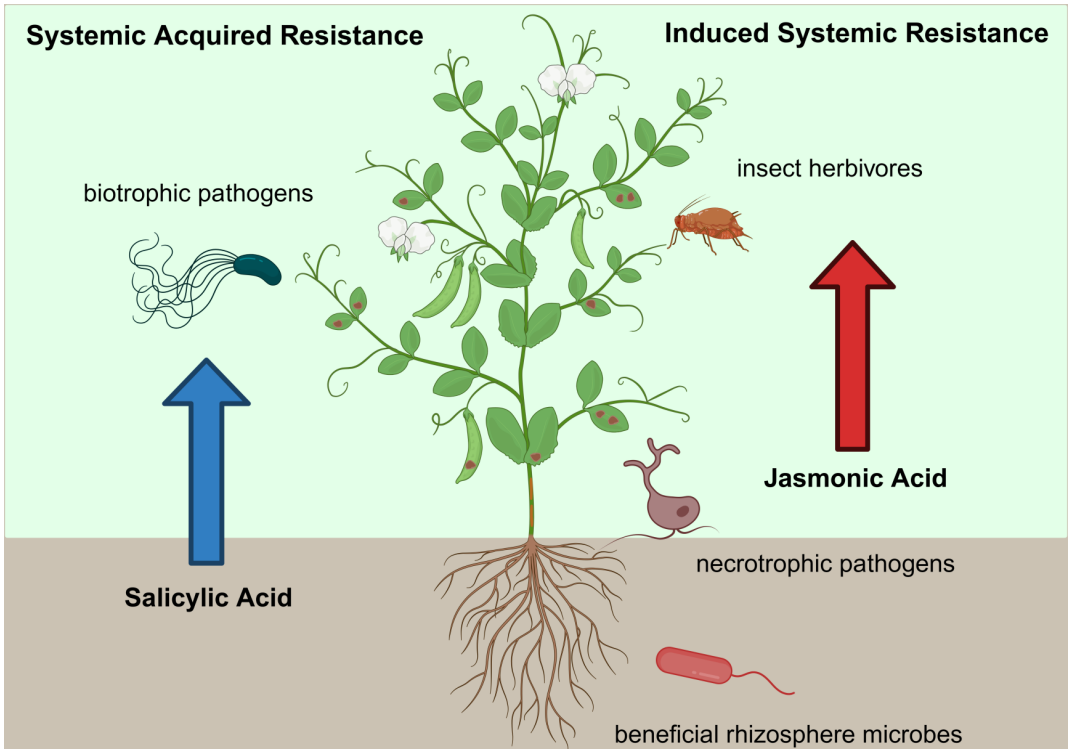


Figure 1.5: A diagram showing a very simplified view of resistance induced by SA and JA. Plants typically activate the salicylic acid pathway in response to biotrophic pathogens, in what is called systemic acquired resistance. In contrast, plants typically activate the jasmonic acid (JA) pathway in response to necrotrophic pathogens and herbivores, in what is called induced systemic resistance. Induced systemic resistance and the JA pathway can also be activated by beneficial rhizosphere microbes. Cartoon adapted from Chen et al 2014 (Chen et al. 2014)

root tissues, used as soil drenches or worked into seed treatments or the composition of the soil (Conrath 2009, Mauch-Mani et al. 2017, Pozo et al. 2009).

A key component of induced resistance is the direct induction of defense responses like production of antimicrobial compounds and corresponding changes in gene expression (Mauch-Mani et al. 2017). Sometimes after resistance has been induced and the plant's initial response has subsided, the plant can still respond more quickly and more strongly, and sometimes in an altered way, to future infection and can mount a systemic response when perhaps previously it only reacted locally to resistance inducer. When this happens, it is termed priming. It is generally accepted that priming is an inherent part of induced resistance, but testing for priming requires waiting for initial induced resistance responses to subside before exposing the plant to another challenge (Mauch-Mani et al. 2017).

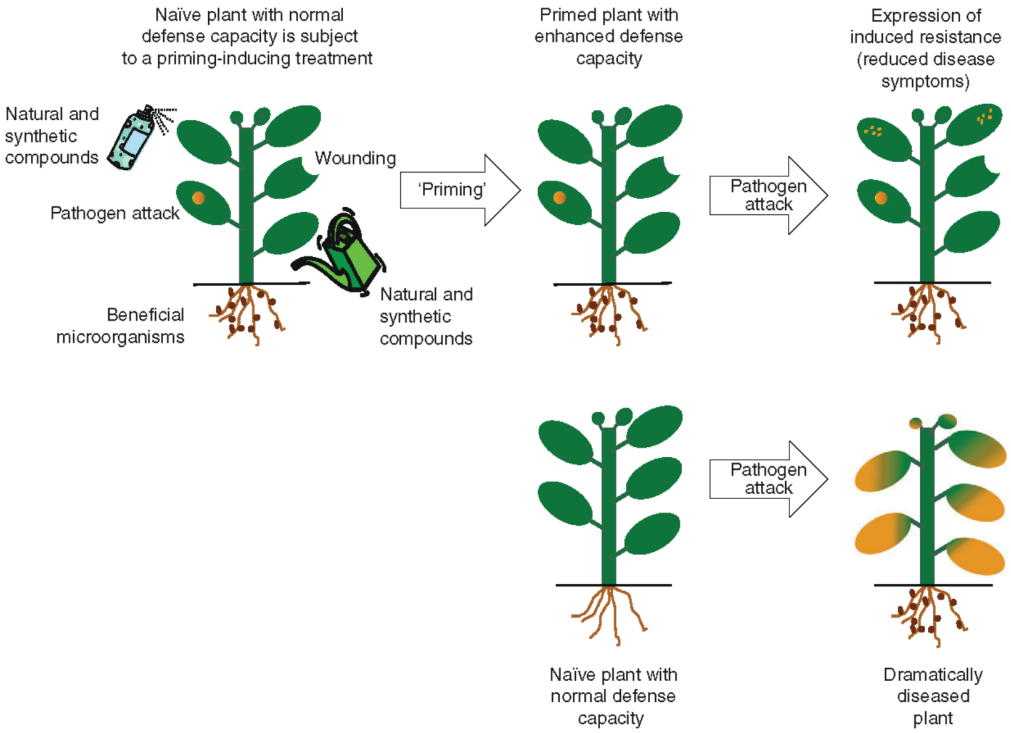


Figure 1.6: Diagram describing induced resistance and priming in plants. Treating a naive plant with a natural or synthetic compound, control wounding or pathogen attack, or beneficial microorganisms can induce upregulation of plant defense responses, termed induced resistance. If time has elapsed and this initial response has ebbed, upon later exposure to pathogens, herbivores or abiotic stressors a primed plant will react more quickly and more strongly to combat that stressor, and will often much reduced symptoms, than if it had not been primed. Adapted from Conrath 2009 (Conrath 2009)

Like induced resistance, priming is effective against both pathogens and insect herbivores and abiotic stressors. Newman et al found that exposing pepper plant leaves to lipopolysaccharides induced enzymatic changes in the plant and then later, after the induction phase, when challenged with *Xanthomonas campestris* pv. *vesicatoria* the plants were able to response with increased transcription of PR proteins and increased synthesis of antibacterial compounds (Newman et al. 2002). Exposing grape plants to the plant growth-promoting rhizobacteria *Burkholderia phytofirmans* PsJN resulted in no major metabolic or transcriptomic induced changes, but post-induction phase allowed the plants to have greater resistance to cold, increasing expression of stress response genes as well as increasing proline, malondialdehyde, aldehyde, and reaction oxygen species (ROS) accumulation

(Theocharis et al. 2012). Allowing oviposition by *Spodoptera exigua* on *Nicotiana attenuata* leaves resulted in no changes in defense metabolite production during the induction phase but later exposure to *Spodoptera exigua* larvae or wounding and excretions from feeding resulted in increased defense metabolite production of caffeoyl putrescine and trypsine protease inhibitors as well as enhanced transcription of NaMyb8, which regulates production of several anti-herbivore defense compounds (Bandoly et al. 2015). Application of MeJA at low levels, 10 μmol/L, induced H_2O_2 generation and enhanced expression of a defense related gene VvNPR1.1 in grapes and after the induction phase resulted in a priming response increasing grape plant survival against *Botrytis cinerea* (Wang et al. 2015).

There is even evidence that priming can be transgenerational. Experiments in Arabidopsis priming with β-amino-butyric acid (BABA) showed that both S1 and S2 progeny of these plants inherented a 'primed' state, showing a faster and higher accumulation of defense transcripts when exposed to *Pseudomonas syringa*e as well as resistance to the oomycete pathogen *Hyaloperonospora arabidopsidis* (Slaughter et al. 2012). Interestingly, they also found that these plants inherent a 'primed to be primed' phenotype, where pre-primed descendants respond with an even stronger primed phenotype upon priming exposure in their own generation (Slaughter et al. 2012). Rasmann et al observed a similar response in the descendants of Arabidopsis and tomato plants which had been primed with either caterpillar herbivory, mechanical damage, or MeJA application (Rasmann et al. 2012). López Sánchez et al found that this transgenerational priming was associated with altered DNA methylation patterns in Arabidopsis (López Sánchez et al. 2016).

1.7 Costs of Induced Resistance and Priming

However, in any biological system there is limited resources and thus often trade-offs associated with activating defense responses versus pursuing growth and reproduction (Heil and Baldwin 2002, Huot et al. 2014). This appears to also hold true in cases of transgenerational immune priming; in further study by López Sánchez et al, primed plants and their descendants incurred ecological fitness

costs where plants primed for JA pathway activation were more susceptible to SA-dependent stressors (López Sánchez et al. 2021).

Similarly, priming can also come at a cost. Plants primed with BABA have been shown to have both a lower growth rate and lower total seed production than unprimed plants, however, the costs associated with priming were lower than the costs associated with induced resistance using higher concentrations of BABA (van Hulten et al. 2006). This indicates that priming may (i) require smaller 'inducing' doses of compounds and (ii) come at a lower fitness cost than induced resistance (van Hulten et al. 2006).

Induced resistance classically can come with costs to growth and development. JA-induced resistance in tomato plants has been shown protect plants against *Manduca sexta* but also reduces fruit-set and number of seeds per unit of fruit weight as compared to control plants (Redman et al. 2001). Other costs of induced resistance include susceptibility to other stressors. For example in broccoli and Arabidopsis plants primed with SA and chitosan plants showed a higher resistance to drought and the hemibiotrophic pathogen *Pseudomonas syringae* DC 3000 but increased susceptibility to the chewing insect *Ascia monuste* and the necrotrophic fungus *Alternaria alternata* (Venegas-Molina et al. 2020).

1.8 Potential Applications of Induced Resistance and Priming

Incorporating compounds which can induce disease resistance and or prime plants into seed treatments could offer significant improvements to disease management systems in agriculture. Even with some costs to growth and development, induced resistance and priming could massively improve overall yields by preventing early losses (Rhaman et al. 2020, Tiwari et al. 2022, Reglinski et al. 2023). This is particularly relevant in the context of emerging diseases and changing climactic and thus ecological and growth conditions. Changing climatic conditions shift the geographic ranges of pests and pathogens, exposing plants that have no evolved resistance to these new threats (Anderson et al. 2004, Bebber et al. 2013). Increased

globalization means plants, plant products, and carriers of plant pests and pathogens are shipped around the world, even more quickly than changing climatic conditions can shift geographic ranges (Bebber et al. 2015, Brasier 2008). Changing climatic conditions also correlate with local increases in extreme and unpredictable weather like increasing maximum or minimum temperatures and changing precipitation patterns which can cause significant stress to plants or crops unaccustomed to these new and unpredictable conditions (Velásquez et al. 2018, IPCC 2022). Stressed plants are less resistant to pests and pathogens (Cohen and Leach 2020, Velásquez et al. 2018). Taken together, these effects of climate change on crops create enormous vulnerabilities in agriculture (Ristaino et al. 2021, Singh et al. 2023).

Inducing resistance or priming a plant could make a big difference in plant and agriculture resiliency in the face of a changing climate. A primed plant or one with induced resistance may both be more flexible in terms of which pathogens it can respond to as well as have enhanced abiotic stress tolerance (Fones et al. 2020, Gupta and Kumar 2020, Conrath et al. 2006). In this way, defense priming can act as a sort of vaccination for plants, allowing increased disease resistance that bypasses the potential for fungicide or biocide resistant pathogens (Luna-Diez 2016).

Unlike vaccines, however, immune priming and induced resistance in plants can be broad spectrum (Conrath et al. 2006). A broad spectrum of resistance could allow plants to be more adaptable to changing and unpredictable agricultural disease futures. For example, induction of induced systemic resistance by activators of the jasmonic acid pathway can provide resistance to a variety of fungal and arthropod infection – Worral et al. found treating tomato plants with jasmonic acid can induce resistance to herbivory by spider mites, caterpillars and aphids as well as against the fungal pathogen *Botrytis cinerea* (Worrall et al. 2012). However, most studies have looked at priming for individual plant-pathosystems, so it has to be inferred that treatment with exogenous inducers of resistance, like JA and SA, can induce broad-spectrum resistance. For instance, JA can induce resistance to bacterial blight (*Xanthomonas oryzae* pv. *oryzae*) in rice; fall armyworm in soybeans; cotton bollworm (*Helicoverpa armigera*) in groundnut; cotton aphids, two-spotted spider

mites and western flower thrips in cotton plants; and thrips and wheat blossom midges in wheat (Yamada et al. 2012, Lanka et al. 2021, War et al. 2011, Omer et al. 2001, El-Wakeil et al. 2010). SA in turn can induce resistance to *Fusarium oxysporum*, *Alternaria solani*, and root knot nematodes in tomato; *Gaeumannomyces graminis* var. *tritici* and *Fusarium graminearum* in wheat; *Erysiphe pisi* in peas; and *Magnaporthe grisea* in rice (Mandal et al. 2009, Spletzer and Enyedi 1999, Molinari et al. 2014, Sari and Etebarian 2009, Qi et a. 2012, Frfy and Carver 1998, Daw et al. 2008). The list provided here for JA- and SA-induced resistance is not exhaustive, but aims to provide an overview of the variety of plants and pathogens in which exogenous hormone application has induced resistance.

Within this project, jasmonates were chosen as the primary hormone of interest so they will be discussed in additional detail.

1.9 Jasmonates

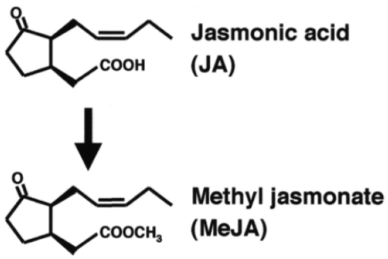


Figure 1.7: Chemical structures of jasmonic acid (JA) and methyl jasmonate (MeJA). Methyl jasmonate is derived from jasmonic acid in planta and is used for inter- and intra-plant communication (Sasaki et al. 2001).

Jasmonates are oxylipins derived from chloroplast membrane lipids. Phospholipase enzymes release α-linolenic acid from chloroplast membranes which is then converted step-wise by lipoxygenase (LOX), allene oxide synthase (AOS) and allene oxide cyclase (AOC) into 12-Oxo-phytodienoic acid (12-OPDA) (C. Wasternack and Hause 2013, Avanci et al. 2010W). 12-OPDA is the primary precursor to jasmonic acid and increasing evidence shows it can activate its own unique subsets of jasmonate-responsive genes (Lie and Park 2021). 12-OPDA then transfers

to the peroxisome and undergoes a series of three β-oxidations leading to the formation of JA. From there, JA can be converted into MeJA and JA-Ile via JA carboxyl methyltransferase (JMT) and jasmonate resistant-1 (JAR1), respectively (Ruan et al. 2019).

JA-Ile is the primary bioactive form of JA and binds to coronatine insensitive 1 (COI1) triggering the degradation of the jasmonate ZIM-domain (JAZ) transcriptional repressors. These, in turn, release a variety of transcription factors like ethylene-responsive factor (ERF), WRKY, MYB, MYC, and NAC proteins which regulate JA-responsive genes (Ruan et al. 2019).

Jasmonates are involved in many aspects of plant development, growth, and defense (Campos et al. 2014, Avanci et al. 2010, Glazebrook 2005, Wang et al. 2020, Thaler et al. 2004). Jasmonates were first discovered as the volatile compounds which give *Jasminum grandiflorum* (jasmine) flowers their scent. They were later found to be an integral signaling hormone in plants and signals in the major pathway mediating responses to necrotrophic pathogens, herbivores, and wounding (Huang et al. 2017, Heil and Ton 2008). The primary jasmonate is jasmonic acid (JA), which is produced within cells for circulation within the plant. JA is often derivatised into methyl jasmonate (MeJA) which is the form in which jasmonates are able to cross membranes and is the form secreted by plants for intra- and inter-plant communication (Wasternack and Song 2017, Heil and Ton 2008). Figure 1.7 shows the chemical structures of JA and MeJA. There are several other biologically active derivatives of JA, like jasmonic acid isoleucine, JA-Ile, and cis jasmone. These will not be discussed here, as JA and MeJA are the primary forms of jasmonates and the forms most commonly used in studies of exogenous application of jasmonates.

Jasmonates are key regulators of floral development and fertility as well as germination (Huang et al. 2017, Wasternack and Hause 2013). Additionally, jasmonates play several roles in root development and regulation, including promoting lateral root formation, induction of root regeneration, and inhibition of primary root growth (Cai et al. 2014, Ye et al. 2020, Zhang et al. 2019, Chen et al. 2011). Jasmonate treatments have been studied for their effects of modulating

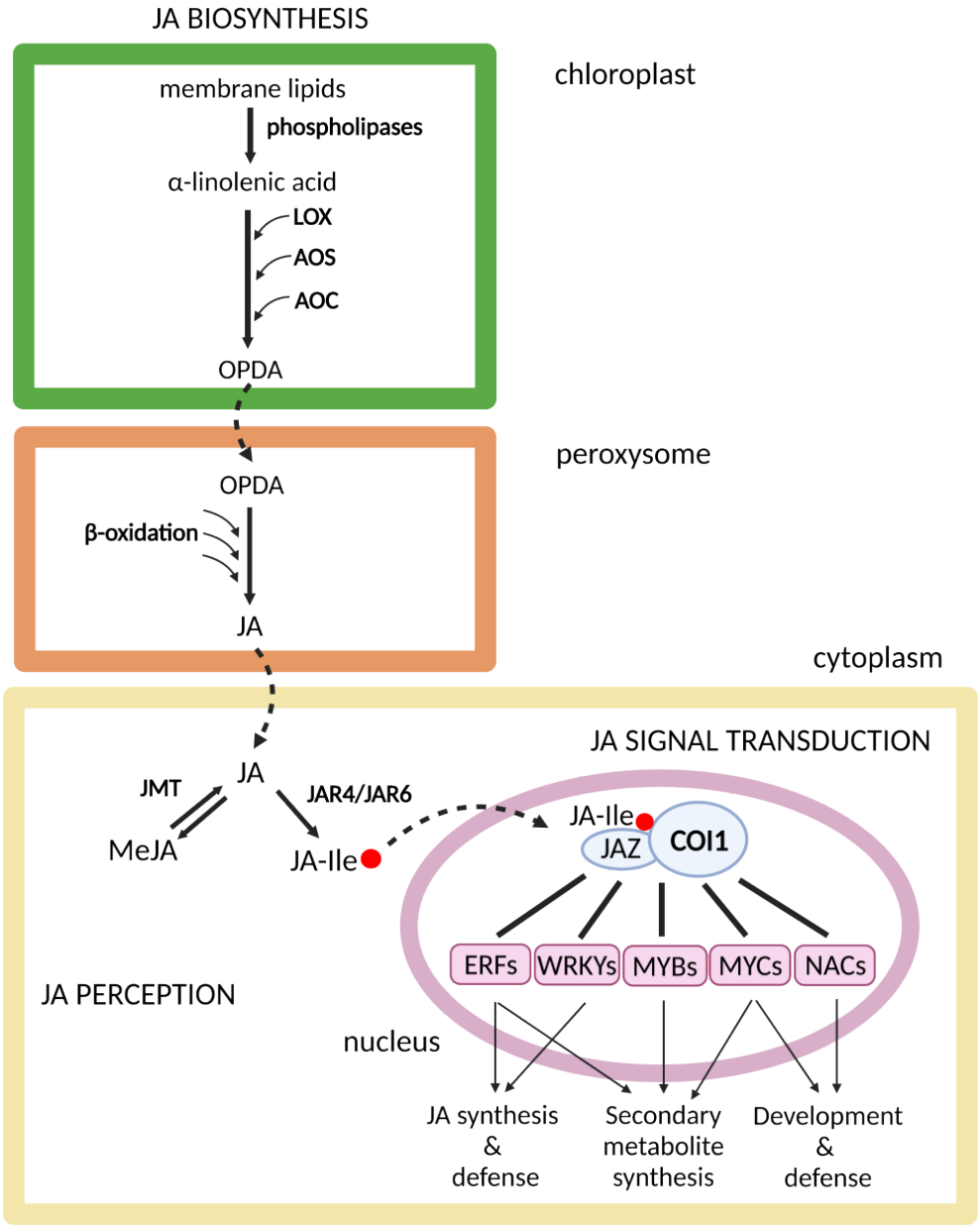


Figure 1.8: Jasmonate biosynthesis and signaling pathway. Figure from Ruan et al 2019 (Ruan et al. 2019).

leaf senescence, photosynthetic activity and abiotic stress tolerance in a variety of species (Yang et al. 2012, Yang et al. 2019, Qi et al. 2015, Sirhindi et al. 2020, Huang et al. 2017).

Jasmonates regulate a wide variety of plant growth and development factors in the context of environmental stress, including the activation of defense responses which involve tissue damage or wounding and lead to repression of growth and

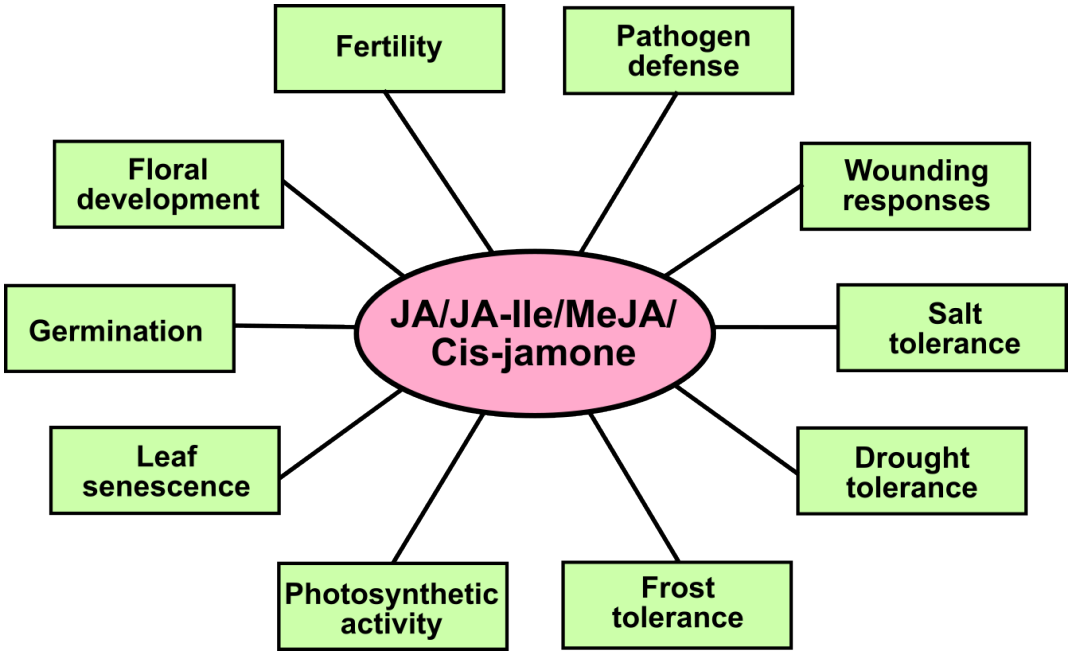


Figure 1.9: Plant processes modulated by jasmonic acid (JA), methyl jasmonate (MeJA), cis-jasmone, or the isoleucine conjugate of jasmonic acid (JA-Ile). These include growth and development functions like fertility, floral development, germination, photosynthetic activity and leaf senescence as well as abiotic and biotic stress responses like pathogen defenses, wounding responses, and salt, drought, and frost tolerance, Adapted from Ali & Baek 2020 (Ali and Baek 2020).

development when the plant is under attack (Wasternack and Song 2017, Guo et al. 2018, Campos et al. 2014). Figure 1.10 illustrates the various routes, including via foliar or root attack by necrotrophic pathogens or herbivores, defense priming by beneficial microbes, and exposure to exogenous methyl jasmonate or other JA elicitors via foliar, soil (root), or seed exposure. JA-mediated defense responses are inducible as opposed to constitutive which allows the plant to grow and develop normally until incidents of wounding or tissue damage, when it can then temporarily re-route resources from growth and development into defense and stress tolerance. A key component of the plant wound response is that, up until a certain threshold of activation, it appears to be temporary, and, after having dealt with the tissue damage or pathogen threat, the plant can return to normal growth and development (Guo et al. 2018, Pozo et al. 2004). This allows the plant to divert energy into defense responses only when it needs to, as opposed to continually spending energy on functions it may not need.

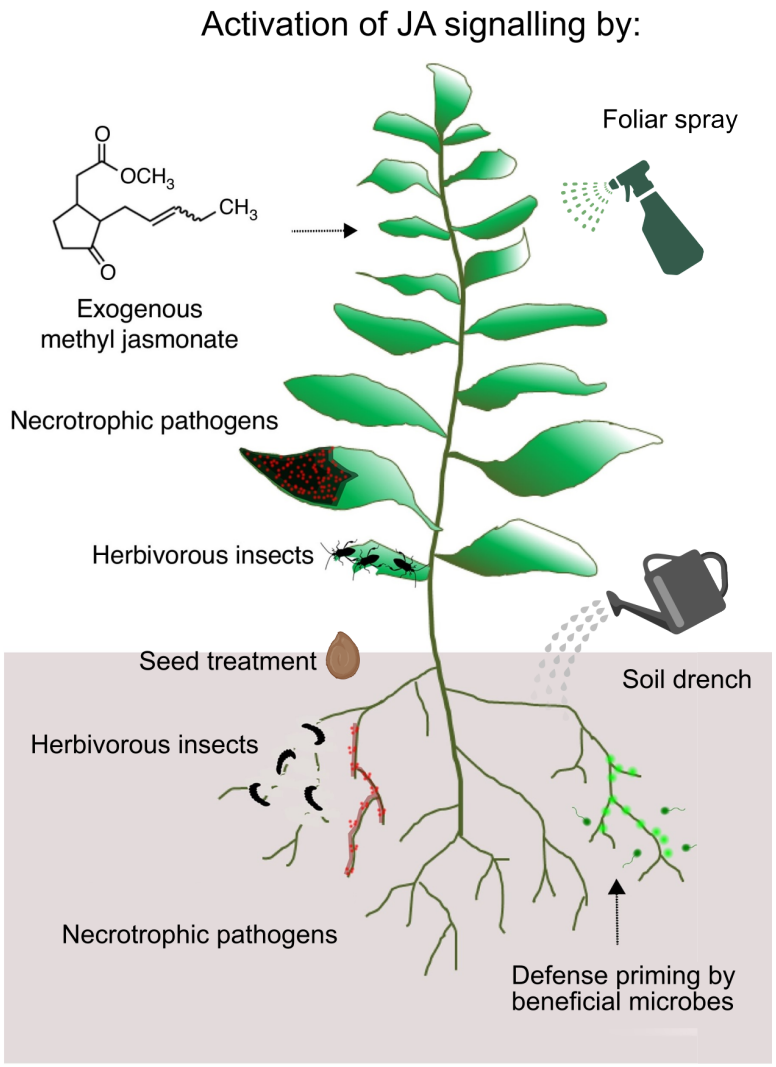


Figure 1.10: Activation of JA signalling by various routes. Attack by necrotrophic pathogens or herbivorous insects either on foliar, stem, or root tissues induces jasmonate signalling. Additionally, jasmonate signalling can be activated by beneficial microbes in the rhizosphere or via exposure to exogenous methyl jasmonate. Finally, JA signalling can be activated by foliar spray, soil drench, or seed treatment of JA elicitors. Adapted from Carvalhais et al 2017.(Carvalhais et al. 2017).

1.10 Jasmonate-Based Seed Treatments

Jasmonate-based seed treatments have been shown to confer broad-range pathogen and pest resistance in a variety of plants, including tomatoes, cowpeas, norway spruce, rice, wheat, pearl millet, and chinese cabbage (Worrall et al. 2012, Bali et al. 2020, Pankaj et al. 2013, Berglund et al. 2016, Kraus and Stout 2019, Motallebi et al. 2015, Motallebi et al. 2017, Zhang et al. 2013, Deepak et al. 2007). JA-based seed

treatments can provide long-term defense against pathogens, depending on the plant and the dose of JA. However, it is not yet clear what exact combinations of plants, doses, and timings of exposure can reliably lead to long-term defense activation. Additionally, some plants are not responsive to JA-based seed treatments or have not been tested. For example, Pankaj et al found JA and MeJA treatments effective in inducing resistance in cowpeas and tomato but not in soybean (Pankaj et al. 2013).

Only a few studies have looked at the defense activation and resistance over the course of a plant's lifetime. Kraus and Stout found that methyl jasmonate treatments induced resistance to rice water weevil in rice but that the effects decayed over time, within two months of treatment. They also found that there were delays in growth and development, like rice heading and seeding, as well as overall biomass, though grain yield per plant was not affected (Kraus and Stout 2019). A follow up study by the same group found that both JA and MeJA seed treatments induced resistance to rice water weevil in rice but this time found reductions in both growth and grain yield per plant, though the effects were less pronounced with the JA treatments (Bhavanam and Stout 2021). Baldwin found that treating roots of *Nicotiana attenuata* with MeJA induced herbivore resistance under moderate herbivory and allowed plants to produce more yield weight of seeds under herbivory conditions than those not treated with MeJA, but came at costs to growth and seed yield when plants were treated but then faced no herbivory (Baldwin 1998). This study was done over the course of a growing season and the actual length in days or weeks was not specified, though it can be guessed to be approximately 8-12 weeks based on propagation information for *Nicotian attenuata* (Hartke 2014).

Most of the data supporting 'long-term' induction of resistance refers instead to the 'long-term' between a seed being sown and the plant reaching a maturity at which it is both less susceptible to diseases that affect young plants and at which it has already started producing the bulk biomass or yield for which it is cultivated. For example, in melon, tomatoes, and Norway spruce several studies have shown induced resistance up until the fruit production stage or seedling plant-out stage in the case of the trees, but have not then continued to monitor the plants after

this point (Buzi et al. 2004, Berglund et al. 2016, Kępczyńska and Paulina Król 2012, Kozlowski et al. 1999, Król et al. 2015).

There are a variety of factors which can potentially affect the efficacy of a seed treatment. The surface and physical characteristics of the seed, including the surface architecture of the seed surface – relative smoothness or roughness, presence of ridges or other variations in surface structure – and the permeability of the seed coat (both to water and other compounds) can affect the quantity and time frame (dose) of the active agent received by the seed (Mouden et al. 2020, Haferkamp et al. 1984, Mills 1972, Taylor and Harman 1990).

It is a known phenomenon that different cultivars can respond differently to seed treatments. Smart et al found that JA treatment efficacy varied heavily between tomato cultivars, with 'Moneymaker' variety showing no difference between JA seed-treated plants and control plants in defense against herbivorous mites while 'Carousel' varieties saw a significant decrease in incidences of herbivory with JA seed-treated plants as compared to controls (Smart et al. 2013). In a follow up study Mouden et al found that variations in seed coat permeability between cultivars of tomato affected the ability of JA seed treatments to induce resistance against western flower thrips. However, the ability of the plants to respond to resistance inducers did not correlate with the amount of water the seeds from each cultivar could absorb, and this water absorbance appeared to be independent of the permeability of the seed coat to the compounds of interest (Mouden et al. 2020).

A study in barley which used a combination of elicitors (aci-benzolar-S-methyl, β-aminobutyric acid, and cis-jasmone) also found variations in induced resistance elicitation based on cultivar. They found elicitor induced disease resistance against *Rhynchosporium secalis* and *Blumeriagraminis* f. sp. *hordei* varied between 32 – 67% depending on cultivar in both field and controlled conditions (Walters et al. 2011). They found this to be true independent of existing cultivar genotype resistance within the plant (Walters et al. 2011).

While some of this variation can be attributed to the permeability of the seed coat even the genetic resistance of the cultivar do not fully explain the differences

observed. For example, in some cases resistant cultivars respond better to resistance-inducing seed treatments – as in 2,6-dichloroisonicotinic acid induced resistance in cucumber to *Sphaerotheca fuligenea* (Hijwegen and Verhaar 1995) – and in some cases susceptible cultivars respond best to resistance-inducers – as in soybean, where acibenzolar-S-methyl induced resistance to *Sclerotinia sclerotiorum* (Dann et al. 1998). This indicates that other factors, including metabolism and the exact composition of seed coats can affect seed treatment efficacy between cultivars.

The soil medium in which a seed is grown could also affect the effectiveness of seed treatments. Seed treatments often work differently in soil vs hydroponic systems, even if the seeds themselves were started in soil before being transferred to a hydroponic system (Saldinger et al. 2023, Korkmaz et al. 1998). Additionally, some soils may be more or less suppressive of diseases generally, based on their nutrient content and the presence of growth promoting or growth suppressive microorganisms and this could affect the relative efficacy of seed treatments (Mazzola 2002, Postma et al. 2008, Mousa and Raizada 2016, Ghorbani et al. 2008).

JA-induced disease resistance often comes at a cost to growth and development, for example, activation of JA defense responses can slow or reduce germination, reduce growth, and cause early senescence (Kim et al. 2015, Qi et al. 2015, Seltmann et al. 2010). In several cases where JA seed treatments increased disease resistance the JA responses also produced delays in germination or costs to growth, decreased root length, biomass, or lower seed yield (Buzi et al. 2004, Berglund et al. 2016, Kępczyńska and Paulina Król 2012, Kozlowski et al. 1999, Król et al. 2015, Kraus and Stout 2019, Bhavanam and Stout 2021, Baldwin 1998). Whether or not a plant responds in this way appears to be highly dose dependent. Several studies have been performed using tomato seeds soaked in different concentrations of either JA or MeJA from 4-24 hours, all of which lead to an increase in disease resistance, though the effects were more pronounced at moderate doses of either hormone, whereas low or high doses either did not induce a strong response or came at such a high cost to growth or germination efficiency that the treatment was not thought worthwhile (Worrall et al. 2012, Okada et al. 2015, Bali et al. 2020). Thus there is

evidence that, while JA and MeJA can be effective inducers of plant resistance to pathogens, achieving an optimal dose which does not incur high growth or yield costs is crucial to developing these seed treatments for real-world applications.

Even the studies which monitored a plant's disease resistance throughout the plant's full lifetime noticed a diminishing of the resistance over time. For example, in rice seeds treated with MeJA, plants that were first exposed to pests 15 days after treatment had a higher resistance than those first exposed to pests 29 days after treatment (Kraus and Stout 2019). Additionally, there are variations in plant responses to jasmonates between plant species. For example, soybeans and cowpeas were found to barely tolerate jasmonate treatments, dropping germination efficiency by almost 80% (Pankaj et al. 2013), while tomatoes have consistently shown strong resistance induction following jasmonate exposure while only incurring small costs in germination or defense (Bali et al. 2020, Kępczyńska and Paulina Król 2012, Karban 2011, Worrall et al. 2012). Taken together, these results indicate that, though jasmonate induction of pathogen and pest resistance is a promising approach, there is significant room for optimization of these jasmonate-based seed treatments.

It is unclear at what point the priming occurs; whether it is when the seed is initially soaked in the jasmonate treatment, when the seedling is undergoing germination, or as a young plant. In fact, results from the existing studies on jasmonate seed treatments suggest that seeds and seedlings are responsive to jasmonate treatments at all of these time points. Some studies have shown induction of defense responses in cases where the seeds were soaked in JA or MeJA and then thoroughly rinsed before planting (Bhavanam and Stout 2021, Pankaj et al. 2013). Others in cases where the seeds were soaked and then either dried or planted directly; i.e. the treatment was not necessarily for the period of soaking only (Bali et al. 2020, Berglund et al. 2016). And still others where germinated seedlings were exposed to jasmonates either via foliar spray or injection near the roots (Kozlowski et al. 1999, Motallebi et al. 2015, Motallebi et al. 2017, Singh et al. 2019). This suggests that a seed treatment in which seeds could have a more controlled exposure over a longer time, i.e. - if the jasmonates were encapsulated and slowly released,

might allow for effective defense response induction while minimizing total dosage and any associated growth and development costs.

There is evidence that MeJA has a stronger negative impact upon germination and growth than JA. This makes some sense, given that MeJA is the volatile, biologically active form used for distance signaling and inter plant communication, while JA is produced for circulation within a plant (Wasternack and Song 2017, Heil and Ton 2008). For example, rice seeds were found to have a stronger decrease in germination and emergence when exposed to MeJA vs JA (Bhavanam and Stout 2021), while tomatoes, wheat, cowpeas, and soybeans were all found to have a stronger decrease in germination and growth measurements to MeJA as compared to JA (Pankaj et al. 2013). However, because MeJA is the volatile form, it is correspondingly most often used in seed treatments, and so there are relatively few studies directly comparing the effects of MeJA and JA.

When resources are moved to defense, rather than growth or development, there are corresponding costs (Cipollini et al. 2003, Conrath 2009). The patterns seen in the existing studies on jasmonate treatments support this. Both positive effects, successful induction of resistance, and negative effects, costs to growth or yield, of jasmonate treatments vary according to the concentration and length of time of the seed treatment as well as the plant species upon which the treatment was tested. Higher concentrations of both JA and MeJA inhibit germination and growth, and often do not reduce pathogen or herbivore load in plants. Likewise, a low concentration often does not impact disease, though it may also not impact growth or development. A moderate dose and one which is perhaps applied multiple times or sustained throughout germination may thus provide the best induction of defense responses at minimal cost to yield. This corresponds to existing theories and data on resistance priming in plants (Cipollini et al. 2003, Conrath 2009).

Jasmonates have also been found to be key factors in mitigating abiotic stress in plants. Jasmonates have been shown to help mitigate stress from cold, drought, light, salt, and heavy metals, both through indirect and direct defenses. See Figure 1.9 for more examples (Wyenandt 2020, Raza et al. 2021).

Thus solutions to the problems of dosage and delivery of jasmonates could allow effective use of jasmonates and enhance both biotic and abiotic stress responses. A well-delivered and dosed jasmonate seed treatment could increase overall plant health and resiliency, a quality already key in agriculture and that will be increasingly important in uncertain climatic futures.

1.11 Silicates in agriculture

One potential solution for dose and delivery of jasmonates is the use of a carrier. Within this work, two types of silicate are tested as potential carriers for a jasmonate-based seed soaking treatment.

Silica is not an essential nutrient for plants but has been shown to have multiple beneficial effects across different taxa. These include studies showing silica inducing broad-spectrum resistance in rice, assisting with amelioration of aluminum toxicity in maize, chromium toxicity in peas, UV-B stress in wheat, and salt stress in aloe, wheat, rice, peas, and sorghum (Luyckx et al. 2017, Zhu et al. 2019, Kim et al. 2017, Mostofa et al. 2021).

Silicates are used in a variety of ways in agriculture, both as slag and in nanoformulations (Liang et al. 2015). They are used as soil amendments (Swoboda et al. 2022, Zarebanadkouki et al. 2022, Galindo et al. 2021), animal and fish feed amendments (Öz and Aral 2023, EFSA Panel 2017, Di Gregorio et al. 2014, Ikusika et al. 2019), carriers for nutrients or minerals (Tayade et al. 2022, Rastogi et al. 2019, Naaz et al. 2023), as treatments against insect herbivores and plant pathogens (Fauteux et al. 2005, Keeping and Kvedaras 2008, Van Bockhaven et al. 2013), and as binding agents for remediation efforts (Puppe et al. 2023, Chen et al. 2021). Silicates such as vermiculite and perlite are often used as soil amendments in potting soil mixes, to add structure to the soil and assist with drainage and water and nutrient retention. Zeolites, perlite, bentonite and vermiculite are also used as fill to bulk animal and fish feed and as soil conditioners to bind or filter nutrients, fertilizers, or other compounds from the soil (Swoboda et al. 2022, Ikusika et al. 2019, Chen et al. 2021, Ghasemi et al. 2018). There are many commercially

available types of silicate and aluminosilicate feed, soil, and plant supplements available as well as numerous patents.

Agricultural plant species are often defined via their silicic acid uptake, as either active, using silicon transporters , passive, or restrictive, resulting in varying levels of silica accumulation (Epstein 1999). This silica accumulation is highly correlated with phylogeny (Hodson et al. 2005, Trembath-Reichert et al. 2015). Among agriculturally important plants rice, wheat, and sugar cane contain high concentrations of silica (up to 10% of dry biomass), and plants such as peas, green onions, radish, and tomato have low silica accumulation (Takahashi 1968, Epstein 1999, Hodson et al. 2005).

Silicon functions biochemically within plants as silicic acid and is deposited from plants and animals as amorphous silica, SiO_2NH_2O. Amphorphous silica can be taken up both from slag and nanoformulations as silicon dioxide (Liang et al. 2015b). Silica in the form of silicon dioxide is bioavailable and is essential for bone development in livestock and humans (Martin 2007, Jugdaohsingh 2007, Rondanelli et al. 2021). Additionally, silicon appears to play a large role in plant abiotic and biotic stress tolerance, including in priming defense responses to incur disease and herbivore resistance (Chérif et al. 1992, Fauteux et al. 2005, Tripathi et al. 2014, Van Bockhaven et al. 2013).

Silica appears to confer disease and herbivore resistance through multiple mechanisms in plants, including activating defense-related genes and enzymes, inducing antimicrobial compound production, and regulating signal pathways (Wang et al. 2017). In the forms of kaolin and diatomaceous earth it also prevents insect herbiviory by drying out the insects and forming a physical barrier between plant tissues and fungi and insects (Korunić Zlatko 2013, Subramanyam and Roesli 2000, Eroglu et al. 2017, Arthur and Puterka 2002).

1.11.1 Silica nanoparticles in agriculture

Nanoparticles are employed in a variety of agricultural contexts as biosensors and biomolecule nanocarriers. Nanoparticles have been used for nutrient supplementation,

delivery of herbicides, pesticides, and fertilizers, and for plant genetic engineering by the delivery of miRNA or siRNA (Rastogi et al. 2019, Sanzari et al. 2019). Nanoparticles have a high surface-to-volume ratio and can be engineered to have a variety of physiochemical properties, often differing considerably from their bulk counterparts. They can be metal-based, metal-oxide based, carbon based, quantum dots, or silicon based (Sanzari et al. 2019). Of particular interest in this project are hexagonal mesoporous silica nanoparticles (HMSNPs), which have an internal hexagonal structure that gives them a large surface to volume ratio, can be easily capped in order to contain volatile compounds, and may confer some of the beneficial effects of silica supplementation (Siddiqui et al. 2020, Bravo Cadena et al. 2018a, Bravo Cadena et al. 2018b). Capping is the process of stabilizing the surface and thus the contents of a nanoparticle. Capping allows for control over how long a loaded nanoparticle is stable or viable and also under which conditions its contents can be released (Javed et al. 2020). For example, chitosan and alginate are commonly used as capping agents for nanoparticles used in plant systems because they are biodegradable, non-toxic, compatible with plant systems, and inexpensive and easy to source (Bravo Cadena et al. 2018a, Javed et al. 2020).

Silica nanoparticles (SiNPs) have been used as direct silica supplementation as well as carriers for pesticides, herbicides, fertilizers, and natural biocides like essential oils (Rastogi et al. 2019, Bravo-Cadena 2018). Many studies have found SiNPs to be effective silica supplementation, increasing plant height, root length, and photosynthetic activity in larch, tomato, maize and wheat (Rastogi et al. 2019). They've also been used as carriers for pesticides and found to provide slow release and more targeted effects for both avermectin and pyoluteorin (Li et al. 2007, Chen et al. 2011). SiNPs have also been used to deliver both DNA and chemicals into plants (Torney et al. 2007, Mushtaq et al. 2018, Liu et al. 2006, Rastogi et al. 2019, Siddiqui et al. 2020). Nanoparticles are particularly useful for delivering volatile compounds (Liu et al. 2006). Bravo-Cadena found SiNPs to be effective carriers for biocide essential oils (EOs) and found the SiNP-EOs to be more effective than the oils alone on controlling bacterial disease in peas (Bravo-Cadena 2018).

Incorporating nanoparticles into seed coatings can assist with targeted delivery by preventing loss due to evaporation and releasing the contents over time as the seed coating itself degrades (Bravo Cadena et al. 2018a).

However, silica nanoparticles have several draw backs for use in agriculture or even in larger scale lab studies of plants. One, the particles must be synthesized in a lab by highly trained technicians or scientists. Two, the synthesis, barring a few examples in the literature (Zhang et al. 2013, Paryab et al. 2024), is generally performed in small batch sizes, around 120 mg per batch (Huang et al. 2014a, Bravo-Cadena 2018). Three, the synthesis takes two to three days to complete (Hom et al. 2010, Zhang et al. 2013, Candela-Noguera et al. 2024).

For these reasons, it was thought an alternative silicate carrier could be useful. Ideally, it would be either a naturally occurring silicate or quick and easy to produce in bulk and would have a porous structure allowing a large surface area and thus high potential loading of compounds like jasmonates. Zeolite clinoptilolite was selected as a test alternative carrier and trialled alongside silica nanoparticles in this work. Zeolite clinoptilolite hits all of these ideal requirements: it is a cheap, easy to secure, naturally-occurring porous silicate and is already used in various capacities in agriculture.

1.11.2 Zeolites in agriculture

Most silicates used in agriculture come from either industrial slag or are SiNPs. However, an interesting option as a carrier is the natural volcanic aluminosilicate zeolite.

Zeolites are volcanic sedimentary aluminosilicate deposits. They are crystalline with repeating open three-dimensional structure (Mumpton 1999). The slightly negatively charged structure allows selective binding of cations, and the large structural cavities and the entry channels leading into them allow reversible water uptake. In addition, the large internal surface area is slightly polar and preferentially adsorbs polar molecules, water most sensitively, allowing ‘loading’ of a variety of compounds in addition to its selective binding of cations (Mumpton 1999).

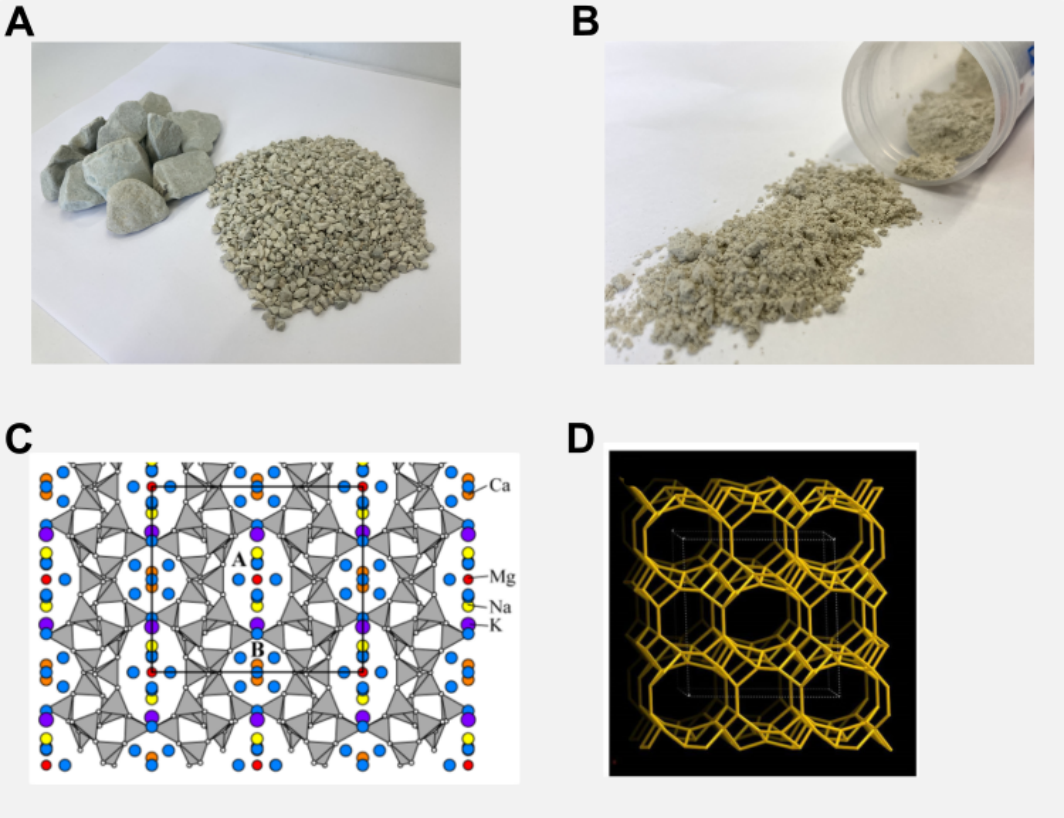


Figure 1.11: Zeolite clinoptilolite. Panel **(A)** shows clinoptilolite in various sizes, large 3-6 cm length aggregate and smaller 3-10 mm length gravel. Panel **(B)** shows ground zeolite, such as was used in the following chapters. Panel **(C)** shows the crystal structure of zeolite clinoptilolite. Cation positions shown are from the Koyama & Takéuchi 1977. Typically clinoptilolite contains 4 to 7 cations per unit cell (Deer et al. 2004). Panel **(D)** shows the clinoptilolite framework without the cations. Images in panels C and D from IZA 2003 (IZA 2003, Koyama and Takéuchi 1977, Deer et al. 2004).

Zeolite is used in building materials, in water and wastewater treatment, and in agriculture as soil conditioners (Cataldo et al. 2021, Mumpton 1999). In addition to their water retention properties zeolites are also useful in agricultural soils to help reduce nitrate leaking and ammonia volatilization, as they selectively bind ammonia cations.

Zeolites have been used as particle films, similar to kaolin, for controlling pests and diseases. Zeolites are inert and likely to have no direct biochemical effects on the plants, pathogens, or pests (De Smedt et al. 2016). Zeolites' high water adsorption likely prevents water films forming on the plant surface upon which the zeolites is applied, i.e., leaf surface or in the case of this project the seed and seedling

surface. This can prevent fungal and pathogen growth simply by removing the water and making the seed or leaf-surface environment unfavorable to the pathogen (De Smedt et al. 2016D). It also has a range of uses in commercial products as a deodorizing agent, as a component of portable dialysis systems, and off-label as a wound healing agent (Bacakova et al. 2018, Mumpton 1999).

Zeolites were first discovered in 1756, but large natural deposits not discovered until the 1950s (Mumpton 1999). Natural zeolites were the inspiration for synthetic zeolites/artificial sieves, and zeolites are in part the inspiration for the MCM class of synthesized molecular sieves, of which MCM-41 is the basis of the hexagonal mesoporous silica nanoparticles used in this thesis (Kresge and Roth 2013).

Zeolite's cation exchange capacities (CEC) vary in direct proportion to the amount of aluminum (Al) that substitutes for Si in the tetrahedral framework (Mumpton 1999). The greater the Al content the more extra-framework cations are needed to balance the charge. The hydration spheres of high field-strength cations prevent their close approach to the seat of charge in the framework; hence, cations of low field strength are generally more tightly held and selectively exchanged by the zeolite than other ions. Clinoptilolite has a relatively small CEC ≈ 2.25 meq/g, and its ion selectivity is: Cs > Rb > K > NH^{4+} > Ba > Sr > Na > Ca > Fe > Al > Mg > Li (Mumpton 1999).

This preference for larger cations like NH^{4+} has been extensively exploited for removing nitrogen water and sewage treatment as well as a carrier for fertilizers and bioavailable NH^{4+} in agriculture.

Zeolite's cation exchange and adorption propeties made it an early component of many types of remediation, particularly in aqueous solutions. Clinoptilolite can also be modified with additional amines for adsorption of heavy metals, and so it also used extensively in remediation of heavy metal contamination and for removing contaminants from nuclear waste. (Mumpton 1999). Surface modifications on clinoptilolite allow it to adsorb components of petroleum spills like benzene, toluene, and xylene from aqueous solutions and so it has been a valuable component of oil spill remediation since the 1990s (Gao et al. 1991, Haggerty and Bowman 1994).

Agricultural soils currently face major problems with heavy metal accumulation and heavy metal accumulation in the resulting produce (Chang et al. 2014, Mortvedt 1995, Adhikari et al. 2021, Alengebawy et al. 2021, Neely and Baker 1989).

Clinoptilolite and other zeolites are also used for adsorption and catalysis in petroleum refining, though this has been phased out somewhat since the creation of synthetic zeolites (Mumpton 1999).

In agriculture zeolites are used to improve soil characteristics like water holding capacity, conductivity, infiltration rate and cation exchange capacity (Cataldo et al. 2021). They can also help reduce nitrate leaking and ammonia volatilization within soils (Mumpton 1999). Nitrate leaking causes acute and long term poisoning in animals, including livestock, birds, insects, and humans, and is a major health implications for agricultural ecosystems, water systems, and the environment (Ward et al. 2018, Singh et al. 2022, Bouwman et al. 2002, Pan et al. 2016, Savcı 2012). Ammonia volatilization is the leading cause of nitrogen loss in agricultural systems worldwide (Bouwman et al. 2002) and leads lower fertilizer nitrogen use efficiency and to indirect nitrous oxide emission, both of which pose serious threats to ecological, climatic, and human health (Pan et al. 2016, Cataldo et al. 2021). The growing intake of synthetic fertilizers has doubled the flow of nitrogen and phosphorous into ecosystems. Increasing environmental concentrations of fertilizer change the soil humic-mineral content and the microbial biospheres of soil and water systems, causing progressive loss of fertility, desertification, and toxicity to plant, marine, animal, and human health (Cataldo et al. 2021, Sharpley et al. 1987, Passaglia and Prisa 2018, Kassas 1995).

1.12 Aims and Objectives

Treating seeds with plant-defense inducing compounds to confer broad-spectrum pathogen and pest resistance is increasingly common and a promising line of inquiry. However, many gaps still exist. More work still needs to be done towards understanding the balance between costs and benefits in induced resistance and priming. Induced resistance and priming seem to provide broad-spectrum protection

but literature to date has revealed some exceptions to this. It is important to continue to test various combinations of resistance and priming inducers in different agronomically important plant-disease-abiotic stress systems. Induced resistance and priming are not silver bullets. For this to be a useful tool in agriculture combined treatments, like seed treatments containing a plant growth promoter and a resistance inducer, need to be tested in a variety of crop-stress systems.

Silicates form a large percentage of the world's soils and their roles in plant and soil health are still being explored. There is some evidence they can act as inducers of resistance (and potentially as priming agents) on their own; however, the same concerns apply to silicate inducers as to inducers generally - more research is needed to understand the soils and plant-stress systems in which they are applicable, the impacts of combined treatments, and the mechanisms behind observed effects. Combining the use of silicates with jasmonates in seed treatments could be a very useful tool for mitigating biotic and abiotic stressors in crop plants, in a relatively inexpensive and sustainable way.

Pythium ultimum is a virulent and devastating pathogen. Control methods rely heavily on pesticides. The world increasingly needs to move away from pesticide use and so research into alternative management strategies and treatments is of vital interest. Legumes are a key part of strategies for future food security and sustainability. Thus, testing a disease control method in an agronomically important legume species and a virulent, common pathogen is important work. If a jasmonate or silicate seed treatment could be useful in reducing *Pythium ultimum* disease symptoms in *Pisum sativum* this could form the basis of a promising further treatment for controlling *Pythium ultimum* disease in both peas and potentially in other legumes.

This works investigates the potential of mesoporous silica nanoparticles (MSNPs) and zeolite clinoptilolite as silicate carriers and inducers combined with jasmonates as a soaking treatment for seeds to induce disease resistance. The effects of the seed treatments will be measured as induction of resistance to the necrotrophic pathogen *Pythium ultimum* which causes damping-off disease in a wide variety of

crop species. Jasmonates have been used successfully as seed treatments directly against damping off pathogens such as *Fusarium*, *Pythium*, and *Phytopthora* in a variety of species, including wheat, Arabidopsis, Norway spruce, tomato, and rice (Motallebi et al. 2017, Thaler et al. 2004, Vijayan et al. 1998, Kozlowski et al. 1999, Verbeek et al. 2019). It is proposed that the use of silicate particles will enhance a jasmonate treatment for inducing resistance, and potentially overcome some existing limitations of jasmonate-based seed treatments, specifically providing a longer release time frame and combating the volatility of jasmonate treatments.

Specific hypotheses:

(i) A jasmonate seed soaking treatment might induce resistance to *Pythium ultimum* in *Pisum sativum*

(ii) Jasmonates can be loaded into mesoporous silica nanoparticles.

(iii) Zeolite may be a reasonable substitute for silica nanoparticles as a loading substrate for a jasmonate-silica preparation.

(iv) combining a jasmonate seed treatment with a silicate may confer more disease resistance or growth benefits than either a jasmonate or a silicate alone on *Pisum sativum* when exposed to*Pythium ultimum*.

Specific objectives:

(i) Compare loading jasmonates (JA and MeJA) into silica nanoparticles and zeolite.

(ii) Test the efficacy of jasmonate seed soaking treatments on *Pisum sativum* growth, development, and disease resistance to *Pythium ultimum*.

(iii) Test the efficacy of combined jasmonate-silica seed soaking treatments on *Pisum sativum* growth, development, and disease resistance to *Pythium ultimum*.

2

Development of Jasmonate Loaded Nanosilicates

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2.1 Introduction

This chapter will show the development of jasmonate-loaded mesoporous silica nanoparticles. This includes synthesis of the nanoparticles, a method for detecting jasmonates, and characterizing the loading and release of the jasmonates to and from silica nanoparticles and zeolite.

2.1.1 Silicates in agriculture

Si is not considered an essential nutrient for plants but it has been shown to improve growth and yield under abiotic and biotic stress conditions and is a key growth promoter for crops such as sugarcane and rice, which grow on low-Si soil (Savant et al. 1996, Savant et al. 1999, J. F. Ma and Yamaji 2006, Meena et al. 2014, Tripathi et al. 2014). Increasing evidence points to the importance of silicon cycling in ecosystems, including agricultural ecosystems; despite not being considered an essential nutrient all plants grown in soil contain silicon (Epstein 1994, Vandevenne et al. 2012, Cooke and Leishman 2011, de Tombeur et al. 2023).

Silicon's biocompatibility, beneficial impacts on biological organisms and it's huge variety of sizes and structures, including many porous micro- and nanoparticles, make it a useful carrier for a wide variety of compounds across biological disciplines (Bruckmann et al. 2022, O'Farrell et al. 2006 Rastogi et al. 2019). Silicon particles are of special interest in agriculture because of silicon's beneficial micronutrient properties in plants and the increasing evidence of its contribution to abiotic and biotic stress tolerance (Emanuel Epstein 1999, Fauteux et al. 2005, Galindo et al. 2021, Keeping and Kvedaras 2008, Mahmoud et al. 2023, Mostofa et al. 2021, Wang et al. 2017). Various types of silicon particles are used as carriers for fertilizers, pesticides, and other compounds like DNA, proteins, and growth-promoting compounds in agriculture (Rastogi et al. 2019, Reháková et al. 2004). The forms of silica used vary widely, ranging from microporous zeolites and

vermiculites, and kaolin clays to various synthesized silica nanoparticles (Sharma et al. 2022, Sharma et al. 2021, De Smedt et al. 2016, Rastogi et al. 2019).

2.1.2 Mesoporous silica nanoparticles

Mesoporous silica nanoparticles (MSNPs) are used in a wide variety of of biological contexts as carriers and delivery agents. Their high surface area, large pore density, rigid framework and adjustable surface chemistry allow loading and stabilisation of many compounds, including DNA, proteins, cancer drugs, targeting molecules, imaging probes, pesticides, fertilizers, micronutrients, and essential oils (Bravo-Cadena 2018, Kong et al. 2021, Niculescu 2020, Goswami et al. 2022, Mahawar et al. 2023).

Hexagonal mesoporous silica nanoparticles (HMSNPs) were chosen for this project as they are widely used in delivery of drugs in cancer biology contexts and have a high biocompatibility because of their neutral surface properties (Niculescu 2020, Huang et al. 2014b, Li et al. 2012, Sreeharsha et al. 2022, Najafi-Hajivar et al. 2016). However, their use in agricultural contexts is increasing and have most notably been used for delivery essential oils in in plant systems (Rastogi et al. 2019, Bravo Cadena et al. 2018a, Bravo Cadena et al. 2018b).

One major drawback of HMSNPs, however, is their production. They must be synthesized in a lab, either on-site or purchased commercially, and require both specialized equipment and 2-3 days to produce a single batch. It is difficult to scale them up outside of a commercial lab, and their synthesis, as will be seen later in this chapter, requires very specific conditions in order to produce the standard size and pore dimensions. Additionally, the reactions produce a heterogenous mix of particles, with anywhere between 65-80% of the particles the standard size and shape.

Thus, it was thought a natural porous silicate might be a useful alternative carrier to the synthesized HMSNPs. One common, inexpensive porous silicate which is already used in a number of industrial purposes, both for its pores and sorption capabilities, is zeolite.

2.1.3 Zeolite clinoptilolite

Zeolite is an abundant natural porous silicate found in volcaniclastic sediment and volcanic rocks, such as tuff, basalts, andesites and rhyolites. Zeolite has the general formula $(Na,K,Ca)_{2\text{-}3}Al_3(Al,Si)_2Si_{13}O_{36}12H_2O$ (Smyth et al. 1990). Zeolite's structure is a three dimensional tretrahedral framework and the structure itself is formed mainly of silica, with some aluminum ions. The resulting structure has a slight to moderate negative charge and thus selectively binds cations. Additionally, zeolites have large internal pores or channels which hold water and other components dissolved in the water. It is commonly used as a molecular sieve because of its ion exchange capacity. It is widely used in industrial applications like water purification, wastewater remediation, and as an aggregate additive in soils and building materials. Zeolites are also used as soil conditioners to improve water retention and as components of slow-release fertilizers. Zeolites have a high thermal stability, large cation exchange capacity, surface area, porosity and water-holding capacity and it is thought these properties contribute to zeolite's efficacy as a soil additive (Szatanik-Kloc et al. 2021).

2.1.4 Detection techniques for jasmonates

Reliable detection of jasmonates is required in order to investigate the volatility of the compounds and their rate of release from the nanoparticles. Established methods to detect jasmonates include Liquid Chromatography-Mass Spectrometry (LC-MS), Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (LC-MS/MS) (Forcat et al. 2008, Liu et al. 2010), Gas Chromatography-Mass Spectrometry (GC-MS) (Rawlinson et al. 2015, da Silva et al. 2018, Meyer et al. 2003, W. Ma et al. 2013, Huang et a.. 2015), specialized extractions plus High Performance Liquid Chromatography (HPLC) (Zhang et al. 2007, Zhang and Chen 2013, Wu and Hu 2009), and fluorescently labelling jasmonic acid or methyl jasmonate for detection using either an absorbance plate reader, HPLC, or fluoresence microscopy (Xiong et al. 2012, Kitaoka et al. 2012).

Unfortunately, due to lack of access it was not possible to utilize LC-MS/MS for this project. Instead, methods for detecting jasmonates using GC-MS were adapted for use in this project, primarily from Rawlinson et al. 2015 and Huang et al. 2015. The resulting method combined the rapid extraction and methyl chloroformate derivatization from Rawlinson et al. 2015 with the use of dihydrojasmone as the internal reference from Huang et al. 2015.

2.2 Materials and Methods

2.2.1 Synthesis of HMSNPs

HMSNPs were synthesized using the sol gel synthesis method described by Hom et al. (Hom et al. 2010). 100 mg cetyl trimethylammonium bromide (CTAB, Aldrich; 99%) was dissolved in 48 mL ddH2O and 350μL of 2M NaOH. The solution was stirred vigorously, around 500 rpm, at 80°C in a round-bottom flask. After the temperature was stabilized (circa 20 minutes), 500 μL tetraethyl orthosilicate (TEOS, Aldrich) was added dropwise. After a further two hours incubation, the synthesis solution was cooled to room temperature and the nanoparticles collected by centrifugation. The nanoparticles were washed twice with methanol. The CTAB surfactant was removed by overnight reflux in acidic methanol (40 mL methanol, 2 mL 37 % hydrochloric acid) at 80°C. The solution was cooled to room temperature and then the nanoparticles collected via centrifugation, washed twice with methanol, and washed once with ethanol before being dried in a vacuum desiccator for 24 hours. Before use the nanoparticles were ground to a find powder using a mortar and pestle.

2.2.2 Preparation of Zeolite

Zeolite clinoptilolite (Finest-Filters, UK) was prepared by grinding 100 g zeolite gravel in a coffee grinder for 4 minutes, until smooth, and then sieving through a 1 mm^2 sieve.

Zeolite size was determined by sifting through an industrial sieve set using a 621-7110 (78-700) Fieldmaster Sieve Set (Cole-Parmer, UK). The sieve set contained a 35 mesh, 60 mesh, 120 mesh, and 230 mesh standard sieve sizes. These equated

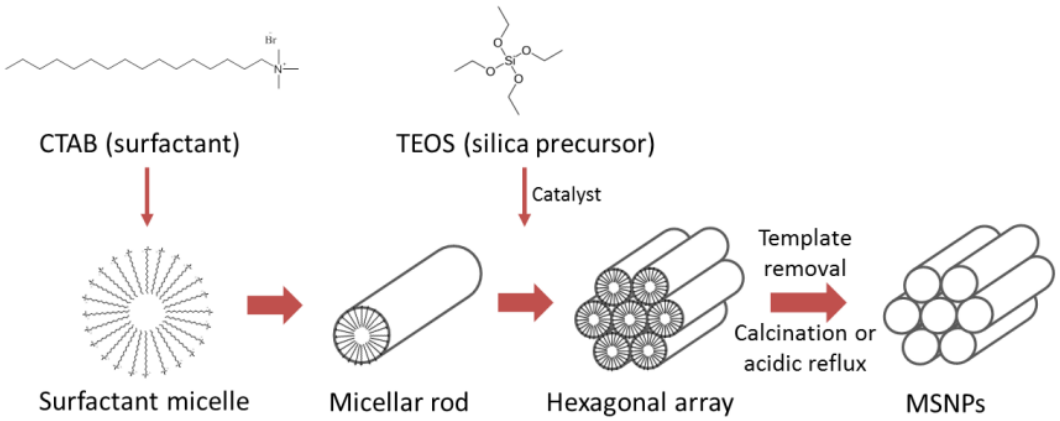


Figure 2.1: Sol gel synthesis of HMSNPs. CTAB surfactant is added to its critical micellular concentration to form micelles. These form into micellular rods, which then stack into a hexagonal array. TEOS is added a silica precursor. This interacts with the catalyst in the solution and causes the silica to hydrolyse and condense around the template. Creating a silica-coated hexagonal array. The CTAB template is then removed by acidic reflux, leaving hollow porous hexagonal silica nanoparticles.

to 500 micron, 250 micron, 125 micron and 63 micron pores respectively. 200 grams of pre-ground zeolite was placed in the top sieve and sieved through each successive layer until no more particles were moving between layers. This could be performed manually or with the used of a shaker, being careful not to let any of the powder spill out of the sieves. It typically took 45 minutes to 1 hour to fully sieve 200 grams of zeolite. The zeolite present in each layer was then measured and reported to get the size distribution of the zeolite.

2.2.3 Characterization of Nanosilicates

Size and charge were confirmed via dynamic light scattering using a Malvern Zeta Sizer. Size and porous structure were confirmed via transmission electron microscopy (TEM).

2.2.3.1 Transmission Electron Microscopy

Samples for TEM analysis were prepared by sonicating powdered silicates for 5 mins with a Soniprep 150 (MSE Sanyo, UK) then drop casting 10 μl of the solution onto copper carbon coated TEM grids (Agar Scientific, UK). Bright field imaging was performed using a JEOL JEM-2010 operating at 200kV and imaged using a Gatan OneView Camera, a JOEL JEOL 1400 Flash operating at 120kv and imaged with a Gatan Rio camera, or a Tecnai T12 operating at 120 kv and imaged with a Gatan OneView camera.

2.2.3.2 Scanning Electron Microscopy

Samples for scanning electron microscopy (SEM) were prepared by pressing a small amount of powdered silicate onto a 12 mm specimen carbon tab (Agar, UK) on a 0.5" aluminum specimen stub (Agar, UK) and then coated with gold to a final coating thickness of approximately 15 nm at a rate of 20 mA and a density of 19.32 g/m^3 for one hundred and twenty seconds using a Q150R ES rotary pumped coater (Quorum, UK). After coating, samples were imaged using the Everhart-Thornley secondary electron detector on a Zeiss Sigma 300 Field Emission Gun SEM operated at 2kV.

2.2.3.3 Dynamic Light Scattering for Size and Zeta Potential

The size and surface charge of the HMSNPs and zeolite preparation were measured using a Zetasizer Nano ZS (Malvern, UK) or a Zetasize Nano S (Malvern, UK). To determine the electrokinetic potential, or zeta potential (which represents the surface charge of a colloidal suspension), 10 mg or less of silicate particles were suspended in 4 mls ddH2O and sonicated as listed under the TEM methods. 1 ml of the sonicated silicate-water solution was then added to a disposable folded capillary cell DTS 1070 (Malvern, UK). 30 runs were read before the calculation of size and surface zeta potential.

2.2.4 Detection of Jasmonates

The GCMS extraction and detection method for JA and MeJA was adapted from Rawlinson et al. 2015 and Huang et al. 2015 (Rawlinson et al. 2015, Huang et al. 2015).

Reagents and jasmonates were purchase from Sigma-Aldrich, Dorset, UK. Catalog numbers were as follows: methyl jasmonate (392707), jasmonic acid (J2500), dihydrojasmone (W376302), methanol (34860), pyridine (360570), sodium hydroxide (S5881)

Extraction and derivatisation method was adapted from Rawlinson et al. 2015 as follows:

Calibration standards and other sample solutions were prepared from 100 mM ethanol stocks of either methyl jasmonate or jasmonic acid. Dihydrojasmone was used as the internal reference. Calibration standards were made up in methanol to 0.1, 0.5, 1, 10, 25, 50, 100, and 150 µg/mL for a total volume of 700 µL of each standard, before aliquots were taken for extraction and derivatisation.

Blanks consisted of 150 µL of ddH2O. 150 µL of each sample, standard or blank were transferred to a 2ml microcentrifuge tube. To each standard, sample, and blank 4.5 µL of 1 mM dihydrojasmone in ethanol was directly added and the sample suspended in 200 µL of a sodium hydroxide (1% w/v) solution. 147 µL of methanol and 34 µL of pyridine were added to the mixture before vigorous mixing by vortex for 25–30 s. Tubes were capped with reusable microcentrifuge tube cap locks before vortexing and tubes were opened carefully due to pressure build up in the methyl chloroformate steps. An additional 20 µL of methyl chloroformate was added and vigorously mixed by vortex for 25-30 s, making a total of 40 µL of methyl chloroformate added in two batches. 400 µL of chloroform was then added and mixed by vortex for 10 s before 400 µL of 50 mM sodium bicarbonate solution was added. Samples were then mixed by vortex for a further 15 s. Samples were separated into phases via centrifugation for 30 s at 17,000 x g. The lower organic layer was carefully transferred by pipette to a new 1.5 mL microcentrifuge tube. Anhydrous sodium sulphate was then added until the crystalline sodium

sulphate appeared dry upon further addition. 120 μL of the water-free solution was transferred to a glass analytical vial for GC–MS analysis.

Detection method was as follows:

All method testing and development was carried out on an Agilent Intuvo 9000 gas chromatograph with a split/splitless injector and an Agilent 5977B mass selective detector with an eletron ionisation (EI) source (Agilent, Stockport, UK). The GC inlet was equipped with an Agilent splitless single taper glass wool deactivated liner and an Agilent 5ul tapered fixed needle 23-26s/42/HP ALS syringe (Agilent, Stockport, UK). Two Agilent J&W DB-5ms Ultra Inert 15 m, 0.25 mm, 0.25 um columns connected by an Agilent column ferrule were used for all analyses (Agilent, Stockport, UK).

Injection temperature and transfer-line were both set to 250°C. A 2.5 μL injection was used with a 26.09 psi pressure pulse held for 42 sec. The column was set to initial temperature of 100°C held for 5 min and then programmed to increase 7.5°C min-1 to 250°C for a 25 min total run. Helium was used as the carrier gas at 2 ml min-1. The EI ion source was maintained at 230°C and the quadrupole at 250°C. The solvent delay was 7.5 min. The MS was run in selective ion monitoring (SIM) mode for 67, 83, 110, 137, 151, 162, and 224 m/z. The dwell time for each ion was 50 s.

2.2.5 Loading of jasmonates into silicate particles

Jasmonic acid and methyl jasmonate were purchased from Sigma-Aldrich, Dorset, UK.

Jasmonates were loaded into silicate particles in two different set ups of loading solution, labeled 'small volume' or 'large volume' as adapted from methods developed by Bravo-Cadena 2018 (Bravo-Cadena 2018).

Small volume loading method: Jasmonic acid or Methyl Jasmonate was dissolved with 1 mL dimethylsulfoxide (DMSO) into a total volume of 5 mL ddH2O, to a final JA or MeJA concentration of either 1, 10, or 50 mM. 60 mgs of either HMSNPs or prepared zeolite was then added to the mixture and sonicated with a Soniprep 150 probe sonicator (MSE Sanyo, UK) for 3 minutes at 5 second intervals. The

combined silicate particle-loading solution was then sealed and stirred at 250 rpm for 24 hours in dark conditions.

Loaded HMSNPs or zeolite were centrifuged at 3500 x g for 10 minutes. The supernatant was then centrifuged at 17,000 x g for 5 minutes to removed any remaining nanoparticles, before being processed for gas chromatography-mass spectrometry (GCMS). The concentration of JA or MeJA was analyzed by GCMS and compared to a calibration curve to estimate the loading capacity of the HMSNPs and zeolite. A control without silicate particles added was used to account for jasmonates lost from the system due to evaporation.

Loaded HMSNPs or zeolite were washed twice with 45 mls ddH2O before being frozen and then freeze-dried in a vacuum lyophiliser for 16-24 hours or until all liquid had evaporated. The freeze dried particles were then collected and stored at 4°C for up to two months.

Large volume loading method:

Jasmonic acid (cat no. J2500, Sigma-Aldrich, UK) or Methyl Jasmonate (95%, cat no. 392707, Sigma-Aldrich, UK) was dissolved with 6 mL DMSO into a total volume of 27 mL ddH2O, to a final JA or MeJA concentration of either 1, 10, or 50 mM. 60 mgs of either HMSNPs or prepared zeolite was then added to the mixture and sonicated with a Soniprep 150 probe sonicator (MSE Sanyo, UK) for 3 minutes at 5 second intervals. The combined silicate particle-loading solution was then sealed and stirred at 250 rpm for 24 hours in dark conditions.

Large volume loaded HMSNPs and zeolite and their loading supernatants were then collected and freeze-dried in the same way as the small volume loaded HMSNPs and zeolite.

2.2.6 Rate of concentration over time

The volatility of JA and MeJA, defined here as the rate of concentration loss over time in steady conditions, was evaluated by incubating 100 ug/ml of either JA or MeJA in 50 mLs ddH2O at 22°C, stirring at 150 rpm, sealed, initially over one day but up to four days, or 100 hours. Five replicates were set up for each jasmonate.

At each time point, including an initial timepoint, T0, two 150 ul samples were taken, one to process for GCMS and one to freeze and store as backup. No volume was replaced over the course of the experiment. With up to six measurements over the course of 100 hours, this equated to a total of 1.8 mLs or 3.6% of the initial volume removed over the course of the experiment.

Loading, volatility, and release tests were conducted using a GCMS, see 2.2.4.

2.2.7 Release studies

Freeze-dried, loaded HMSNPs or zeolite were added to either 27 mL or 5 mL ddH2O, depending upon whether loaded in small volume method or large volume method, and resuspended with a probe sonicator before stirring at 250 rpm in the dark, sealed, for 24 hours. 150 ul of solution was sampled for processing for GCMS detection of jasmonates. Initial measurements were taken at 4 and 6 hour timepoints, with the release solution re-sealed after each sampling timepoint but no volume replaced.

2.2.8 Statistical Analyses

Statistical analyses in this chapter varied, depending on the input format of the data and the recommendation of the statistical analysis software, GraphPad Prism 10. In some cases a mixed effects model was used and in others a Two-Way ANOVA. Following a two way ANOVA sometimes a Tukey's multiple comparison's test was used and sometimes a Holm-Šídák multiple comparisons test. This depended on the recommendations of the software and on whether or not a confidence interval for each multiple comparison was sought.

2.3 Results

2.3.1 HMSNP Characterization

Hexagonal mesoporous silica nanoparticles were synthesized following the sol gel method re Hom et al. 2010. Correct or standard batches were those which matched the specifications given in the literature: roughly spherical and 130-180 nm in diameter as measured by a zeta sizer DLS, and with a zeta potential average surface

charge of approximately -29 mV (Hom et al. 2010, Bravo Cadena et al. 2018b, Kumar et al. 2001, Trewyn et al. 2007, Huang et al. 2014a. However, any small change in the conditions of the protocol, including stir bar to flask ratio, spin speed, temperature, and age of reagents, specifically CTAB and TEOS, affected the synthesis of the nanoparticles, leading to two major 'incorrect' morphologies, dubbed HMSNP_V2 and HMSNP_V3. Correct nanoparticles are denoted HMSNP_V1. Figure 2.1 for size, shape description, and zeta potential of the different HMSNP morphologies.

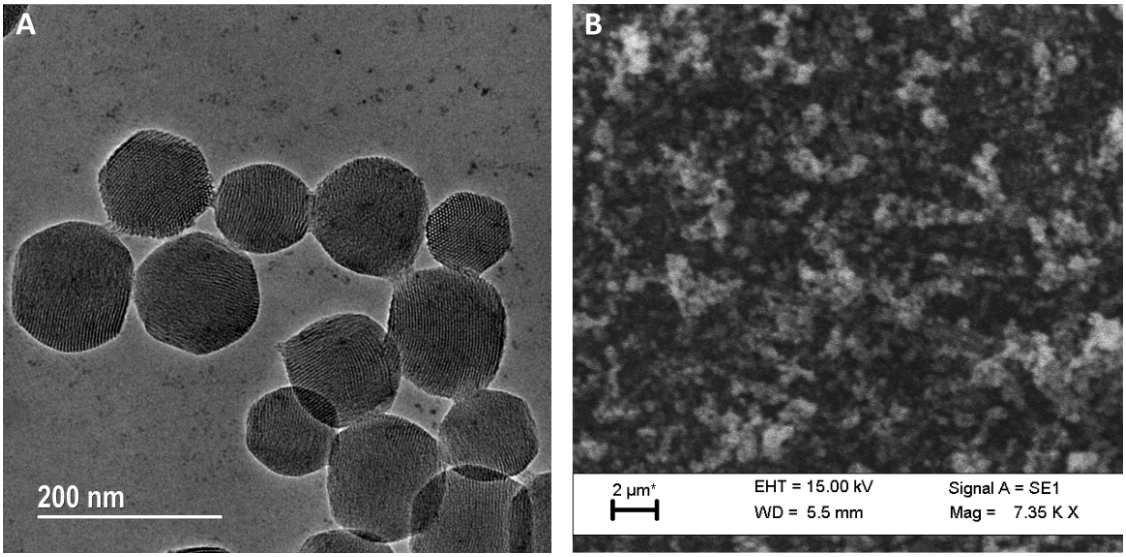


Figure 2.2: Representative TEM (left) and SEM (right) images of HMSNPs. Particles were approximately 150 - 180 nm in diameter. TEM images show hexagonal porous internal structure of HMSNPs.

HMSNPs size and morphology was confirmed by TEM imaging, as shown in Figure 2.2. Standard particles (as defined by characteristics given in Hom et al.) were round with a hexagonal internal porous structure showing both pores and channels depending on the orientation of the particle. Standard particles as here on labelled as type HMSNP_V1

HMSNPs were further characterized using SEM imaging. Standard HMSNP_V1 type showed moderately uniform size and morphology within each batch, see Figure 2.2.

Non-standard HMSNP morphologies broke into broadly two categories, HMSNP_V2 which was large, oblong, and often folded over, forming a bean shape

and affecting two major sizes, those averaging around 185 nm in diameter and those averaging around 550 nm in diameter while the other incorrect morphology, HMSNP_V3, were short and oblong and showed a distinct 'ball of wool' surface texture, showing some evidence of channels but no clear porous structure but were most uniform in size, averaging about 200 nm in diameter.

HMSNP_V2 nanoparticles imaged via TEM displayed a porous structure, usually along one axis, but the shape and regularity of the pores was indeterminate or variable, though occasionally resembling the hexagonal array expected of HMSNP_V1. See panels A and C of Figure 2.3 for examples of the HMSNP_V2 type and panels B and D of Figure 2.3 for examples of the HMSNP_V3 type.

HMSNP_V2 particles had a bi modal size distribution, with one peak around 185 nm comprising $\tilde{1}5\%$ of the total particles in a batch and one size peak around 550 nm, comprising $\tilde{8}5\%$ of the total particles in a batch. Both size distributions had an average surface zeta potential of -31.3 mV. See Table 2.1 for details on nanoparticle size, shape, structure, and surface zeta potential.

2.3.2 Zeolite Particle Characterization

Zeolite was characterized with the same techniques as the nanoparticles but additionally with a set of standard soil grading sieves, as the zeolite produced much larger particles and with much greater size variation than the synthesized nanoparticles. See Table 2.1 for details on the shape, pores, size, and zeta potential of zeolite, as compared to those of the HMSNP varieties.

To grade the soil, 200 grams of prepared zeolite was sieved through the sieve set. This was repeated a total of three times. However, up to twenty-five grams were lost through the process, presumably as very fine powder that escaped the stack and lid of the sieves.

The largest portion of the prepared zeolite was $\leq$ 63 um, the next largest proportion was $\geq$ 125 um but less than 250 um.

SEM imaging of the prepared zeolite showed a wide range of sizes and clumping in the zeolite particles, including a distinct flaky texture.

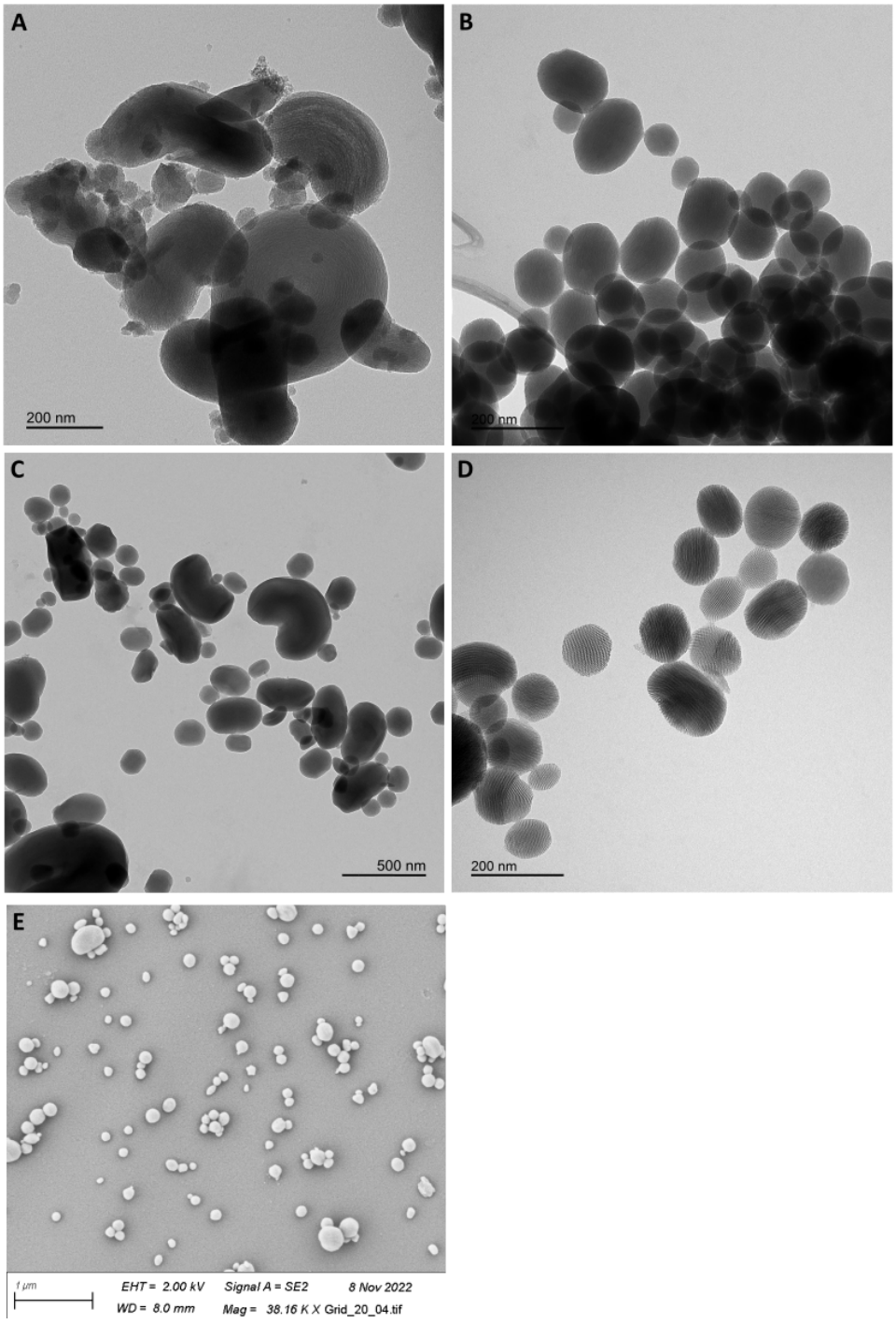


Figure 2.3: Examples of incorrect morphologies of HMSNPs. **(A)** HMSNP_V2 morphology, showing the high variability of size and shape, including the large oblong, folded over, bean-shaped morphology and the smaller, non-descript particles. **(B)** HMSNP_V3 morphology, showing close to the correct morphology but slightly more oblong and with no clear hexagonal porous structure, instead showing a mottled and 'fuzzy' surface.

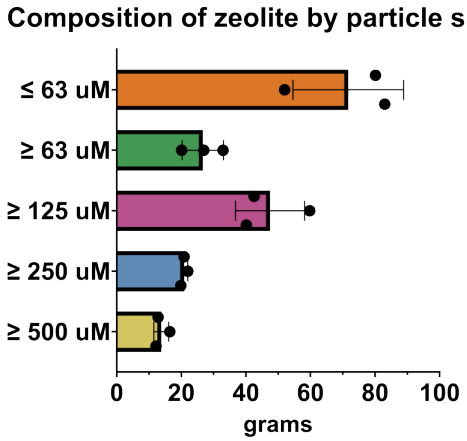


Figure 2.4: Zeolite size data from sieving with a standard US soil size sieve set. Data from three replicates of 200 grams prepped zeolite.

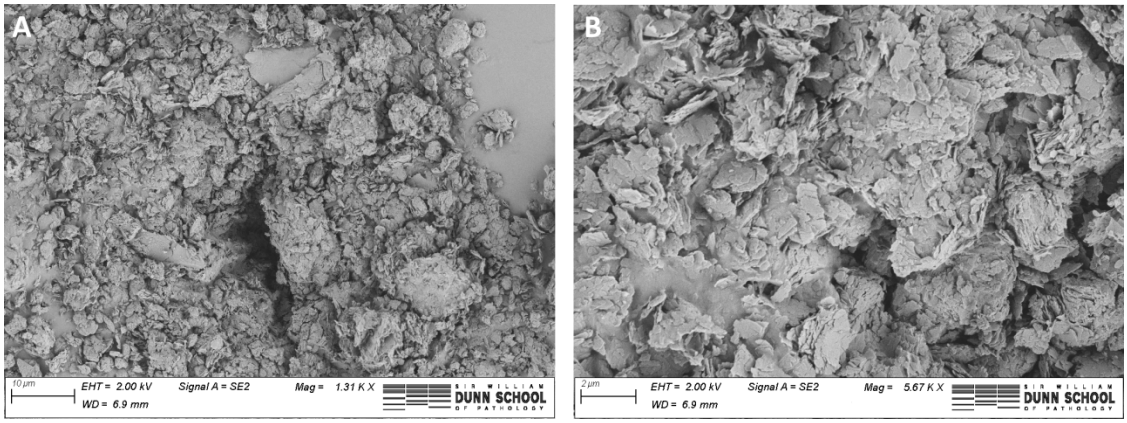


Figure 2.5: Examples of Zeolite SEM images, at various magnitudes. **(A)** & **(B)** SEM images of zeolite showing flakes and particle clusters of prepared zeolite, at moderate micrometer scale **(A)** and micrometer scale **(B)**.

TEM imaging of the zeolite showed additional variation in micro to nano scale particles, some crystalline, clumped or flaky. The μm scale shows the distinct holey microporous structure of zeolite. At the nanometer scale crystals and particles of zeolite are distinct.

Prepared zeolite was also analyzed for size and surface zeta potential using a zeta sizer. The zeolite had a wide variety of sizes and it was difficult to get clear delineation on size on the zeta sizer, results showing averages between 280 and 1700 nm. Surface zeta potential was consistent at -4.2 $\pm$ 2.1 mV (See Table 2.1).

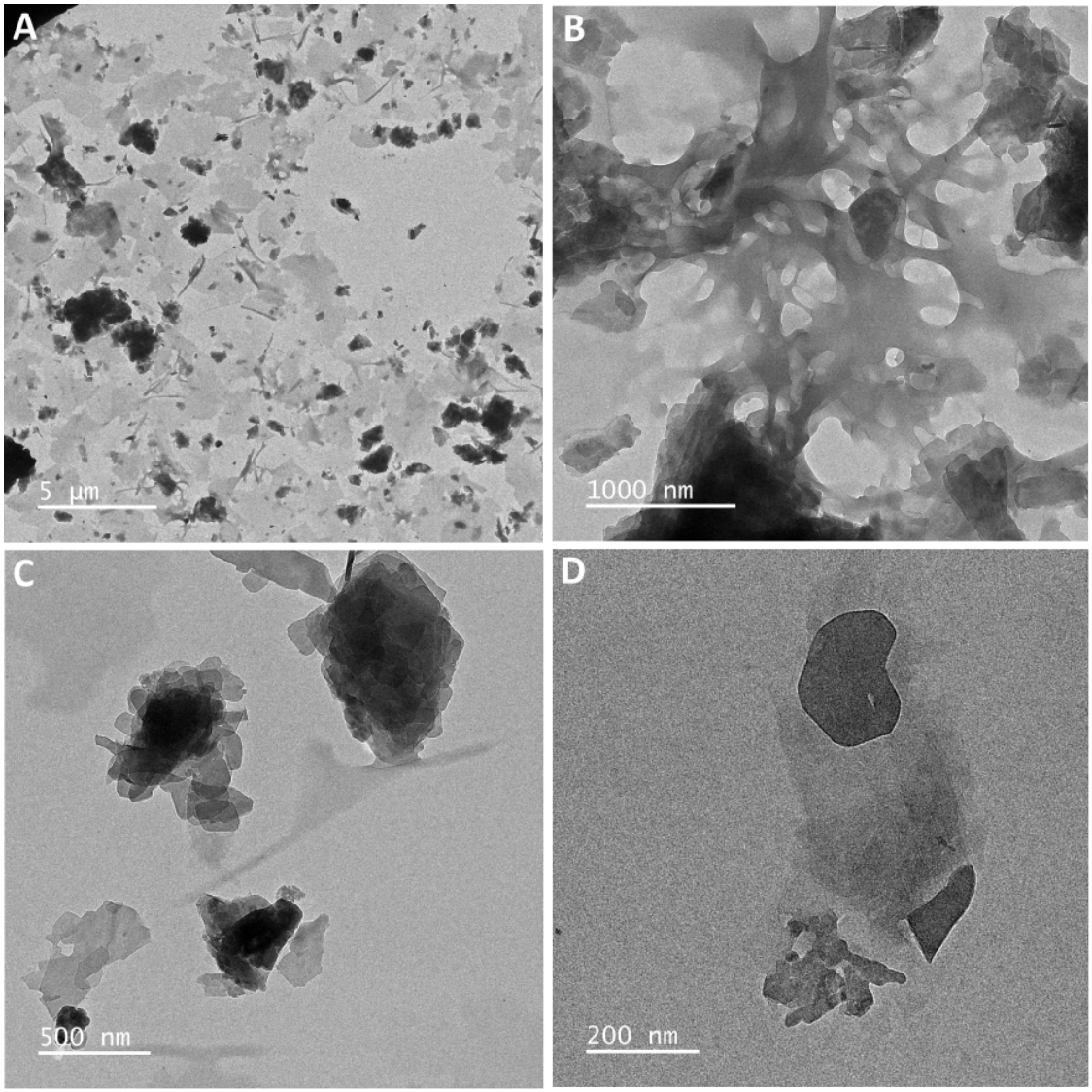


Figure 2.6: TEM images of prepared zeolite, at various magnitudes. **(A)** Image showing zeolite on μm scale, showing some of the flaky texture observed under SEM.**(B)** Image showing microporous structure of zeolite. **(C)** Image showing approximately 500 - 850 nm size crystals of zeolite. **(D)** Image showing typical smallest particles of zeolite, irregular in shape and varying from approximately 100 - 350 nm in size.

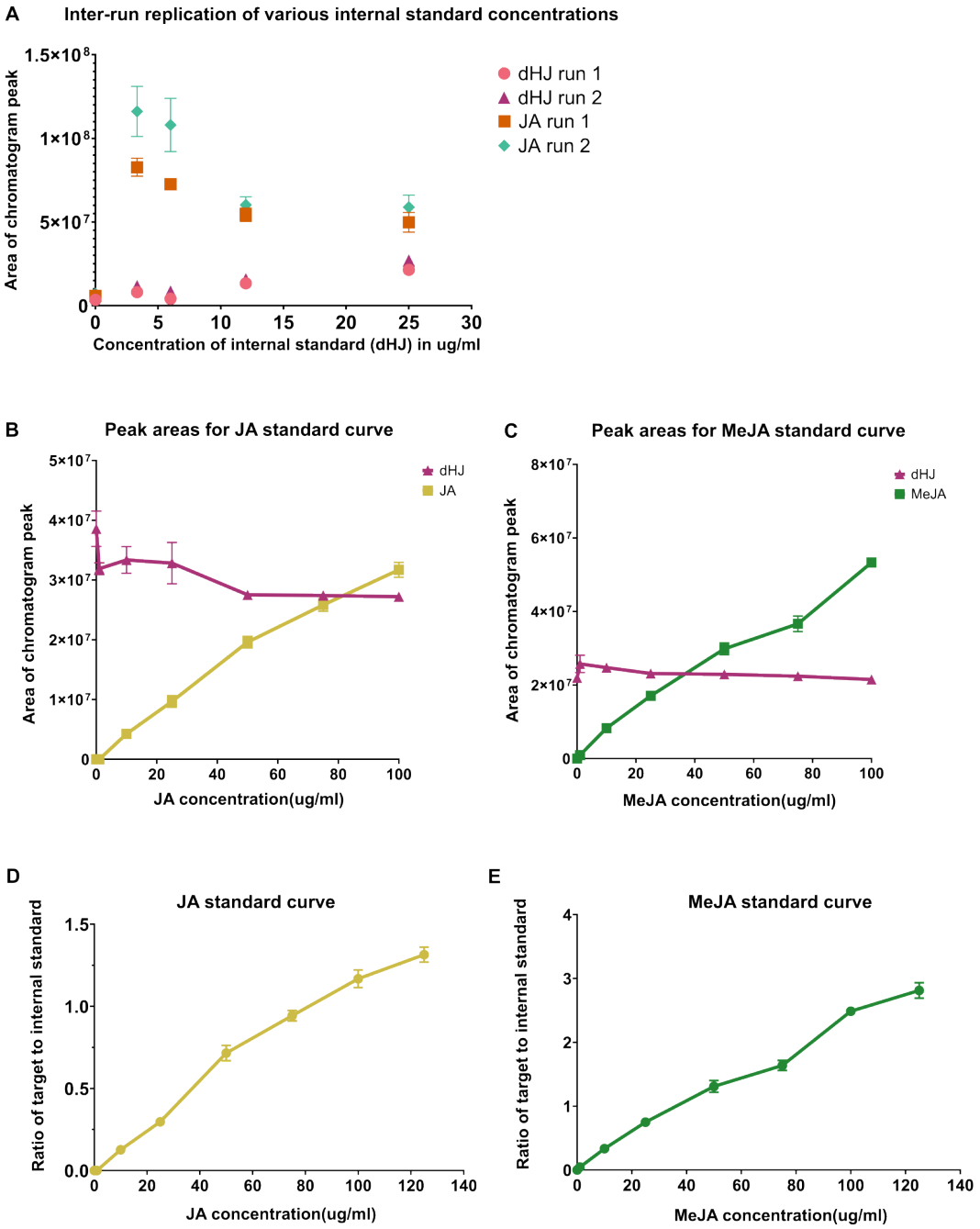


Figure 2.7: Development of standard curves for JA and MeJA via GCMS. **(A)** Inter-run replication of various internal standard concentrations. A set of samples containing 100 ug/ml JA and varying concentrations of internal standard, dHJ, were run twice through. Five replicates per concentration. Error bars show standard deviation. Despite visible variance multiple paired t-tests with Two-stage step-up (Benjamini, Krieger, and Yekutieli) revealed no statistical differences between runs one and two. **(B)** Chromatogram peak areas for dHJ and JA for a typical JA standard curve. **(C)** Chromatogram peak areas for dHJ and MeJA for a typical MeJA standard curve. **(D)** A typical example of a JA standard curve showing the ratio of JA to dHJ. A typical example of an MeJA standard curve showing the ratio of MeJA to dHJ.

Table 2.1: Silicate particle characterization.

Silicate	Shape	Pores	Size	Zeta Potential
HMSNP_V1	Round	Hexagonal porous structure with pores and channels	183 $\pm 26nm$	-29.7 mV
HMSNP_V2	long oblong, folded over, beanshaped	Porous structure along one axis, shape indeterminate	185 nm (17%), 550 nm (83%)	-31.3 mV
HMSNP_V3	short oblong, 'fuzzy' appearance	Some channels, indeterminate surface texture, no obvious pores	200 nm	23.5 mV
Zeolite	crystalline and fractured, irregular shapes	Open crystal structure but no regular pores	280-1700 nm	-4.2 $\pm 2.1mV$

2.3.3 Developing Jasmonate Detection Protocol

Jasmonate detection was attempted with an HPLC, but results were unreliable for MeJA and did not show for JA. A GCMS was then used, following the protocol set out by Rawlinson et al., 2015. The protocol was published as extraction from freeze dried plant tissue. This protocol was adapted to fit extraction from aqueous solutions, with significant loss of sensitivity. Many attempts were required to optimize the protocol for detection of JA and MeJA from aqueous solutions.

As part of the optimization a different internal standard was used from the one in Rawlinson et al. 2015, in this case dihydrojasmone, as used in Huang et al.'s GCMS method for jasmonate detection (Huang et al. 2015).

While optimizing the use of the GCMS different combinations of derivatized dHJ and JA were tested for inter-run replication. An example of this can be seen in panel A of Figure 2.7. There were no statistically significant differences between runs for either dHJ or JA in these experiments.

Once the protocol was optimized it was intended to be used to generate calibration curves for quantification of future JA and MeJA samples. However, both accuracy and precision were difficult to attain. Shown in Figure 2.7 are examples of the peak areas for JA and MeJA and dHJ values used to compute the standard curves, shown in panels B and C, and the standard curves generated by these values, made using the ratio of the JA or MeJA to that of the internal standard, the dHJ, shown in panels **(D)** and **(E)**. There was often variation in these values and despite repeated adjustments and tests to the protocol, the standard curves were unreliable and did not provide suitable accuracy or precision for use in interpolating concentration values from unknown samples. Panel **(B)** shows the chromatogram peak areas for dHJ and JA for the different concentrations of JA used to generate a standard curve. For each JA concentration the a standard concentration of dHJ, 30 ug/ml, was added as an internal standard. As shown in panel **(B)** the peak area corresponding to the dHJ showed large variation, indicating the GCMS method was not producing highly accurate or precise measurements of the dHJ. If it were, each dHJ point should have had the same peak area. The same is shown in panel **(C)**, which shows the dHJ and MeJA peak areas used to produce a standard curve. Again, there is significant variation in the peak area readout for the dHJ values. Panels **(B)** and **(C)** also show the peak areas for JA and MeJA, respectively. If the GCMS method were sufficiently accurate, these JA and MeJA values should produce a straight line. This is not the case for either graph.

Panels **(D)** and **(C)** show the standard curve for JA and MeJA, respectively, as the ratio between the peak area of the analyte of interest – JA or MeJA – to the peak area of the internal standard – dHJ. If the GCMS method were sufficiently accurate, these should also be straight lines. However, in neither case is that true. The data shown in panels (A), (B), (C), and (D) are representative of many attempts at standard curve generation.

Shown in Figure 2.8 are several trial of JA and MeJA volatility over fifty to one hundred hours. Panels A through D show volatility calculated as percentage of initial concentration based upon interpolating concentration from standard curves. JA

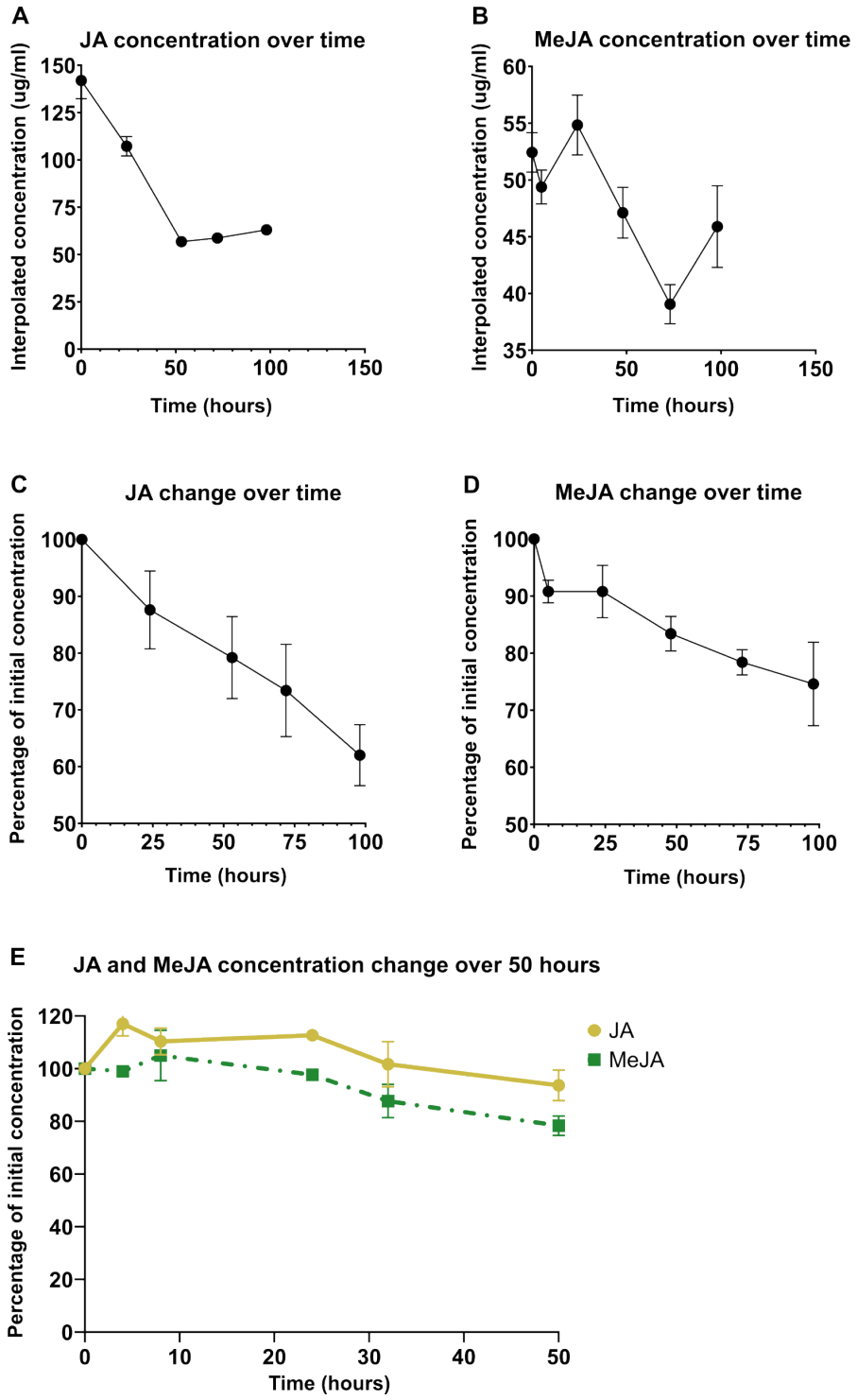


Figure 2.8: JA and MeJA concentration change over time in water, as measured by interpolation from a standard curve and as percentage of initial concentration over time. **(A)** Interpolated JA concentration in ugml over 100 hours **(B)** Interpolated MeJA concentration in ugml over 100 hours. **(C)** JA concentration change over time as percentage of initial concentration, over 100 hours. **(D)** MeJA concentration change over time as percentage of initial concentration, over 100 hours. **(E)** JA and MeJA concentration change over time shown as percentage of initial concentration over 50 hours. Three replicates per time point. Error bars show standard deviation. Panels A, B and E show data from the same experiment. Panels C and D show data from a different experiment.

consistently lost about fifty percent of initial concentration over one hundred hours and MeJA showed approximately twenty five percent loss of concentration over time.

Taken together, these results show that JA and MeJA have an approximately equal level of 'volatility' or rate of evaporation out of a solution. These results also show the large variability, and at times nonsensical data produced by this GCMS method. For instance, in Panel **(A)**, the starting concentration as calculated via a standard curve shows the starting concentration to be 130 ug/ml when it was in fact 100 ug/ml - a result both wrong and physically impossible. Likewise, panel **(B)** shows the interpolated concentration of a sample of concentration 100 ug/ml as 53 ug/ml, which then increases to 55 ug/ml after twenty hours, another incorrect and physically impossible result.

It was determined from this data that quantification via interpolating from a standard curve using this GCMS method was not possible.

In order to better estimate the loss of concentration over time, the peak areas were instead converted into percentage of initial concentration. These outputs are seen in panels **(c)**, **(D)**, and **(E)**. In this case there is still large variability between replicates but there is clearer relationships between time and percent of initial concentration.

Another way to look at the data is without standard curve interpolation just as the area of chromatogram peaks measured over time. In Figure 2.9 volatility is shown over 11 days as the area of chromatogram peak on each day. There were five replicates per jasmonate. At each sampling time point one aliquot was removed and this volume was not replaced so that the total volume of each replicate decreased by 150 ul after each sampling period. Each aliquot had a standard amount of dHJ added before being derivatized and run on the GC-MS. Panel A shows JA change over time, and Panel B shows MeJA change over time. In each panel the internal standard, dHJ, is shown on the left. Despite being prepared in the same way fresh on each day the dHJ values vary widely and some are statistically significantly different. Even accounting for errors in preparation – which were minimized as much as possible – the values for dHJ should be the same on each

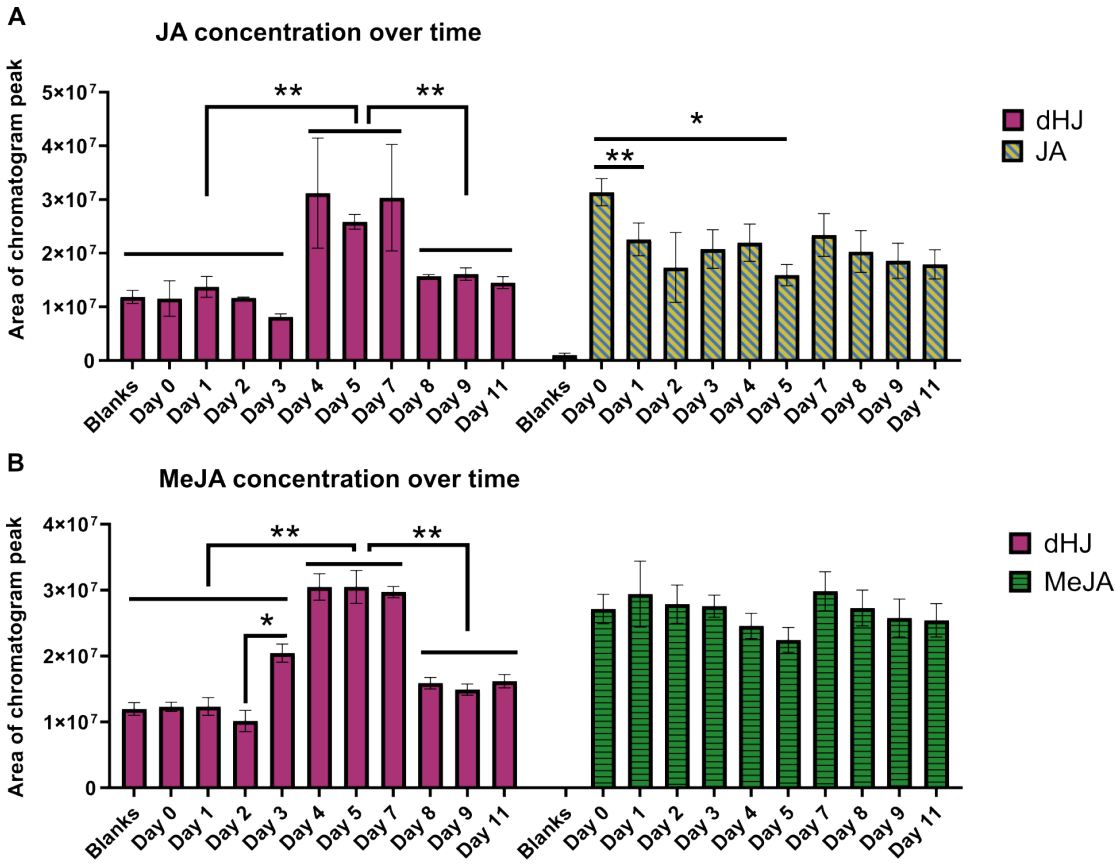


Figure 2.9: (A) Right: blanked areas for jasmonic acid (JA) concentration over time. Left: internal reference concentration for dihydrojasmone (dHJ), not blanked. Days 0 - 5 (and corresponding blanks) and Days 7 - 11 (and corresponding blanks) run on separate GC-MS runs. Results of mixed-effects analysis shown comparing each day within either dHJ or JA values. Right: * p-value < 0.05, ** p-value < 0.005. **(B)** Right: blanked areas for methyl jasmonate (MeJA) concentration over time. Left: internal reference concentration for dihydrojasmone (dHJ), not blanked. Days 0 - 5 (and corresponding blanks) and Days 7 - 11 (and corresponding blanks) run on separate GC-MS runs.

day. This, again, indicates that the method and machine used for GCMS detection could not provide accurate, precise results.

The JA and MeJA values show some variation between days 0 and 11. However, the data appears to show an increase in JA and MeJA after day 5. This is a physical impossibility, as each solution started with the same concentration of JA or MeJA and no jasmonates were added to the samples over the course of the 11 days. Given the previous evidence for loss of concentration due to volatility, shown in Figure 2.8, there should be a steady decrease in concentration and not a plateau. This is further evidence that the GCMS method could not produce accurate or precise results.

However, tests with the GCMS did show that it had a negligible false positive rate (data not shown). So this data does indicate that jasmonates were still present in the solutions up to eleven days after initially being dissolved in solution. This shows that both JA and MeJA show a relatively low volatility – or the ability to evaporate from solution over time.

2.3.4 Loading and Release of Jasmonates into HMSNPs and Zeolite

The GCMS technique used could not accurately or precisely quantify either JA or MeJA. However, the GCMS could detect jasmonates. The GCMS was thus used to (i) determine whether JA and MeJA could be loaded into the HMSNPs or zeolite and (ii) how quickly any loaded jasmonates could be released from the HMSNPs or zeolite.

Nanoparticles or prepared zeolite were sonicated in the loading solutions, a mixture of DMSO, ddH_2O and either JA or MeJA to set concentrations of the jasmonates, and then stirred, sealed, in the dark and at room temperature for 24 hours, before isolating the now loaded silicates via centrifugation. The isolated, loaded silicates were then washed twice with ddH_2O before being frozen and freeze-dried in a vacuum lyophilizer. This two wash steps was the standard approach and what was used to prepare the nanoparticles and zeolite used as seed treatments in later chapters, but as shown by data in Figure 2.11, it was later discovered the number of wash steps directly correlated with the amount of detectable jasmonates released from the loaded zeolite.

The initial aim was to determine loading by measuring the jasmonates present in the loading solution before the particles were added and incubated and then again after twenty-four hours of incubation. Set up in parallel was a control in the absence of particles to account for loss of jasmonates due to evaporation. However, the GC-MS method available was not sensitive enough to show any reliable differences between the control solution and the solutions used to load HMSNPs or zeolite, see Figure 2.10.

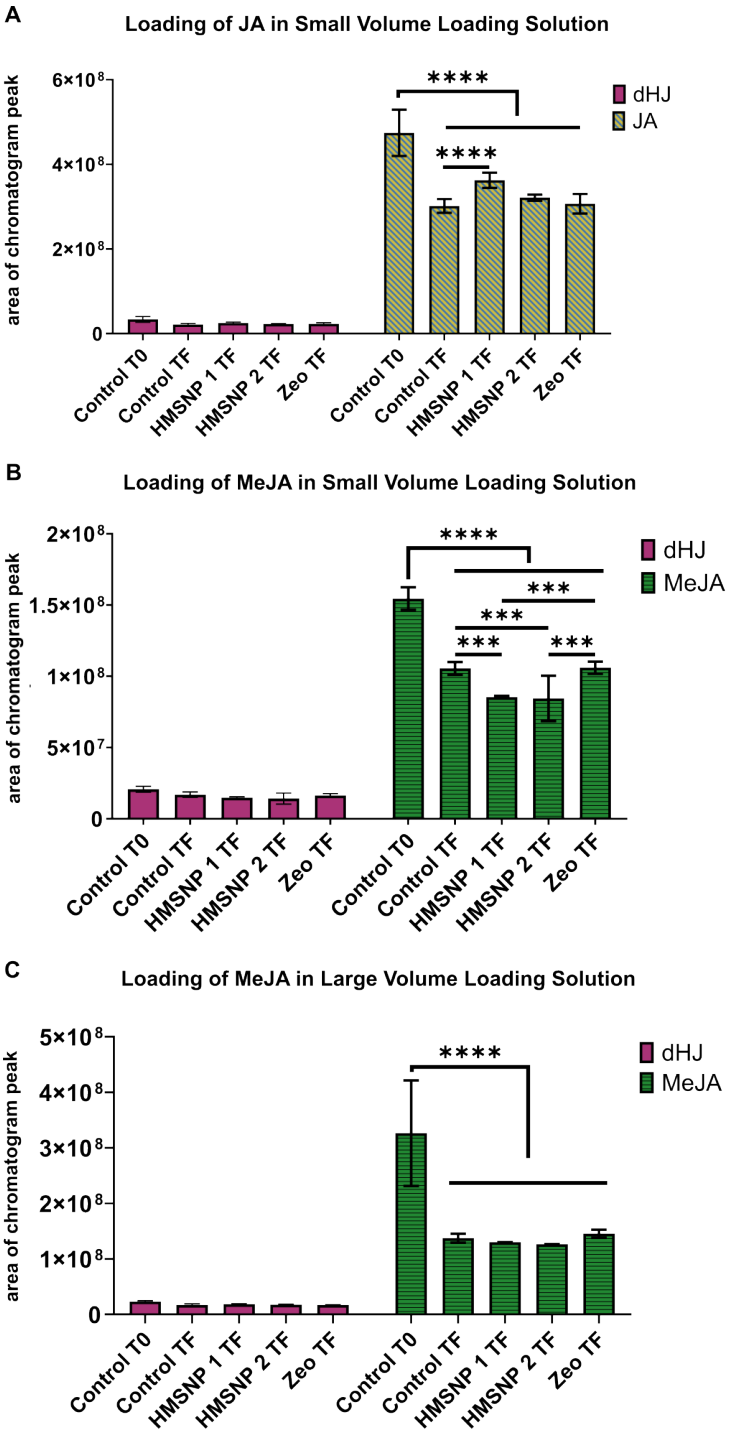


Figure 2.10: **(A)** Loading of JA into different batches of HMSNP or Zeolite using the small volume method. **(B)** Loading of MeJA into difference batches of HMSNP or Zeolite using the small volume method. **(C)** Loading of MeJA into difference batches of HMSNP or Zeolite the large volume method. Three replicates per sample type. Error bars show standard deviation. Results of a two-way ANOVA with the Holm-Šídák multiple comparisons test, where columns marked 'a' are not significantly different from one another. 'b' columns are different from 'a' columns with p-values < 0.001. 'c' columns are different from 'a' columns with p-values < 0.001

In order to confirm if any jasmonates had been loaded into the particles the particles were harvest, washed, freeze dried and then placed in ddH_2O, stirring, in the dark, for twenty-four hours and the final solution tested via GC-MS for the presence of jasmonates. In this instance jasmonates were detected, albeit with low signal - blanks and any background noise was removed from the signals so although low it is highly likely to show real presence of jasmonates and not simple artifacts - from all batches of nanoparticles and zeolites for both JA and MeJA, see Figure 2.11.

Figure 2.10 shows the results of loading tests with different compositions of loading solutions, referred to as 'Small Volume' and 'Large Volume' respectively. These refer to the ratio of DMSO:ddH_2O:particles used in the loading solutions. The different loading solution ratios were taken from Bravo-Cadena 2018.

Panel A of Figure 2.10 shows the loading results of JA with the Small Volume loading solution, where Control is the loading solution set up in absence of particles. T0 refers to the initial measurement of the control solution. And TF shows the final measurement of the solution. If jasmonates had bound to the silicate particles and thus been removed from solution that should be a statistically significant difference between the Control Final Timepoint (TF) and the HMSNP or Zeolite TF, indicating that some jasmonate were removed from the solutions beyond which can be accounted for by evaporation alone. However, this was only seen in Panel B which shows the loading of MeJA in Small Volume loading solutions. For Panels A and C, which showed loading for JA in Small Volumes solutions and loading for MeJA in Large Volume solutions, respectively, there is either no significant difference between Control TF and the any of the silicate particle TFs or, as in the case of the JA Small Volume loading, there is a statistically significant *increase* in JA detected from nanoparticles samples compared to that of the Control TF. As it is baring on physically impossible for jasmonates to be spontaneously created this is taken to be inaccurate and unreliable data.

Also included on the right hand sides of Panels A, B, and C in 2.10 is the dHJ internal standard detected for each of the samples. This is displayed separately

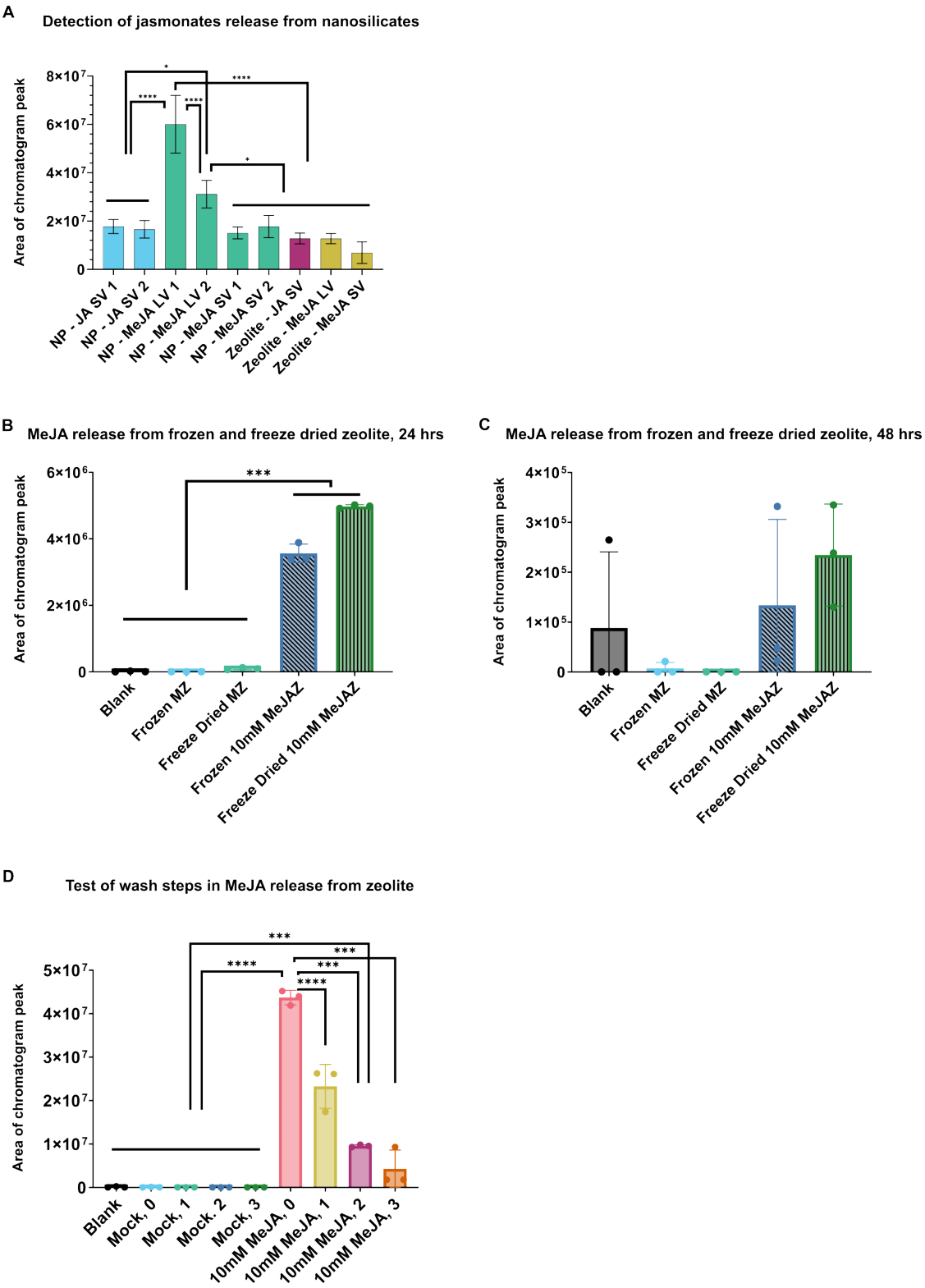


Figure 2.11: **(A)** Release of JA and MeJA from different batches of HMSNP or Zeolite that had been loaded with either small volume or large volume loading solutions. Data shown have been blanked. **(B)** Loading of MeJA into different batches of HMSNP or Zeolite using the small volume method. **(C)** Loading of MeJA into difference batches of HMSNP or Zeolite the large volume method. **(D)** Wash step tests. Three replicates per sample type. Error bars show standard deviation. Results of a two-way ANOVA with with the Holm-Šídák multiple comparisons test, where columns marked 'a' are not significantly different from one another. 'b' columns are different from 'a' columns with p-values < 0.001. 'c' columns are different from 'a' columns with p-values < 0.001.

to show the variation present in the dHJ detection, though none of the variation seen was statistically significant.

Figure 2.11 shows three different types of experiment, used to understand, in Panel A, if HMSNPs and zeolite could load JA or MeJA, and in panels B, C, and D, how different changes to the treatment of the loaded zeolites after loading affected the release of jasmonates from the loaded zeolites.

The experiment in panel **(A)** was to test if jasmonates could be detected from previously loaded, washed, and freeze dried HMSNPs and zeolite. The two different types of loading tests are designated as 'SV' for small volume and 'LV' for large volume are compared with JA and MeJA loading in HMSNPs, shortened to 'NP', and zeolite. The LV test was not completed with JA as it required a prohibitively large quantity of JA.

The results were not compared to one another because of the previously established unreliability in the GCMS method. However, the results were used to confirm that JA and MeJA could be loaded into both HMSNPs and zeolite.

An experiment was run to test the effects of freezing versus freeze drying on the relative amounts of MeJA released from 10 mM MeJA loaded zeolite as well as the approximate time frame in which the release occurred. Previously loaded, washed, and then either frozen or freeze dried zeolite was put in sterile water for up to 48 hours. Soaking solution and zeolite were collected at 48 hrs. Zeolite was then added to new sterile water for a further 24 hours. Panel **(B)** shows the results for detection at 24hrs and panel **(C)** shows the detection results at 48 hours. The results are compared to a blank and a frozen Mock-loaded – has DMSO and water but no jasmonate – and a freeze dried mock-loaded.

The results show that at 24 hours, panel **(B)**, significantly more MeJA has been released from the frozen and freeze dried MeJA zeolite than from the blank and mock controls. While the freeze dried samples visually appear to have released more MeJA than the frozen samples this is not statistically significant.

By 48 hours, panel **(C)**, the same level of MeJA was detected in all the samples, including the blanks and the mocks. The chromatogram peak areas were also very

low, indicating that all of the loaded jasmonates had left the zeolite by the end of 24 hours when the solution was changed.

A final experiment was done to test the effects of the number of wash steps on the amount of MeJA released from freeze-dried zeolite. 0, 1, 2 or 3 wash steps – each consisting of 45 mLs dd_2O – were tested on both 10 mM MeJA loaded zeolite and mock loaded zeolite. The results show no difference between any of the mock samples and the blanks. The results do show a statistically significant difference between the 0, 1, and 2-3 wash steps for the 10 mM MeJA loaded zeolite. There was no statistically significant difference in MeJA released from the 2 and 3 wash step preparations.

2.4 Discussion

Silicate carriers

HMSNP synthesis takes three days and requires specialized equipment and technical expertise. The purpose of this research project is to generate and test a mesoporous silicate seed treatment loaded with jasmonates and test it in planta. Each batch of HMSNPs produces approximately 120 mg of HMSNPs and takes three days. Each treatment group in the in planta pathogen system used in this thesis uses approximately 600 mg of loaded silicate. There were a minimum of five treatment groups per experiment. With three experimental replicates that equates to 216 days required to produce enough HMSNPs for one set of experimental replicates. Ways to minimize the amount of nanoparticles needed were considered, however, the limitations outlined above are the minimum required for robust testing and replication.

As the HMSNPs were being used primarily for their large surface area, zeolite clinoptilolite was tested as a cheap, scalable alternative to the HMSNPs, since the zeolite cost about £10 per kilogram and could be prepared in under ten minutes using a coffee grinder and a sieve.

Additionally, while the reagents required for HMSNP synthesis are relatively inexpensive the process produces hazardous chemical waste. In contrast, the zeolite is similarly inexpensive but can be produced at the gram to kilogram level within one hour as opposed to the HMSNPs which require three days and produces only in the mg scale.

Once it was determined that both JA and MeJA could load into the zeolite preparation, zeolite was determined as the best silicate candidate for moving forward within the time, cost, and equipment constraints of the PhD project.

The first sets of experiments in this chapter were to characterize the nanoparticles and the zeolite preparation. This involved using a Zeta Sizer to determine surface zeta potential, a TEM to determine size and structure and an SEM to determine overall uniformity and to view aggregates of the particles at scale. These characterization methods revealed three major types of particle produced by the HMSNP synthesis reaction. Only one of these was the standard or 'correct' type described in the various sources which use this type of nanoparticle (Hom et al. 2010, Huang et al. 2014b, Cai et al. 2001). 22 batches of HMSNP were trialled before the adjustments required to get reliable standard batches were determined. This involved using a specific stir bar and flask combination, using fresh TEOS, storing the TEOS in an air-tight container, and very accurately measuring the NaOH used – even a drop too much or too little affected the outcome.

This size and shape, round, $\tilde{1}$85 nm in diameter, hexagonal internal porous structure, are one of the most commonly produced sizes of MSNP and allow maximum loading via surface area adsorption while still being small enough to be taken up into most biological tissues, including plant root tissue and various transformed cell lines (Bharti et al. 2015, Hom et al. 2010, Kumar et al. 2001, Sun et al. 2014).

The HMSNP morphologies noted in this section are not without precedent, Cai et al. 2001, which refers to what is here called HMSNP_V1 or correct morphology as MCM-41 silica, showed various types of MCM-41 morphologies depending on the type and quantity of the solvent - they used either sodium hydroxide as was used here

or aqueous ammonia - and the temperature of the reaction (Cai et al. 2001, Zhao et al. 1996). Additionally, Candela-Noguera et al. 2024 recently published and in depth study of the factors afffecting MCM-41 morphologies (Candela-Noguera et al. 2024).

MCM-41 has an interesting history, it was first developed by the mobile corporation as mesoporous molecular sieve to replace zeolites for catalytic cracking, but quickly became a whole class of molecular sieves and nanoparticles, all based upon the MCM-41 structure and synthesis method and used for a huge variety of applications, including in catalysis and separations (Beck et al. 1992, Zhao et al. 1996). The liquid crystal method, now called sol-gel, first creates micelle formation using a surfactant. The micellular rods then form into a hexagonal array and the addition of a silica precursor plus a catalyst causes calcination of silica over the template. Finally, the template is removed to create hollow porous particles - see Figure 1 in this chapter. These 'zeotype' materials took off and within a few years over a hundred papers had been published elaborating on and adapting this basic structure to create whole classes of particles (Zhao et al. 1996, Kumar et al. 2001). Variations of MCM-41 nanoparticles have been used for drug delivery as early as 2000. Their large surface area to volume ratio, mesoporous size, and the relative ease of adding surface modifications has made them a key technology for encapsulation and delivery of compounds in biological systems (Kneuer et al. 2000, Bhattacharyya et al. 2006, Bharti et al. 2015, Trewyn et al. 2007).

Quantification of Jasmonates

One of the initial aims of the project was to understand the volatility, in this case defined as the rate of concentration change over time, for JA and MeJA in aqueous solutions. This is turn can help elucidate the dynamics of seed exposure to JA and MeJA in both soaking seed treatments and combined nanoparticle-jasmonate or zeolite-jasmonate seed soaking treatments.

GC-MS systems are not ideal for high resolution detection and quantification of jasmonates, but the combined method of Rawlinson et al. 2015 and Huang et al. 2015 provides a non-deuterated set up for detection of jasmonates on GCMS

using dihydrojasmone as a derivatizable internal standard. Many of the jasmonate detection protocols involved extracting from freeze dried plant tissue, including Rawlinson et al. 2015, but for the purpose of this project the extraction was adapted to aqueous solutions containing jasmonates, which likely accounts for both some of the low precision and low accuracy results obtained with this method. Additionally, the GC-MS available for use was older and used for a variety of compound detection, incurring heavy use, and thus less able to produce high accuracy results.

As part of this aim, several sets of assays were performed to measure volatility for JA and MeJA. A lot of time ($>$ 10 experiments spread over 8 months) was spent adapting and optimizing a GCMS based method for detecting and quantifying jasmonates. The method could reliably detect jasmonates but quantification proved challenging. Standard curves were generated for each of the jasmonates prior to each volatility experiment to try to determine the concentration changes. Figures 2.7 and 2.8 contain typical examples of these experiments. In both figures high variability is seen both for the internal standard, dHJ, and JA or MeJA, in all points across the standard curve. To test the accuracy of the standard curves measurements of known concentrations of JA or MeJA were interpolated from the standard curves. These resulted in wildly inaccurate results, some showing 30% higher values than the actual value and some showing nearly 50% less than the actual values. This range of variation is unacceptable for quantification. Several changes to both the extraction method and the GCMS method were trialled but none of these resulted in major improvements to the outcomes. This lack of sensitivity and accuracy is likely due to several factors. One, is that the original extraction method was designed for extracting out of freeze-dried plant tissue and not aqueous solutions. Sensitivity could be lower when extracting from dilute aqueous solutions versus in dry tissues. Lower sensitivity can affect the accuracy of quantification (Fialkov et al. 2007). GCMS requires high analyte volatility to get full separation and thus detection on the gas chromatography. This oftens requires derivatization of hydrophilic groups with a silylating agent – in this work, methyl chloroformate (Balcke et al. 2012). This requires a lot of starting material to raise the signal within dynamic range

of the mass spectrometer. One way to overcome these limitation is through the use of high performance liquid chromatography (HPLC) which eliminates the need for derivatization coupled with tandem mass spectrometry (MS/MS) (Balcke et al. 2012). Unfortunately, the only system available for the duration of this project was a GC-MS and not an LC-MS/MS. The GCMS method and system used in this project could not obtain accurate or sensitive quantification. This is particularly clear in Figure 2.9 in which there is (i) large and statistically significant variability in internal standard concentration, despite the internal standard always being prepared and added at the same concentration, and (ii) in which there appears to be jasmonate accumulation in a system in which that is physically impossible. A known starting concentration of jasmonate was added to an aqueous solution and measured over 11 days – the jasmonate concentration could only stay the same or decrease. No jasmonates could be made in the system nor were any jasmonates added to the system over time. However, some estimates of jasmonate presence and relative rate of concentration change still needed to be made. Despite its lack of accuracy and sensitivity, the GCMS system had a nearly negligible false positive rate for jasmonates (data not shown) and could therefore be used to determine whether or not jasmonates were present in a given solution.

Taken together, the data in Figures 2.9, 2.7, and 2.8 show that while JA and MeJA can be detected reliably using the GC-MS but not for accurate or precise quantification.

The data gathered indicates that JA and MeJA, while often referred to as volatile plant hormones, are still detectable with fairly strong signals up to day eleven post being place in aqueous solutions. This is important information for understanding the loading and release of the jasmonates into the nanoparticles or zeolites as well as for understanding if and when seeds might receive a dose of jasmonates in a seed treatment.

The most accurate and robust methods for detecting jasmonates are via LC-MS/MS as methods like HPLC, ELISA, and GC-MS have either limited sensitivity and/or require derivatization.

To better characterize the volatility, loading and release of jasmonates from the silicates in future a method using solid phase microextraction or monolithic material sorptive extraction combined with GC-MS (Meyer et al. 2003, W. Ma et al. 2013) or a method utilizing liquid chromatography tandem mass spectrometry (Liu et al. 2010, Forcat et al. 2008, Wu and Hu 2009) should be used instead of the GC-MS method used here. This would overcome the limitations of the GC-MS system used in these studies and likely allow for precise quantification of loading and release.

Loading and Release of Jasmonates onto Silicate Carriers

The next portion of the project was to attempt to characterize the loading and release of jasmonates from the HMSNPs and zeolite. This was made somewhat difficult due to the lack of accurate and precise GC-MS detection, but some estimates of (i) whether jasmonates can be loaded into HMSNPs or zeolite and (ii) how quickly any loaded jasmonates release from HMSNPs or zeolite were made using this technique. Different loading solution ratios were also tested, as previous work by Bravo-Cadena 2018 indicated that different water:DMSO:compound-of-interest ratios could produce better loading results (Bravo-Cadena 2018).

The data in Figures 2.10 and 2.11 show the result of these loading and release tests for HMSNPs and zeolite. HMSNPs and zeolite loaded using either the SV or LV loading solution ratios with either JA or MeJA were compared. Included in all the graphs is the internal standard data as a control. In this case loading was estimated by setting up a control solution which was the same as the loading solutions but did not contain any silicate particles. An initial and a final measurement were taken from these controls to account for any jasmonate concentration lost to volatility. The silicate particles were loaded and then removed from the solution. This remaining solution was then tested for jasmonate concentration. By comparing the jasmonate detection in the loading solutions post-loading to that of the control at the final time point an indirect measure of loading into the particles could be estimated. Because quantification could not be determined jasmonate detection was measured as the area of the chromatogram peak.

However, the GCMS system did not have the accuracy or sensitivity to do this, as seen in the data in Figure 2.10, where only two loaded-silicate samples show a lower detection level than the control at the final timepoint – see panel B.

To then confirm that some jasmonates were loaded onto the silicates a release test was needed. In the release tests, shown in panel A of Figure 2.11, the silicate samples from the loading tests were washed and freeze dried before being added to an aqueous solution for unloading. The particles were stirred, sealed, for 24 hours before being removed from solution by centrifugation. 24 hours was used because this was the length of time chosen to test the soaking seed treatments. The solution was then tested for the presence of jasmonates. This showed that both JA and MeJA had been loaded onto both HMSNPs and zeolite.

Figure 2.11 also shows two additional experiments measuring release in panels B, C and D. Panels B and C show an experiment where freeze drying vs freezing the particles post-loading was measured. This was to see if there were any large differences in the amount of MeJA retained on zeolite with or without freeze drying and whether this affected release post 24 hours.

Unfortunately, due to the lack of sensitivity and accuracy in the GCMS method, no real differences can be interpreted between loading in freeze dried versus frozen zeolite. However, the results do show that all loaded MeJA was released within the first 24 hours. The water was changed after 24 hours and the 48 timepoint saw no difference in MeJA detected between blanks, mocks, and loaded particles. This helped determine the length of seed soaking treatment used in the following chapters. A 24 hour soak was used because all jasmonates loaded onto the particles would have been released into the solution and thus been available to be taken up by the plant. Towards the end of the project more considerations on how to best prepare the loaded zeolite emerged. One factor that could affect the amount of MeJA released by the loaded zeolite would be the number of wash steps used after loading. Figure 2.11 panel D shows the results from a test comparing 0 - 3 wash steps for both mock loaded and 10 mM MeJA loaded zeolite. The results indicate fewer wash steps result in more MeJA release. Given the lack of sensitivity

and accuracy in the GCMS results, these data can only be estimates, but they do indicate that moving from two to one wash step could result in more MeJA being released to the plant during the seed soaking treatments. In all of the tests done in Chapter four with MeJA loaded zeolite the zeolite was prepared with two wash steps. It was hoped there would be time to test a single wash step preparation in the non-sterile soil systems tests of Chapter 4, but unfortunately the project ran out of time and funding before this could be tested.

No surface modifications were added to either the nanoparticles or the zeolites. Any loading or unloading of the jasmonates was presumed to occur due to adsorption and desorption. It was assumed that surface area was a key factor in the relative amounts of jasmonates loaded or unloaded. However, pore size, density of pores (porosity), surface charge, loading compound properties – lipophobic, hydrophobic, hydrophilic, etc– and solvent properties – polarity, vapor pressure, eluotropic value, etc – can all affect loading and unloading in silicates (Seljak et al. 2020, Riikonen 2018, Guo et al. 2021).

Jasmonate-Loaded Zeolite

A key novelty of this work was the development of jasmonate-loaded zeolite and different considerations to its preparation. There are no studies of jasmonates loaded into zeolites but there are examples of jasmonates being loaded into PLGA nanocarriers for use in grapes, though this was tested only in cell culture and did not include any silicates (Chronopoulou et al. 2019). Zeolites are commonly used as carriers and nanocarriers for ammonia and phosphorus in fertilizer formulations; several patents exist on zeolite formulation for fertilizer as well as various unpatented commercial products (Foereid et al. 2019, Sharma et al. 2022). Zeolites can also be modified or synthesized for specific characteristics, including better loading or sorption of agrochemicals and fertilizers and there is robust literature on synthetic zeolites (Andrunik et al. 2023, Cieśla et al. 2019).

The zeolite used in these experiments was produced rapidly and had a large variety in size, see Figures 2.5 and 2.4. Some of the variability in loading and even the

comparatively low loading of the zeolite versus the HMSNPs could be due to these large variations in particle size. Standardizing the zeolite preparation by grinding for longer, using only the smallest particle size range, and further narrowing the sizes by additional filtering could produce a product with both more consistent particle size and surface area, and may improve the loading efficiency of the zeolite preparation.

2.5 Summary

Investigations in this chapter adapted and optimized a GC-MS detection technique for jasmonates in aqueous solutions, synthesized mesoporous nanoparticles in a variety of morphologies, and adapted zeolite clinoptilolite for loading with jasmonates to test as a seed treatment preparation. This included characterizing the nanoparticles and zeolite preparation as well as attempting to characterize the loading and unloading of jasmonates from both types of silicates. Due to cost, time, and equipment restrictions, the jasmonate loaded zeolites will be the primary seed treatment tested in future chapters.

3

Development of non-sterile soil *Pisum sativum* and *Pythium ultimum* Disease System with Jasmonate Treatments

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3.1 Introduction

A key component of the project was to establish a damping off disease system in order to test the effects of jasmonates and jasmonate based seed treatments. *Pythium ultimum* was chosen as the damping off pathogen and pea, *Pisum sativum*, was chosen for the model plant. In addition to the growth and disease assay RT-qPCR was also used to detect gene expression changes.

3.1.1 Jasmonates and Plant Growth and Development

Jasmonic acid and its precursors and derivatives, here referred to as jasmonates, were first discovered in 1957 through detection of MeJA, which is a significant aromatic component in several plant essential oils, notably *Jasminum grandiflorum* (Demole et al. 1962). Its initial functions were characterized as senescence-promoting and growth-inhibition in 1980 and 1981 (Ueda and Kato 1980, Dathe et al. 1981). It was not until 1990 that its accumulation in response to wounding, and thus its role in plant defense, was described (Farmer and Ryan 1990).

Since then the potential roles of jasmonates in the literature have expanded, and there is evidence both that MeJA and JA can induce senescence in leaves and that they are formed as by-products during leaf senescence (Kim et al. 2015, Seltmann et al. 2010, Zhu et al. 2015). JA application can inhibit elongation in seedlings and can impair root and stem growth (Han et al. 2023, Corti Monzón et al. 2012). This role in growth inhibition likely plays a role in defense responses, signalling plants to reduce growth to focus on defense (Li et al. 2022).

Jasmonates play multiple roles in seed germination. Jasmonate have been observed to induce germination in dormant seeds and also to inhibit germination in non-dormant seeds (Yildiz et al. 2008, Worrall et al. 2012, Finch-Savage et al. 1996).

Jasmonates also interact with growth patterns and root and stem architecture. JA has been linked to phenotypes like coiling, adventitious and lateral root formation, gravitropic responses, and even fibre elongation in cotton plants (Gutjahr et al. 2005, Hu et al. 2016, Cai et al. 2014, Weiler et al. 1993).

3.1.2 Jasmonates and Plant Disease

JA's role in defense appears to be primarily in response to necrotrophic pathogens or herbivory, though it has also been shown to mediate rice plant defense against *Xanthomonas oryzae*, a biotrophic pathogen (Howe and Jander 2008, Campos et al. 2014, De Vleesschauwer et al. 2013, Lu et al. 2015). This is contrasted by salicylic acid (SA) which is a major regulator of defense against biotropic and hemibiotrophic pathogens. Canonically SA and JA are considered antagonistic in regulating defense responses but there is evidence that both hormones can confer drought, salinity, and cold tolerance (Górnik et al. 2014, Khan et al. 2012, Sayyari et al. 2011, Pieterse et al. 2012). Additionally, there are a whole suite of regulatory proteins that respond to both SA and JA, allowing fine-tuning of defense responses under a variety of environmental and pathogen conditions (Wasternack and Hause 2013, Shim et al. 2013).

3.1.3 *Pythium ultimum* and damping off disease

Oomycetes are eukaryotic organisms which resemble filamentous fungi but are Stramenopiles in the proposed chromista kingdom which also contains brown algae and diatoms (Cavalier-Smith and Chao 2006, Riisberg et al. 2009). *Pythium* spp are soil-borne oomycetes which cause root rot or damping off disease in over 300 host species (Farr and Rossman 2024). Root rot and damping-off are disease symptoms characterized by non-germination, prevention of emergence after germination, rotting of seeds, and rotting and collapse of seedlings at soil level (Lamichhane et al. 2017). Damping-off was first coined as a term in horticultural manuals in the early 20$^{\text{th}}$ century (Hartley and Pierce 1917, Hartley 1921, Tillotson 1917). Crop losses due to *Pythium* and other damping off diseases include loss of seeds and seedlings,

costs of replanting and lower yields due to later planting dates. One literature review estimated incidence of damping off disease worldwide varies from 5-80% (Lamichhane et al. 2017). *Pythium* spp. alone are responsible for up US $25 million loss in corn production annual in the United States and Ontario (Bickel and Koehler 2021) and up to 60% loss at seedling stage in tomatoes, chillies and aubergine just within the Indian subcontinent (Manoranjitham and Prakasam 2000, Jadhav and Ambadkar 2007). Losses due to *Pythium* infection were estimated at over US $1 billion in 2003 and since then more host plants and losses due to *Pythium* have been reported in the scientific literature (van West et al. 2003, Farr and Rossman 2024). The economic impact of *Pythium* diseases is therefore significant and a growing threat to food security and other agronomically important crops.

Effective management of *Pythium* diseases requires an integrated management approach, where cropping practices, seed treatments, use of resistant or tolerant cultivars, and intervention with chemical or biocontrol agents are employed in conjunction with one another.

Chemical and biocontrol agents include traditional pesticides and fungicides but also include adding beneficial microorganisms and or activation of host plant defenses.

3.1.4 Disease resistance and gene expression

Like all organisms, plants change their gene expression, i.e., which signalling pathways they are activating and which proteins they are producing, when faced with environmental changes or stressors. This allows them respond in near real time, minutes, and over longer time periods, days to weeks to months, to changing abiotic and biotic conditions (Larrieu et al. 2015, Koo et al. 2009). This is a crucial ability in all species but even more so in plants, as they are sessile and unable to move away from stressful environments or towards ideal environments (van Loon 2016, Gatehouse 1997).

Jasmonic acid and its derivatives are part of larger signalling networks within the plants which allow environmental signals like water stress, heat stress, herbivory,

and the presence of pathogens to be translated into cellular responses (Spoel et al. 2007, C. Wasternack and Hause 2013). Rapid gene expression changes occur in response to jasmonic acid signalling which can allow the plant to produce defense compounds, initiate callose or lignin synthesis, activate reactive oxygen species, or regulate other hormone or growth factors (Kaur et al. 2022). These gene expression changes can be observed through detection of mRNA.

Techniques like reverse transcriptase polymerase chain reaction allow detection and quantification of mRNA and study and comparison of gene expression under different conditions. This provides valuable insight into the regulatory and signalling mechanisms behind abiotic and biotic stress responses but can also serve as a detection method to see if key pathways or responses have been induced without having to extract any downstream defense compounds or protein production from plants. It also allows detection of cellular responses before visible phenotypes occur (Mai et al. 2014, Knopkiewicz and Wojtaszek 2019).

In this study, RT-qPCR is used for two purposes, (i) to see if the plants are showing upregulation of jasmonate-related genes after exposure to the seed treatments and (ii) to better understand the molecular basis for any disease resistance or phenotypic changes that might be observed in response to the seed treatments.

3.1.4.1 Jasmonate related gene expression

Figure 3.11 shows a simplified version of the JA perception, biosynthesis, and activation pathway. JA biosynthesis occurs in the chloroplast where membrane lipids are converted to alpha linolenic acid which is then converted into a key JA precursor OPDA by the lipoxygenase (LOX) genes. OPDA then undergoes beta oxidation into JA (and somewhat interchangeably MeJA) which are bound by the Jasmonate Response Locus genes, in peas primarily JAR4/6, which catalyze JA into JA isoleucine, the primary bioactive form of JA. JA-Ile then binds to COI1, which then can bind to the JAZ protein complex, triggering release of transcription of jasmonate responsive genes and the degradation of COI1. One set of jasmonate response genes that are released by the degradation of COI1 and

release of JAZ binding are the MYC2 (myelocytomatosis) transcription factors, which regulate many key downstream jasmonate responses. DRR230 is a pea defensin gene which has been shown to have broad host-range antifungal activity and which is downstream of jasmonate response genes (Lai et al. 2002, Almeida et al. 2000, Hadwiger 2008, Lacerda et al. 2014).

3.1.5 Aims

A damping-off disease assay was required to test the efficacy of the proposed JA and MeJA treatments. *Pythium ultimum* was chosen because it infects peas and is a well-established pathogen of over 300 crop species (Verbeek et al. 2019, Agrios 2005). As it is an oomycete, it does not produce aerosolized spores but instead produces oospores in water or dry medium which means it is both easier to contain and quantify than many plant pathogenic fungi. *Pythium ultimum* is a necrotrophic pathogen and thus relevant to JA-based seed treatments. The work in this chapter aimed to establish a *Pisum sativum-Pythium ultimum* pathosystem and use that pathosystem to test the effects of jasmonates. Additionally, RT-qPCR was used to look at any gene expression changes in response to the jasmonate treatments.

3.2 Materials and Methods

3.2.1 *Pythium ultimum* culture

Three strains of Pythium ultimum were obtained (p194, p195, and p220; a kind gift from the University of Warwick). Both p194 and p195 were isolated from carrots and p220 was isolated from parsnips. *Pythium ultimum* was grown at 22°C on potato dextrose agar (PDA) or corn meal agar (CMA) on 90 mm tissue culture dishes for 3 days, then switched to PDA for the experiments. PDA, CMA and Potato Dextrose Broth (PDB) were purchased from Merck Millipore, Watford, UK.

3.2.2 *Pythium ultimum* DNA extraction and Sequencing

Strains arrived on CMA plates and were stored at 4°C. To confirm that the strains were *Pythium* cultures were grown for DNA extraction and Sanger sequencing.

Mycelial mats were grown for DNA extraction by propagation using a small agar cube from the original culture plants on liquid Potato Dextrose Broth (PDB). The mats were grown for 1-2 days or until mycelial mats were $\tilde{4}$ cm in diameter. The mycelial mats were carefully removed from the liquid using sterile forceps and placed on sterile filter paper. The paper was folded and gently blotted using blue roll. Once excess moisture was removed, the pressed mycelium was moved into a two ml Eppendorf. Three to four holes were made in the lid using a sterile needle. The sample was then freeze-dried for 12 hours. The freeze-dried *Pythium* was stored at -20°C.

Total DNA was extracted using the QIAGEN Purification of Total DNA from Plant Tissue - Mini Protocol (QIAGEN Sciences, Manchester, UK). Extract quality and concentration were measured on a Nanodrop Microvolume Spectrophotometer (Loughborough, UK), data not shown.

A PCR using Meridian Bioscience MangoTaq DNA polymerase (London, UK, Cat. No. BIO-21083) was performed using the following universal internal transcribed spacer (ITS) region primers:

ITS1 (F) : TCCGTAGGTGAACCTGCGG

ITS4 (R) : TCCTCCGCTTATTGATATGC

The cycle conditions of the PCR were: 1x (94°C 2min), 40x (4°C 35s, 55°C 55s, 72°C 60s), 1x (72°C 10min, 12°C hold) (White et al. 1990, Gardes and Bruns 1993). Primers were ordered from Invitrogen (Therm Fisher Scientific, Loughborough, UK).

The PCR products were run on a 1% agarose gel with 1X Tris- acetate - Ethylenediaminetetraacetic acid (TAE) Buffer plus 0.4 μg/ml ethidium bromide. Product bands were visualized using a UV gel imager, cut out using a sterile razor blade, and extracted using QIAquick PCR Purification Kit (QIAGEN, Manchester, UK). The extracted DNA concentration was then measured on a NanoDrop Microvolume Spectrophotometer (Thermo Fisher Scientific, Loughborough, UK). before being sent to Source Bioscience (Source Bioscience, Nottingham, UK) with the appropriate primers - see ITS F and R primers above - for Sanger sequencing.

The resulting .seq files were checked for quality in ApE Plasmid Editor (Davis and Jorgensen 2022) and aligned using a nucleotide query using NCBI BLAST and the standard NCBI nucleotide database (Altschul et al. 1990, NCBI 1988).

3.2.3 Disc diffusion assays

Pythium ultimum was grown on PDA for four days at 25°C. A 1 mm^2 cube of pre-grown *Py. ultimum* was then placed in the center of either a PDA plate or a 2% (w/v) water agar (WA) plate. Blank, 6.25 mm diameter sterile sensitivity discs (Sigma-Aldrich, Gillingham, UK) were soaked in either water, 1% (v/v) ethanol, 1.0 mM JA, 10.0 mM JA, 1.0 mM MeJA, or 10.0 mM MeJA solutions for five minutes until saturated. A 1% ethanol disc and a treatment disc were then placed equidistant from the agar cube onto each plate. The jasmonate treatments were made up with a small amount of ethanol for solubility in water, the highest ethanol concentration in the jasmonate treatments was 1% in the 10.0 mM treatments. Each plate had a control disc of 1% ethanol as well as a test condition - either water or one of the jasmonate treatments. There were five replicate plates per test condition and five plates containing only water-treated discs as a control for *Pythium ultimum* growth.

2% WA plates were grown for five days at 25°C. PDA plates were grown for three days at 25°C. Plates were imaged on the final day of growth.

3.2.4 In vitro pathosystem

The in vitro pathosystem was adapted from Benavent-Celma et al 2021, following the nomenclature used in that paper (Benavent-Celma et al. 2021). This system is referred to as in vitro because the *Pythium* and plants are grown on tissue culture plates instead in soil. This differentiates it from the sterile soil system, where the pathogen and plant are grown in magenta vessels in a sterile soil system and the non-sterile soil system where the pathogen and plant are grown in trays in a non-sterile soil system. Please see Figure 3.1 for a visual of the in vitro system set up.

Pisum sativum Kelvedon wonder seeds were surface-sterilize seeds in 2.5% (v/v) sodium hypochlorite for five min. Standard over-the-counter bleach is 4.5%

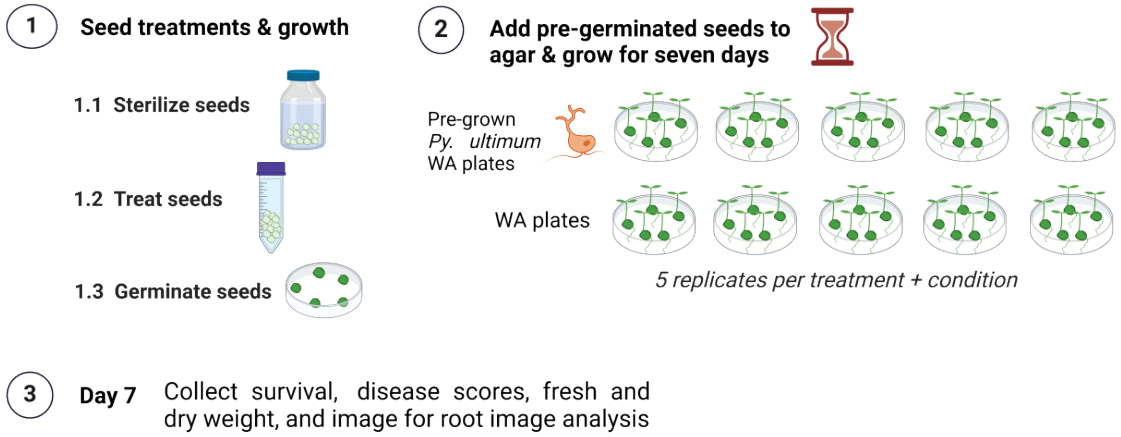


Figure 3.1: Set up of the in vitro *Pisum-Py* system. Step one, sterilize, treat, and germinate seeds. Step two, add the pre-germinated seeds to 2% water agar plates either with or without a two day pre-growth of *Pythium ultimum* and grow for seven days. Step three, collect survival percentages per plate, disease scores of seedlings, fresh weight, image seedlings for root analysis, and dry seedlings to obtain dry weight. These results are then averaged per plate, with five replicate plates per treatment and condition combination

(v/v) sodium hypochlorite so 2.5% sodium hypochlorite was prepared as a 50:50 bleach:sterile MiliQ double distilled water (ddH_2O) solution. Seeds were washed three times in sterile ddH_2O and soaked in either sterile ddH_2O or a 1.0 mM JA, 1.0 mM MeJA, or 0.1% ethanol solution for 24 hours in the dark at 25°C. Imbibed seeds were placed on 2% water agar (WA) in 90 mm diameter Petri dishes at room temperature for two days (pre-germination).

Py. ultimum was grown on 90 mm PDA plates in the dark at 25°C for 2 days until the mycelia covered the plate. Five pre-germinated seeds were placed equally spaced on the pre-grown *Pythium* culture surface. For controls, five pre-germinated seeds were placed on 2% WA without *P. ultimum* cultures. The plates were sealed with two rounds of micropore tape (3M, Binfiled, UK) and then incubated at 25°C with a 12-h photoperiod at a light intensity of 30–50 µmol/m2/s for 7 days. 5 replicate plates were used per treatment and culture condition combination. The experiment was repeated a total of two times.

On day seven the plants were harvested, survival and disease severity evaluate and seedlings weighed for fresh weight, imaged for root length, and dried at 60°C in a Gallenkamp Hotbox (Gallenkamp, Cambridge, UK) for one to two days until

constant weight before recording final dry weight.

The root images were analyzed using RhizoVision Explorer v2.0.3 (Seethepalli and York 2020) using algorithms described by Seethepalli et al. 2021 (Seethepalli et al. 2021). Roots were imaged in 24 bit color with images saved as .tif files at 400 DPI. The images were then converted to black and white and inverted for analysis. The following settings in RhizoVision Explorer were used in all root analyses for all in vitro, sterile soil, and non-sterile soil assays to keep root measurements consistent across experiments: Broken roots selected, pixels converted to physical units where 23 pixels equaled 1 mm, image thresholding set to 176, non-root objects filtered and maximum size set to 2 mm^2, holes in root object filled and maximum size set to 1 mm^2, root pruning threshold set to 12, and initial root diameter range set to 0.00 - 0.30.

Disease Severity (DS) was assessed by visual evaluation of each seed on the day of harvesting. Seeds were rated for the presence of rot using a categorical 0–4 disease severity scale where 0 = seed germinated without visible infection and symptoms (a seed is defined as germinated when the primary root length equals the seed length); 1 = seed germinated with reduced growth and light discoloration or lesions; 2 = seed germinated with severely reduced growth and strong visible discoloration and lesions on roots; 3 = seedling died after germination with partial colonization by mycelial growth; 4 = seed died before germination with complete colonization of seed by mycelium.

The disease severity scores were converted to a continuous Disease Severity Index (DSI) value per plate, using the following formula:

$$\text{DSI} = \frac{\sum \text{Severity rating x seeds per rating}}{\text{total seeds x highest severity rating}} \times 100$$

3.2.5 Sterile soil pathosystem

The sterile soil pathosystem was adapted from Benavent-Celma 2021 (Benavent-Celma et al. 2021).

Sterile soil assays were carried out in Magenta boxes (74.79 × 74.79 × 99.08 mm) containing 70 g sterile substrate (All Purpose Growing Medium, Sinclair). Boxes were autoclaved with the growing medium for 60 min two times, at an interval of 24 hr.

Seeds were surface sterilized, washed, and soaked overnight in either control or jasmonate solutions as in the plate growth system. The imbibed seeds were then placed on 90 mm 2% WA plates and sealed with two rounds of micropore tape. The plates were then incubated in the dark at 25°C until germination, usually between 2-4 days.

Py. ultimum was grown on PDA in the dark at 25°C for seven days. Mean while, 200 g millet (50 g per flask for 4 total flasks) was autoclaved for 60 min two times, with a 24 hr interval in between runs. 150 mL 20% unclarified V8 broth (Campbell Soup Company, Nottingham, UK) (35 ml per flask for 4 total flasks) was autoclaved once for 15 min.

Py. ultimum inoculum was prepared as follows: Six 5 mm^2 plugs from the seven day *Py. ultimum* cultures were added to 150 mL glass vessels containing 50g autoclaved millet grains and 35 mL 20% unclarified autoclaved V8 broth under sterile conditions. The flasks were incubated in the dark at 25°C for 15 days prior to soil inoculation. Cultures were shaken vigorously by hand every two days to ensure homogeneous distribution of inoculum growth.

Magenta boxes were prepared as follows: to 70g sterile substrate 1g of *Py. ultimum* inoculum was added and mixed with a sterile spoon to to ensure homogeneity of the inoculum in the soil.

One germinated seed was placed in each Magenta box, inserted 3 cm into the soil and lightly covered. The magenta box lids had two holes drilled into the top which were covered by sterile 3 uM sticky filter discs (Biomed Solutions, Sawbridgeworth, UK). The vessels were incubated at 25°C with a 12 h photoperiod

at a light intensity of 50-70 µmol/m2/s for 10 days. Plants were watered as needed to retain soil moisture. Eight replicates were set up for each treatment - jasmonate or control - and condition - *Pythium* or Soil only - combination. For an overview of the set up of the sterile soil system see Figure 3.2.

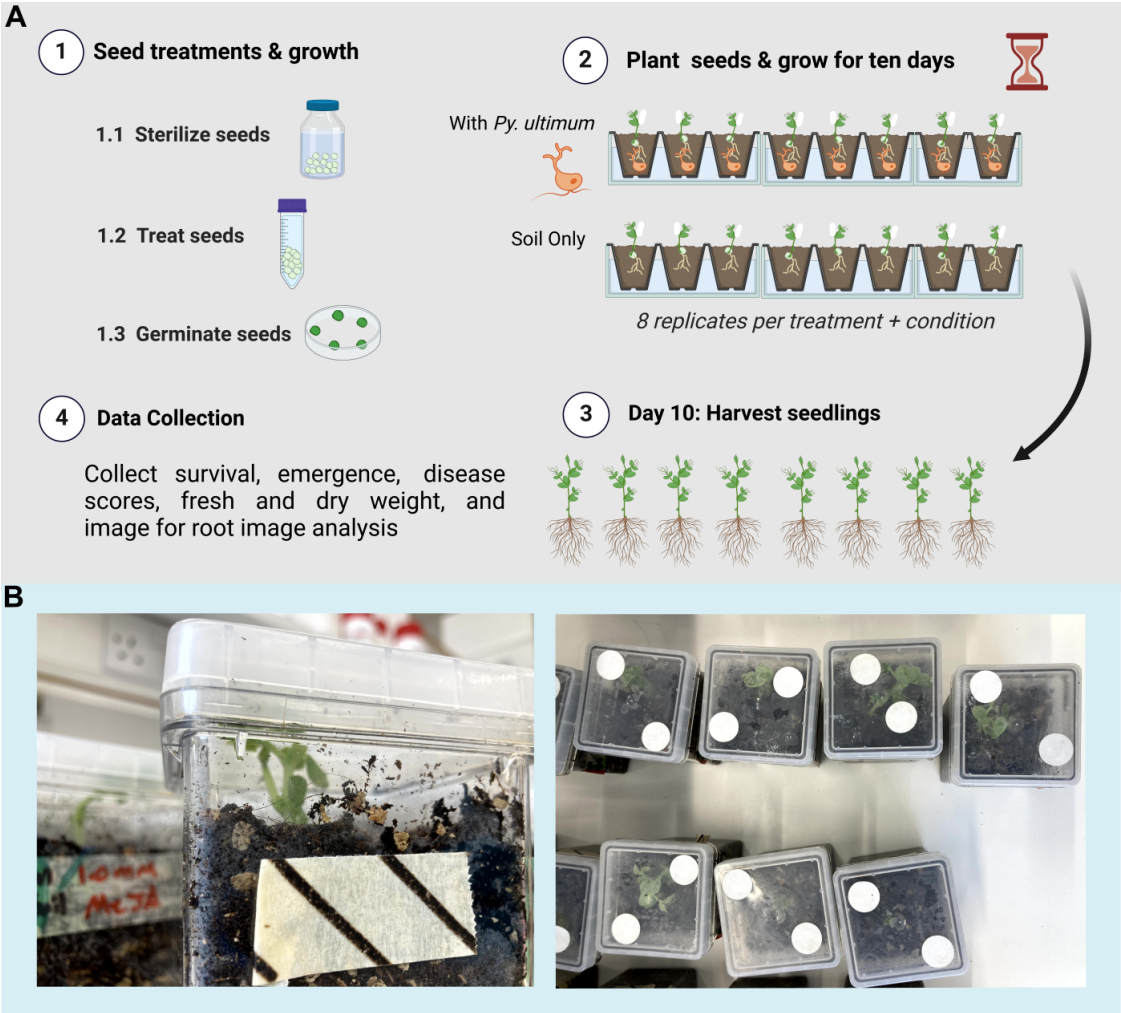


Figure 3.2: Set up of sterile soil system *Pisum satvium-Py. ultimum* pathosystem. **(A)** Schematic overview of the sterile soil pathosystem. Step one, sterilize, treat, and pre-germinate seeds. Step two, plant seeds into magenta vessels containing either 70 g sterile 2:1 compost:vermiculite or 70 g sterile 2:1 compost:vermiculite plus 1 g *Pythium ultimum* inoculum. Grow for seven days. Step three, harvest seedlings. Step four, collect survival and emergence data, disease scores, fresh weight, dry weight, and image roots for root image analysis. Eight replicate seedlings per treatment condition combination. **(B)** Pictures show 70 mm x 70 mm magenta vessel set up, with each seed in an individual magenta vessel. Left image shows a seed in its box. Right image shows boxes from the top, each with two holes covered with 0.3 um filter stickers.

On day ten numbers of emerged/not emerged and alive/dead plants were recorded

and visible disease severity evaluated for both foliar and root tissues. Harvested plants were gently washed to remove soil, patted dry and then weighed, imaged for root analysis and dried as in the in vitro set up to obtain dry weight.

Disease severity was assessed with a modified version of the in vitro disease severity scale:

Foliar symptoms: 0 = foliage without visible infection symptoms; 1 = foliage yellowing; 2 = foliage yellowing and tip wilting; 3 = yellowing, tip and total foliage wilting; 4 = plant death.

Root rot disease severity: 0 = roots without visible infection or discoloration; 1 = roots with light discoloration and light root and stem rot; 2 = short roots with discoloration; 3 = short roots with severe discoloration and root rot; 4 = plant death.

Unlike in the in vitro and non-sterile soil system assays, disease severity ratings were not converted to a continuous disease severity scale, as each disease rating was treated as a separate replicate.

All variables were treated as continuous in the statistical analyses. Root length, fresh weight and dry weight were determined for the living, germinated seeds. Data were analysed using Prism 10 (GraphPad Software Inc, Boston, MA USA). Root images were analyzed as in the in vitro pathosystem.

3.2.6 Non-sterile pathosystem

The non-sterile pathosystem was adapted from Mullins et al 2019 (Mullins et al. 2019). Figure 3.3 shows an overview of the non-sterile pathosystem.

Non-sterile pathosystem assays were carried out in non-sterile conditions in trays containing 60, 3.25 cm^2 wells. M2 soil and M2 vermiculite were mixed 2:1 soil:vermiculite to produce the potting mix.

Seeds were sterilized, washed, and soaked overnight in treatment solutions as in in vitro and sterile soil system assays.

Py. ultimum was cultured on PDA in the dark at 25°C for three days. The PDA plates were prepared using 18 mLs PDA per plate.

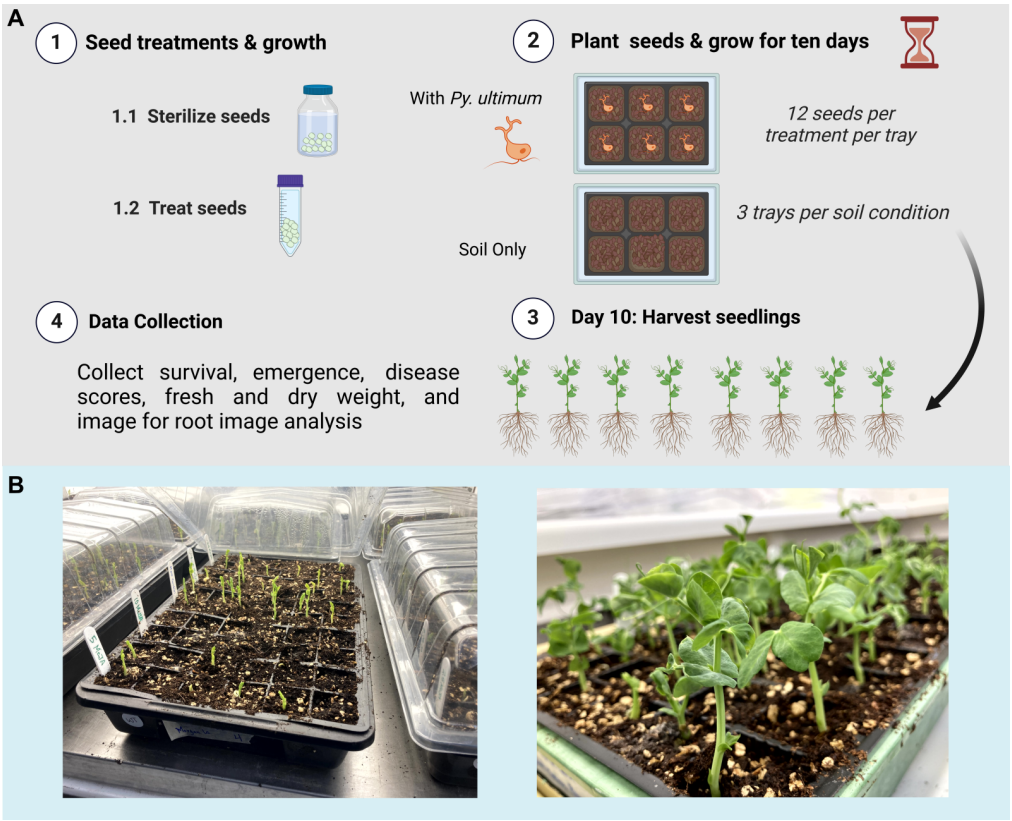


Figure 3.3: Set up for the non-sterile *Pisum sativum-Pythium ultimum* pathosystem. **(A)** Schematic showing overview of non-sterile soil disease system. Step one, sterilize and treat seeds. Step two, plant treated seeds (with no pre-germination) into either non-sterile 2:1 compost:vermiculite or into a 1:100 mix of non-sterile 2:1 compost:vermiculite and *Py* inoculum. Seeds grown in 55 mm Vacapot 60 tray inserts (H. Smith Plastics Ltd, Wickford, UK). Twelve seedlings used per treatment + condition combination per tray with three trays total per treatment + condition combination. Five different treatment + condition combinations grown on each tray, either all soil only or all *Py* only, to prevent spread of *Pythium* to control plants. All plants grown for ten days before harvesting. Step three, harvest seedlings, gently washing root systems to remove soil. Step four, collect survival, emergence, foliar and root disease scores, fresh and dry weight, and image root for root analysis. **(B)** Images showing the non-sterile soil pathosystem set up. Left image shows the tray set ups, with seed markers at early growth stage. Right image shows an up-close of a tray on day ten, before harvest.

Infested soil was produced by mixing the agar and surface mycelia from the PDA agar plates into a well-watered 2:1 medium compost:medium vermiculite mixture (one 90-mm PDA Petri dish per 120 g soil) and incubating the soil at 25°C for three days.

Potting mix was composed of 1% (w/w) *Pythium*-infested soil in unsterilized non-infested soil or 100% unsterilized non-infested soil for a non-infested control.

Seeds were planted one seed per well in either *Pythium*-infested soil or non-infested soil. Trays were covered with clear plastic humidity domes for the duration of the experiment. Trays were grown in a growth cabinet at 25°C with a 12 h photoperiod at a light intensity of 90 - 105 μmol/m2/s for 10 days and watered as needed. Three replicate trays were used per condition, with 12 seeds planted per treatment per tray. Growth, development, and disease assessment values are shown as three replicates per treatment-condition combination, where each replicate value is the average of twelve seeds.

On day ten the numbers of emerged/not emerged and alive/dead plants were recorded. Visual disease symptoms were evaluated for both foliar and root tissues using the same disease severity scales as in the sterile soil experiments.

Unlike the sterile-soil system assays, the disease severity scores were converted to a continuous Disease Severity Index (DSI) value per tray, using the following formula:

$$\text{DSI} = \frac{\sum \text{Severity rating x seeds per rating}}{\text{total seeds x highest severity rating}} \times 100$$

Roots were gently washed and patted dry then plants were weighed, imaged and dried as in previous assays. One plant per treatment per tray was taken post-symptom evaluation and pre-weighing and flash frozen in liquid nitrogen for RNA extraction and processing. The frozen plants were chosen from the healthy, surviving plants on each tray and stored at -80°C until RNA extraction.

A note on the length of experiments in the sterile soil system and non-sterile soil pathosystems: A time point of ten days was chosen to study the effects of the seed treatments, based upon the time points studied by Benavent-Celma et

al 2021, Mullins et al 2019, and Bera et al 2020 (Benavent-Celma et al. 2021, Mullins et al. 2019, Bera et al. 2020). Ten days was enough time to catch even quite delayed germination in the peas and presented an opportunity to study gene transcripts at a later time point (ten days) than those previously reported in the literature (seven days) (Bera et al. 2020).

3.2.7 Germination Tests

The impacts of JA and MeJA concentration on seed germination dynamics were assessed using modified forms of the in vitro and non-sterile soil disease assay systems. In this case, seeds were sterilized and treated in the aforementioned ways and then left on 2% WA plates in the dark at 25°C for ten days. Germination was assessed on days five and ten. An initial trial was run to determine the time frame of seed germination. Under control treatments and conditions *Pisum sativum* seeds will germinate and emerge from the soil within eight days. A ten day time frame was selected here to make sure all germination events were recorded.

For in vitro germination tests five treated but not germinated seeds were placed per plate, with ten replicate plates per jasmonate concentration. For non-sterile soil tests fifteen seeds per tray and six trays total per treatment were used where each replicate was the average emergence for all fifteen seeds on one tray.

3.2.8 Reverse transcriptase quantitative PCR

Previously flash-frozen seedlings, including roots and shoots but no seeds, were ground into a fine powder using a sterile mortar and pestle under liquid nitrogen. RNA was extracted from 100 mgs of the powdered whole-plant tissue using a Qiagen RNeasy Plant Mini Kit (Qiagen, Manchester, UK, Cat. No. 74904). RNA concentration and purity was determined using a nanodrop and then synthesized into cDNA using the PCR Biosystems UltraScript™ cDNA Synthesis Kit (PCR Biosystems Ltd, London, UK, Cat. No. PB30.11-02).

The following primers were used:

Housekeeping genes:

Beta Tubulin:

(F) GTAACCCAAGCTTTGGTGATC

(R) ACTGAGAGTCCTGTACTGCT

(Bera et al. 2020, Basu et al. 2021)

PP2A:

(F) CGGCTGCTGCGTGATAATG

(R) TGGTAGAATATGCTGAATGGCTAGTT

(Knopkiewicz and Wojtaszek 2019)

Jasmonate-related genes:

LOX2:

(F) GCAACCAAGTGACGAAGTCTA

(R) GGAGACCCGATTGTAAGGTATTT

(Basu et al. 2021)

COI1:

(F) GCGGCGATGTTTAACTTGAT

(R) CGAAAGTGCAACGACTTCAA

(Bera et al. 2020)

DRR230:

(F) TTGCAGGAACAACGAGCACTT

(R) GCACCAGCAGCGAAAATCAT

(Basu et al. 2021)

Jar4:

(F) TCATGCTTTCAGAGCGTTTG

(R) CTGGATTCGGTTTGAGCAAT

MYC2:

(F) AACAACCATGGGAGTTCTCG

(R) TGACGAGGATGATTGGAACA

qPCRs were run using qPCRBIO SyGreen Mix Hi-ROX (PCR Biosystems Ltd, London, UK, Cat. No. PB20.12-05) on a StepOne Plus™ Real-Time PCR System (Applied Biosystems, Oxford, UK, Cat. No. 4376600). Results were analyzed using the 2-ΔΔCT method from Livak and Schmittgen 2001 using the geometric mean of the two housekeeping genes.

Graphing and statistics were done in Prism 10 (GraphPad Software Inc, Boston, MA USA).

3.3 Results

3.3.1 Developing a *Pisum sativum*-*Pythium ultimum* Disease assay

Pythium ultimum isolates were received from Warwick Crop Sciences centre, in Warwick UK. It was necessary to confirm: (i) that all three strains were *Pythium ultimum*, (ii) that they could infect peas, (iii) to select one strain to move forward with, and (iv) to set up infection assay in which 50% of the plants would succumb to *Py. ultimum* to provide a usable sensitivity range for future assays.

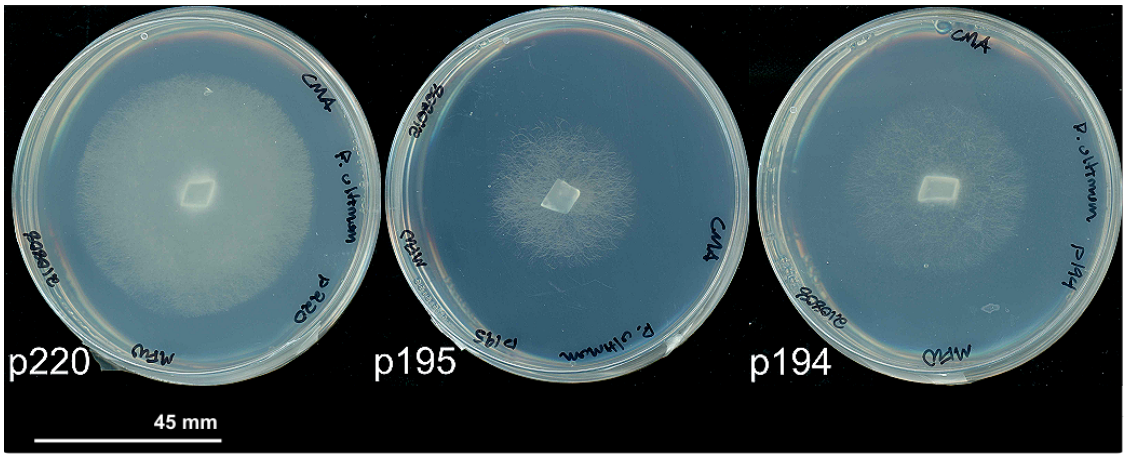


Figure 3.4: Images of the three *Pythium ultimum* cultures after three days of growth on 90 mm PDA plates at 16°C. From left to right: pathovar p220, pathovar p195, pathovar p194. Based on growth rates pathovar p194 was chosen for future experiments.

Each strain was grown in liquid PBB in 90 mm diamter sterile tissues culture dishes until the mycelial mat was approximately 40 mm in diamter. The mats were then removed and freeze dried for DNA extraction. DNA from all three

strains were sent for sequencing and all came back as 99% or more identity match to *Pythium ultimum* and *Globosporangium ultimum* with e-values below 0.0, see Table 3.1 for details.

The Percent Identity, Query Cover, and E-Value for the top hits of the forward and reverse sequences of all three strains are shown in Figure 3.4 **(B)**. Results confirm all three strains as *Pythium ultimum*.

Table 3.1: Percent Identity of Oomycete Cultures for *Globisporangium ultimum* and *Pythium ultimum*

Sequence	Percent Identity	Query Cover (%)	E-Value
p194-F	98.85	93	0.0
p194-R	99.54	99	0.0
p195-F	99.77	100	0.0
p195-R	99.19	99	0.0
p220-F	99.19	99	0.0
p220-R	99.70	99	0.0

Table detailing percent identity of ITS region sequences from sequencing data. Each strain is shown with both its forward (F) and reverse (R) ITS primers. Sequence homology is shown to both *Globosporangium ultimum* and *Pythium ultimum*. At the time of running, sequence homologies were the same for both organisms.

After *Py. ultimum* identity was confirmed, it was tested against pea seeds and seedlings to confirm pathogenicity against the selected plant species. All three strains of *Py. ultimum* infected pea seeds and seedlings. The virulence was quite strong in the conditions tested and caused 100% mortality among all seeds and seedlings tested (data not shown).

p194 was chosen as the strain for use in future assays based on its growth rate. See Figure3.4 Part **(A)** for a visual of the relative growth of the three *Py. ultimum* cultures after three days of growth on PDA at 16°C.

Initial assays were adapted from Benevant-Celma et al 2021, for both in vitro seed rot and sterile soil damping off disease systems.

The in vitro system used involved pre-germinated seeds to WA plates either with or without a pre-growth of *P. ultimum*. A schematic of the in vitro system set up in shown in Figure 3.1. Please see methods in 3.2 for details.

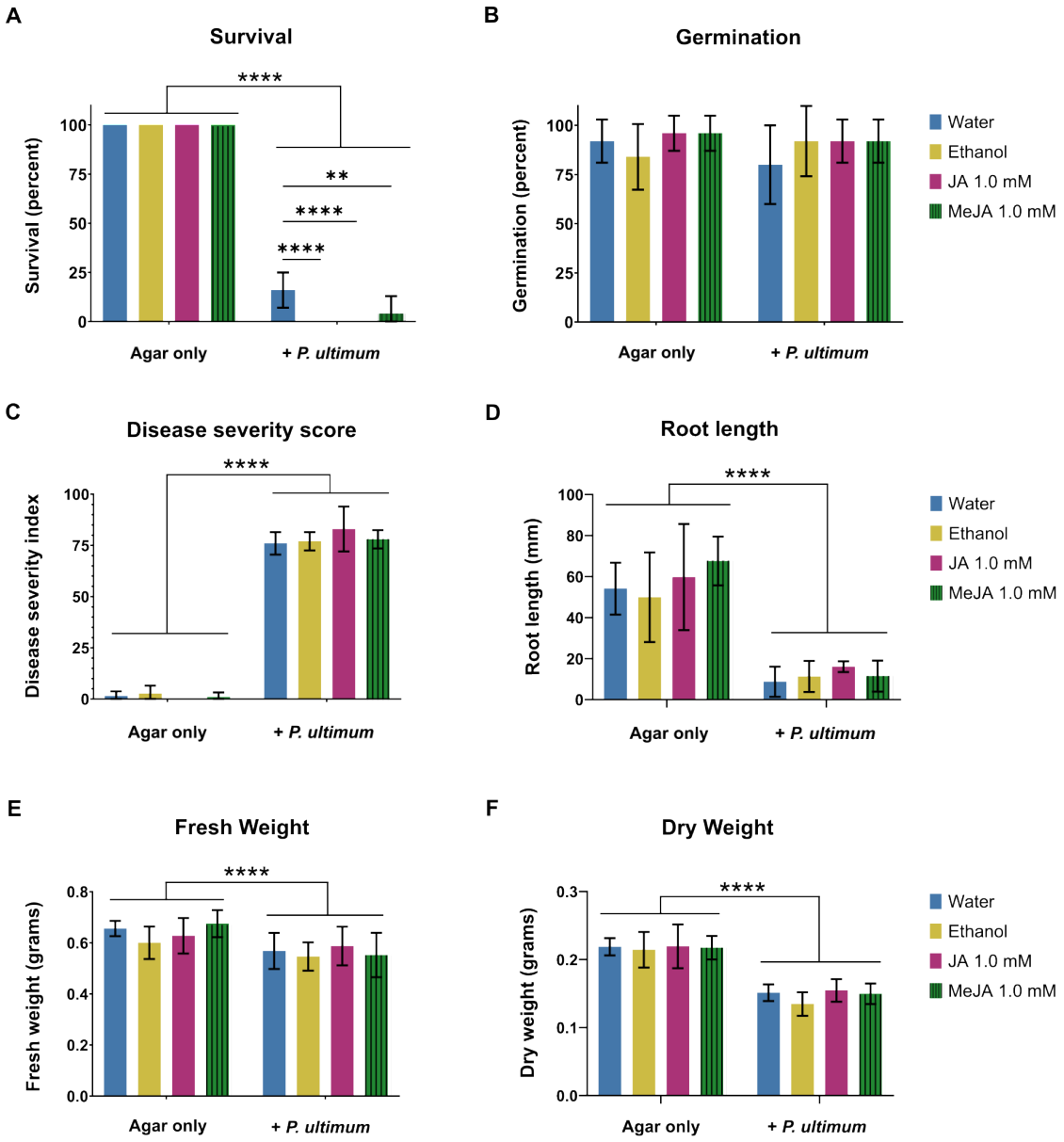


Figure 3.5: Representative results for JA and MeJA seed treatments tested in the in vitro *Pisum satvium-Py. ultimum* pathosystem. Results from day seven of in vitro seed rot assay. Treated seeds were grown for seven days on conditions of either agar alone (Agar only) or on agar with a pre-growth of *Pythium ultimum* (*P. ultimum*). Treatments include a water control (Water), a 0.1% ethanol control (Ethanol), 1.0 mM JA and 1.0 mM MeJA. Error bars show standard deviation. **(A)** Percent survival per plate of seedlings. **(B)** Percent germination per plate of seedlings. **(C)** Disease severity score, scored 0-4, 0 being healthy with no symptoms and 4 being dead and then converted into a disease severity index (see methods). **(D)** Root length of seedlings. **(E)** Fresh weight of seedlings, in grams. **(F)** Dry weight of seedlings, in grams. All graphs show means of five replicate plates per treatment condition combination, each plate value is the average of five seedling on that plate. Data checked for normality and analysed by two-way ANOVA with Tukey's post hoc tests for multiple comparisons. * p-value < 0.05, ** p-value < 0.005, *** p-value < 0.0005, **** p-value < 0.0001.

Figure 3.5 shows typical results for the in vitro *P. ultimum-Pisum sativum* pathosystem. All treatments tested resulted in full survival under control (agar-only, no *Pythium*) conditions. Water treated seeds showed a slightly higher survival rate than ethanol control, 1.0 mM JA, or 1.0 mM MeJA treated seeds, see panel (A). Survival was measured as presence of whole seed with small radical and/or hypocotyl formation. A seed was considered germinated when the primary root (radicle) length equaled the length of the seed. Germination varied for both both control and disease-exposed conditions, with no statistically significant differences between conditions or between treatments within a condition, see panel (B).

Disease severity score, shown in panel (C), was assessed based on yellowing of root or shoot and discoloration or sponginess/softness of seed - a spongy or soft seed indicated *Pythium* infection and breakdown of seed material. Agar-only seeds showed minimal to no discoloration or yellowing of shoots and roots. *Pythium*-exposed seeds had a high disease score, averaging around 75 (out of 100) for all treatment groups. There were no statistically significant differences between the treatment groups, though the 1.0 mM JA treatment showed the largest variability. There was a statistically significant difference between the two conditions.

Total root length, shown in panel (D), was measured from root images. Seedlings under control conditions show variability in seed length but no statistically significant differences between treatment groups. The largest variability was seen in the ethanol control and 1.0 mM JA treatment groups. Root length was significantly shorter in *Pythium*-exposed seedlings, for all treatment groups. There were no significant differences between treatment groups.

Fresh weight of seedlings, including seeds, is shown in panel (E). Treatment group had no statistically significant effect on fresh weight within either condition. However, the presence of *Pythium* did lower total fresh weight by a small but significant amount.

Dry weight of seedlings, shown in panel (F), was not affected by the treatments within each condition. As with the fresh weight, the presence of *Pythium* decreased

the dry weight by a moderate amount. Variability within treatment groups for both fresh and dry weight were smaller than that seen for germination or root length.

Results comparing seed soaking treatments in the sterile soil system are shown in Figure 3.6. In these assays, a control treatment of water 0.1% ethanol (Control) was compared to a 1.0 mM JA and a 1.0 mM MeJA treatment. As in the in vitro system, a seedling was labeled as 'survived' when the seed was (i) still present (not melted or broken down), and (ii) still had either a growing root or shoot.

The survival in the soil only conditions was 100%. The survival in the *Pythium* conditions was significantly lower than that of the soil only conditions. There was high variability in all treatment groups under the *Pythium* conditions and the most variability was seen with the 1.0 mM JA treatment, however, there were no statistically significant differences between the treatments groups within the disease condition.

Root length showed high variability within treatments under the control condition, and the difference between the control treatment and the 1.0 mM JA treatment was statistically different. Root length was significantly longer under the control conditions than under the *Pythium* conditions. There was much less variability within the treatment groups under the disease condition than under the control condition and this was statistically significant. There was no difference in root length between treatments within the disease condition.

Foliar disease score was zero for all treatments under the control condition while it was significantly higher in all the treatments under the disease condition. There were no differences between treatments for either the control or the disease condition.

The roots had some non-zero root disease scores under the control condition and large variability within each treatment group. The root scores for the control condition were significantly less than those seen in the *Pythium* condition. The treatments under the *Pythium* condition showed some variability within each treatment but there were no statistically significant differences between the treatment groups.

Fresh weight was significantly higher in the soil only condition than in the *Pythium* exposed condition for all treatment groups. There were no differences

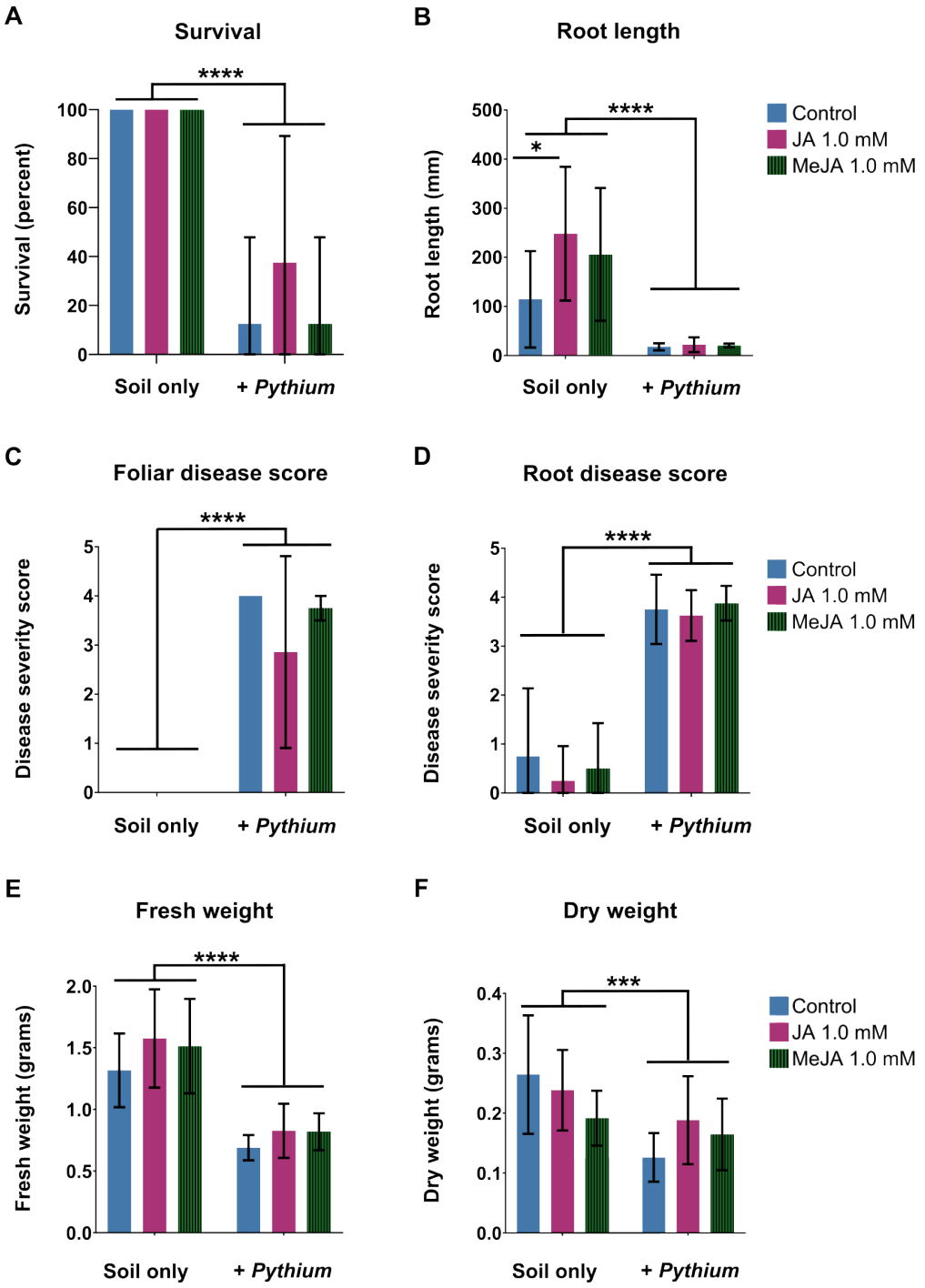


Figure 3.6: Representative results for JA and MeJA seed treatments in the sterile soil *Pisum sativum-Py. ultimum* pathosystem. Results shown for survival, emergence, root length, foliar disease score, root disease score, fresh weight and dry weight. Data shows mean of eight replicates per treatment + condition combination. Treatments are either a 0.1% ethanol control, 1.0 mM JA or 1.0 mM MeJA. Error bars show standard deviation. Data checked for normality and analysed by two-way ANOVA with Tukey's post hoc tests for multiple comparisons. * p-value < 0.05, ** p-value < 0.005, *** p-value < 0.0005, **** p-value < 0.0001. **(A)** Percent survival. **(B)** Root length. **(C)** Foliar disease score, measured on a scale of 0-4 where 0 is healthy and uninfected and 4 is dead from infection. **(D)** Root disease score, measured on a scale of 0-4 where 0 is healthy and uninfected and 4 is dead from infection. **(E)** Fresh weight, in grams. **(F)** Dry weight, in grams.

between treatment groups within either condition, though there was more variability within treatments for the soil only condition than within treatments for the disease condition.

Dry weight was also higher in the soil only condition than in the disease condition. There was moderate variability within all treatment groups for both conditions and no statistically significant differences between the treatment groups within each condition. There did appear to be slightly higher dry weight for the 1.0 mM JA treatment under *Pythium* exposure but this was not statistically meaningful.

3.3.2 Effects of Jasmonates on *Pisum sativum* Growth and Development

In an effort to get clearer results and increase the replication power of the disease assays an additional assay was adapted from Mullins et al 2019 (Mullins et al. 2019). This assay was referred to as the 'non-sterile soil' assay to differentiate it from the other assays. A schematic of the non-sterile soil system is shown in Figure3.3. Panel A shows an overview of the procedure and panel B shows two images of the trays - on on the left showing a tray with its humidity cover removed – only for imaging – and the right image showing an up close of typical day ten seedlings.

In order to determine the concentration of jasmonate treatments to trial it was important to understand the concentrations of both JA and MeJA in seed soaking treatments that changed germination and emergence behavior in the seedlings under control – no disease – conditions. Emergence was defined as a seedling germinating and emerging from the soil. This was tested in all three systems, but only data from the non-sterile soil system, the most replicable of the three systems (data not shown) is shown here, in Figure 3.7. JA and MeJA seed treatment solutions were tested at 1.0, 2.5 5.0, and 10.0 mM and number of emerged seedlings counted at day 5 and day 10. All tests were done in control soil - no *Pythium*.

Treatments of 1.0 mM JA or 1.0 mM MeJA did not impact pea seed emergence over either five days or ten days as compared to the ethanol control. 2.5 mM JA and

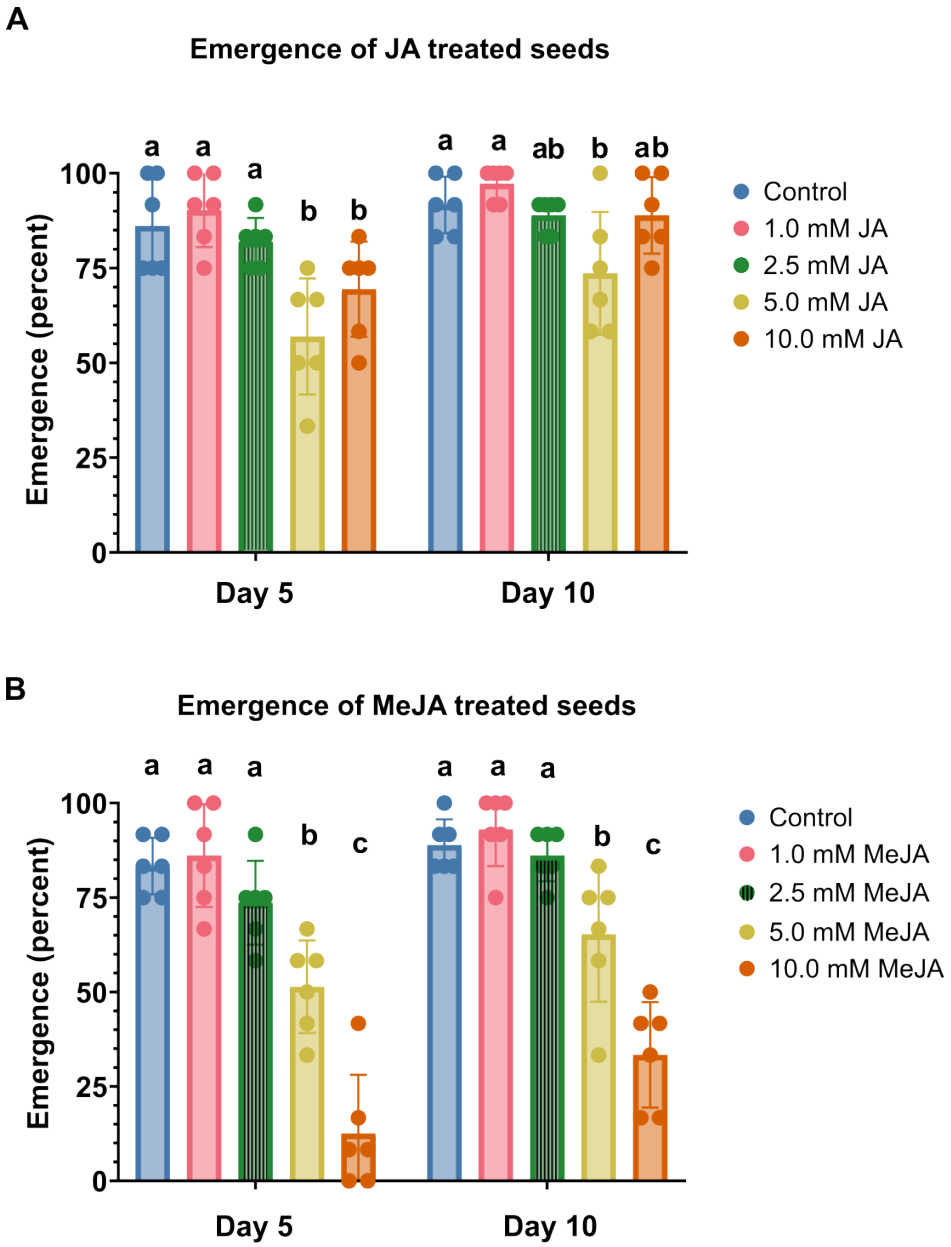


Figure 3.7: Emergence tests for various JA and MeJA concentrations using the non-sterile soil system set up. **(A)** Emergence data for days five and ten post planting for seeds treated with either 1.0 mM, 2.5 mM, 5.0 mM, 10.0 mM JA or a 1% ethanol control solution. Data shows emergence means of six replicate trays per treatment with each tray's value the mean of twelve seedlings. Error bars show standard deviation. * p-value < 0.05, ** p-value < 0.005. **(B)** Emergence data for days five and ten post planting for seeds treated with either 1.0 mM, 2.5 mM, 5.0 mM, 10.0 mM MeJA or a 1% ethanol control solution. Columns marked 'a' are not significantly different from one another. 'b' columns are different from 'a' columns with a p-values < 0.05. Columns marked 'c' are different from 'a' columns by p-value <0.001 and from 'b' columns by p-value < 0.005. Data shows emergence means of six replicate trays per treatment with each tray's value the mean of twelve seedlings. All data checked for normality and analysed using a two-way ANOVA with Tukey's post hoc tests for multiple comparisons.

2.5 mM MeJA visually showed slightly lower rates of emergence than the control treatment but this was not statistically significant.

5.0 mM JA treatments caused delays in emergence at both five and ten days. The 5.0 mM JA treatment was statistically different than the control and 1.0 mM JA treatments at ten days but not statistically different than the emergence results for 2.5 mM or 10.0 mM JA treatments.

10 mM JA treatments also caused delays in emergence at five days as compared to the control, 1.0 mM and 2.5 mM JA treatments. By ten days the emergence was no longer statistically different from any of the other treatments.

MeJA treatments produced a different pattern of effects. 1.0 mM and 2.5 mM MeJA treatments showed no differences in emergence at either day 5 or ten as compared to the control. 5.0 mM MeJA treatment showed a decrease in emergence at both time points and was statistically different than all the other treatment groups. The same was true for the 10.0 mM MeJA treatment which resulted in a significant decreased in emergence at both time points and which was statistically different than all of the other treatments.

Based on this data, 1.0 mM concentrations of JA and MeJA were used as basic seed soaking treatments. 2.5 mM was going to be tested but was not due to time constraints.

3.3.3 Effects of Jasmonates on *Pisum sativum* resistance to *Pythium ultimum*

Once the replicable, robust non-sterile soil test system was developed and the ranges of non-germination inhibiting doses for both jasmonates, the effects of jasmonates on pea growth and defense were investigated.

As part of this, disc diffusion assays were run to test for any direct effects of the jasmonates against *Pythium ultimum*.

Figure 3.8 shows two panels of plates - panel A grown on PDA and panel B grown on 2% WA. Both figures compare a 1% ethanol control to different concentrations of JA or MeJA and water alone.

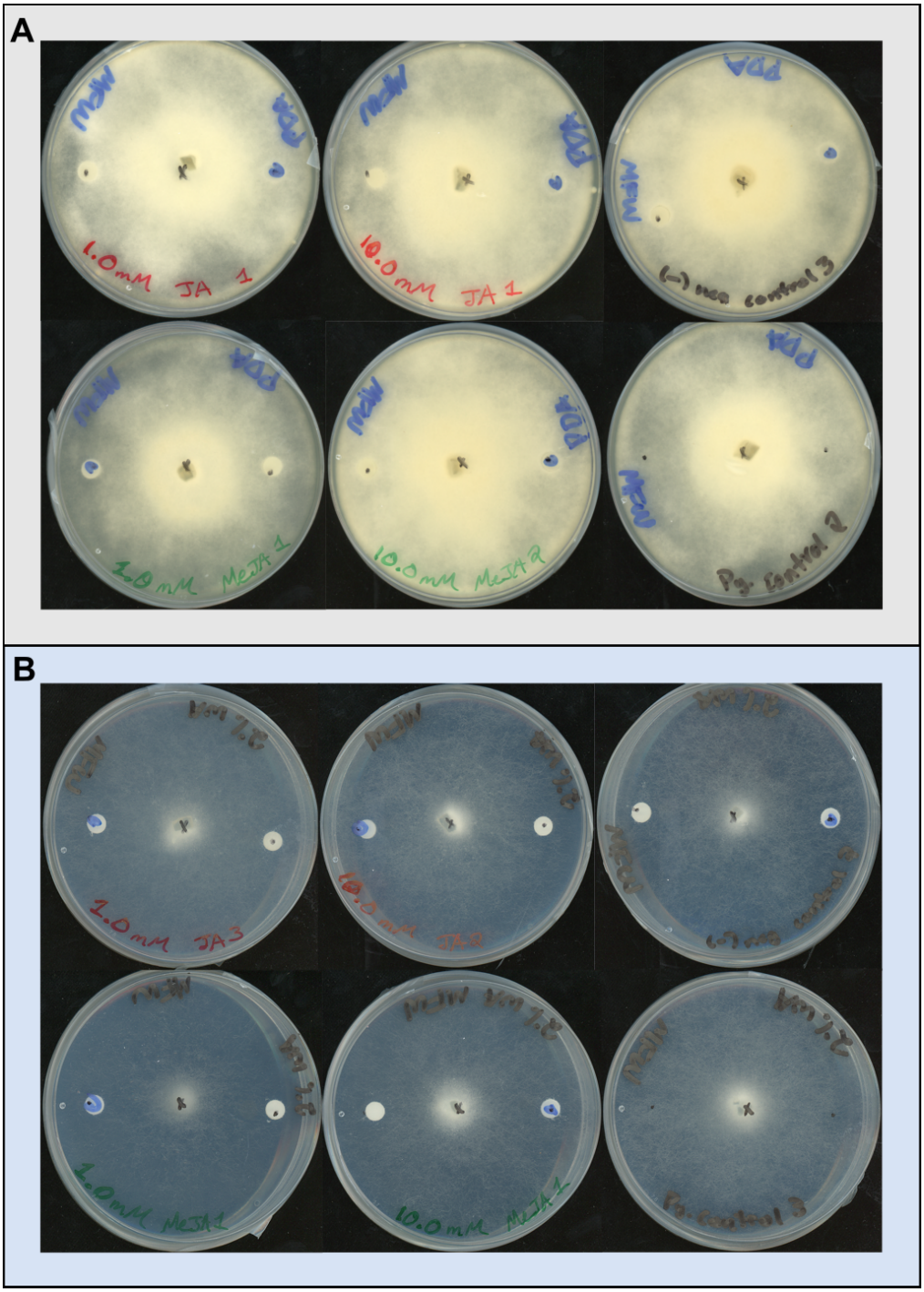


Figure 3.8: Disc diffusion assays, testing the effects of jasmonates on *Pythium ultimum* directly. For both images, blue dots indicate control solutions of 1% ethanol (EtOH) and black dots indicate test conditions. For both images, from left to right: Top row: 1.0 mM JA (black dot) vs EtOH control (blue dot), 10.0 mM JA (black dot) vs EtOH control (blue dot), ddH2O (black dot) vs EtOH control (blue dot). Bottom row, from left to right: 1.0 mM MeJA (black dot) vs EtOH control (blue dot), 10.0 mM MeJA (black dot) vs EtOH control (blue dot), *Pythium* control with ddH2O (black dot) and ddH2O (black dot). **(A)** *Pythium ultimum* grown on PDA with discs soaked in either a control solution or a jasmonate solution. **(B)** *Pythium ultimum* grown on 2% WA with discs soaked in either a control solution or a jasmonate solution.

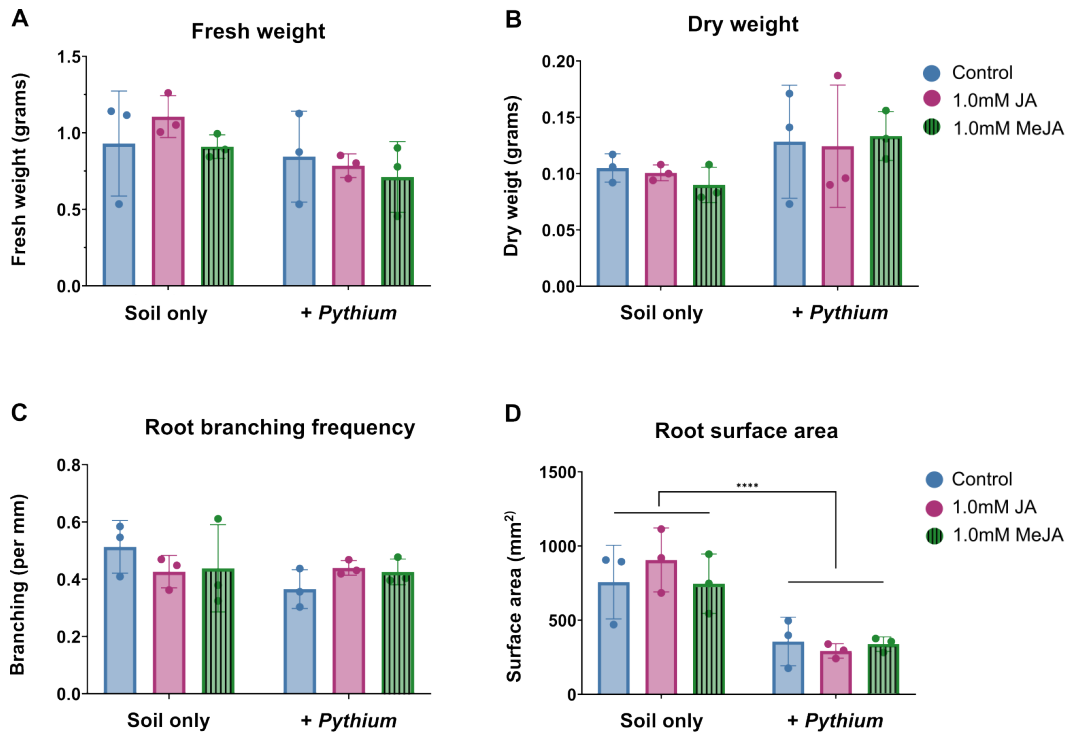


Figure 3.9: Growth and development effects of jasmonate seed treatments on *Pisum sativum* within the non-sterile soil pathosystem. Data shows means of three replicate trays, each tray value as the mean of twelve seedlings. Error bars show standard deviation. Data tested for normality and analyzed with a two-way ANOVA and Tukey's post hoc tests for multiple comparisons. **(A)** Fresh weight in grams of seedlings grown with or without *Pythium ultimum*. **(B)** Dry weight in grams of seedlings grown with or without *Pythium ultimum*. **(C)** Root branching frequency per mm of seedlings grown with or without *Pythium ultimum*. **(D)** Root surface area of seedlings grown with or without *Pythium ultimum*.

There were no effects on mycelial growth pattern or plate coverage for either of the control conditions nor for any of the JA and MeJA concentrations. Within two days the *Pythium* was observed to grow into and over the paper discs for all treatments. This was true both when the *Pythium* was grown on its preferred substrate of PDA and when it was grown on a minimal substrate of 2% water agar.

Moving forward, any effects seen of the jasmonate or combined jasmonate-silicate treatments in future chapters are likely not due to any direct interaction of the jasmonate(s) with the *Pythium*.

Using the non-sterile soil disease system the effects of 1.0 mM JA and 1.0 mM MeJA on *Pisum sativum* growth and development factors as well as disease factors in response to *Pythium ultimum* were tested.

Figure 3.9 shows the results for growth and development factors in the soil only vs *Pythium* conditions.

Fresh weight varied within and between treatment groups for both soil only and *Pythium* conditions but none of these differences were statistically significant. This was true of the overall difference between the two conditions as well, despite the fresh weight visually appearing to be less in the disease conditions than in the control conditions.

Dry weight showed low variability in the soil only condition and higher variability within the control and 1.0 mM JA treatment groups under the disease conditions. There were no statistically significant differences between treatment groups within either the soil only condition or within the *Pythium* exposed condition. The dry weights for the *Pythium* exposed condition appeared visually to be higher than those for the soil only condition but this was not statistically significant.

Root branching frequency had high variability within the 1.0 mM MeJA treatment under the soil only condition. All other treatments under the two conditions had little variability and there were no statistically significant differences between the conditions or between treatments within each condition.

Root surface area was lower under the disease condition as compared to the soil only condition. There was moderate variability within the treatment groups under the soil only condition but no statistically significant differences between the three treatment groups. There was much less variability within the treatment groups under the disease condition and also no statistically significant differences between the three groups.

Disease severity metrics were also measured using the non-sterile soil system. These results are shown in Figure 3.10.

Both survival and emergence were lower in the *Pythium* exposed condition than the control condition. There was large variability in survival and emergence for the control and 1.0 mM JA treatments under soil only conditions though this did not result in any statistically significant differences between treatment groups.

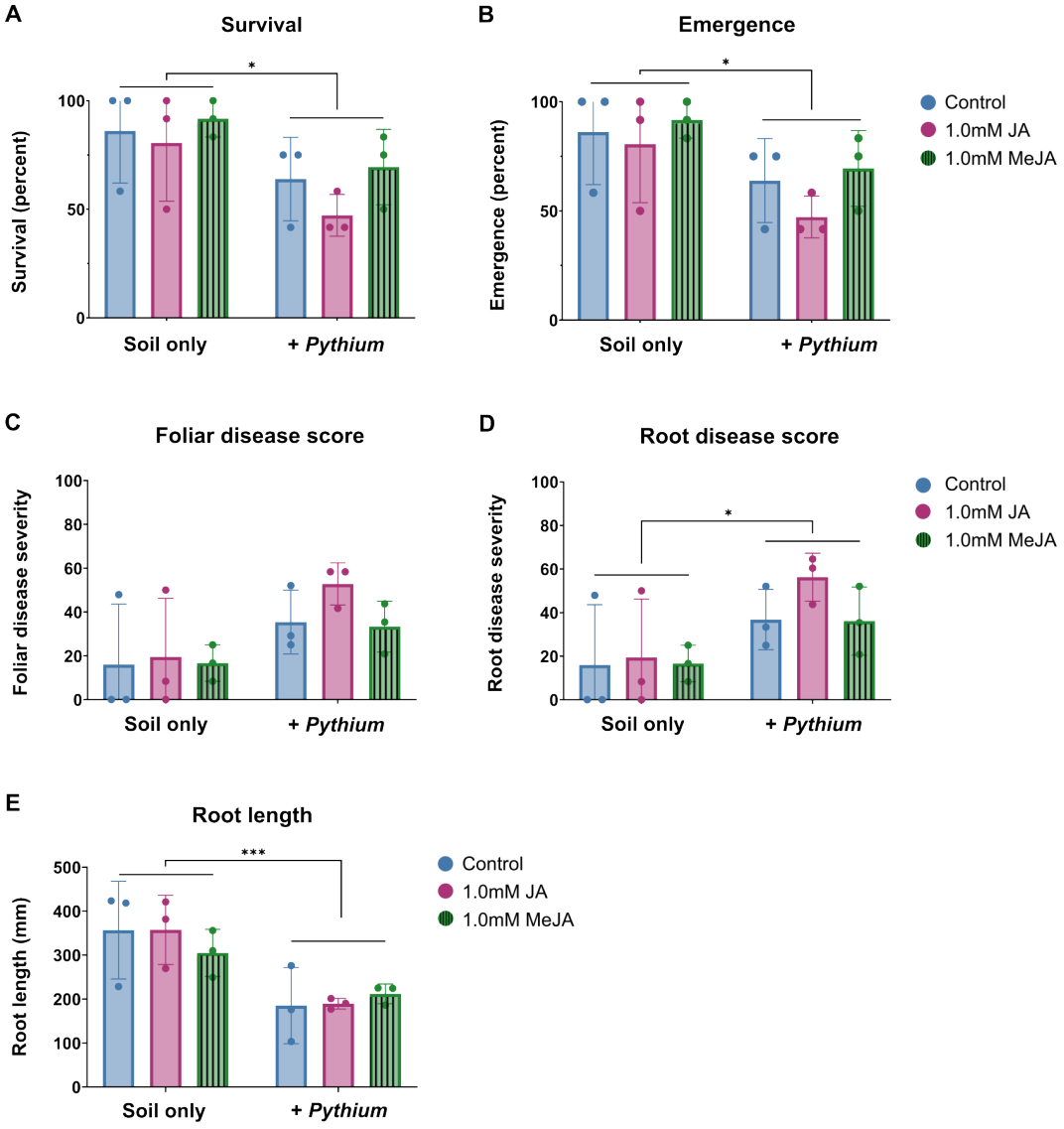


Figure 3.10: Disease assay results of of jasmonate seed treatments on *Pisum sativum* within the non-sterile soil pathosystem. Data shows means of three replicate trays, each tray value as the mean of twelve seedlings. Error bars show standard deviation. Data tested for normality and analyzed with a two-way ANOVA and Tukey's post hoc tests for multiple comparisons. **(A)** Percentage survival of seedlings grown with or without *Pythium ultimum*. **(B)** Percentage emergence of seedlings grown with or without *Pythium ultimum*. **(C)** Foliar disease score on a scale of 0-4 where 0 is alive with no disease and 4 is dead from infection for seedlings grown with or without *Pythium ultimum*. **(D)** Root disease score on a scale of 0-4 where 0 is alive with no disease and 4 is dead from infection for seedlings grown with or without *Pythium ultimum*. **(E)** Root length of seedlings grown with or without *Pythium ultimum*.

There was also large variability within the control and 1.0 mM MeJA treatments under the *Pythium* exposed conditions for survival and emergence. Both of these treatments also appeared to result in higher levels of survival and emergence than the 1.0 mM JA treatment but none of these differences were statistically significant.

There was some foliar disease symptoms under the the control condition for all treatment groups. There were no signs of *Pythium* or other obvious infection in the soil despite the plants showing yellowing at the tips of the leaves and some drooping. Water levels were routinely checked to confirm yellowing was not due to over or under watering. These symptoms appeared on one replicate tray primarily. The data was left in this analysis, despite the large variability it gives the control and 1.0 mM JA treatments. Foliar disease symptoms appeared higher and more consistent under the *Pythium* exposed condition though there was no statistically significant difference between the two conditions.

Pythium exposure resulted in higher root disease severity than soil only conditions. This difference was statistically significant. As with the foliar disease symptoms, there was some root disease symptoms – shorter roots or discoloration – in all three treatments under the soil only condition. This was seen in the same trays that the foliar symptoms were seen. There were no obvious signs of *Pythium* or other infection in the soil. The largest variability was seen in the control and 1.0 mM JA treatment groups. There were no statistical differences between treatment groups under the soil only condition.

There was moderate variability within the treatments groups under the *Pythium* condition. 1.0 mM JA appeared to result in a slightly higher root disease score as compared to the control or the 1.0 mM MeJA treatment but these differences were not statistically significant.

As with the root surface area shown in Figure 3.9 panel **(D)** root length in Figure 3.10 panel **(E)** was 35 - 50% shorter under the *Pythium* exposed condition as compared to the soil only condition. There was large variability in root length within the control treatments for both conditions but no statistically significant differences between any of the three treatments under either condition.

3.3.4 Effects of Jasmonates on *Pisum sativum* gene expression

In addition to the growth and disease assays we wanted to investigate whether the jasmonate treatments were having any effects on gene expression. I.e., were the plants receiving and reacting to the jasmonates, even if they weren't increasing disease resistance to the *Pythium*? This was investigated using RT-qPCR.

A panel of genes was selected to measure jasmonate perception, biosynthesis, and jasmonate pathway activation, including downstream defense responses.

LOX2, JAR4, COI1, and DRR230 were chosen for gene expression analysis using RT qPCR with Beta tubulin and PP2A as housekeeping genes.

Figure 3.11 shows a simplified jasmonate biosynthesis and expression pathway, focusing on the genes of interest used in this study. JA biosynthesis begins in the chloroplast and finishes in the cytoplasm. JA can then be converted into MeJA and vice versa. The JAR4/6 complex converts JA into the bioactive JA-Ile, which then binds to the JAZ-COI1 complex, initiating degradation of COI1 and releasing jasmonate-dependent transcription factors, like MYC2. DRR230 is a pea defensin peptide downstream of MYC2.

Figure 3.12 shows relative gene expression for the jasmonate pathway genes for *Pisum sativum* seeds treated with either a 1% ethanol control, 1.0 mM JA, 1.0 mM MeJA, or 10.0 mM MeJA and grown in the in vivo system. 10.0 mM MeJA treated seedlings had only a 30% emergence rate so this concentration was only used one time, for this experiment.

Upregulation was as relative increase in expression as compared to the control treatment. For Lox2 and DRR230 the 10 mM MeJA treatment resulted in a 6.5 fold increase in expression. This was significantly different to the other treatments for LOX2. For DRR230 there was high variability within the 1.0 mM JA replicates but only small variability in the 1.0 mM MeJA treatments. The 10.0 mM MeJA treatment was statistically different from the control and the 1.0 mM MeJA but not the 1.0 mM JA treatments.

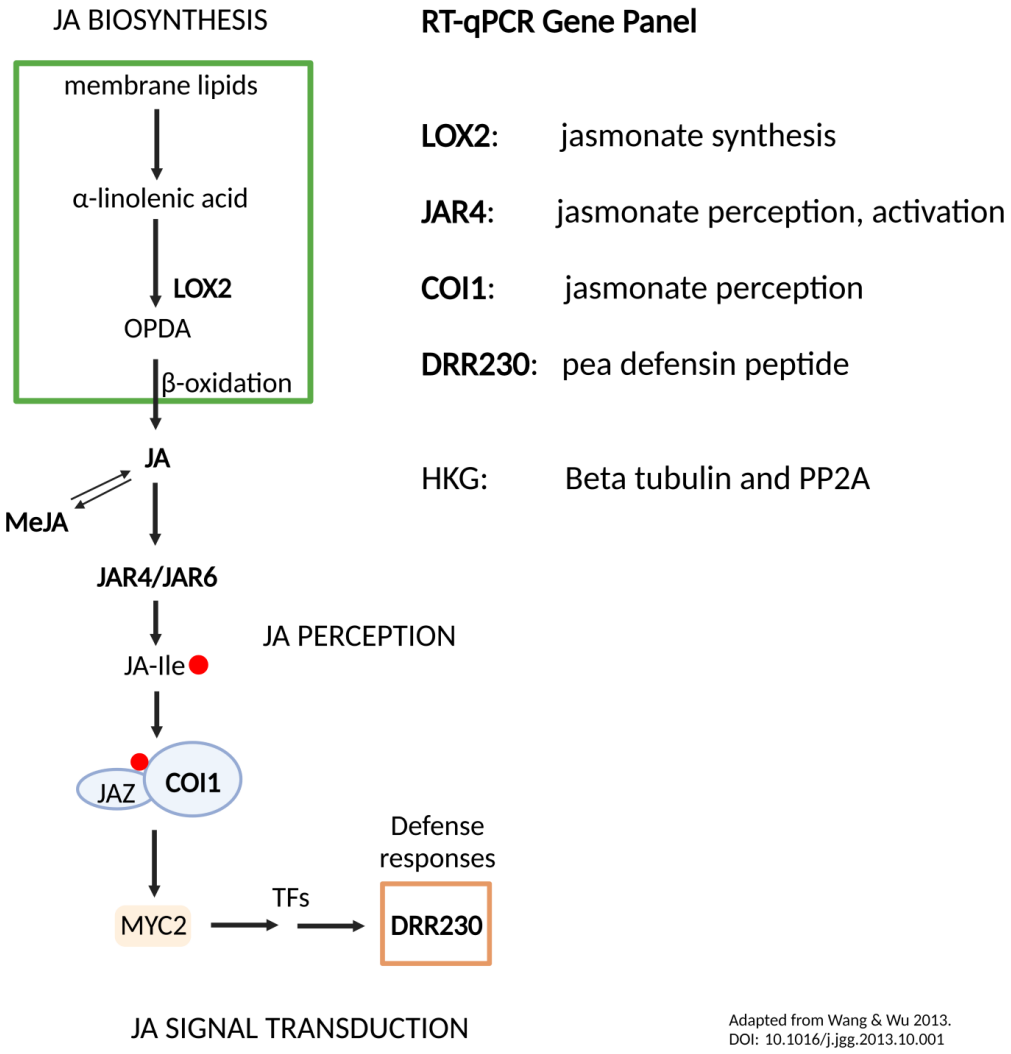


Figure 3.11: Gene expression pathway and genes used for RT-qPCR. Green box indicates biosynthesis steps which occur within the chloroplast.

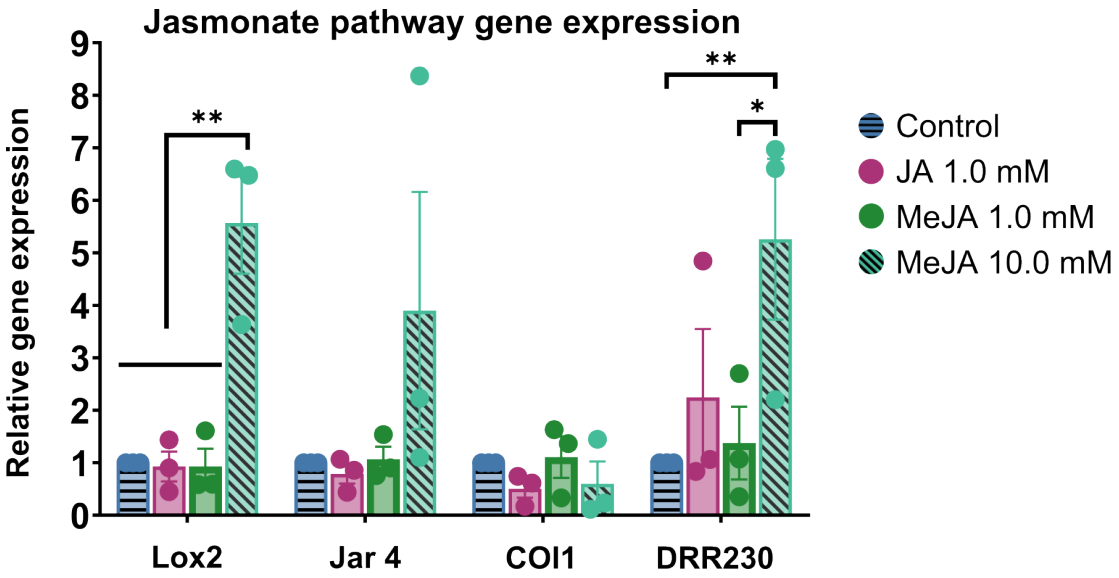


Figure 3.12: Gene expression results for sterile soil system showing relative gene expression values from RT-qPCR of pea seedlings treated either JA or MeJA or a 1% EtOH control. Data was analysed using the 2-ΔΔCT method from Livak and Schmittgen 2001 and the geometric mean of the two housekeeping genes. Data is shown as expression relative to the control treatment. Data showing means of technical replicates for three biological replicates per treatment. Error bars show standard deviation. Data analyzed with a two-way ANOVA and Tukey's post hoc tests for multiple comparisons. * p-value < 0.05, ** p-value < 0.005.

There was high variability in Jar4 expression within the 10.0 mM MeJA treatment group. However, this was not statistically different from the Jar4 expression levels for the other treatments groups. COI1 had little variability within treatment groups and no difference between treatment groups.

3.4 Discussion

In vitro system

The in vitro system showed *Pythium ultimum* as an effective and virulent pathogen in peas but did not reveal any significant effects of jasmonate treatments on pea survival or disease resistance to *Py. ultimum*. Interestingly, while *Py. ultimum* impacted all other metrics measured it did not alter germination percentage in a statistically significant way. There was a lot of variability between the in vitro assays overall and so this could be due simply to that variability. It also could be

because, despite the plate having a pre-growth of *Pythium*, the seed could have had minimal exposure while it was germinating, as the *Pythium* inoculum would have still been growing and primarily confined to one surface plane of the seed. However, the most likely explanation is due to the nature of the protocol. In the in vitro assays the seeds were soaked in the seed treatments for 24 hours and then plated for two days as a pre-germination stage before being transferred to the assay plates. Signals for initiation of germination may already have been under way in the seeds and then the inhibitory effects of the *Pythium* not strong enough in the first few days of exposure to fully block germination.

An increase in disease severity score between agar only plates and those with *Pythium* is expected. There were minimal, but still non-zero, disease score for the control plates. This was not unusual for any of the pathosystems tested in this work. Any control plates that were found to have visible bacterial or fungal/oomycete growth were discarded and those experiments repeated. Therefore, any disease-like symptoms seen were assumed to be from non-disease causes.

To be as objective as possible any discoloration or stunting in growth among the control plants was recorded under the disease severity scale. Some seedlings had double or triple stems or broken seeds indicative of mechanical damage to the seeds prior to treating and planting (Karagić et al. 2010). Some seeds will simply germinate slower. Others had rolled closer to the edge of the plate when the plates were moved – plates were not reopened after initial sealing – and thus had different physical constraints on their growth than did other seeds. All these factors could cause disease-like symptoms in control plants.

Decreases in survival, root length, fresh weight, and dry weight are expected in plants under pathogen stress. The data in Figure 3.5 thus fits expectations about both the pathogenicity and virulence of *Pythium* disease.

The in vitro system was a useful starting point but it was thought a soil-based assay might reveal more dynamics between the peas and the *Pythium* and show any effects the jasmonates treatments might be having.

Sterile soil system

The sterile soil system also did not reveal many effects of jasmonate seed treatments on *Pisum sativum* disease resistance to *Pythium ultimum*, though it showed both strong virulence and pathogenicity of *Py. ultimum*. 1.0 mM JA treatment resulted in high variability in survival and foliar disease score of *Pythium* exposed seedlings but otherwise was no different than the 0.1% ethanol control treatment. The roots showed some yellowing and discoloration under the control conditions. There were no other signs of infection and it is likely this is due to physical constraints on the roots' physical growth from the magenta boxes. Additionally, some of the seeds, though alive, germinated later than others and had comparatively much smaller roots. These were scored according to the disease system and so some of the disease scores reflect the shortness of the roots but not any disease.

Sterile soil system was limited to 8 seedlings each as individual replicates. It was suggested when presenting some of this work in a group meeting that a non-sterile soil system in trays might be more useful and help increase the replication power of the assays. A non-sterile soil system with three to five replicate trays and where each tray contained 12 twelve seedlings was eventually settled on as the most feasible method. The results are shown here in this chapter.

Non-sterile soil system

Emergence tests were conducted to better understand the effects of a range of concentrations of JA and MeJA on seedling emergence within the non-sterile soil system. 1.0 mM and 2.5 mM JA and MeJA had no effects on timing or total percentage of emergence in the pea seeds. This makes both 1.0 mM and up to 2.5 mM concentrations of either jasmonate potential concentrations for seed treatments. Unfortunately, due to time constraints, only 1.0 mM treatments were taken for further testing. If there was time for further work 2.5 mM and 5.0 mM treatments of JA and MeJA would have tested to see if these concentrations had any effects on the plants.

In some assays there were no differences in foliar score between *Pythium* exposed and non-pythium exposed plants. While damping off diseases like *Pythium* often enter through roots and cause the most notable damage in root systems, in quite young plants foliar symptoms can appear early on in infection (Lamicchane et al. 2017). Why there is still some foliar damage in control plants is another question. There are a few factors that could be affecting this, similar to in the sterile soil system assays: (i), Variations in growth speed. Some plants grow more quickly and robustly and thus the plants they are being compared to look slightly 'sick'. These plants then get marked with a low disease score, despite there being no actual disease. Aside from subtle genetic variation and the myriad of factors which can impact germination and development speed, including conditions when the seed was forming, microenvironment, physical condition of seed coating, etc. (Mitchell et al. 2017), bet-hedging can also be present. Bet-hedging strategies, when isogenic plants produce different phenotypes in the same environment, are advantageous in unpredictable environments but also very common in seed dormancy and germination, even in stable environments (Abley et al. 2024). Thus, a combination of factors could be influencing germination and growth rate in the control seeds.

(ii), Edge effects in the trays. Plants towards the edge of the trays often dried out more quickly than plants in the centre. They were also more likely to be touching the clear plastic covers which were placed over the trays. Plants whose leaves touched the clear plastic lid were more likely to develop tip yellowing (data not shown). For consistency, any yellowing or shortening of growth was recorded under the disease severity scale.

(iii), Variations within growth chambers. Due to equipment failures and competition for growing space, different experimental replicates and types of growth assays had to be completed in a variety of growth chambers. Light intensity, temperature and humidity were monitored and controlled but it is a known phenomenon that there will still be variations in conditions within a climate-controlled chamber (Potvin et al. 1990).

The presence of *Pythium* often did not effect dry weight between the conditions, especially when fresh weight differences between conditions were also not statistically significant. It is not clear why sometimes fresh weight was significantly lower in the *Pythium* condition and sometimes not. There could be a variety of factors here, including relative inoculum load of the *Pythium*. Though the inoculum production was standardize as in the methods, there could still be variation in the level of *Pythium* inoculum which could account for why sometimes the disease affected fresh weight – and not just disease scores – and sometimes it did not. Experiments to test variation in *Pythium* inoculum were just starting to be tested towards the end of the PhD but these were unfortunately not finished due to time constraints.

Shorter, smaller roots are a key indicator of damping off diseases and *Pythium* infection so these results are expected.

None of the three types of assays revealed any significant effects of either JA or MeJA on *Pisum sativum* resistance to *Pythium ultimum* when applied by seed soaking. It is possible that the concentrations used were not high enough, that using 2.0 or 2.5 mM, or even 5.0 mM, JA or MeJA would have induced stronger effects. Equally, there were no consistent effects of the jasmonate seed treatments on any pea growth or development factors. This could mean the concentrations used were below detection/response thresholds in the plants or it could mean they were detectable by the plant but simply below the levels required to change the growth and development factors. Further testing of higher concentrations of jasmonates would help elucidate this. Effective doses of jasmonates range widely in the literature. 2.5 mM and 5.0 mM MeJA have both been shown to induce resistance to rice water weevils in rice, albeit with germination delays and some biomass decrease (Kraus & Stout 2019). This seems to be somewhat host species-specific, as another study in tomato found 100 nM doses of JA effective for inducing defense responses against root knot nematodes (Bali et al. 2020) while 45 μM MeJA was found to be effective in reducing disease symptoms in Melon from *Didymella bryoniae*, *Sclerotinia sclerotiorum* and *Fusarium oxysporum f. sp. melonis* (Buzi et al. 2004).

Additionally, there is evidence in the literature that lower treatment concentrations of MeJA, 0.1 mM, increased disease resistance to *Fusarium oxysporum* in tomatoes but 1.0 mM increased susceptibility (Kępczyńska and Paulina Król 2012, Król et al. 2015). It is possible a similar effect is occurring in the Pea-*Pythium* system. Further testing of lower jasmonate concentrations would be useful in determining this.

Gene expression results

The RT qPCR results indicate that while at ten day the seedlings are still upregulating jasmonate response genes for the 10.0 mM MeJA treatment they are not doing so for either the 1.0 JA or 1.0 MeJA treatments. The 10.0 mM MeJA treatments here are an interesting case. 10.0 mM MeJA was shown to dramatically reduce emergence, down to 30% of emergence of control seeds. During initial tests 10.0 mM MeJA was trialed and samples taken for later RT-qPCR but the 10.0 mM MeJA treatment was ultimately abandoned because of its strong inhibitory effects on germination and growth. However, out of curiosity, the collected 10.0 mM MeJA plants were still tested via RT-qPCR, and these are the results shown in Figure 3.12.

The RT-qPCR results here were only taken at 10 days post seed-soaking treatment and were also taken under the sterile soil system. Unfortunately, there was not time to complete further RT-qPCRs and so the data, and thus its interpretations, is limited. From these results only the 10.0 mM MeJA can be confirmed to induce any response in the plant at ten days. This does not tell us whether the 1.0 mM JA or MeJA treatments have been perceived by the plants or have any effect on the plants. Testing at much earlier timepoints would be needed to determine this. Testing under pathogen stress conditions would be needed to determine if the jasmonates treatments were inducing any resistance in the plants. Finally, for any priming effects to be observed, plants would need to be treated, tested for perception of jasmonates and/or induction of response and then at a later timepoint when any induction had ceased, tested under pathogen conditions for priming. Unfortunately,

none of this was possible under the time constraints of the project and it leaves a major gap in our understanding and interpretation of this data.

There are various explanations for why no changes in defense gene expression were seen in this experiment. The 1.0 mM JA and MeJA treatments could be below the threshold of activation for the peas either by ten days or altogether, i.e., the peas could detect 1.0 mM in the early stages of germination and then afterward the signal is not strong enough to induce longer term responses, or the signal could be below the response threshold, and no jasmonate gene responses would be detected at earlier time points either. Jasmonate pathway gene upregulation has been detected in response to wounding and infestation as early as 3-4 hours post exposure in peas (Ishiga et al. 2003, Bera et al. 2020) and as early as 2 minutes post wounding in *Arabidospsis* (Larrieu et al. 2015) and as late as 7 days post exposure in peas (Basu et al. 2021). Most results in the literature investigating jasmonate upregulation in peas do so between 24 – 96 hour time points (Bera et al. 2020, Mai et al. 2014, Patel et al. 2016, Sanchez-Arcos et al. 2016, Saha and Vandemark 2012). At the time of writing, the author was unable to find any studies examining JA-related transcripts in peas at ten days or later. It is not clear why pea transcripts have not been examined post-seven days, there is certainly evidence of phenotypic effects from jasmonates in rice and cowpeas at much later time points, weeks and months post-treatment and in Scots pine up to a year after jasmonate exposure (Bhavanam and Stout 2021, Kraus and M. J. Stout 2019, Pankaj et al. 2013, Heijari et al. 2008). The studies in this thesis opted to use ten days as time points for gene expression assays to see if any could be detected, as there were not clear reasons why transcripts would not be detected post-seven days given in the literature.

Limitations and other potential tests

None of these tests show any effects in gene expression or plant development or disease resistance due to either 1.0 mM JA or 1.0 mM MeJA on the peas. These same treatments are used in comparison in the following chapters, despite the lack of evidence of effect. This is due to timing and logistics of finishing a coherent

project – most of the work in chapters 3 and 4 of this thesis was completed in the final six months of experimental work time. Originally, the project was to have a more nanoparticle and analytical chemistry focus and major delays in the testing of nanoparticle synthesis and development of GCMS detection methods led to a change in direction. This, combined with the multiple iterations of testing and development for the pathosystems meant the key questions of this work could only be tested at the very end of the project. Ideally, we would have (i) tested these different concentrations as soil drench as well, and (ii) tried higher concentrations, and/or (iii) tried a different pathogen-plant combination.

At the time of this writing there were no available studies on jasmonate treatments in peas for inducing defense responses but there are a few studies using jasmonate treatments to help relieve cadmium stress in peas. One study reported foliar treatments of 1, 5 or 10 uM MeJA reduced cadmium stress in three varieties of peas while also increasing activity of antioxidant enzymes superoxide dismutase, peroxidase, and catalase (Manzoor et al. 2022). Another study found a combined foliar spray of 5 M potassium and 0.5 mM JA treatment effective in reducing cadmium stress in peas (Abbas et al. 2022).

Another experiment that would have been useful to our understanding of this work would be tests to verify that the JA was taken up by the seeds. Within this work a visual assessment was used to confirm the seeds had received the seed treatment – after soaking 24 hours, sealed, in the seed soaking treatments the seeds had grown by 50 -80% in volume and the soaking solution had decreased by > 70% in volume. This, while it confirms the seeds were imbibed, does not directly confirm that the jasmonates within the seed soaking solutions were taken up by the plants. To fully confirm the jasmonates were taken up by the plants, early timepoint RT-qPCRs could have been used. Additionally, a jasmonate biosensor line of Arabidopsis has been developed by Larrieu et al., and this could have been used as a proxy for understanding whether plants had perceived the jasmonates (Larrieu et al. 2015). Many tests were trialled with these biosensor Arabidopsis plants but time constraints meant the work was too preliminary to be included in this thesis.

Furthermore, it may be that *Pythium ultimum* is not the ideal pathogen to study the effects of jasmonates in peas and/or peas may not be particularly responsive to jasmonates. Other pathogens or insects, like *Rhizoctonia* spp., *Fusarium* spp. or aphids could form a better pathosystem or other crops could be investigated, like barley, wheat or maize.

Root analysis is still an emerging field within plant science and new understandings about how metrics such as root length, branching patterns, surface areas, and total dry mass relate to aspects of plant growth, defense and reproduction are still being discovered (Freschet et al. 2021, Urfan et al. 2022). RhizoVision Explorer v2.0.3 (Seethepalli and York 2020, Seethepalli, Dhakal, et al. 2021) was chosen for image analysis of roots because it produces a wide variety of data outputs and has many citations and lots of publicly available data backing up its reliability and functions (91 citations as of Feb 2024 via Dimensions https://badge.dimensions.ai/details/id/pub.1140975354).

3.5 Summary

Investigations in this chapter adapted and tested several *Pisum sativum-Pythium ultimum* disease systems, one in vitro, one sterile soil, and one non-sterile soil. After settling on the non-sterile soil system as the most replicable and accurate different concentrations of JA and MeJA were tested for germination inhibition in peas. Initial tests using 1.0 mM JA and 1.0 mM MeJA seed treatments in the non-sterile soil system revealed minimal effects of either jasmonate on plant growth and development factors or disease resistance in Pea-*Py.* system. Finally, RT-qPCR was used to investigate the effects of the seed treatments on jasmonate related gene expression and downstream defense response gene expression, through COI1, JAR4, LOX2, MYC1, and DRR230.

4

Effects of Jasmonate-Loaded Zeolite on *Pisum sativum* gene expression and resistance to *Pythium ultimum*

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4.1 Introduction

This chapter examines the effects of jasmonate-loaded silicates on disease resistance and plant growth and development metrics in peas exposed to *Pythium ultimum*. RT-qPCR was also used to examine gene expression responses to the loaded silicates.

4.1.1 Silicates and Plant Growth and Development

Elemental silicon is the second most abundant element in the earth's crust and in average mineral soil forms about 28% of the soil matrix (Wedepohl 1995). Silicon is rarely found by itself, it is often bonded to oxygen in the form of silicon dioxide or to other minerals in crystalline structures (RSC 2017). Silicon in soil is mainly found as monosilicic acid (H_2SiO_4) and this also appears to be the primary form of silicon taken up by plants (Liang et al. 2015b). Monosilicic acid is actively transported via roots into plant tissues and precipitates as silicon oxide, SiO_2, in which form it is deposited in various biological structures, including cell walls and epidermal tissues (Luyckx et al. 2017, Liang et al 2015b). The concentration of monosilicic acid in soil is strongly dependent upon soil pH and redox potential and for these reasons the available silicon in soil varies heavily with flood and drought conditions (Liang et al. 2015b). Silicon content varies widely between highly weathered soils and highly productive agricultural land and thus the threshold for bioavailable soil silicon also varies but is broadly considered to be above 20 ppm or 95 mg SiO_2 kg-1 (Liang et al. 2015b). However, evidence is accumulating of the positive impacts of Si fertilization on rice even in soils two to three times above the bioavailable threshold value. Threshold values for use in other crop species are still being defined, suggesting room for revision on the threshold targets for bioavailable Si (Liang et al. 2015b).

Silicon has been for a long time considered non-essential for plant growth, as it was not a required element for solution culture techniques when they were first being developed in the 1860s. At this time, elements were included or excluded based upon the question, will lack of this element prevent a plant from completing the vegetative or reproductive stage of its life cycle? (Emanuel Epstein 1999). However, this view is being increasingly questioned as evidence accumulates for the

positive role of silicon in strengthening cell walls, ameliorating drought, heat, and cold stress, priming immune responses for disease resistance, and increasing growth and yield in several key crops such as rice, sugarcane, soybean, rapeseed, potato, and wheat (Artyszak 2018, Schaller et al. 2023, Liang et al. 2015b).

Silicon supplementation has been shown to directly impact several metrics of plant growth and development in a variety of plant species. Cucumber plants grown with higher vs minimal silicon supplementation grew darker, larger leaves with a higher rigidity, had a higher dry weight per area, and had higher RuBisCO activity (Adatia and Besford 1986). However, the fruit yield was the same between plants grown on minimal versus high silicon (Adatia and Besford 1986). Rice, cotton, loblolly pine, and pointsetta plants have all been found to benefit in growth and development metrics like plant height, leaf area, and seed yield from silicon supplementation (Emanuel Epstein 1999, J. F. Ma and Yamaji 2006).

4.1.2 Silicates and Abiotic and Biotic Stress Tolerance

One of the major ways silicon appears to promote plant growth and development is through mitigating the effects of abiotic stressors like drought, heat stress, cold stress, salinity, and heavy metal toxicity. Two major mechanisms appear to be responsible for these positive effects of silicon on abiotic and biotic stress tolerance: (i) the physical and mechanical functions of silicon deposits within the plant, and (ii) biochemical responses triggered by the uptake of silicon, like those responses that induce biotic stress tolerance (Luyckx et al. 2017).

SiO_2 deposits in the stem of rice plants strengthen the stem and improve lodging resistance - the ability of a plant to not topple over during wind or other adverse weather conditions (Liang et al. 2013), SiO_2 deposits in the leaf epidermis form a physical barrier and enhance UV protection (Goto et al. 2003) as well as form a double cuticle layer which can reduce water loss (Gong et al. 2003). SiO_2 deposits in the exodermis and endodermis in rice can alleviate salinity toxicity by acting as a physical filter system and reducing transpirational bypass flow (Yeo et al. 1999).

Silicon treatment has been shown to induce anti-fungal aglycones in wheat (Rémus-Borel et al. 2005), and to induce defense-associated enzymes such as polyphenol oxidase and peroxidase as well as increasing expression of phenylalanine ammonia lyase and lipoxygenase genes when plants were under infection (Rahman et al. 2015). Silicon application has also been shown to prime JA anti-herbivore immune responses in rice (Ye et al. 2013).

The majority of evidence for silicon priming plant defenses is for biotrophic or hemibiotrophic pathogens. Silicon effectively controls powdery mildews in a broad range of plants as well as *Magnaporthe grisea* and *Bipolaris oryzae* in rice by both mechanical means, i.e., forming a physical barrier to infection, and, specifically when applied to root tissues, by inducing defense response upregulation in the plants (Bowen et al. 1992, Liang et al. 2015a, Guével et al. 2007, Ning et al. 2014, Liang et al. 2015b, Wang et al. 2020). There are a few examples of silicon priming reducing disease of *Rhizoctonia solani* on rice (Rodrigues et al. 2003) and *Pythium ultimum* in cucumbers (Chérif et al. 1992). It is unclear if this bias towards biotrophic and hemibiotrophic pathogens is simply due to these types of plant-pathogen interactions being more commonly studied or if silicon is genuinely more effective against pathogens which have a biotrophic phase of their lifestyle (Liang et al. 2015a).

4.1.3 Zeolites and Biotic and Abiotic Stress Tolerance

Zeolites have been shown to provide similar benefits to silica supplementation in various crop species, with added benefits of improving water retention and soil structure, due to zeolites' water sorption characteristics (Ghanbari and Ariafar 2011, Ozbahce et al. 2015, Shahsavari et al. 2014, Chen et al. 2017, Aainaa et al. 2018, Zheng et al. 2018). Zeolite has primarily been used for mitigating effects of drought, heat, salinity and other abiotic stressors; there are limited tests of its efficacy for crop protection against biotic stressors. Poulaki et al found zeolite soil amendments increase resistance to *Sclerotinia sclerotiorum* in lettuce though it was not found to be effective against *Rhizoctonia solani* (Poulaki et al. 2020).

Drench applications of zeolite were found to be effective against *Plasmopara viticola* downy mildew in table grapes (Polat et al. 2020).

The primary methods of zeolite providing crop protection differ from those of standard slag silicon, however. Zeolite appears to work similarly to kaolin and other aluminosilicate clays by providing both a physical barrier and desiccation properties which prevent insects and pathogens from growing (Percival and Boyle 2009, De Smedt et al. 2016, Eroglu 2014). These are most effective as foliar treatments or drenches. There are few tests of zeolite as a soil amendment or zeolite's ability to produce soluble monosilicic acid but those available show minimal increase in soluble monosilicic acid from zeolite (Rashad and Hussien 2020). However, zeolite has been shown to increase polyphenol production in plants, similar to that induced by silica treatment, which suggests some similarity in zeolite perception and perhaps uptake of zeolite-weathered soluble silicon into the plants (Moale et al. 2021).

There has been little study of zeolite in plant-pathogen interactions and biotic stress tolerance, though there is substantial supporting evidence of its beneficial effects for water use efficiency, nitrogen use efficiency and the ability of soils to retain water and nutrients (Babaousmail et al. 2022, Chen et al. 2017, Ghanbari and Ariafar 2011, Ozbahce et al. 2015, Shamili et al. 2020, Szatanik-Kloc et al. 2021, Mondal et al. 2021, Hazrati et al. 2022).

4.1.4 Silicates and Gene Expression

Silicon treatment is associated with a wide variety of gene upregulation, including genes related to defense, drought response, heat stress, and photosynthetic activity. Silicate treatment in tomato plants exposed to *A. solani* increased expression of defense-related genes *PR1*, *PR2*, *PR3*, *LOXD*, *JERF3*, and *WRKYII*, with large upregulation in the JA-pathway genes *PR3*, *LOXD* and *JERF3* (Gulzar et al. 2021). Muhindi et al found silicon treatment increased expression of thirteen *Nicotiana tabacum* histidine-rich defensin 1 (NtHRD1) family genes in Nicotiana tabacum (Muhindi et al. 2023). Rice under dry cultivation showed an increase in photosynthetic and antioxidant enzyme activity as well as upregulation of genes

associated with flavonoid biosynthesis and photosystems I and II upon treatment with silicon (Jiang et al. 2022). These plants also showed improved drought tolerance (Jiang et al. 2022). Silicon supplementation has additionally been shown to increase phenylalanine ammonia lyase (PALa and PALb) and lipoxygenase (LOXa) gene expression as well as polyphenolic compound production following infection with *Magnaporthe oryzae* in perennial ryegrass (*Lolium perenne L.*) (Rahman et al. 2015).

Soybean plants supplemented with silicon showed upregulation of auxin biosynthesis and transporter genes without any drought or pathogen stress while wheat seedlings treated with silicon showed high upregulation in genes involved in secondary biosynthetic processes (Tripathi et al. 2022, Hao et al. 2021). Auxin transporters were also found to be upregulated with silicon treatment in rice seedlings under arsenic stress (Khan et al. 2023). An RNA-sequencing experiment in lentils found silicon supplementation under drought conditions increased expression of genes related to photosynthesis, osmoprotection, antioxidant systems, signal transduction, cell wall biosynthesis, vascular development and biosynthesis of alkaloids and flavonoids (Biju et al. 2023). Additionally, drought, metal toxicity, and salt stress responsive genes are upregulated in rice and sorghum upon treatment with silicon (Yin et al. 2016, Song et al. 2014, Khattab et al. 2014).

4.1.5 Zeolites Gene Expression

There are fewer studies on zeolite treatments and gene expression than on silicates and gene expression. Poulaki et al found zeolite treatment increased expression of LOX and ERF1 in lettuce exposed to hemibiotrophic *S. sclerotiorum* but decreased expression of LOX and ERF1 in lettuce exposed to necrotrophic *R. solani* (Poulaki et al. 2020). Some gene expression changes can be inferred by increased production of secondary metabolites like anthocyanins and polyphenols, but few studies have looked specifically at gene expression in relation to zeolite treatment in plants. This may be because zeolite is primarily used in agriculture as scaffold for fertilizers or for increasing water holding capacity of soils. For example, zeolite combined with compost was shown to increase anthocyanin content, photosynthetic activity and

reduce water stress, and leaf temperature in grape vines as compared to compost alone, while application of nanozeolite was shown to increase essential oil contents in commercial coriander plants and to increase activity of antioxidant enzymes in sweet potato under salinity stress (Cataldo et al. 2021, Mahmoud et al. 2020, Mahmoud et al. 2023). A zeolite treatment was found to slightly upregulate PR1 (pathogenesis related protein 1) and downregulate PR4 (pathogenesis related protein 4) in aubergine exposed to *Verticillium dahliae* while upregulating Chi1 (chitinase 1) and PAL1 (Phenylalanine ammonia-lyase 1) in melon plants exposed to *Fusarium oxysporum* f. sp. melonis (Kefalogianni et al. 2017). PR1 is a marker of the SA-dependent defense pathway and Chi1 and PAL1 are markers of secondary metabolite pathways. The evidence from Kefalogianni et al and Poulaki et al taken together presents evidence for zeolite activating a wide variety of defense responses in plants, including secondary metabolite production and both the JA and SA dependent plant defense pathways. Though there are few studies on zeolite induced gene expression they hint towards a similar broad spectrum of plant defense and abiotic stress tolerance functions as that of silicon. Zeolite is thus a promising candidate for exploring further in plant defense functions.

4.2 Methods

4.2.1 Preparation of Loaded Silicates

The zeolite loading protocol was adapted from the mesoporous silica nanoparticle loading protocol in Bravo-Cadena et al 2018 (Bravo-Cadena 2018). Loading solutions of 1.0 mM, 10.0 mM, or 50.0 mM methyl jasmonate were prepared in ddH_2O with a small amount of methanol, up to 0.5% to allow solubility in water (Sigma Aldrich, Gillingham, UK).

50 grams of zeolite clinoptilolite (Finest Filters, 1kg clinoptilolite) was added to a CookWorks Coffee & Spice Grinder (Argos, Milton Keynes, UK) and ground on full speed for 2 minutes until smooth. The ground zeolite was then sifted through a 1 mm^2 strainer. The powder was stored in a sealed 50 mL falcon tube at room temperature.

600 mg zeolite was added per 50 mLs loading solution. Loading solution proportions were maintained at 10 mL DMSO plus up to 40 mL ddH2O with the appropriate concentration of MeJA added to reach the sought loading solution concentration and a total solution volume of 50 mL.

Mock zeolite was prepared in the same ratios as above, with only water and DMSO (40 mL ddH2O and 10 mL DMSO). Mock zeolite was used as a control treatment, along with water alone and untreated (and unsterilized) zeolite.

The zeolite was added to the loading solutions and stirred at room temperature (22°C) for 24 hrs.

After 24 hrs the solution, including all sediment, was transferred to a 50mL falcon tube and centrifuged at 3050 x g for 9 minutes. The supernatant was discared.

The zeolite sediment was then washed 2X with 45 mL sterile water. A stripette and a vortex were used to resuspend the zeolite between centrifuging for each wash step. Zeolite was centrifuged at 3050 x g for 9 minutes for each wash step. The final wash step supernatant was discarded.

The loaded, washed zeolite was freeze dried in a Christ Alpha 2-4 LD freeze drier (Martin Christ Gefriertrocknungsanlagen, Osterode am Harz, Germany) and stored at –20°C until use.

4.2.2 Non-sterile Soil System

The assay used to assess the loaded zeolite seed treatments was the non-sterile soil system described in Chapter 3.2. Seeds were sterilized as described in Chapter 3.2. Sterilized seeds were added to solutions containing 20 mg/mL mock or 20mg/mL loaded zeolite. Sterile ddH2O was included as a control treatment. Solutions were prepared in a sterile environment using sterile ddH2O. Seeds were soaked in the sterile treatment solutions for 24 hrs in the dark at 25°C. After treatment, seeds were drained and immediately planted into either soil or *Pythium* inoculated soil, as described in 3.2. Seeds were harvested and metrics of growth, development, and disease resistance measured and analyzed as in 3.2.

4.2.3 Reverse Transcriptase Quantitative PCR

RT qPCR was completed as in 3.2.

4.3 Results

The aim of this chapter was to examine the effects of jasmonate-loaded zeolites on plant disease resistance and growth and development metrics.

Several tests were performed to test different combinations of jasmonate-zeolite or jasmonate-HMSNP seed treatments. The primary test was an non-sterile soil growth and disease test, developed in Chapter 3. Additionally, germination tests using the basic non-sterile soil set up were also performed as preliminary tests to study the effects of zeolites and different loading solutions on pea germination. Finally, RT-qPCR was used to test for gene expression changes in the different tests groups, to complement the non-sterile soil tests.

4.3.1 Disease Resistance and Plant Growth Results

Initial tests using mock, 1 mM, 10 mM, and 50 mM loading solutions of either JA or MeJA were completed using the non-sterile soil system. These tests were repeated a total of three times for the different JA loaded zeolites and five times for the MeJA loaded zeolites. In each case, the concentration of the jasmonates listed is the concentration of the loading solution for the zeolites. After the zeolites were loaded, washed, and freeze dried they were then added at 20 mg/ml to ddH_2O to create the seed soaking solution. The final concentration of jasmonates in the seed soaking solution is unknown.

The JA loaded zeolite treatments had no effect on fresh or dry weight as compared to the mock loaded control zeolite in either the soil only or *Pythium* exposed conditions. See panels (A) and (B) in Figure 4.1. Both the fresh weights and the dry weights were lower in the *Pythium* exposed condition than in the control condition and this difference was statistically significant.

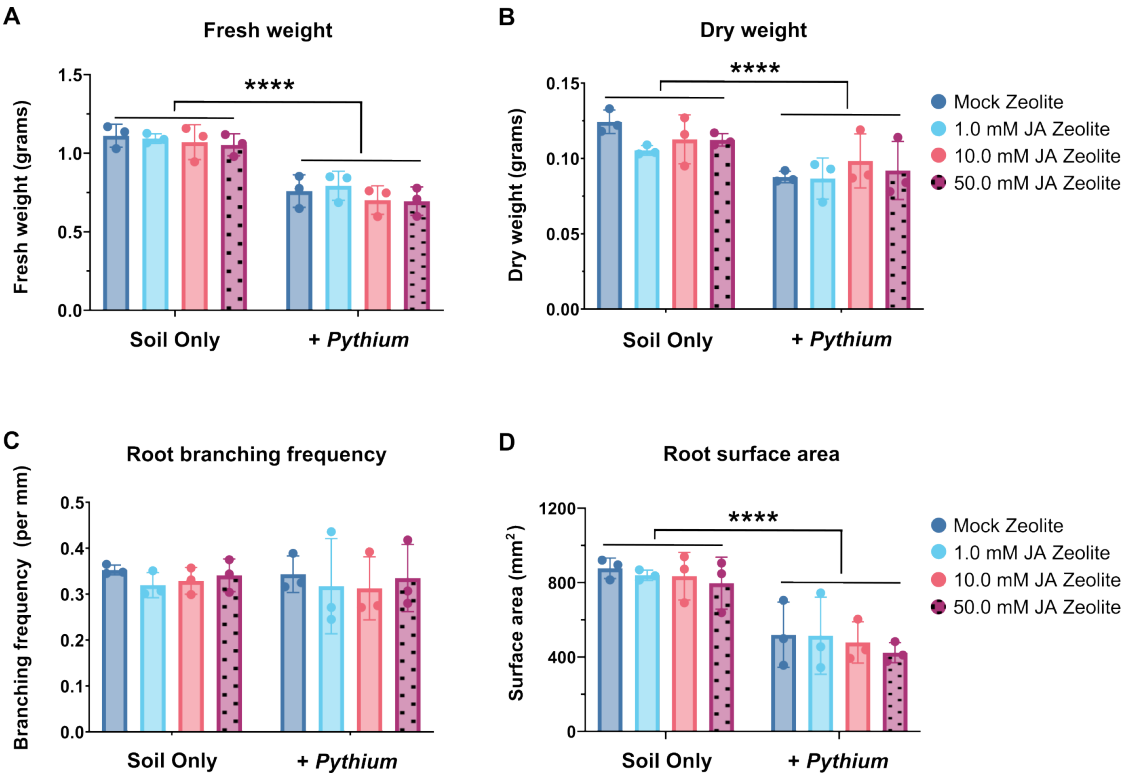


Figure 4.1: Growth and development outcomes from non-sterile soil tests of mock, 1.0 mM, 10.0 mM and 50.0 mM JA loaded zeolite seed treatments. Seeds were surface sterilized and then soaked for 24 hours in the dark at 25°C before planting in either control soil (soil only) or soil with a *Pythium* innoculum mixed in (+ *Pythium*). The plants were then grown in a growth chamber under a 12 h photoperiod at a light intensity of 90 - 105 µmol/m2/s for 10 days and watered as needed. Plants were harvested on day 10. Representative data showing **(A)** yield of fresh weight in grams, **(B)** yield of dry weight in grams, **(C)** root branching per mm, and **(D)** root surface area in mm^2 for the different JA loaded zeolite seed treatments. Data shows means of three replicate trays where each tray value is the mean of twelve seedlings. Error bars show standard deviation. Data tested for normality and analyzed with a two-way ANOVA and Tukey's post hoc tests for multiple comparisons.

None of the JA loaded zeolite treatments had an effect on root branching frequency, either under the control condition or under the disease condition. There was no difference between either condition.

Root surface area was also not affected by any of the treatments within either condition though root surface area was lower under the *Pythium* condition overall.

Survival, emergence and root length were all lower under the *Pythium* exposed condition than the soil only condition. Within each condition there were no differences between the treatment groups. Root length showed some variability

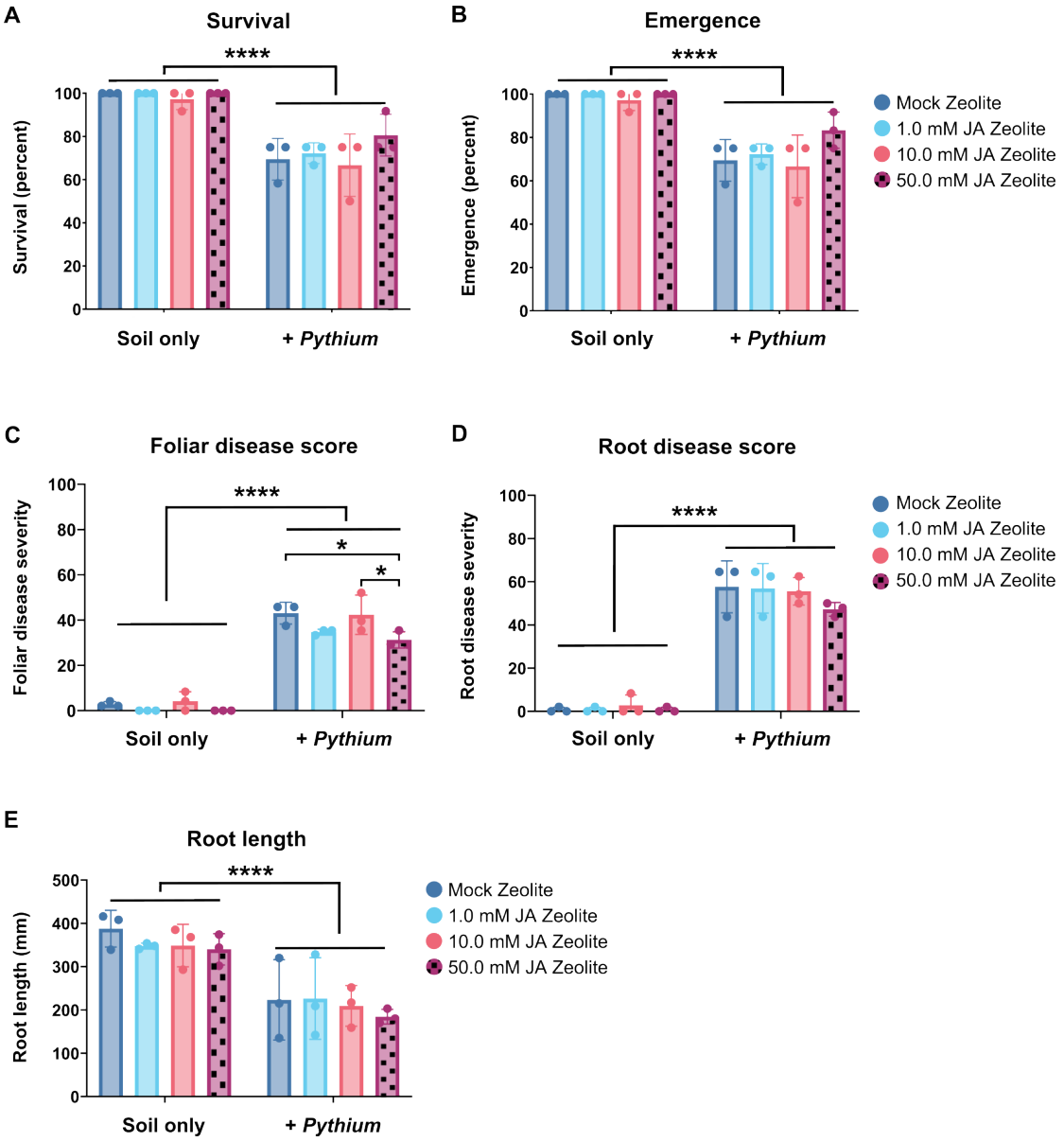


Figure 4.2: Disease resistance outcomes from non-sterile soil tests of mock, 1.0 mM, 10.0 mM and 50.0 mM JA loaded zeolite seed treatments. Seeds were surface sterilized and then soaked for 24 hours in the dark at 25°C before planting in either control soil (soil only) or soil with a *Pythium* innoculum mixed in (+ *Pythium*). The plants were then grown in a growth chamber under a 12 h photoperiod at a light intensity of 90 - 105 µmol/m2/s for 10 days and watered as needed. Plants were harvested on day 10. Representative data showing **(A)** percent survival, **(B)** percent emergence, **(C)** foliar disease index, **(D)** root disease index, and **(E)** root length in mm for the different JA loaded zeolite seed treatments. Data shows means of three replicate trays where each tray value is the mean of twelve seedlings. Error bars show standard deviation. Data tested for normality and analyzed with a two-way ANOVA and Tukey's post hoc tests for multiple comparisons.

within the mock and 1.0 mM JA loading solution groups but these differences were not statistically significant.

Foliar disease and root disease were both much higher under the *Pythium* condition as compared to the soil only condition. The soil only condition had very low foliar and root disease scores overall. Within the *Pythium* condition foliar disease was lower in the 50.0 mM JA loading solution than in the 10.0 mM JA loading solution and the mock but not different from the 1.0 mM JA loading solution. There were no differences between treatment groups for root disease score within the *Pythium* exposed group though the 50.0 mM JA group had the lowest variability overall.

The JA loaded zeolites produced roughly the same results for each experimental repeat - no effect of the JA-loaded zeolites on pea survival or disease symptoms either with or without *Pythium* exposure. However, the MeJA loaded zeolites, shown in Figures 4.3, 4.4, and 4.5 produced two conflicting sets of results.

Survival and emergence were lower in the *Pythium* exposed conditions as compared to the soil only conditions for both the summer and the fall/winter trials, seen in Figure 4.4. However, in the summer trials both survival and emergence were higher in the 10.0 mM MeJA zeolite treatments than in the controls and 50.0 mM MeJA zeolite treatments. There was no difference between treatment groups under either condition for the fall/winter trials. There was additionally very little variation in survival and emergence within groups under soil only conditions for the summer trials, shown in panels (A) and (B). This was not the case in the fall/winter trials, where there was much higher variability within groups across treatment groups and conditions.

Additional disease severity metric for the two types of trials are shown in Figure 4.5. Again, half of the panels show data from the summer trials – panels (A), (B) and (E) – and half of the panels show data from the fall/winter trials – panels (C), (D) and (F).

Both foliar and root disease severity were higher under *Pythium* conditions than in soil only conditions for both summer and fall/winter trials. However, the

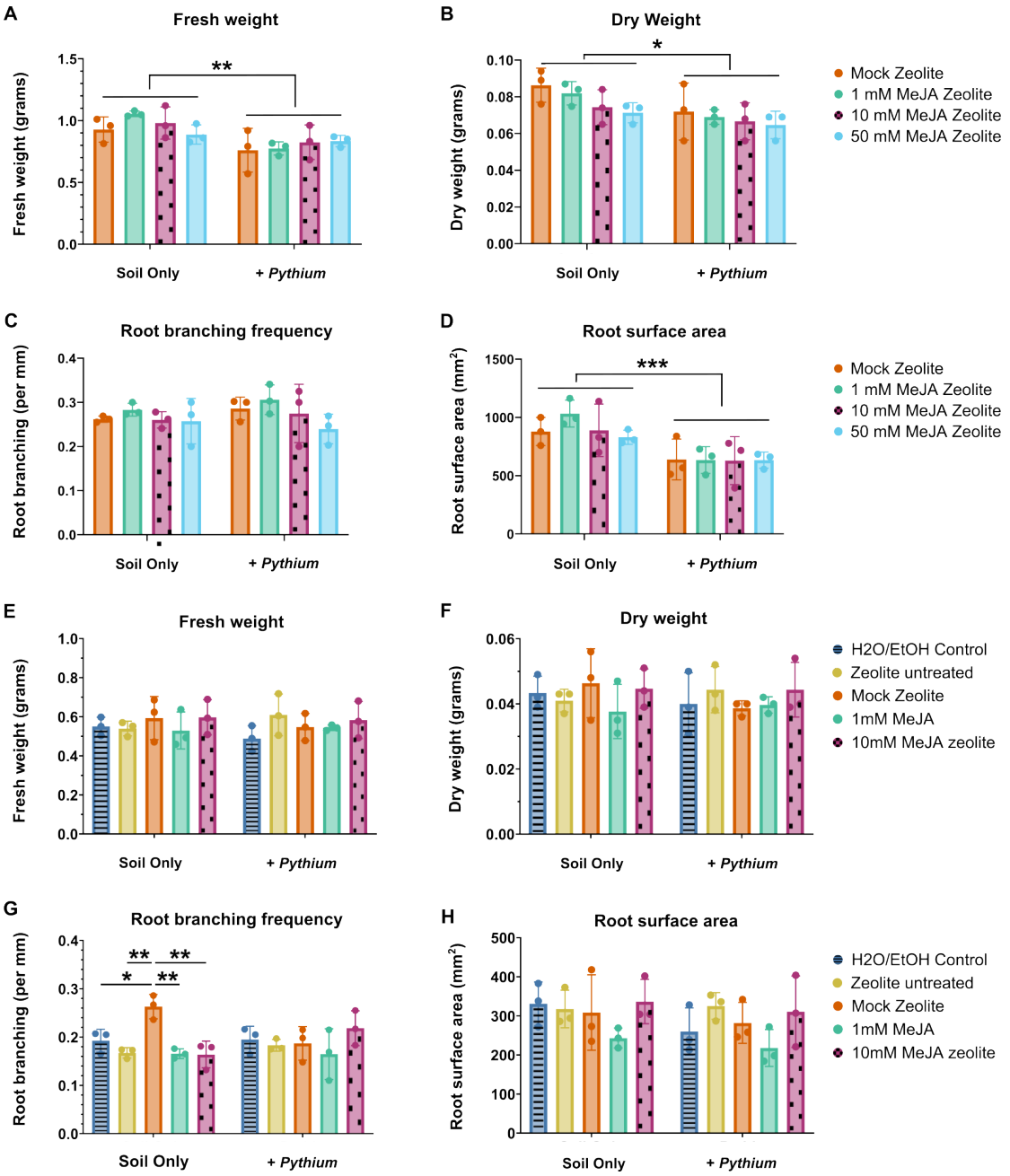


Figure 4.3: Growth and development outcomes from non-sterile soil tests of mock, 1.0 mM, 10.0 mM and 50.0 mM MeJA loaded zeolite seed treatments. Seeds were surface sterilized and then soaked for 24 hours in the dark at 25°C before planting in either control soil (soil only) or soil with a *Pythium* innoculum mixed in (+ *Pythium*). The plants were then grown in a growth chamber under a 12 h photoperiod at a light intensity of 90 - 105 µmol/m2/s for 10 days and watered as needed. Plants were harvested on day 10. Representative data showing the two types of experimental results. Panels **A, B, C** & **D** representative of experiments where 10 mM MeJA conferred some disease resistance and panels **E, F, G** and **H** representative of experiments where there were no effects of any of the seed treatments. **(A)** and **(E)** yield of fresh weight in grams, **(B)** and **(F)** yield of dry weight in grams, **(C)** and **(G)** root branching per mm, and **(D)** and **(H)** root surface area in mm^2 for the different JA loaded zeolite seed treatments. Data shows means of three replicate trays where each tray value is the mean of twelve seedlings. Error bars show standard deviation. Data tested for normality and analyzed with a two-way ANOVA and Tukey's post hoc tests for multiple comparisons.

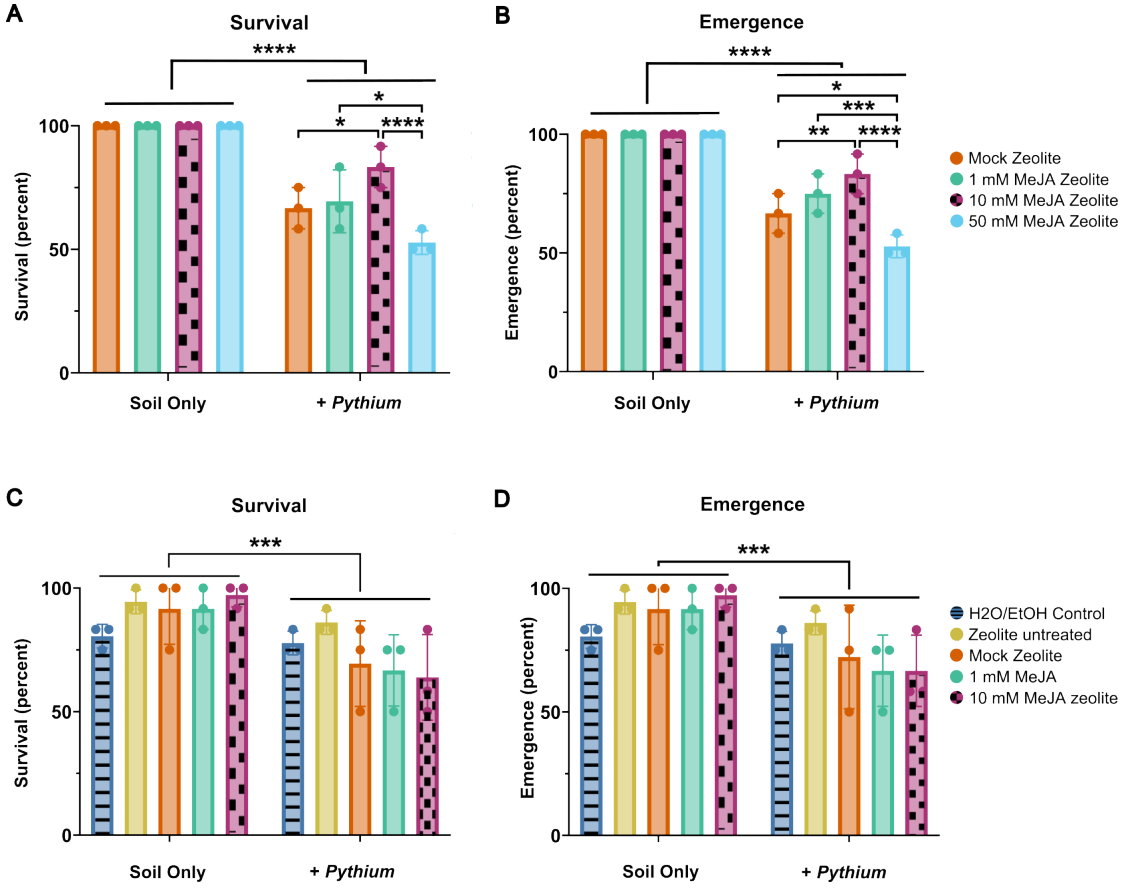


Figure 4.4: Disease resistance outcomes Part I from non-sterile soil tests of mock, 1.0 mM, 10.0 mM and 50.0 mM MeJA loaded zeolite seed treatments. Seeds were surface sterilized and then soaked for 24 hours in the dark at 25°C before planting in either control soil (soil only) or soil with a *Pythium* innoculum mixed in (*Pythium*). The plants were then grown in a growth chamber under a 12 h photoperiod at a light intensity of 90 - 105 µmol/m2/s for 10 days and watered as needed. Plants were harvested on day 10. Representative data showing the two types of experimental results. Panels **A** & **B** representative of experiments where 10 mM MeJA conferred some disease resistance and panels **C** and **D** representative of experiments where there were no effects of any of the seed treatments. Panels **(A)** and **(c)** show percentage survival. Panels **(B)** and **(D)** show percentage emergence for the different MeJA loaded zeolite seed treatments. Data shows means of three replicate trays where each tray value is the mean of twelve seedlings. Error bars show standard deviation. Data tested for normality and analyzed with a two-way ANOVA and Tukey's post hoc tests for multiple comparisons.

foliar and root disease severity under control conditions in the summer trials is minimal and much lower than that of the fall/winter trials. Additionally, in the summer trials the 10 mM MeJA loaded zeolite had a lower foliar disease score than the 50 mM MeJA zeolite treatment group did under *Pythium* conditions. Root disease severity was also higher by a statistically significant margin for the 50 mM MeJA zeolite treatment under *Pythium* conditions.

In the fall/winter trials, there was a much higher baseline for both foliar and root disease severity as well as more variation within treatment groups under both conditions. In the fall/winter trials an unloaded zeolite was tested as an additional treatment group. In these trials, the unloaded zeolite appeared to result in the lowest foliar and root disease severity under *Pythium* conditions though this difference was not statistically significant.

Root length for both summer and fall/winter trials was lower under *Pythium* conditions as compared to control conditions. There were no statistically significant difference in root length between treatment groups within conditions for either summer or fall/winter trials. However, root lengths were on average shorter for the fall/winter trials as compared to the summer trials for both conditions by nearly 50%.

The first trials of the MeJA loaded zeolites, performed in June and July 2023, showed increased disease resistance and decreased disease symptoms with the 10 mM MeJA zeolite seed treatment. However, when these results were repeated in October and December 2023 no effects on disease resistance or disease severity were seen. The different trials had slightly different treatment groups: Mock, 1.0 mM MeJA, 10.0 mM MeJA, and 50.0 mM MeJA in the first sets of trials and a non-zeolite seed soak control, an untreated – not loaded –, 1.0 mM MeJA, and 10.0 mM MeJA in the second set of trails. Figure 4.3 compares the growth and development data from these two different types of experimental outcome.

In panels (A) and (B) fresh weight and dry weight data are shown for the first sets of trials. In these cases, both fresh weight and dry weight were lower under *Pythium* conditions than under soil only conditions. There was relatively small variation within treatments excepting the 10 mM MeJA zeolite under the *Pythium condition*.

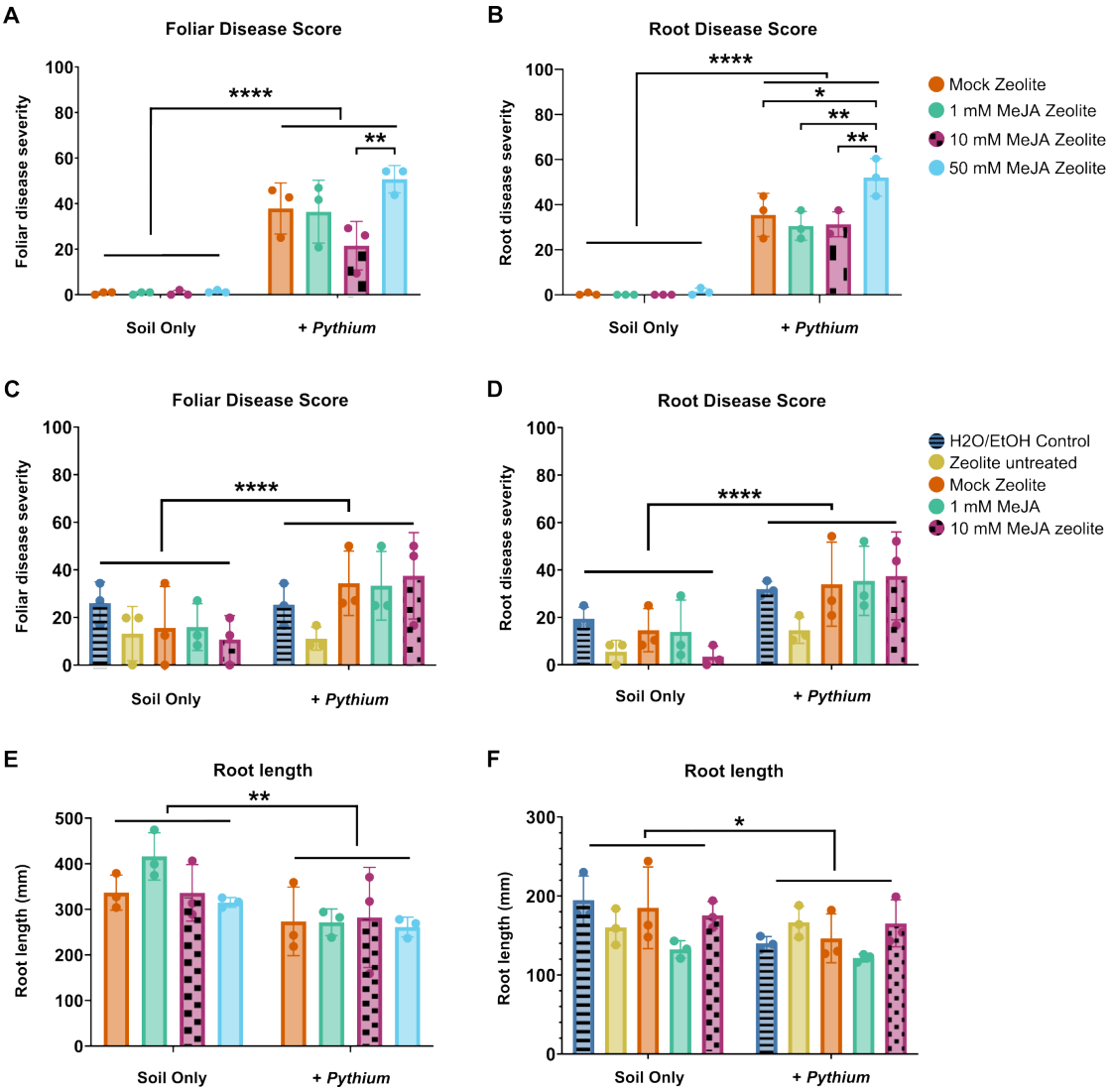


Figure 4.5: Disease resistance outcomes Part II from non-sterile soil tests of mock, 1.0 mM, 10.0 mM and 50.0 mM MeJA loaded zeolite seed treatments. Seeds were surface sterilized and then soaked for 24 hours in the dark at 25°C before planting in either control soil (soil only) or soil with a *Pythium* innoculum mixed in (*Pythium*). The plants were then grown in a growth chamber under a 12 h photoperiod at a light intensity of 90 - 105 µmol/m2/s for 10 days and watered as needed. Plants were harvested on day 10. Representative data showing the two types of experimental results. Panels **A, B** and **E** representative from experiments where 10 mM MeJA conferred some disease resistance and panels **C, D** and **F** representative of experiments where there were no effects of any of the seed treatments. Panels **(A)** and **(C)** show foliar disease severity index. Panels **(B)** and **(D)** show root disease severity index. Panels **(E)** and **(F)** show root length. Data shows means of three replicate trays where each tray value is the mean of twelve seedlings. Error bars show standard deviation. Data tested for normality and analyzed with a two-way ANOVA and Tukey's post hoc tests for multiple comparisons.

Comparing these to the data in panels (E) and (F), which were representative of later trails, both fresh weight and dry weight were higher in panels (A) and (B) and variation within treatment groups was also lower for the summer trials in panels (A) and (B). In the fall/winter trials shown in panels (E) and (F) there was no difference between conditions or between treatment groups within conditions.

Panels (C) and (D) show the root branching frequency and root surface area for the summer trials. In this case, root surface area is lower under the *Pythium* condition with moderate variability within treatment groups. There are no statistically significant differences between groups within either the soil only or *Pythium* condition. Root branching frequency showed no difference between soil only and disease conditions and no differences between groups within either condition.

In the fall/winter trials there were no difference in root surface area between control and disease conditions, unlike that of the summer trials. Variability within treatment groups was higher for the fall/winter trials than in the summer trials, for both conditions. Root branching frequency had no differences between the two conditions. Within the soil only condition, mock zeolite showed a statistically significant increase compared to all other treatment groups.

4.3.2 Relative Gene Expression Results

As part of the investigations into the effects of the seed treatments on plant defense responses RT qPCR experiments were performed on non-sterile soil pea samples for various jasmonate and defense pathway genes, see Figure 3.11 for an overview of the genes detected.

Figures 4.6 and 4.7 show gene expression data for both control condition (soil only) and *Pythium*-exposed pea seedlings. Figure 4.6 shows relative expression values, calculated according to the $2^{-\Delta\Delta Ct}$ method. Figure 4.7 show the Ct values for the same experiment as in Figure 4.6.

Relative expression of LOX2, Jar4, and DRR230 for the 10.0 mM MeJA Zeolite treatment had high variability under soil conditions, shown in panel (A) of Figure 4.6. Under *Pythium* exposed conditions the 10.0 mM MeJA zeolite also showed

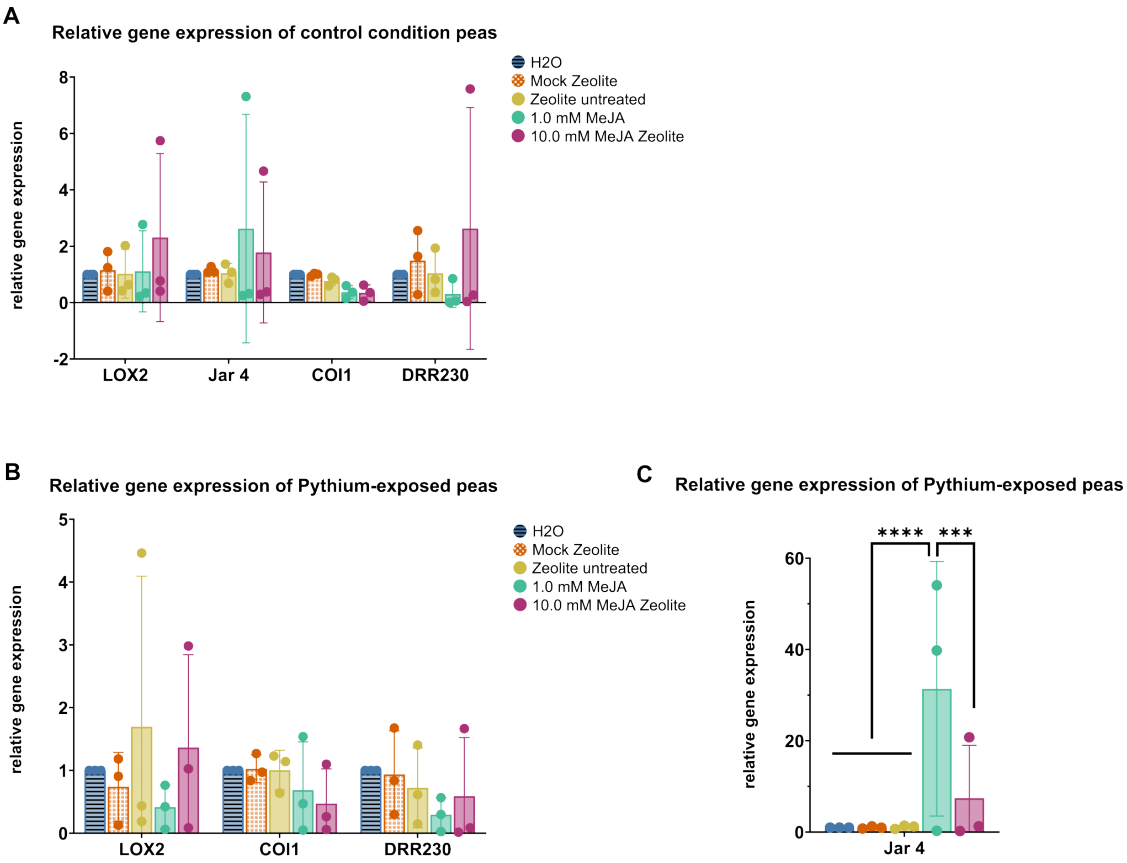


Figure 4.6: $2^{-\Delta\Delta Ct}$ relative gene expression for non-sterile soil peas, for LOX2, JAR4, COI1 and DRR230, relative to H_2O treatment expression levels. Seeds were surface sterilized and then soaked for 24 hours in the dark at 25°C before planting in either control soil (soil only) or soil with a *Pythium* innoculum mixed in (+ *Pythium*). The plants were then grown in a growth chamber under a 12 h photoperiod at a light intensity of 90 - 105 µmol/m2/s for 10 days and watered as needed. Plants were harvested on day 10 and flash frozen in liquid nitrogen before undergoing RNA extraction. **(A)** Relative expression for peas in control (soil only, no *Pythium*) conditions, shown on a linear scale. **(B)** Relative expression of LOX2, COI1 and DRR230 for peas in *Pythium*-exposed conditions, shown on a linear scale. **(C)** Relative expression for peas in *Pythium*-exposed conditions, shown on a linear scale. Data shows means of three technical replicates each for three total biological replicates. Error bars show standard deviation. Data tested for normality and analyzed with a two-way ANOVA and Tukey's post hoc tests for multiple comparisons.

some variability for LOX2 and high variability for Jar4, shown in panels (B) and (C) of Figure 4.6, respectively. DRR230 levels were similar on average to those seen in the H_2O control group under *Pythium* conditions, shown in panel (B) of Figure 4.6. None of these differences were statistically different from one another or from the H_2O control treatment within their conditions.

10.0 mM MeJA zeolite resulted in low variability in COI1 under soil conditions and low variability in COI1 and DRR230 under *Pythium* conditions.

The 1.0 mM MeJA treatment resulted in high variability in Jar4 expression under both soil only and *Pythium*-exposed conditions. The Jar4 expression values for 1.0 mM MeJA were higher than all other treatment groups under *Pythium* conditions by the statistically significant margin. 1.0 mM MeJA had low variability in gene expression for LOX2, COI1, and DRR230 under both conditions.

Untreated zeolite (zeolite neither mock-loaded nor washed) resulted in some LOX2 variability under *Pythium* conditions. This difference was not statistically significant. Untreated zeolite had low variability for all other genes under *Pythium* conditions and all genes under soil only conditions.

Water was the control treatment to which values were compared and so had a relative expression of 1 for all genes under both conditions. Mock zeolite resulted in low variability for all genes under both conditions.

In addition to relative expression results the threshold cycle (Ct) values for the same experiment are included in Figure 4.7. A Ct value is the cycle at which a transcript is first detected. Panel (A) shows the Ct values for the control condition peas. Like with the relative expression results there are no statistically significant differences between the treatment groups within each gene. Differences between genes were not measured. Beta tubulin (Bt) and PP2A were the housekeeping genes used and these have relatively lower Ct values for all treatments. COI1 and DRR230 also had lower Ct values. There is significant variation in Ct value with the H_2O, Mock zeolite, and untreated zeolite treatment groups for DRR230. 1.0 mM MeJA and 10.0 mM MeJA loaded zeolite show moderate variation for COI1 transcripts. LOX2 and Jar4 show very little variation within all treatment groups.

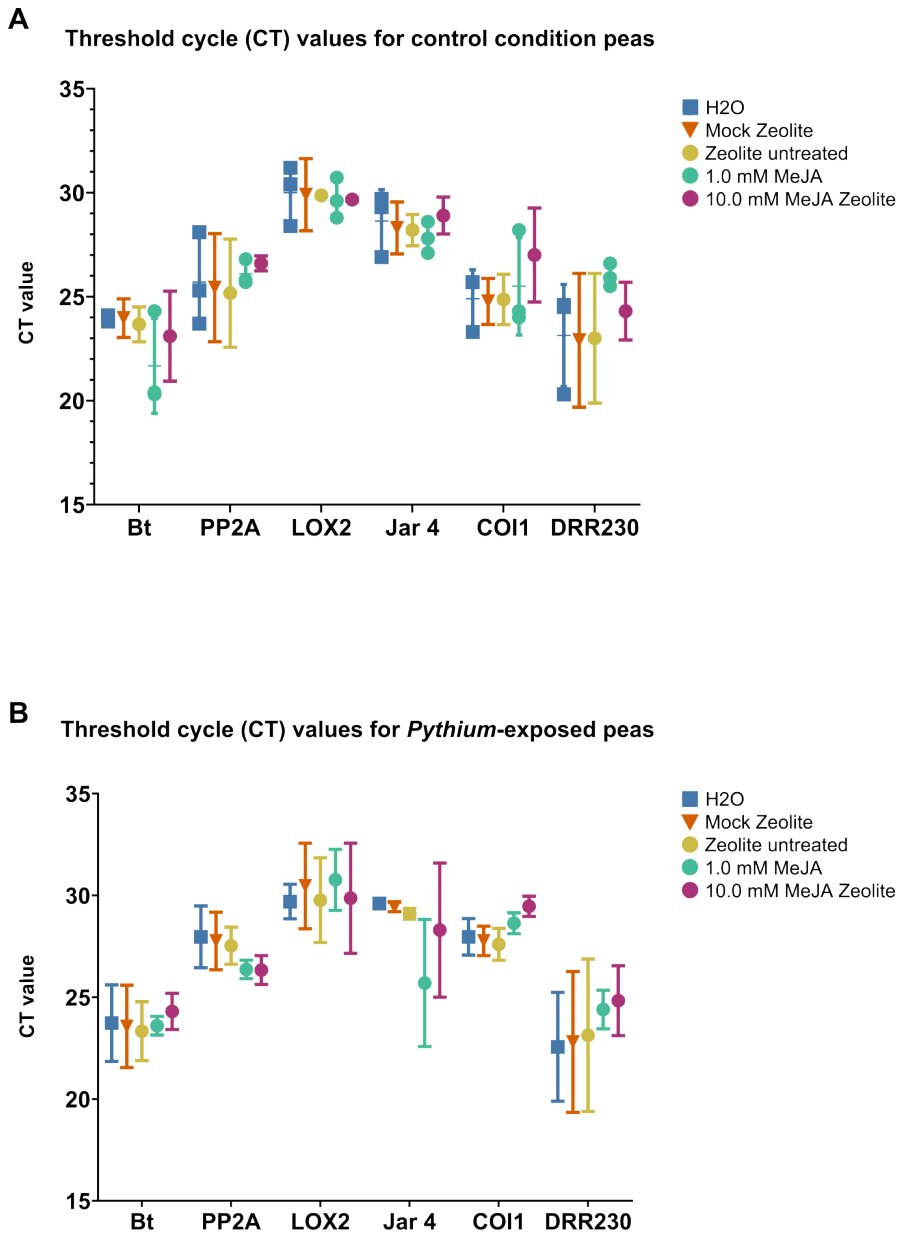


Figure 4.7: RT-qPCR threshold cycle (Ct) values for non-sterile soil pea experiments, showing when transcripts were first detected. The lower the Ct value the more abundant the transcripts. **(A)** Ct values for peas in control (soil only, no *Pythium*). **(B)** Ct values for peas in *Pythium*-exposed conditions. Data shows means of three technical replicates each of three total biological replicates. Error bars show standard deviation. Data tested for normality and analyzed with a two-way ANOVA and Tukey's post hoc tests for multiple comparisons. Seeds were surface sterilized and then soaked for 24 hours in the dark at 25°C before planting in either control soil (soil only) or soil with a *Pythium* innoculum mixed in (*Pythium*). The plants were then grown in a growth chamber under a 12 h photoperiod at a light intensity of 90 - 105 µmol/m2/s for 10 days and watered as needed. Plants were harvested on day 10 and flash frozen in liquid nitrogen before undergoing RNA extraction.

Panel B shows the Ct values for the *Pythium*-exposed peas. There were no statistically significant differences between the treatment groups within each gene. Bt and PP2A each showed moderate variation within H_2O, Mock loaded zeolite, and untreated zeolite treatment groups. Lox2 Ct values with the mock loaded zeolite, untreated zeolite, 1.0 mM MeJA, and 10.0 mM MeJA loaded zeolite treatment groups was also moderate to high. Variation within the H_2O treatment group for LOX2 Ct values with minimal. Jar4 transcripts showed minimal variation with the H_2O, mock loaded zeolite, and untreated zeolite treatment groups. Jar4 variation was quite large, spanning 7-8 cycles for the 1.0 mM MeJA and 10.0 mM MeJA loaded zeolite treatment groups. This correlates with the large differences in relative expression seen in panel (C) of Figure 4.6.

All treatment groups under the *Pythium* conditions, shown in panel (B) of Figure 4.7, resulted in minimal variation in COI1 Ct values. H_2O, mock loaded zeolite, and untreated zeolite treatments all resulted in highly variable DRR230 Ct values. 1.0 mM MeJA resulted in minimal variation in DRR230 Ct values and 10 mM MeJA loaded zeolite resulted in moderate variation in DRR230 Ct values.

4.3.3 Additional Tests Using Loaded Zeolites

Additional emergence tests were completed in order to test other factors of the loaded zeolite preparations. These included two experiments done in the non-sterile soil system, (i) testing different concentrations of MeJA in the loading solutions, shown in panel (A) of Figure 4.8, and (ii) testing different concentrations of untreated zeolite alone as a seed treatment, shown in panel (B) of Figure 4.8. Emergence for these experiments was defined as seedlings which had germinated, emerged, and survived the ten days of the experiment.

A choice test using loaded zeolites was also completed in order to detect any direct effects of the zeolites on the *Pythium*, shown in Figure 4.9.

Figure 4.8 panel A shows emergence results from seeds treated with either a H_2O control, mock loaded zeolite, or MeJA loaded zeolite prepared using different concentrations of MeJA in the loading solution, either 10.0 mM, 20.0 mM, or 30.0 mM.

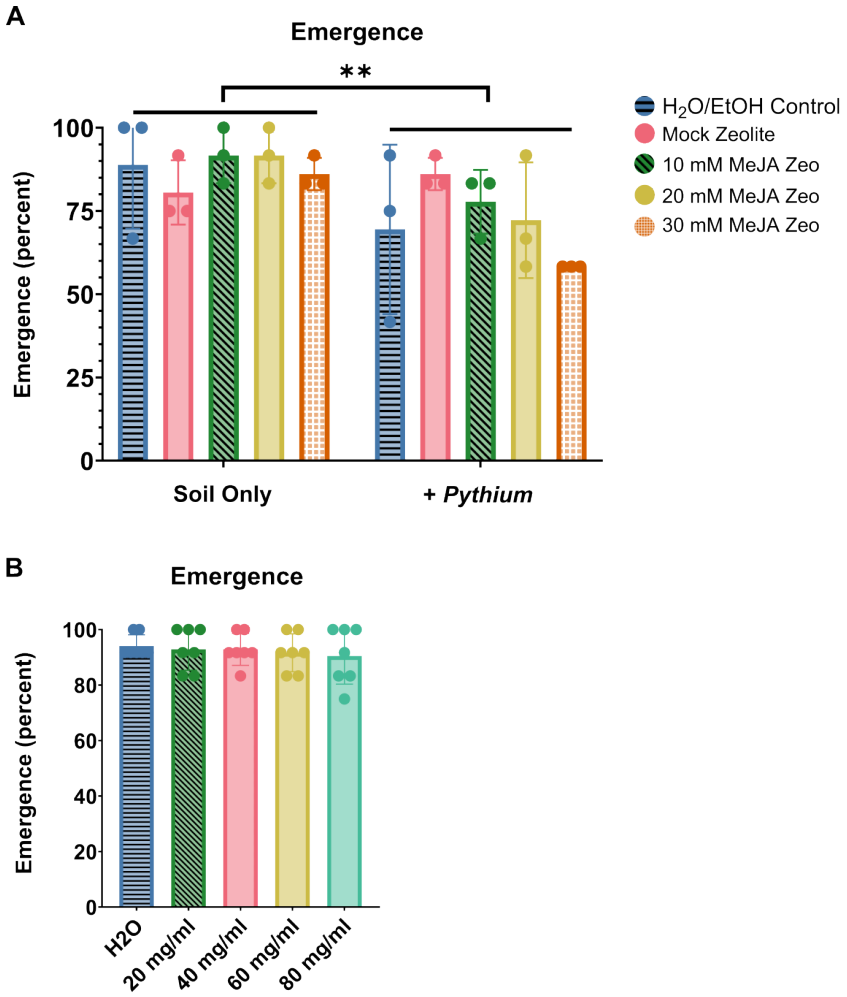


Figure 4.8: Emergence Tests for additional concentrations of MeJA loading solutions and additional concentrations of zeolite. **(A)** Percent emergence for pea seedlings treated with 20 mg/ml of zeolite loaded with various concentrations of MeJA. Data shows means of three replicate trays where each tray value is the mean of fifteen seedlings. Error bars show standard deviation. Data tested for normality and analyzed with a two-way ANOVA and Tukey's post hoc tests for multiple comparisons. **(B)** Percentage emergence for pea seedlings treated with 20, 40, 60, or 80 mg/ml of zeolite. Data shows means of six replicate trays where each tray value is the mean of fifteen seedlings. Error bars show standard deviation. Data tested for normality and analyzed with a two-way ANOVA and Tukey's post hoc tests for multiple comparisons.

Emergence varied within the H_2O control, mock loaded zeolite, 10.0 mM MeJA loaded zeolite, and 20.0 mM MeJA loaded zeolite treatment groups under the soil-only condition. There was minimal variation within the 30.0 mM MeJA loaded zeolite group under soil only conditions. There were no differences between groups within the control condition.

There was small to moderate variation within the H_2O control, mock loaded zeolite, 10.0 mM MeJA loaded zeolite, and 20.0 mM MeJA loaded zeolite treatment groups under *Pythium*-exposure conditions. There was minimal variation within the 30.0 mM MeJA loaded zeolite treatment group under disease conditions.

Panel (B) of Figure 4.8 shows emergence results in soil only for different concentrations of untreated zeolite. All other experiments used a 20 mg/ml concentration of zeolite – whether untreated, mock loaded, or JA/MeJA loaded. In this experiment, an H_2O control soaking treatment was compared to soaking treatments of either 20, 40, 60 or 80 mg/ml zeolite in H_2O. There was minimal to moderate variation within the 20 mg/ml, 40 mg/ml, 60 mg/ml and 80 mg/ml treatment groups. There were no significant differences between any of the treatment groups.

In order to determine if there was any direct effect of the loaded zeolites, either those loaded with a 10 mM MeJA solution or with a mock - DMSO and water only - solution a choice test was set up on agar plates.

In order to determine if there was any direct effect of the loaded zeolites, either those loaded with a 10 mM MeJA solution or with a mock - DMSO and water only - solution a choice test was set up on agar plates.

Figure 4.9 shows the results. The *Pythium* showed no preference for the water control or either of the zeolites and grew in a continuous mycelial mat over the plates. Figure 4.9 parts **(C)** and **(D)** show the water vs water control and the mock loaded zeolite vs water control, respectively. Part **(B)** shows water versus the 10 mM MeJA loaded zeolite and part **(A)** shows 10 mM MeJA loaded zeolite vs mock loaded zeolite. In the images with zeolites, Parts **(A), (B)**, and **(D)**, the zeolite forms a opaque deposit on the plate. The plates were imaged from the

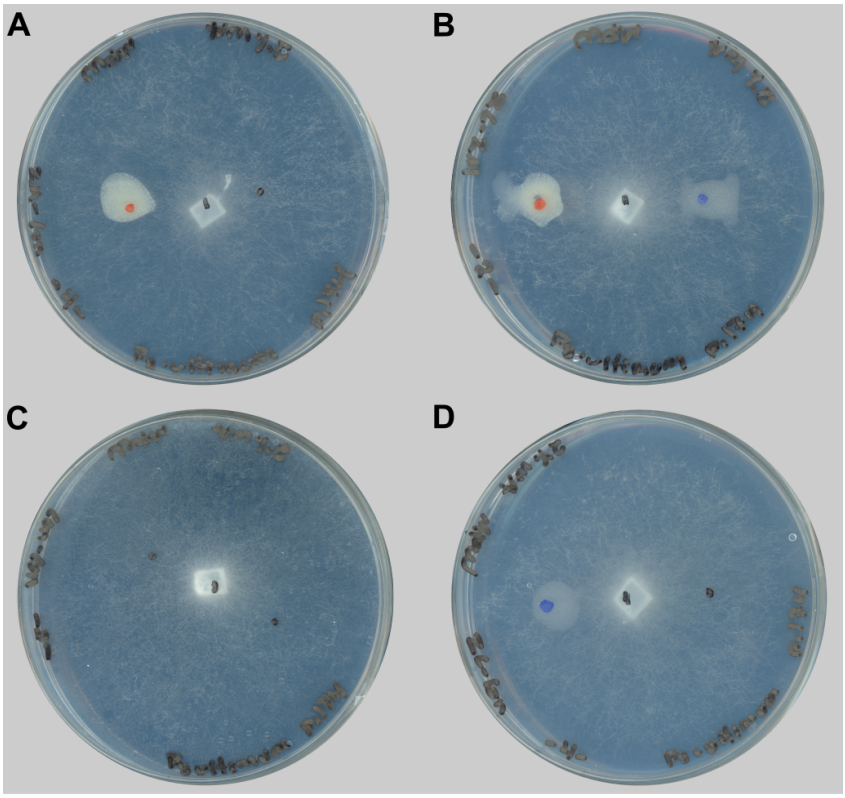


Figure 4.9: *Pythium ultimum* vs mock loaded zeolite, 10 mM MeJA loaded zeolite, and control solutions. **(A), (B), (C), and (D)**: images showing five day cultures of *Pythium ultimum* grown on 2% WA at 25°C. **(A)** 20 ul of 10 mM MeJA loaded zeolite at 20 mg/ml in H2O (red dot) 20 ul of ddH2O (black dot). **(B)** 20 ul of 10 mM MeJA loaded zeolite at 20 mg/ml in H2O (red dot) vs 20 ul of the mock loaded zeolite at 20 mg/ml in H2O (black dot). **(C)** 20 ul of H2O (black dot) vs 20 ul of H2O (black dot). **(D)** 20 ul of mock loaded zeolite at 20 mg/ml in H2) (blue dot) vs 20 ul of H2O (black dot).

bottom so it is not clear in the image but the *Pythium* mycelium grew over the tops of the zeolite deposits and did not avoid the zeolite.

4.4 Discussion

Loading of zeolite and zeolite as a carrier

The data in this chapter shows the effects of MeJA-loaded zeolites as a proof-of-concept system for loading zeolites with plant defense priming or growth promoting compounds.

Within this chapter, the concentration of jasmonates listed is for the loading

solutions and this is not equivalent to the concentration in the seed soaking solutions. Unfortunately, the concentration of the seed soaking solutions is not known, due to lack of robust GCMS quantification, but it is likely much lower than the loading solution. This is why much higher concentrations were used as loading solutions in this chapter than were tested for as direct soaking solutions in chapter 3.

For an example of the discrepancy between loading solution and resulting release solution concentrations, Bravo-Cadena loaded hexagonal mesoporous silica nanoparticles in a solution of 7.34 g/L cinnamaldehyde and found the maximum release concentration was 6.5 mg/L (controlling for the same volume of loading solution as release solution) (Bravo-Cadena 2018). This is over a 1000-fold difference between loading solution concentration and the concentration produced by the loaded nanoparticles. Bravo-Cadena also loaded HMSNPs in a loading solution of 2.754 g/L allyl isothiocyanate and found the maximum release concentration was 34.7 mg/L – again, controlling for the same quantities of nanoparticles and same volume of loading solution and release solution (Bravo-Cadena 2018). This is a 79-fold difference between the loading solution concentration and the final concentration produced by the nanoparticles. Calculated per mg of nanoparticle, cinnamaldehyde loaded 0.0945 mg per mg nanoparticle and allyl isothiocyanate loaded 0.74 mg per mg of nanoparticle. No quantification was available for the jasmonates used in this thesis, so unfortunately no estimates could be made of the quantity of loading or release. Given the wide potential range of loading seen in the examples above, a conservative estimate could still guess the release concentration likely to be a minimum of 10-50 times lower than the loading concentration. As in previous chapters, many questions about loading efficiency, delivery, and concentration of jasmonates released to the plants could be answered with an accurate LC-MS/MS quantification protocol for detection jasmonic acid and methyl jasmonate.

In lieu of being able to quantify the release concentrations and thus the relationship between the loading solution concentration and the release solution concentration, different loading solution concentrations were tested towards the very end of the time allotted for experimental work. These tests only progressed to

emergence tests, shown in Figure 4.8 panel (A). The results indicated no statistically significant differences between the different loading solutions tested and emergence under soil only or *Pythium* conditions. However, the data does seem to indicate that mock loaded zeolite, 10.0 mM MeJA loaded zeolite, and 20.0 mM MeJA loaded zeolite result in lower variability and slightly higher emergence than do H_2O alone or 30.0 mM MeJA loaded zeolite. These results would need to be repeated to make any definite conclusions but they indicate that at least in germination and emergence tolerance a higher concentration of MeJA loading solution could be used without adverse effects upon the seeds early growth and development.

The loading seen appeared to be less in zeolite than in the HMSNPs – data shown in Chapter 2. However, the lack of robust GCMS quantification means even statistically significant differences on the GCMS could be artifacts – see data in Chapter 2. Assuming this difference were real and not an artifact there are a few considerations. (i), This was loading as measured by the release of jasmonates from pre-loaded particles, so it is unclear if the loading was in fact less per milligram than that of the HMSNPs or if the zeolite released the loaded compounds more easily and thus much of the pre-loaded compounds were removed during wash steps. (ii), Without calculations of the surface area of the zeolite vs that of the HMSNPs per milligram of material it is difficult to compare directly between the two. These questions could be answered with the use of an accurate and reliable LC-MS/MS quantification method for the jasmonates and a BET surface analysis, respectively. To fully understand the dynamics of this MeJA-zeolite system and whether it has any potential as a resistance inducing treatment in either this or another plant-pathosystem, these analyses would need to be done.

A further consideration to the loading of zeolite is the relative presence of unfavourable surface interactions compared to those seen with mesoporous silica nanoparticles. The zeolite could be loading the JA and MeJA at a lower weight-weight ratio due to unfavourable surface interactions.

High drug loading in porous silicates is dependent on hydrophobic interactions, electrostatic interactions, hydrogen bonding and *van der* Waals interactions (Liu et

al. 2020). Weakly acidic conditions have been found to be important for improving drug loading in mesoporous silica nanoparticles for many compounds, including doxorubicin hydrochloride and salicylic acid, two common drug-loading model molecules (Lestari et al. 2021, Rosenholm and Lindén 2008). Rosenholm and Lindén found that salicylic acid loaded significantly better at pH 3.5 than pH 2 or pH 5 (Rosenholm and Lindén 2008). While these results were for mesoporous silica, which has much more neutral surface properties than does zeolite, they do suggest that adjusting the pH of the loading solution could improve loading of compounds onto other silicates, like zeolite nanostructures.

Steric hinderance is a much stronger factor in zeolite loading than in mesoporous silica loading, as the negatively charged structure of the zeolite slightly repels negative charges and attracts positive charges. However, various modifications have been used to improve this loading capability, including lowering the pH of the loading solution environment which helps facilitate hydrogen bonding, and coating with functionalization molecules like polydopamine, which binds readily to silicates and has different binding properties depending upon pH, meaning you can load compounds with unfavourable steric interactions onto polydopamine-coated zeolite frameworks simply by raising or lowering the pH of the loading solution (Yang et al. 2020, Hu et al. 2023, Ryu et al. 2018, Menichetti et al. 2024). Zeolite has shown promise as a carrier compound and modifications such as these could expand its loading capabilities, both within the system trialled in this thesis and in other agricultural and drug-loading contexts.

Solvent properties can also affect loading and release capacity within silicate frameworks. In this instance, a solvent other than DMSO could be trialled to see if this improves loading and retention on the zeolite. Solvents like dimethylformamide (DMF) and acetonitrile have similar solubility in water as DMSO, but both compounds are much more toxic and less compatible with biological systems. DMF is a strong base though and may not create the appropriate binding interactions within the solution. Acetonitrile is neither acidic nor basic and may be more suitable. Despite higher toxicity, DMF or acetonitrile could be trialled with various

wash steps and a quantification system to see if loading could be improved while keeping biotoxicity low.

Within the DMSO-MeJA-Water loading system used in this thesis there are other adjustments which could be made to improve loading and retention of the jasmonates on the zeolite. These include adjusting various aspects of the washing. Adjusting the pH of the washing solution to both lower and higher than the optimal range could prevent MeJA from washing off as readily in the washing process. Additionally, data in Chapter 2 suggests that using only one wash step could retain more MeJA than the two wash steps used throughout the zeolite preparation in Chapter 3 and 4. Trialling these changes would be a simple way to try to improve loading capability of the zeolite, both with the use of MeJA and other compounds, opening up further opportunities for loaded-zeolite as a delivery system for plant growth-promoting and/or defense inducing compounds.

While silica nanoparticles have been trialled as carriers for a wide variety of compounds within agriculture, including abscisic acid, fertilizers, and agrochemicals, zeolite is primarily used as a carrier for fertilizers (D. Sun et al. 2018, An et al. 2022, Shelar et al. 2023, Sanzari et al. 2019). This is due in part to zeolite's preferential binding for cations but also stems from its early uses in waste remediation. An interesting future path for zeolite could be in combining fertilizer like NaH^{4+} with other plant-growth promoting compounds like jasmonates, other plant hormones involved in growth, development, and defense regulation like salicylic acid, auxins, abscisic acid, cytokinins, gibberellins, or brassinosteroids, or even in combination with plant growth promoting bacteria. Finally, zeolite could be combined with another carrier to increase the retention of jasmonates.

Direct effects of zeolite on *Pythium ultimum*

To confirm that the jasmonates, zeolites, and jasmonate-loaded zeolites were not having any direct effects on the pathogens disc diffusion assays – shown in Chapter 3 – and choice assays – shown in this chapter – were performed with *Pythium* cultures. The disc diffusion assays in Chapter 3 showed no effects of the

jasmonates on *Pythium* growth patterns or total growth. Notably, the *Pythium* grew into and consumed the cellulose paper discs used for the assay, whether or not they had been treated with JA, MeJA, or EtOH.

Figure 4.9 in this chapter shows the results of the choice assay, where a drop of either water or an untreated, mock loaded, or MeJA loaded zeolite were place on opposite sides of a 2% WA plate. *Pythium* cultures were added to the centre of the plate and allowed to grow under standard conditions for three days. Plates were then imaged. If the *Pythium* were affected by one of the zeolite preparations it should show a marked preference in growth pattern and distribution for the water control. The *Pythium* instead showed no preference or avoidance of any of the zeolites, loaded or otherwise, or the water treatment and grew a confluent lawn over the whole plate, including over the zeolite particles. Any effects of the seed treatments were thus determined to be due to effects of the treatments upon the plants and not upon the *Pythium* directly.

However, an additional factor to consider and that was only indirectly tested for in the choice assays, is any potential effect of the DMSO used in the loading process on the *Pythium*. The zeolite samples used for the choice assay were prepared the same as those used in the non-sterile soil system and so presumably any effects of the residual DMSO used in the loading process would have been observed in the *Pythium*'s behavoir between the untreated zeolite and the loaded and mock loaded zeolites. No differences were seen between these groups, which leads the author to tentatively conclude the DMSO had no direct effects upon the *Pythium* within the systems tested.

However, there is some evidence that DMSO can inhibit growth and infection status of *Pythium insidiosum*, a human and animal pathogen, in both in vitro inhibition assays as well as when applied topically to *P. insidiosum* lesions on horses (Yolanda et al. 2022). DMSO has been shown to have anti-fungal activity against *Botrytis cinerea* but has never been tested directly against any of the plant pathogenic *Pythium* species as an anti-oomycete (**v_petruccelli_antifungal_2020**). The

overwhelming use of DMSO in the literature is as a solvent for testing other anti-fungal or anti-oomycete compounds and controls in these studies show no effect of the DMSO on its own in their various study systems (Hazen 2013, Pimentel et al. 2022, Sheikh et al. 2023, Loreto et al. 2011). To confirm effects seen are due to the compound tested and not the solvent, solvent susceptibility testing should be completed with any biological use loaded nanoparticle system. Any future work building upon the work in this thesis should include these tests as they were not included in this thesis.

JA Loaded Zeolite and the Pisum sativum-Pythium ultimum system

There were no effects on growth, development or disease resistance for any of the JA loaded zeolites tested, with the exception of a small statistically significant difference in foliar disease score under Pythium conditions between the 50.0 mM JA loaded zeolite and the mock loaded zeolite and 10.0 mM JA loaded zeolite – shown in Figure 4.2. However, this difference was not present for the 1.0 mM JA loaded zeolite nor were there any other statistically significant differences between the treatments, making this affect difficult to interpret. If the 50.0 mM JA loaded zeolite was truly lowering foliar disease score one would expect to see it lower compared to all treatment groups, including the 1.0 mM JA loaded zeolite, which was not the case. Additionally, if the JA combined with the zeolite were having an affect one would expect the disease scores to decrease slightly with the increase in JA, which also did not occur. As a result, no strong conclusions about the impacts of JA loaded zeolites on foliar disease score can be concluded from this data.

The other statistically significant differences seen were those between the soil only and *Pythium* conditions. Both survival and emergence were lower in the *Pythium* exposed condition than in the soil only condition. This was expected as *Pythium* disease both supresses germination and kills off plants.

Root length, fresh weight, dry weight, and root surface area were also lower under *Pythium* conditions as compared to the control condition. Fresh weight and dry weight are not always lower in plants exposed to *Pythium*. Various factors can

impact whether fresh and dry weight are lower and these include the suppressiveness of the *Pythium*-infected soil – higher suppressiveness leads to lower germination and emergence and less plant biomass overall. The density of the inoculum can also impact total biomass – lower density inoculum can take longer to infect the plants, meaning more plants germinate, emerge and grow before becoming infected and starting to lose root or foliar tissue. The *Pythium* also adds its own biomass to the plants and because it grows its mycelial network within and across plant tissues it can be difficult to separate the *Pythium* from the plant material for weighing, thus skewing the measurements higher. *Pythium* also induces water soaking in root and stem tissues, which can increase the fresh weight of tissue (Golińska and Świecimska 2020).

Root length and surface area are also expected to be decreased under *Pythium* exposure, as *Pythium* primarily targets roots and prevents growth as well as destroying existing tissues. Because surface area is not only dependent upon length but also width, sometimes surface area is not as affected by *Pythium* disease as root length – if thick tap roots are still present under *Pythium* infection, even if they are damaged and discoloured, the surface area could show a relatively smaller decrease than root length.

The data show that, while JA-loaded zeolite, even up to loading solution concentrations of 50 mM JA, is generally well-tolerated among *Pisum sativum* seedlings it does not confer any disease resistance to *Pythium ultimum*.

MeJA Loaded Zeolite and the Pisum sativum-Pythium ultimum system

Notable in the data presented in this chapter is the discrepancy between the outcomes of the summer trials, which indicated some protective effect of the 10.0 mM MeJA loaded zeolite, and the fall/winter trials which showed no effects for this treatment. Figure 4.3 shows the growth and development responses and Figures 4.4 and 4.5 show the disease resistance results from these two types of outcomes.

Under *Pythium* conditions, survival and emergence appeared to be increased in the 10.0 mM MeJA loaded zeolite compared to the mock and 50.0 mM MeJA loaded zeolite treatment groups for the summer trials. This was promising result and taken with the root and foliar disease scores seemed to indicate a slight protective effect of the 10.0 mM MeJA treatment. However, these results only replicated twice, during the summer months. Further trials in the fall and winter showed no evidence of any of the treatments affecting disease severity or survival in the peas.

Disease symptoms were higher overall for the fall/winter trials as compared to the summer trials. Foliar disease varied widely within the soil only condition for the fall and winter assays. While damping off diseases like *Pythium* often do not cause immediate foliar symptoms in plants, they certainly can cause foliar symptoms (Golińska and Świecimska 2020, Kraft et al. 1988). A higher inoculum density could cause more pronounced foliar symptoms however that does not account for the higher foliar symptoms in the control soil-only plants. Variations in growth chamber light intensity and watering could cause some yellowing and disease-like symptoms in the foliar tissues. However, these results remain somewhat unexplained.

Additionally, root length was nearly 50% shorter for all treatment groups in the fall/winter trials than in the summer trials and the difference between the soil only condition and the disease condition was significantly less in the fall/winter trails than in the summer trials.

The initial tests were completed in June, July and August 2023 while the later tests were completed in November and December. The initial effects seen could simply be natural variability in the system, however, there are many other potential factors which could have affected these outcomes.

One possible explanation is that the peas responded more strongly to the seed treatment in the summer months when they were more vigorous in their growth as compared to the early winter months, when the MeJA may have had a more suppressive effect. The plants are grown in growth chambers and not in green houses but consistent effects have been noted of seasonality on growth in the plants within our lab, despite being in climate controlled chambers year-round. Seasonal

effects on growth and development factors have been noted in plant tissue cultures grown in controlled environments (Sharma et al. 2005).

Another possibility is variation within and between growth chambers. Unfortunately, due to space constraints and some broken growth chambers, not all of the experiments in this thesis were completed in the same growth chambers. Conditions including light programming, light intensity, temperature, and humidity were controlled and independently measured for all growth chambers used. Summer trials were completed in two primary growth chambers. However, fall and winter trials were split between those two chambers and a third chamber. Inter-chamber positional variation in light and humidity is a known phenomenon which affects plant growth (Measures et al. 1973, Downs and Hellmers 1975a, Downs and Hellmers 1975b, Porter et al. 2015, Proietti et al. 2021, Chiang et al. 2021). To control for this plant trays were spaced in a randomized block design and rotated through positions in the growth chambers every three days. However, despite these attempts at consistency, variation within and between chambers could account for the different response seen in the two sets of data.

The concentration of the *Pythium* inoculum is another potential factor. The *Pythium* inoculum was prepared in a standardized way – see the Methods section of Chapter 3 – but within that preparation there is still some variability. In particular, the preparation of the inoculum involves growing confluent lawns of *Pythium* on agar plates for three days and mixing the agar and surface mycelium into the soil. The amount of agar poured per plate was standardized at 18 mLs, however, the coverage of surface mycelia varied somewhat plate to plate. Some initial tests were completed in December to test the variability in mycelial content between plates and these indicated that consistency in initial inoculum may have played a role in relatively severity of disease seen in the assays. More mycelium could result in a denser concentration of *Pythium* in the inoculum and thus a higher inoculum load in the *Pythium* condition. However, the preparation of the *Pythium* inoculum did not change between summer and fall/winter trials, and so it seems less likely

to the author that this alone would count for the seasonal discrepancies and more likely for this to have caused variation between all the assays.

It was also thought perhaps the preparation of the loaded zeolite was affecting the received does of the MeJA to the seeds, and that this in turn was affecting the results. Several tests comparing freeze drying versus freezing alone during the loaded zeolite preparation were completed with no changes in results (data not shown).

Root branching frequency was included as a metric in all of the non-sterile soil system experiments. In general, root branching frequency did not change between soil only and *Pythium* conditions within an experiment, nor was it affected by the various treatments trialled, both JA and MeJA on their own and the combined zeolite treatments. The one exception was the fall/winter trials of the MeJA loaded zeolites, where mock zeolite showed a significant increase in root branching frequency under soil only conditions compared to all of the other treatment groups. Additionally, the root branching frequency for the fall/winter trials was on average 30% lower than that seen in the summer trials. This is a curious finding, and difficult to explain. Root branching frequency is controlled by many factors, including genetics, water availability, temperature, pH, microbial communities, relative concentrations of nitrogen, phosphorus, and potassium, and hormonal modulation (Lynch 1995, Walch-Liu et al. 2006, Camilo et al. 2021, Saleem et al. 2018, Bao et al. 2014, Verbon and Liberman 2016). It typically increases with soil resource variability when thin roots proliferate while the plant searches for higher concentrations of nutrients. This higher branching increases local soil exploration but may be a disadvantage for longer-range soil exploration where lower branching frequency and longer, thicker roots are more suitable (Freschet et al. 2021). The zeolite could be having multiple affects. It could be changing the soil chemistry immediately around the seed, as zeolite is known to increase both water retention and cation capacity of soil, causing and uneven distribution of nutrients and triggering the seed to induce more branching (Szatanik-Kloc et al. 2021). It could also be driving some kind of internal mechanism in the plant either through silicic acid uptake or physical abrasions to the root. Silica supplementation is known to increase

auxin and abscisic acid production in wheat and rice (Li et al. 2023, Tripathi et al. 2021). Zeolite could be doing something similar. Of course, to draw robust conclusions from this data the experiments would need to be repeated seasonally to confirm the outcomes seen in the fall/winter trials.

Worth noting is the use of different control groups between the summer and fall/winter trials. It was difficult to include more than five treatment groups in the non-sterile soil assays, due to the large amount of set up and harvesting work required. The summer trials were done with only a mock-loaded zeolite as a control. For the fall/winter trials, a water/EtOH control and an untreated zeolite control were added and the 50 mM MeJA loaded zeolite treatment removed – the 50.0 mM MeJA zeolite treatment slightly increased disease scores and decreased survival and emergence in the summer trials. Including or not including these different controls is unlikely to have produced the discrepancy in the results seen, however, as the initial increase in survival seen for 10.0 mM MeJA loaded zeolite was compared to the mock zeolite control, which was included in all of the experiments.

Interestingly, there did appear to be some beneficial effects of the zeolite on its own when applied as a seed treatment. This could be for a few reasons, (i) it could form a physical barrier that prevents the *Pythium* from infecting the seeds as quickly, lengthening the amount of time the seedling has to grow and establish itself before being infected. (ii), similar to one, it could be changing the physio-chemical environment around the seed, which could in turn affect the *Pythium*'s ability to establish itself immediately around the seed. Finally, it could be inducing stress responses in the plant or otherwise causing an up-regulation of JA defense responses in the plant.

All of the experiments completed in this thesis were done using 20 mg/ml of whatever mock or MeJA loaded zeolite preparation was being tested – 20 mg/ml of xzeolite in water was the basis of the seed soaking solutions used in all the in vitro and in planta tests. It was hypothesized that a higher concentration of the zeolite may be useful in conferring disease resistance. Unfortunately, testing only proceeded to an initial emergence test under control conditions before time for experiments

ran out. These results are shown in Figure 4.8 panel (B), where 20, 40, 60 and 80 mg/ml concentrations of untreated zeolite were tested in seed soaking solutions for germination inhibition and total emergence. No statistically significant differences were observed between any of the zeolite concentrations or the water control, though the 80 mg/ml zeolite concentration had an higher internal variability than did the other treatment groups. The next step was to test these zeolite concentrations on their own in the full non-sterile soil pathosystem but unfortunately there was not time to do so. Future work focusing on zeolite as a carrier should also include tests of zeolite itself as a plant defense promoting compound. Future work to clarify these results would be to repeat the assays during the summer and see if the initial results repeated. If not, it is likely the first two trials were simply showing variation – perhaps some defense upregulation is occurring but it is not enough to combat the *Pythium*. Increasing from one to three people is a three-fold increase but three people won't stop a bulldozer any more effectively than one person. To fully understand this data much more gene expression data would be needed, specifically showing jasmonate and defense-related gene expression at early timepoints post-seed soaking treatment such as 2 to 3 days and more replicates for day 10.

Relative gene expression

Figure 4.6 Panel A shows relative gene expression results from control-condition peas. There are no statistically significant differences between treatments in the relative expression of LOX2, JAR4, COI1, and DRR230. Relative expression of COI1 is consistent across treatment groups. Relative expression of LOX2, JAR4 and DRR230 is consistent between H2O, Mock zeolite, 1 mM MeJA, and untreated zeolite treatment groups but shows variability for the 10 mM MeJA loaded zeolite treatment group. An exception to this is JAR4, for which the 1.0 mM MeJA treatment also had one replicate which was several fold different from the other two remaining replicates.

One replicate of the 10.0 mM MeJA loaded zeolite had an upregulation in LOX2, Jar4, and DRR230 under soil only conditions. One replicate of the untreated zeolite caused an upregulation in LOX2 under *Pythium* exposure conditions. It is not

clear if these represent natural variation or perhaps the only plants which were still responding to the seed soaking treatment by day 10.

A similar pattern is seen for JAR4 for *Pythium*-exposed peas, shown in panel C. In this case, there was such high variability in the 1.0 mM MeJA and 10,0 mM MeJA loaded zeolite treatment groups that JAR4 was presented separately on a log scale, with some of the relative expression values for 1.0 mM MeJA treatment group reaching nearly 80-fold higher than that seen in the H2O control treatment group.

Panel B shows the relative expression of the remaining genes of interest, LOX2, COI1, and DRR230 in *Pythium*-exposed peas. While there is some variation in LOX2 seen for the untreated zeolite and the 10.0 mM MeJA loaded zeolite groups the other genes show consistent relative expression.

Treatment group results were compared within each condition. Of primary interest was not what happens to expression in peas exposed to *Pythium* conditions vs those in control conditions but whether within the soil only or *Pythium* conditions are any of the treatments caused an upregulation beyond that of the controls. Panel C is split from panel B because no variation in the other genes is visible, even with a split scale, when the JAR4 is included. This was chosen because it gives the reader the clearest view of the data.

JAR genes catalyze the formation of jasmonyl-isoleucine (JA-Ile), the primary bioactive form of the jasmonic acid (Ruan et al. 2019). The large increase in JAR4 relative transcript abundance is not without precedent - Fukumoto et al. found strong differences in the induction of the two primary JAR genes in rice, OsJAR1 and OsJAR2. OsJAR1 relative transcript abundance increased by about four times after wounding but OsJAR2 increased nearly 90-fold after wounding (Fukumoto et al. 2013). However, the high variation seen between replicates is not consistent with work seen in the literature and it is not fully understood why this occurred.

The Ct values of for the same experiment are a little more revealing. They also show a large variability in the expression of JAR 4 in *Pythium* condition for the 1.0 mM MeJA and 10,0 mM MeJA loaded zeolite treatments but also show relatively high variability of the DRR230, a pea defensin, for the H2O, mock zeolite, and

untreated zeolite treatments, in both the control and *Pythium*-exposed conditions. This is intriguing, for a couple of reasons. One, there is little literature to indicate DRR230 is expressed outside of infection scenarios so Ct values in the control condition similar to those in the *Pythium*-exposed condition seem out of place. The experiments were not done in sterile conditions but the plants used in the RT-qPCR experiments shown in Figures 4.7 and 4.6 are from the same experiment as shown in Figures 4.3, 4.4, and 4.5, where no infection or disease symptoms were noted in the control condition and where experimental notes indicate healthy and otherwise healthy plants. Additionally, the large visible variability seen in the DRR230 Ct values is not reflected in the relative expression values for the same, indicating that perhaps the $2^{-\Delta\Delta Ct}$ method of calculating relative expression is not the only method which should be used for understanding RT-qPCR results and gene expression changes.

Despite rigorous primer testing and optimization of RT-qPCR protocol there is significant variability across the qPCR results, particularly in the 1.0 mM MeJA and 10,0 mM MeJA loaded zeolite treatment groups, both for control condition plants and for *Pythium*-exposed plants, for LOX2, JAR4, and DRR230 expression. The JAR4 results, because of the magnitude of the differences in relative expression, seem likely to indicate real differences in JAR4 expression amongst the peas, both within different condition and treatment group combinations and between the different condition and treatment group combination. However, the sample size is too small to make any strong conclusions from the data. Unfortunately, this work was completed at the very end of the thesis and thus only one experimental replicate was performed. Follow on work would need to include additional RT-qPCR tests, including at both earlier and later timepoints to understand the full dynamics of gene expression changes in response to the treatments.

4.5 Summary

This chapter tested jasmonate-loaded zeolites as potential plant-defense response priming agents using the non-sterile soil system developed in Ch 3. Testing

revealed varied responses - initial tests which occured over summer months showed a protective effect of 10,0 mM MeJA loaded zeolite on pea defense against *Pythium* while follow up tests in fall and winter months showed no statistically significant impacts of any of the loaded zeolites on plant growth and development or defense response. This chapter also included RT-qPCR data which showed highly variable up-regulation of JAR4 between biological replicates of *Pythium*-exposed seedlings.

Finally, the chapter tested both zeolites and jasmonates directly against *Pythium ultimum* cultures to confirm that any effects of seed treatments on defense responses or survival were due to effects on the plants and not direct inhibitory effects of the seed treatment components on the *Pythium*.

5 Discussion

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5.1 Discussion

Pesticide alternatives are increasingly important in modern agriculture, as plant disease incidence continues to rise due to monoculture, climate change, and increasing globalization which create sinks for pathogens and pests, expand pathogen ranges, increase abiotic stress on plants, and spread pathogens beyond their usual ranges (Burdon and Zhan 2020, Chapman et al. 2017, Sarkozi 2019, Garrett et al. 2021). Meanwhile, existing pesticides cause many off-target effects, including killing beneficial microorganisms and insects, changing soil chemistry, and inducing resistance in the targeted species (Sharma et al. 2019, Bass et al. 2015, Powles and Yu 2010, Lucas et al. 2015, Tripathi et al. 2020, Wilson and Tisdell 2001, Zaller

and Brühl 2019). Biodegradable and biocompatible nanomaterials as well as plant growth-promoting or defense-priming agents like BABA are gaining more attention as potential alternatives to pesticides (Shelar et al. 2023, An et al. 2022, Lahlali et al. 2022, Pereira et al. 2021, Catoni et al. 2022, Wilkinson et al. 2018).

If jasmonates can be used effectively in a plant-pathogen system to increase disease resistance it could be a valuable alternative to traditional pesticides or addition to an integrated pest management system. These treatments could provide protection to crops with less off-target effects and minimal build up of compounds in soil. By activating plant defenses the seed treatment would also minimize damage to insect communities. While some insects are invasive the vast majority are native and beneficial to both agricultural ecologies and those ecologies adjacent to agricultural sites (Jankielsohn 2018, Scudder 2017, Busse et al. 2021). The world is experiencing an insect apocalypse, with potentially disastrous consequences for humanity and ecologies worldwide (Forister et al. 2019, Wagner 2020, Wagner et al. 2021, Didham et al. 2020, Goulson 2019, van der Sluijs 2020). Any crop protection system that minimizes both direct killing of insects and build-up of toxic compounds in the soils is key to preserving a future of sustainable agriculture on this planet (Busse et al. 2021, Goulson 2019, Didham et al. 2020).

This thesis aimed to test the idea that nanosilicates like HMSNPs and natural zeolites could be loaded with plant-defense priming agents to create alternative seed treatments. In this case, HMSNPs and zeolite clinoptilolite were trialled with jasmonic acid and methyl jasmonate. The work included setting up a *Pisum sativum-Pythium ultimum* pathosytem, synthesizing the HMSNPs, adapting a GCMS protocol for detecting jasmonates out of liquid solutions, testing jasmonate-loaded nanosilicates in a non-sterile soil pathosystem and performing RT-qPCR gene expression assays to better understand any effects of the seed treatments.

The hypotheses were: (i) jasmonates could be loaded into mesoporous silica nanoparticles, (ii) jasmonates could be loaded into zeolite, (iii) jasmonate seed soaking treatments might induce resistance to *Pythium ultimum* in peas, and (iv)

jasmonate-loaded silicates would be more effective at inducing disease resistance than jasmonates alone.

This thesis also aimed to fill in some of gaps in our current understanding of resistance-inducing seed treatments, the use of silicates in seed treatments, and control treatments for *Pythium ultimum*, a highly virulent and pathogenic organism.

Both JA and MeJA could be loaded successfully into the mesoporous silica nanoparticles. This was expected based upon mesoporous silica's neutral surface properties and broad sorption compatibility (Trzeciak et al. 2021, Kong et al. 2021). Both JA and MeJA could also load into ground zeolite, a more surprising outcome because of zeolite's preference for cation binding. Neither JA nor MeJA induced disease resistance to *Pythium ultimum* in peas within the systems tested. However, MeJA loaded zeolite showed promising initial results in improving survival, emergence, and foliar disease outcomes. These results were slight and inconsistent but did show that zeolites were well-tolerated as components in seed soaking treatments. Gene expression data hinted at a potential priming effect of 1.0 mM MeJA and 10.0 mM MeJA loaded zeolite in the peas exposed to *Pythium* and this could be explored in future work.

The work revealed that both HMNSPs and zeolites can be loaded with jasmonic acid and methyl jasmonate, though zeolite appears to load less efficiently than the HMSNPs. However, it was also discovered that the time and small batch size of the synthesis of the HMSNPs is prohibitory for use in large testing situations, and so only loaded zeolite was tested in the non-sterile soil pathosystem.

5.1.1 Zeolite as a carrier in agriculture

A key finding of this work was the use of zeolite as a carrier for compounds other than fertilizers. Taken together, the data in Chapters 2, 3 and 4 test the idea that plant growth-promoting or defense-inducing compounds can be loaded into zeolite and used as a seed treatment. This is a step forward in both the use of plant defense-priming compounds, like jasmonates, which to date have not been loaded into any

silicate carrier, and in the use of zeolites, which have previously only been used to load fertilizers and small amounts of agrochemicals (Mumpton 1999, De Smedt et al. 2016, Reháková et al. 2004). This is especially useful as zeolite on its own provides many useful benefits to crops, including increasing water retention in soil, preventing nitrogen leaching, and providing small amounts of silica supplementation and its associated growth benefits to plants (Mumpton 1999, Bacakova et al. 2018, Cataldo et al. 2021). Loading jasmonates into biocompatible carriers, like zeolite, could make them a more effective plant-defense priming tool, preserving the hormone until contact with the seed and allowing targeted release of the hormones.

This adds to established work showing zeolite clinoptilolite can be useful for enhancing germination. Zeolite has been used as a seed coating to boost germination in rice, soybean and wheat (Garafutdinova et al. 2021, Di Giuseppe et al. 2018, Palanivell et al. 2015). A zeolite clinoptilolite coating as also been shown to improve germination of alfalfa seedlings under *Phytophthora medicaginis* disease conditions in growth chamber trials and under *Pythium ultimum* and *Pythium paroecandrum* disease conditions in vitro (Samac et al. 2015). There is potential for zeolite to be used as a carrier for plant growth promoting or defense inducing compounds like jasmonates in both a pea-*Pythium* system and other crop-disease systems. These kind of natural, pesticide-free seed treatments could be a cost effective and more environmentally friendly approach to crop protection.

This also creates opportunities for dual-loaded zeolite treatments, like ammonium (NH4+) plus a plant growth-promoting compound or plant defense-inducing hormone. It also opens the door for further testing with other pesticide-alternative compounds, including other plant hormones, immune elicitors like chitosan or coronatine, plant growth-promoting compounds like alginate or micronutrient supplements, or even with plant growth-promoting bacteria or fungi. Zeolite thus may prove a useful alternative to other silicates or carriers which may be more difficult to produce or otherwise unsuitable in certain conditions.

Data in Chapter 2 shows the loading of jasmonic acid and methyl jasmonate into ground, prepared zeolite. Chapter 3 tested the effects of the jasmonates and

zeolite separately on the plants and saw no detrimental effects to either germination, emergence, growth, development, or disease resistance with the zeolite. Finally, data in Chapter 4 show the test of zeolite loaded with 10 mM MeJA as a seed treatment in pea seeds exposed to the plant pathogen *Pythium ultimum*. There were inconsistent effects of this loaded zeolite on plant defense responses, but some indication in both the non-sterile soil data and the RT-qPCR data that the zeolite formulation may cause stronger effects in the plants than the plant hormones on their own.

The loading of the zeolite was compared in chapters 2 and 3 to that of synthesized hexagonal mesoporous silica nanoparticles, a known biocompatible silicate carrier. While the zeolite does not appear to load as well as the HMSNPs in these tests, it is a cheaper more scalable alternative as a carrier.

5.1.2 A *Pisum sativum-Pythium ultimum* pathosystem

Chapter 3 shows the adaptation and development of three *Pisum sativum-Pythium ultimum* pathosystem testing protocols: an in vitro system, a sterile soil system, and a larger-scale non-sterile soil system. The protocols were adapted from existing protocols in the literature to suit peas and *Pythium*. This creates a useful resource for other researchers seeking to test damping-off disease in peas and other legumes. Additionally, these assays could be easily adapted for use in multi-component studies, like the effects of mycorrhizae in damping off disease systems. These assays were then used in Chapters 3 and 4 to test both the components and efficacy of a jasmonate-loaded seed treatment.

There are additional benefits for having a trio of *Pythium ultimum* disease assays, which could be adapted for other legumes. *Pythium ultimum* is the most virulent and widespread of the *Pythium* crop diseases and is difficult to study because it is both a virulent and highly pathogenic disease, infecting over 300 crop species world wide, and often leaves only partially dissolved seeds in soil. Many of the challenges faced in this PhD project involved controlling the *Pythium* inoculum so

that some plants would indeed grow – even at inoculum loads suitable for other *Pythium* species, *Py. ultimum* would cause 80-100% mortality.

A major risk with this work was continuing to work with *Pythium* after it became apparent that even getting a pathosystem where only 50% of the plants died was a challenge. Pythium is fiendishly difficult to treat which is why the list of control measures is so long and requires so many integrative management strategies. Other pathogens were considered but ultimately the risk was deemed worthwhile – if either the jasmonates or the zeolites showed any effects against *Pythium ultimum* it could be enormously useful in the control and treatment *Pythium ultimum*.

Despite increasing literature showing most necrotrophic pathogens do still maintain a biotrophic phase, *Pythium ultimum* causes such quick infection and disease in so many hosts that it appears to be a true necrotrophic pathogen. However, it is possible future work could reveal a biotrophic phase in its life cycle. Furthermore, there is increasing evidence that SA and JA, though often antagonistic, have a subtle and complex interaction and activating both pathways can in some cases be very useful in abiotic and biotic stress tolerance in plants (van Wees et al. 2000, Amil-Ruiz et al. 2016, Flors et al 2007, Wu et al. 2018, Wang et al. 2019, Ullah et al. 2022, Tamaoki et al. 2013). Trialling a combination of activators of SA and JA defense against *Pythiuim ultimum* may be a useful future strategy.

5.1.3 Jasmonates are likely not effective in a *Pisum sativum-Pythium ultimum* pathosystem

Chapter 3 focused on the testing of jasmonate-only seed soaking treatments in three *Pisum sativum-Pythium ultimum* pathosystems. The final pathosystem tested, the non-sterile soil system, was then used in Chapter 4 to test the effects of the jasmonate-loaded zeolites. Data in both chapters showed variability in disease severity caused by *Pythium* even within the most replicable of the pathosystems, the non-sterile soil system. Sometimes the *Pythium* disease was more virulent than others. Various methods to standardize this were tested, including measures of

oospore production and measuring the variability in mycelial growth on the plates used for the inoculum production. These tests continued until the end of time allotted for experimental work and no clear solutions were discovered. Future work would need to continue these tests, measuring the variation in inoculum production to see if this correlated with some of the variations seen in disease severity.

Foliar disease was a factor which varied considerably – sometimes showing little to no disease and sometimes showing disease scores of 20-30%. This is puzzling, and it is not clear why this happened. *Pythium* can and does cause foliar wilting and discoloration. What is unclear is, why, under fairly standard conditions, the disease symptoms produced across experiments were not consistent. Chapter 4 discusses some of the reason why this variability can emerge, but future work would be needed to investigate this variability, especially in the context of non-pesticide seed treatments, where variations in pathogen virulence could have a large effect upon the perceived efficacy of a treatment.

Through the testing in Chapters 3 and 4 we also know that peas do not appear to be particularly sensitive to jasmonates, especially when under stress from *Pythium* exposure. A further direction of this project could include testing either different plant defense-priming compounds in a Pea-*Pythium* system or testing the jasmonate treatments in a different plant-pathogen or invertebrate pest system.

While there is some evidence in Chapter 4 that zeolite on its own may provide some benefit to peas upon exposure to *Pythium* the data in Chapter 3 and 4 shows very little effect of jasmonic acid or methyl jasmonate on pea defense responses in this system. This provides more information about how legumes like peas respond to exogenous jasmonates and complements available data in the literature testing other legumes with exogenous jasmonate treatments. While jasmonates have been shown to be effective upregulators of defense responses to necrotrophic pathogens and insect herbivory in a wide variety of plants like rice, Norway spruce, and tomato, the evidence for legumes show mixed results (Bali et al. 2020, Devos 2020, Kraus and Stout 2019). Cowpeas pre-treated with jasmonates have fewer nematode eggs present on their roots later in life compared to no jasmonate treatment but

soybeans showed little to no change in defense response or nematode load with jasmonate treatments (Pankaj et al. 2013).

5.1.4 Limitations and Future Directions

5.1.4.1 Seed treatment effectiveness

One concern with this project was how to ensure the effectiveness of the seed treatments. A seed soaking treatment was chosen as this most directly compared to the existing JA and MeJA treatments in the literature, which typically involved soaking seeds in a jasmonate solution for 12-24 hours before planting. The seed treatments in this thesis were designed to mimic this as closely as possible. When jasmonates on their own were tested they were dissolved into ddH_2O and used as a seed soaking treatment for 24 hours. The seeds were first sterilized and washed 3X with sterile ddH_2O and then added to 30 mLs of the appropriate JA or control solution, sealed, and allowed to soak in the dark at 25°C for 24 hours. If the seeds were broken or their outer coatings were beginning to peel off after washing, they were removed and not used in the seed treatments. They were then immediately planted. This design was used to try to ensure that the jasmonates did not evaporate away from the solution and that the seed had plenty of time to imbibe and absorb the seed soaking treatment. When silicates were used – loaded or unloaded – 600 mgs of either untreated, mock loaded, or jasmonate loaded zeolites were added to 30 mLs ddH_2O for a final concentration of 20 mg/ml in solution. This was chosen because 20 mg/ml was the concentration of silica nanoparticles in soaking solution recommended by Bravo-Cadena 2018. However, trialling different concentrations of the zeolite could make for a much more effective treatment. This was only tested for emergence under control condition in Chapter 4, but these showed that up to 80 mg/ml concentrations of zeolite in a seed soaking solution did not impair emergence. Trials were started to test these higher concentrations of zeolite within the *Pythium* pathosystem but were unable to be completed due to time constraints. Future work into using zeolites as effective seed treatment and carriers should include further

testing of the concentrations of zeolite tolerated by the plants. More loaded zeolites could deliver more compound of interest to the seeds while also providing a robust barrier to pathogen establishment in the immediate area around the seed.

Many factors can affect the effectiveness of seed treatments, including the genotype of the seeds, the surface and physical characteristics of the seeds, how much liquid they can imbibe, and the length of seed treatments/exposure time.

All seeds used in this thesis were organic, untreated, Kelvedon Wonder pea seeds. This was done for consistency but is a potential limitation of this work. It is possible that Kelevedon Wonder simply isn't responsive to JA treatments and that testing other varieties of peas would produce different results. Future work should include not only different plant species to test but also different varieties of peas.

The peas used were $\tilde{9}0\%$ wrinkled and only about 10% smooth. Both types of seeds were used in the seed soaking treatments, as long as they retained their seed coat after the initial sterilization and wash steps. The surface and physical characteristics of seeds can impact the effectiveness of seed treatments by limiting access to the seed surface or providing rough surface texture where seed coating components can adhere (Souza and Marcos-Filho 2001, Afzal et al. 2020, Taylor et al. 2004, Javed et al. 2022). The thickness of the seed coat so impacts the imbibition of pea seeds, the thinner the coat the greater the water uptake (Lush and Evans 1980). Imbibition is a key component for successful germination in peas, both wild and domesticated, and common seed priming techniques involve water-soaking to initiate imbibition and germination (Smýkal et al. 2014, Hradilová et al. 2019, Nawaz et al. 2013).

5.1.4.2 Jasmonate perception and dose

One question this thesis sought to answer and was unable to is how much of the active agent reaches the seed and over what time frame? Initial tests were completed to measure JA and MeJA volatility in aqueous solutions and there was little indication of major concentration changes within 24 hours. It was thus concluded for the remaining work that the concentrations of the loading solutions

would change very little over the course of the seed soaking treatment and that whatever concentration was used remained steady throughout the 24 hours.

This question was hoped to be addressed using RT-qPCR to measure gene expression for jasmonate detection and biosynthesis genes. Unfortunately, due to time constraints, these tests were not run. Future work involving gene expression tests at early time points would help answer the questions of whether the seeds were in fact absorbing and reacting to the jasmonates and over what time frames. Additional assays measuring jasmonate responses other than gene expression would be very useful to complement the gene expression work. For instance, measures of defense-related enzyme activity like polyphenol oxidase (PPO), superoxide dismutase (SOD), peroxidase (POD), lipoxygenase activity and the accumulation of PR proteins would provide direct evidence of defense responses, and thus JA pathway induction, within the plants. Potential challenges to using these methods are similar to those encountered with the gene expression assays – there is not a lot of plant tissue available at early stages of pea development and so getting enough tissue to get robust results could be difficult. One of the reasons a ten day timepoint was used for the RT-qPCR was because plants had grown sufficiently to produce enough RNA for robust quantification.

Measuring endogenous JA accumulation would be another way to test whether the seeds had received and reacted to the jasmonate treatments. There are methods for extracting and quantifying endogenous hormone production – it was one of these methods that was adapted for the measurements of exogenous JA in aqueous solutions used in this thesis – but the most robust and sensitive of these relies upon use of an LC-MS/MS. Future work using an LC-MS/MS to quantify endogenous JA could provide another level of certainty about whether the seeds had in fact received and responded to the jasmonate treatments.

Another limitation is the lack of additional routes of JA and MeJA treatment. This thesis focused on seed treatments. To verify that the peas could in fact respond to the jasmonate treatments a soil drench assay or a foliar spray would be useful.

A soil drench test would be the most useful and relevant, as its route of activation is more similar to that of a seed soaking treatment.

5.1.4.3 Quantification of Jasmonates and Characterization of Silicate Loading

As mentioned previously, a crucial component to further understanding the data presented in this thesis would be an accurate, reliable LC-MS/MS method for quantifying jasmonates. This method would need to include a method for extracting jasmonates from aqueous solutions. A system like this could help answer remaining questions, such as, could the zeolite loading be improved and what are the dynamics of jasmonate sorption to zeolite surface compared to HMNSP surfaces.

A lot of time (> 2 years) was spent testing and optimizing the GCMS protocol for jasmonate detection. Chapter 2 shows in detail how unreliable the technique was, even after this optimization. Figure 2.9 shows statistically significant increases in the internal standard - dHJ - and for both the JA and MeJA trials, despite this being a physical impossibility - there were set starting amounts of dHJ and JA/MeJA added to the vials at the start of the experiment. No plants or other organic matter where dHJ could have been generated were present in the assay nor was any additional dHJ or JA/MeJA added over the course of the experiment. This data was included to show two points (i) the inherent unreliability of the GCMS for quantification and (ii) the presence of detectable JA and MeJA in a volatility assay up to eleven days after adding the jasmonates into an aqueous solution.

5.1.4.4 Improving silicate carriers

The data in Chapters 3 and 4 indicates there could be some defense priming or otherwise beneficial effect of the zeolite both on its own and as part of a loaded treatment. Due to time constraints, further tests on the quantities of loaded zeolites used and the concentrations of jasmonates loaded in the zeolites were outside the scope of this project. Future directions could include testing different volumes of loaded zeolite, different loading solution concentrations, testing the

jasmonate-loaded zeolites in other plant and pathogen systems, or testing the efficacy of the treatment as a dry additive as opposed to the soaked pre-planting seed treatment tested in this thesis.

Further testing could also be useful in understanding why the treatment appeared to provide some benefit in the summer trials but not in the autumn or winter trials. This could be due to many factors, several of which are discussed in the Chapter 4 discussion section, but one not covered there is variations in the zeolite preparation. Loading capabilities could be improved by obtaining a finer grind on the zeolite and by more uniformity in the zeolite preparation. Additionally, solvents other than DMSO could also be trialled. Various pre-treatments and surface modifications can be performed on the zeolite to increase sorption capacity, which could be useful in improving zeolite's characteristics as a carrier (Straioto et al. 2023, Andrunik et al. 2023, Sayed et al. 2022).

The HMSNPs have a large surface area to volume ratio and are well-established biocompatible carriers. The data in Chapters 2 and 3 show potential for HMSNPs as carriers of jasmonates, however, they could not be produced at lab scale in quantities suitable for non-sterile soil testing. Methods to scale up production of the HMSNPs to the quantities needed for robust non-sterile soil testing could make the HMSNPs a viable future route for jasmonate and other plant-defense priming compound silicate treatments.

Alternatively, different silicate carriers could be trialled, such as slag, which has a higher proportion of silicic acid, the bioavailable form of silica, and is already commonly used as a silica supplementation for rice and maize (Y. Liang et al. 2015).

Finally, non-silicate carriers may be more optimal for jasmonate loading and release. There is a wide variety of nanoparticles and nanoformulations available in the literature that could be investigated.

5.1.4.5 Further investigating plant perception of and responses to jasmonates and silicates

The qPCR results presented in Chapters 3 and 4 are unfortunately only representative of one experimental replicate. Time constraints prevented further investigation in the course of this project, but repeated experiments would prove very useful future work. In particular, more investigation into the functions of JAR4 and improvements to the RT-qPCR method and JAR4 primers may provide additional insights into the interesting JAR4 expression results seen in Chapter 4. JAR4 is a key enzyme for the conjugation of the bioactive JA-Ile from jasmonic acid and methyl jasmonate. If the results presented in Chapter 4 prove to be repeatable and robust then this provides a useful insight into the regulation of internal jasmonate production under biotic stress conditions.

Another way to better understand the dynamics of the jasmonate perception and gene responses within the plant would be tracking either the nanosilicates or jasmonates or both. For instance, the HMSNPs can be labelled with fluorescent proteins like fluorescein isothiocyanate (FITC) and then tracked via fluorescence or confocal microscopy (Rashidi et al. 2017). A proof of concept was successfully tested using FITC labelled HMSNPs on agar and a fluorescence microscope but the data was too preliminary to include in this thesis. Some groups have successfully labelled jasmonates using fluorescent markers, and one particularly promising technique is labelled of jasmonic acid using cadmium-free quantum dots (Liao et al. 2015). A few initial attempts of this method were made, though trials were still in early testing phase when the time and funding of this project ended.

Finally, a plant biosensor for jasmonate pathway activation has been developed in *Arabidopsis thaliana* by Larrieu et al (Larrieu et al. 2015). Initial aims of this project included utilizing these biosensor plants to measure the dynamics of release of jasmonate from loaded silicates and the dynamics of perception of the jasmonates by the plants. Proof of concept tests were completed using a confocal microscope and exogenous application of jasmonic acid and methyl jasmonate but time constraints prevented any further investigation.

5.1.4.6 Induced resistance or priming?

When this project was first proposed it was hoped it could test for priming effects from either the jasmonates or the jasmonate-silicate treatments. However, in the end it tested for induced resistance – the only experiment which tests for priming is shown in Chapter 4 Figure 4.6. To the understanding of this author, priming differs from induced resistance by being (i) removed from the initial stimulus by space and/or time, i.e., the stimulus occurred locally and at a later time point after initial defense responses have receded a defense response is seen systemically, and (ii) by showing a defense response larger in magnitude than that of an un-primed plant. Finally, priming does not always involve strong defense responses around the initial priming stimulus.

This makes priming more difficult to test for than induced resistance. To look for induced resistance one can test immediately after or within a short time frame of the initial stimulus. If there are defense responses, whether that's protein accumulation, enzyme activity, callose deposition or changes in gene expression, the stimulus has induced resistance. In order to test for priming, one would need to wait until these initial responses have receeded and then re-challenge the plant. If the plant responds more quickly and more strongly than it did the first time, and, crucially, it responds more quickly and more strongly than a non-stimulus treated plant, then this would be a priming response.

By these definitions the data in Chapter 4 only just tests for priming, as no initial tests were performed to quantify gene expression changes immediately after stimulus treatment. Additional gene expression tests would be needed to follow up on the JAR4 results seen in Figure 4.6 induced by the 1.0 mM MeJA and 10.0 mM MeJA loaded zeolite treatments.

5.1.5 Concluding Remarks

This thesis developed and tested a novel plant defense-priming seed treatment and adapted and tested several *Pisum sativum-Pythium ultimum* pathosystem

assays. It demonstrated that zeolite clinoptilolite has potential as a silicate carrier alternative to mesoporous silica nanoparticles and may induce or enhance plant-immune priming effects on its own and as part of a seed treatment. Finally, it showed that jasmonic acid and methyl jasmonate on their own and as part of a silicate-based seed treatment did not induce effective resistance to *Pythium ultimum* in pea plants under the conditions tested. Taken together, this thesis adds to our understanding of damping-off disease pathosystems, silicate carriers in agriculture, and the effects and limitations of jasmonates as seed treatments. Finally, it serves a useful proof of concept in developing pesticide-alternatives, showing the possibilities of combing natural silicate carriers like zeolite with plant defense-priming compounds like jasmonate hormones.

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safety, regulatory issues are similar to those for other additives, and a principal benefit of DEs is their removal by normal processing methods. For worker safety, regulatory issues are similar to those for other dusts, such as lime. The proof of potential insecticide values of DE may be assessed by using the analysis of physical and chemical properties of DE and its effect on grain properties and the proof of efficacy may be regulated by bioassay of standard design. Integrated pest management (IPM), a knowledge–based system, is rapidly providing a framework to reduce dependence on synthetic chemical pesticides. The main principle of post–harvest IPM is to prevent problems rather than to react to them. The specific curative measures using synthetic pesticides should be applied only when infestation occurs. DE and enhanced diatomaceous earth (EDE) formulations hold significant promise to increase the effectiveness and broaden the adoption of IPM strategies, thereby reducing the need for synthetic pesticides. By incorporating DE in an effective IPM program, grain is protected against infestation, loss caused by insects is prevented and grain quality is maintained until the grain is processed. Cases study data on the use of DE for commodity and structural treatment show that DE is already a practical alternative to synthetic pesticides in some applications.

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