Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biology

Jakob Irgens Blakstad

# The utilization of frass from the yellow mealworm *(Tenebrio molitor)* as a plant fertilizer and immune stimulant

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Supervisor: Atle M. Bones
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# Acknowledgements

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# Abstract

Insect production is a rapidly growing industry worldwide, as it presents a promising solution for effective recycling of organic waste while being a sustainable protein source for use in both food and feed. The most abundant by-product of insect production is insect feces, scientifically known as 'frass'. Frass is produced in greater volumes than the actual insect products and should be utilized in order to maintain a circular economy. Today's most common use of frass is as a plant fertilizer due to its relatively high nutritional content. However, there is also reason to believe that the chitin content of frass, originating from the insect exoskeleton, leads to activation of immune responses in plants, potentially increasing their resistance towards pests and pathogens through so-called priming. This has not yet been proven by research and is investigated in this thesis, together with an assessment of the fertilizer qualities of frass from the yellow mealworm (*Tenebrio molitor*). Fertilization experiments conducted with tomato plants (*Solanum lycopersicum* var. Moneymaker) confirmed that the frass had a fertilizing effect at 2% by volume. A nutrient deficiency assay with sunflower (*Helianthus annuus*) showed that frass alone could provide sufficient amounts of several macronutrients when applied to the soil at a 5% volume inclusion, but had trouble contributing enough nitrogen to the sunflower plants. A ROS assay showed a triggered immune response in *Arabidopsis thaliana* by pure chitin but not by frass or insect skin residue. Similarly, callose deposition was only observed in roots of *Arabidopsis* treated with chitin. A gene expression analysis of *Arabidopsis thaliana,* grown in frass and insect skin-treated soil and infected with *Botrytis cinerea,* showed higher induction of defense-related genes in the frass and insect skin treatments compared to control treatments, suggesting a priming effect. Pathogen assays with *B. cinerea* and *Pseudomonas syringae* indicated an increased resistance against necrotrophs in plants fertilized with insect skin residue. More research should be conducted, especially on the immune-stimulating aspect of frass, to corroborate the promising results obtained during this project.

# Sammendrag

Produksjon av insekt er en hurtigvoksende industri verden over, siden insekter kan oppsirkulere matavfall til en bærekraftig proteinkilde som kan brukes både som menneskemat og som dyrefôr. Det mest omfangsrike biproduktet fra insektproduksjon er insektavføringen, kalt "frass" på fagspråket. Frass blir produsert i større volum enn de faktiske insektproduktene, og bør bli utnyttet for å opprettholde en sirkulærøkonomi i produksjonen. Den vanligste bruken av frass i dag er som plantegjødsel, grunnet det relativt høye næringsinnholdet i frass. Det er også grunner til å tro at kitininnholdet i frass som stammer fra insektenes eksoskjelett kan aktivere en immunrespons hos planter, som potensielt kan øke deres motstandsdyktighet mot sykdom og skadedyr gjennom såkalt priming. Dette har enda ikke blitt vitenskapelig bevist og blir undersøkt i denne avhandlingen, sammen med en evaluering av gjødslingseffekten til frass fra melorm (*Tenebrio molitor)*. Eksperimenter med tomat (*Solanum lycopersicum* var. Moneymaker) bekreftet at frasset hadde en gjødslingseffekt ved 2% voluminklusjon i jorden. Et næringsmangelforsøk med solsikke (*Helianthus annuus*) viste at frass kan alene tilføre tilstrekkelige mengder av flere næringsstoffer ved 5% voluminklusjon, men ikke nitrogen, som ble tilført i for liten mengde for optimal vekst. Et ROS-forsøk viste en immunrespons i *Arabidopsis thaliana* av en ren kitin-standard, men ikke av frass eller insektskall. Tilsvarende ble callose kun observert i røttene til *Arabidopsis* behandlet med rent kitin. En undersøkelse av genuttrykk i *Arabidopsis thaliana* som var dyrket i jord med frass eller insektskall og deretter infisert med *Botrytis cinerea*, viste en høyere induksjon av forsvarsrelaterte gener i frass- og insektskall-behandlingene sammenlignet med kontrollbehandlingen. Dette kan antyde en priming-effekt. Patogenforsøk med *B. cinerea* og *Pseudomonas syringae* indikerte økt motstandsdyktighet mot nekrotrofe patogener hos planter som var behandlet med insektskall. Ytterligere forskning må gjennomføres, spesielt angående mulige immunstimulerende egenskaper ved frass, for å underbygge de lovende resultatene som ble oppnådd i denne masteravhandlingen.

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# List of abbreviations

| | |
|---|---|
| ABA | Abscisic acid |
| ANOVA | Analysis of variance |
| BC | *Botrytis cinerea* |
| BIK1 | *Botrytis*-induced kinase 1 |
| BSF | Black soldier fly |
| C-soil | Compost soil |
| CatNb | Catalogue number |
| CDPK | $Ca^{2+}$-dependent protein kinase |
| CERK1 | Chitin elicitor receptor kinase 1 |
| Col-0 | The *Arabidopsis thaliana* accession Columbia-0 |
| CTRL | Control |
| DAMPs | Damage-associated molecular patterns |
| dd$H_2O$ | Double-distilled water |
| DP | Degree of polymerization |
| Elf18 | Elongation factor Tu |
| ET | Ethylene |
| ETI | Effector-triggered immunity |
| Flg22 | Peptide domain of bacterial flagellin |
| GE-BC | Gene expression analysis with *Botrytis cinerea* |
| GE-ET | Gene expression analysis with elicitor treatments |
| GHG | Greenhouse gases |
| HAMPs | Herbivore-associated molecular patterns |
| HF | Heat-treated frass |
| hpi | Hours post infection |
| HR | Hypersensitive response |
| IOS1 | Impaired oomycete susceptibility 1 |
| ISR | Insect skin residue |
| JA | Jasmonic acid |

| | |
|---|---|
| L-012 | A luminol analog |
| LCOs | Lipochitooligosaccharides |
| LIK1 | LysM RLK1-interacting kinase 1 |
| LYK1/4/5 | LysM-containing receptor-like kinase 1/4/5 |
| LYM2 | LysM domain-containing GPI-anchored protein 2 |
| LysM-RLK/P | Lysin motif-containing receptor like kinases/proteins |
| MAMPs | Microbe-associated molecular patterns |
| MAPK | Mitogen activated protein kinase |
| MW | Molecular weight |
| NB-LRR | Nucleotide-binding leucine rich repeats |
| NG | Naturgjødsel (organic fertilizer from Hageland) |
| NMR | Nuclear magnetic resonance |
| NPK | Nitrogen, Phosphorus, Potassium |
| PBL27 | Receptor-like cytoplasmic kinase |
| PGPMs | Plant growth promoting microorganisms |
| PR | Pathogenesis-related proteins |
| PRR | Pathogen-recognition receptors |
| PTI | Pattern-triggered immunity |
| qPCR | Quantitative real time polymerase chain reaction |
| RBOHD | Respiratory burst oxidase homolog protein D |
| ROS | Reactive oxygen species |
| RPM | Revolutions per minute |
| RT | Reverse transcriptase |
| SA | Salicylic acid |
| SAR | Systemic acquired resistance |
| SEC | Size exclusion chromatography |
| SF | Sterilized frass |
| UF | Untreated frass |
| VOCs | Volatile organic compounds |

# 1 Introduction

## 1.1 My own motivation for choosing this master thesis

After three years of studying biotechnology, I wanted to try my luck on the job market, to see if this line of work was right for me. I stumbled upon Invertapro, a Norwegian insect start-up producing mealworms in the small town of Voss in Western Norway. After reading up on literature about the topic, I was very fascinated by the incredible potential this novel industry seemed to possess. Invertapro accepted my application, and I ended up working there the whole year, as well as the remaining years of my master study as a side job. I worked with all aspects of insect farming, from breeding of beetles to processing of the final product (ground larvae powder), as well as a wide selection of office tasks ranging from marketing and sales to research project application and fund securing. When it was time for me to choose a master project, it was natural to collaborate with Invertapro. I was very interested in frass and had seen its convincing effects as a fertilizer on my own plants and in growth trials at work. The very small collection of literature that could be found on this topic was also intriguing, as it meant that new discoveries could be made that could actually have an impact, even from an independent master thesis. The proposed, but undocumented benefits of frass on plant disease resistance was an aspect I really wanted to focus on, which is why I joined Atle Bones' research team who have extensive knowledge in the field of plant immunity.

## 1.2 Background: Why insect frass?

Insect feces, scientifically known as 'frass', are considered a promising organic fertilizer (Poveda, 2021). This often comes as a surprise to people, as most have never spared the topic of insect excrements a single thought. To understand why this is of interest, we must begin with the up-and-coming industry of insect production, and why it might help solve several challenges our world faces today (Van Huis et al., 2013).

### 1.2.1 The environmental problems caused by agriculture and human food consumption

Global warming is perhaps the biggest challenge of our time (IPCC, 2019). Livestock production is considered a large part of this problem as this sector alone accounts for about 14.5% of total global greenhouse gas (GHG) emissions. In addition, 26% of global land area is used for production of livestock (Sakadevan & Nguyen, 2017), and it is estimated that the production of 1 kg animal protein requires up to 100 times more water than 1 kg of grain protein (Van Huis et al., 2013). There's a consensus among scientists that a substantial reduction of the global meat consumption will be a very important step towards the UN goals of a more sustainable future, as discussed in the Paris Agreement in 2016 (IPCC, 2019; UNFCC, 2015).

It is estimated that the human population will reach roughly 10 billion by 2050, and that food production has to increase by 70% by 2050 in order to keep up with the growing demand (FAO, 2009). A large proportion of the human population are also suffering from malnutrition, and this is expected to increase in the future due to scarcity of recourses in developing countries (Lindgren et al., 2018).

The dilemma is apparent; we must reduce the environmental footprint of the agricultural sector, while increasing food production by 70%, without compromising the nutritional needs of people in developing countries. An obvious solution to this dilemma is a global shift towards a more plant-based diet, but despite clear scientific results and warnings from researchers, there has been little reduction in the consumption of meat so far. It may seem as if the role of meat in the western food culture is unshakeable, and that the habit of eating meat will be hard to change (Rust et al., 2020).

Another issue regarding our food culture is this: about one third of all food produced globally goes to waste every year (FAO, 2013). This is a waste of valuable nutrients, and of the resources used in production of the food. FAO have estimated that food waste accounts for about 8% of the total anthropogenic GHG emissions globally (FAO, 2013).

### 1.2.2 Insects as a solution

As an approach that might contribute to solving the problems issued above, insect production has been proposed (Van Huis et al., 2013). It is a new, rapidly growing industry, receiving increased attention as a sustainable replacement for meat, as well as feed for livestock. Insects can convert feed to protein a lot more efficiently compared to many conventional livestock such as cattle or pig, due to their ectothermic nature (Van Huis et al., 2013). Insect production consumes very little water (Halloran et al., 2018), and the greenhouse gas emissions of this industry are estimated to be exceptionally low compared to other types of meat production (Van Huis et al., 2013) as shown in Figure 1.1. Insects can be produced indoors in a vertical design

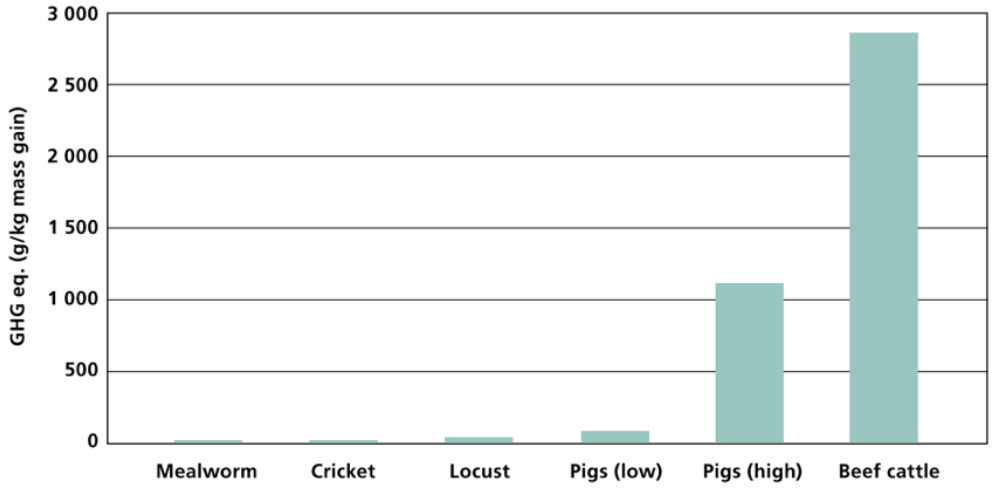



**Figure 1.1:** Production of greenhouse gases (GHGs) per kg mass gain for three different insect species compared to pigs and cattle. From (Van Huis et al., 2013)

by stacking of the rearing boxes. This makes it possible to produce insects virtually anywhere, enabling local production in cities or close to the feed source (such as a food waste processing facility) or the end customer (such as a fish farms), reducing transport (Specht et al., 2019).

Perhaps the most intriguing benefit of many insects is their ability to eat organic waste. These particular insects are exceptionally useful in industrial production as they effectively convert almost all sorts of food waste and other organic wastes into high quality, easily digestible proteins and healthy fats (Van Huis et al., 2013).

Insects are considered a suitable nutrients source for humans, and many edible insects contain similar amounts of protein and fat compared to conventional meat products such as beef (Van Huis et al., 2013). According to a recent review from Nowakowski et al., insect have superior health benefits for both humans and other animals due to high levels of vitamins, minerals, fiber, essential amino acids, omega 3 and 6 fatty acids, and antioxidants. (Nowakowski et al., 2020).

To summarize, food waste can be utilized to produce a sustainable and healthy meat and feed replacement by the use of insects. However, due to cultural challenges, insects are traditionally not viewed as food for human consumption in western countries, even though more than 2 billion people eat insects worldwide (Van Huis et al., 2013). The 'yuck factor' is a serious psychological barrier that can be hard to overcome for many who try insects for the first time (Halloran et al., 2018).

Luckily, farmed animals are less picky. 80% of emissions from the agricultural section are related to livestock production, largely due to production of feed crops (McMichael et al., 2007). Thus, replacing conventional livestock feed with insects farmed on food waste, can greatly improve the carbon footprint from livestock production (Van Huis et al., 2013). Fishmeal, protein sourced from fish, is also a commonly used feed substrate in livestock production, especially in aquaculture. Its production is regarded as unsustainable, leading to overfishing and depletion of fish colonies (FAO, 2020). A transition from fishmeal towards insect protein can result in a more sustainable aquaculture industry (Henry et al., 2015). In Norway, the farmed salmon industry is desperately looking for alternative feed substrates and are already experimenting with insect protein as a replacement for fishmeal (Skretting, 2018).

These benefits of insect production have recently been receiving attention in Western countries, which has led to an exponential growth of the industry during the last decade. The production of insect protein is predicted to continue growing the coming years, and it is estimated to reach half a million metric tons a year by 2030 (Byrne, 2021).

### 1.2.3 Frass

The most abundant by-product in industrial insect production is insect frass. Figure 1.2 displays mealworm frass, which with its low moisture content has a sand-like texture.

Depending on the insect species and its diet, frass can typically make up 80-95% of total production output, i.e. 4-20 times more than insect biomass output (Gärttling et al., 2020; Poveda, 2021). The proposed growth of the insect industry will therefore inevitably lead to a simultaneous increase in frass production. This could be in the range of several million metric tons a year by 2030 and become a serious waste issue for insect producers if it isn't utilized.

Frass contains relatively high levels of plant nutrients and is therefore considered a promising organic fertilizer (Poveda, 2021). It is also thought to be able to stimulate plant immune responses through its natural content of chitin, potentially resulting in an increased resistance against pests and diseases (Gärttling et al., 2020; Poveda, 2021; Quilliam et al., 2020).

It is also worth noting that frass has been considered as an ingredient in feed for livestock, and positive results have been found when fed to farmed omnivore fish (Yildirim-Aksoy et al., 2020), suggesting that alternative applications other than as a fertilizer exist for frass.

**Figure 1.2:** Container with untreated mealworm frass produced by Invertapro. The picture displays the amount of frass generated over 1 week at the factory (approximately 1000 liter). Picture by Invertapro.

## 1.3 Plant nutrition, fertilization, and pest control in agriculture

Plants need water, sunlight, and nutrients in order to function. They can get energy from the sugar compounds produced by photosynthesis but need a steady supply of nutrients absorbed through the roots in order to provide building blocks for continued growth and maintained function (Bhatla et al., 2018; Taiz et al., 2015b). These nutrients are grouped into macronutrients, which plants need in large amounts, and micronutrients, which are sufficient in small doses. The three most important plant nutrients are nitrogen (N), phosphorus (P) and potassium (K), often abbreviated NPK. These are crucial for the development of all plant tissue, and plants will not grow without them. The other macronutrients are sulfur (S), calcium (Ca) and magnesium (Mg). These are required in smaller doses compared to N, P and K. The micronutrients include, among others, iron (Fe), chlorine (Cl) and manganese (Mn) (Bhatla et al., 2018; Taiz et al., 2015b).

A deficiency in one of these nutrients can severely impair the plant's growth, and over time be lethal to the plant. Too much of a nutrient can also be harmful, and nutrient toxicity can lead to reduced growth (Bhatla et al., 2018). Deficiency in one nutrient will limit plant growth and adding more of other nutrients will not help. This principle was made famous by the German chemist Justus von Liebig and is called "the law of the minimum" (UiO, 2016). It is often visualized as a barrel with water, where the shortest plank in the barrel wall determines how high the water level in the barrel can get, regardless of the length of the other planks (Figure 1.3) (Roussel et al., 2018). Similarly, the scarcest nutrient determines how much a plant can grow (Bhatla et al., 2018; UiO, 2016).

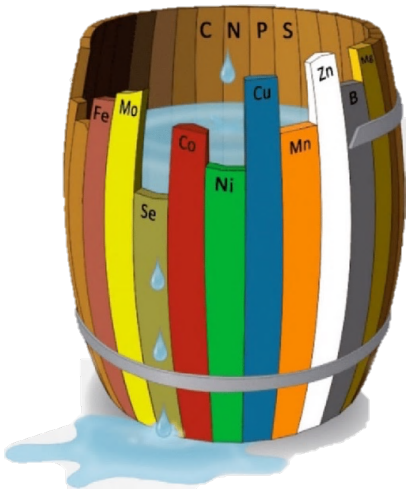



**Figure 1.2:** Illustration of Liebig's law of the minimum. The amount of selenium (Se) in this case limits the growth of the plant, even though other nutrients are in adequate amounts. Similarly, the water cannot rise higher than the lowest plank. From (Roussel et al., 2018)

In natural ecosystems, sufficient amounts of nutrients are often apparent in the soil, due to the constant breakdown of organic matter by insects and microorganisms. However, in most farming practices, the soil does not contain enough nutrients to satisfy the crop's needs, and fertilizers must be applied in order to increase nutrient content of the soil (Poveda, 2021; Taiz et al., 2015b). There are many different types of fertilizers, but they can generally be grouped into organic and inorganic fertilizers.

### 1.3.1 Inorganic fertilizers

Inorganic fertilizers (also called chemical, synthetic, or mineral fertilizers) are produced by extracting nitrogen from the air through a reaction known as the Haber-Bosch process (Smil, 2001), and sourcing phosphorus and potassium from natural reservoirs (Ciceri et al., 2015; Taiz et al., 2015b). Micronutrients and other macronutrients can also be added to the mix. These fertilizers can be tailormade for a specific plant, to supply the optimal proportion of NPK providing the best growth. The nutrients are water soluble and can be readily absorbed by plants (Taiz et al., 2015b).

The widespread use of inorganic fertilizers in agriculture today is crucial in order to provide the world population with enough food. After the discovery of nitrogen synthesis by Fritz Haber in 1909, and the subsequent commercialization by Carl Bosch, the world population started growing at an increasingly faster rate as crop yields suddenly skyrocketed. It has been estimated that without nitrogen synthesis, 2/5 of the world's population could not exist, and that the dependency on synthesized nitrogen will only increase as the world's population continues to grow (Smil, 2001).

The extensive, and often exaggerated, use of inorganic fertilizers today is associated with a wide range of negative environmental impacts (Farooq et al., 2019). Prolonged use can severely reduce soil quality over time by loss of organic matter, erosion and compaction, reduced biodiversity and soil salinization (Farooq & Pisante, 2019; Kotschi, 2015). Crop plants typically absorb less than half of the inorganic fertilizer applied to their soil, which leads to an excess of especially nitrogen and phosphorus in the environment, so-called eutrophication. This can result in harmful algal blooms, disrupting natural ecosystems (Anderson et al., 2002), and also contributes to air pollution and climate change (Taiz et al., 2015b). To top it off, the Haber-Bosch process is very energy demanding and consumes roughly 1% of total energy produced worldwide (Capdevila-Cortada, 2019).

Phosphorus, a key nutrient in fertilizers, is mainly produced from phosphate rock from Morocco or China. It is now apparent that these global phosphate reserves are running low and might be depleted within few decades (Vaccari, 2009). At the same time, the natural content of P in agricultural soils are being depleted due to soil erosion, which in turn leads to more eutrophication (Alewell et al., 2020). In order to secure enough phosphorus for future generations, scientists are calling for a more sustainable P policy, by reduction of soil erosion and P recycling from organic waste (De Ridder et al., 2012; Vaccari, 2009).

### 1.3.2 Organic fertilizers

In contrast to inorganic fertilizers, organic fertilizers are made of natural resources such as manure or organic waste, and will not necessarily contain optimal nutrient proportions, due to their non-synthetic nature. And while inorganic fertilizers provide nutrients in a mineralized, plant available form, organic fertilizers contain a large portion of these nutrients bound to organic molecules. These compounds must be processed by microorganisms in the soil before the nutrients are released in a form that plants can use (Taiz et al., 2015b). This characteristic makes organic fertilizers slower in effect, releasing its nutrients over time rather than providing

them all at once (Wang et al., 2021). Depending on the type of plant cultivation, this can be a positive or negative fertilizer trait. Some fast-growing and nutrient demanding plants may not get sufficient nitrogen from organic fertilizers, while other plants can benefit from the steady release over time (Taiz et al., 2015b).

While inorganic fertilizers have negative environmental impacts, many organic fertilizers are claimed to have the opposite effect. Since the nutrients in organic fertilizers need to be processed by microorganisms in order to be absorbed by plants, they greatly benefit the microflora in the soil, increasing biodiversity of bacteria, protists, fungi, and nematodes (Ikoyi et al., 2020). The importance of these organisms in agriculture are getting increased attention, and research shows that these so-called plant growth promoting microorganisms (PGPMs) can benefit plants in countless ways, including growth and disease resistance (Farooq et al., 2019; Taiz et al., 2015b).

The addition of organic matter to soil in the form of carbon containing compounds can have a wide range of positive impacts, such as improved soil structure, higher water retention, improved root proliferation and higher soil fertility (Farooq et al., 2019).

Also, the slow release of nutrients from organic fertilizers leads to little nutrient pollution, as nutrients are released in portions rather than all at once (Schmitt & de Vries, 2020; Taiz et al., 2015b).

The use of organic waste or animal manure as fertilizers can be a solution to maintain a circular economy in agriculture, as nutrients goes back into the food chain with less pollution to the environment. Such recycling of nutrients can be important in reducing the depletion of especially phosphorus from non-renewable natural sources (Ikoyi et al., 2020; Schmitt & de Vries, 2020).

Many scientists are calling for a shift from inorganic to organic fertilization, or a combination of these, in order to maintain a sustainable agriculture in future years (Farooq & Pisante, 2019; Kotschi, 2015).

It must be noted that not all researcher agree on this, and some of the negative effects of inorganic fertilizers compared to organic fertilizers are under debate (Timsina, 2018). Due to lower nutrient content and slower release of nutrients from organic fertilizers, organic farming often results in lower yields compared to conventional farming (20-50% lower), and organic nutrients alone are not considered sufficient in order to meet the global food demand. Other concerns with organic fertilizers can be high levels of heavy metals and potential pathogens, particularly when using animal manure (Timsina, 2018).

### 1.3.3 Pest and pathogen control in agriculture

A fundamental part of our today's agriculture is the control of pests and diseases. Insect pests alone accounts for 7.9%-15.2% of global annual loss of major crops, depending on location (Nawaz et al., 2019). To counter this, a wide range of pesticides, fungicides, nematicides and bactericides are used globally. However, it is now known that some of these compounds can negatively affect beneficial organisms, both in soil and aboveground, as well as humans (Nawaz et al., 2019). As an example, over 40% of all insect species worldwide are threatened with

extinction, with pollution by synthetic pesticides as one of the main driving factors. This can potentially have devastating consequences for natural ecosystems, as well as for our own food production (Sánchez-Bayo & Wyckhuys, 2019).

New ways of controlling pests and pathogens should therefore be implemented in order to reduce this destructive trend. Biopesticides, which are organisms or natural compounds that can be used to control pests, could be a solution. They are typically beneficial fungi or bacteria that have an antagonistic effect on pathogens or lead to improvement of the plant's own defense systems, thereby limiting negative impacts on non-targeted organisms (Sporleder & Lacey, 2013).

## 1.4 Insect frass composition and its use as a fertilizer

Per definition, frass is the fecal matter of insects (Chavez & Uchanski, in press). However, insect fertilizer products, usually referred to as 'frass', also contains uneaten residues of feed substrate as well as exoskeleton fragments from the insect, rich in chitin. The reason for this is the mechanical sieving process used to separate insects from frass during industrial production, which will not select for only the feces. In this thesis the term 'frass' will be used for the blend of insect feces, exoskeleton, and feed residue, since this is the norm and there is no reason to believe that these substances will be removed from the fertilizer product in the future.

### 1.4.1 Status on the literature

Even though insect farming is a new concept in western countries, it has been going on for decades in Asian and African countries (Halloran et al., 2018). Despite this, little scientific literature has been available on the utilization of frass, until recently. As a by-product of this novel industry, it has naturally received less attention than its main product. This is starting to change; an increasing number of studies have been published on this topic the last 5 years. The focus has been mostly on the fertilizing characteristics of frass, but research on plant defense stimulation by frass has also surfaced the last few years (Poveda, 2021), indicating that this will draw increased attention in the future.

### 1.4.2 Nutrient content of insect frass

The nutrient content of frass will vary depending on the insect species, insect age and life stage, the diet of the insect, rearing conditions, and even the fertilization rate of plants used in the insect diet (Kagata & Ohgushi, 2012). Research on the yellow mealworm (*Tenebrio molitor*) found that the larval frass can have a nitrogen content ranging from 2.7% to 7.8% depending on the diet of the larvae (Poveda et al., 2019). In other words, it's hard to give a general description of the nutrient content of insect frass. However, it is probably sensible to focus on frass from the larval stage of a few insect species used in industrial production today, fed an organic waste diet, as this is how most frass likely will be produced in the future. This is why much of the research published on frass the last few years focuses on frass from the larval stage

of either the yellow mealworm or the black soldier fly (*Hermetia illucens,* abbr. BSF), which are two of the most commonly produced insects today with great potential in animal nutrition and food waste recycling (Varelas, 2019).

The amounts of N, P and K in frass (showed for mealworm and BSF in Table 1.1) makes it suitable as a fertilizer (Poveda, 2021). When expressed in nutritional values per unit dry matter, insect frass has somewhat lower NPK values compared to other animal manures. However, when looking at wet weight (how animal manures are commonly distributed in the field), insect frass which is naturally low in moisture has a higher content of nutrients per kg compared to dairy cow, sheep, and pig (Table 1.1).

Insect frass contains relatively low amounts of free ammonium ($NH_4^+$) and nitrate ($NO_3^-$) (Appendix 1.1), which are the forms of plant available nitrogen. Its nitrogen is mostly bound to organic molecules and cannot be absorbed by plant roots (Beesigamukama et al., 2020). On the other hand, Houben et al. showed that nitrogen content in mealworm frass mineralizes quickly over the first 20 days (Figure 1.4), with a subsequent gradual mineralization of the remaining N over the following months (Houben et al., 2020). Alternatively, in order to achieve a higher amount of plant available nitrogen, inorganic fertilizers has been used together with frass with successful results (Quilliam et al., 2020).

In addition to NPK, frass often contains significant amounts of macro- and micronutrients, while also these will vary with type of insect, diet, etc. (Gärttling et al., 2020; Poveda et al., 2019). Invertapro state that their frass contains low amounts of heavy metals (full nutrient profile in Appendix 1.1), placing the fertilizer in quality class 0 (highest fertilizer quality class in Norway) (Lovdata.no, 2021).

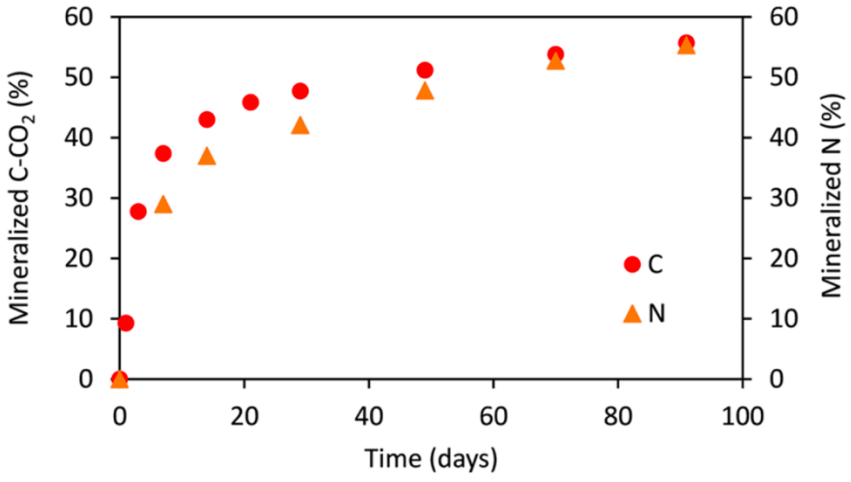



**Figure 1.3:** Mineralization rate of carbon and nitrogen in mealworm frass applied to soil. From Houben et al., 2020.

**Table 1.1:** Comparison of NPK values of different animal manures, based on wet weight or dry weight. All data except for mealworms and black soldier fly (BSF) are from (Daugstad et al., 2012). Mealworm values are as informed by Invertapro (Appendix 1.1) and BSF values are based on (Schmitt & de Vries, 2020) and (Gärttling et al., 2020).

| | **Wet weight** | | | | |
|---|---|---|---|---|---|
| | Dry matter (%) | Total N (g/100 g) | Ammonia-N (g/100g) | Phosphorus (g/100g) | Potassium (g/100g) |
| **Dairy cow** | 6 % | 0.31 | 0.18 | 0.048 | 0.34 |
| **Sheep** | 12 % | 0.55 | 0.32 | 0.11 | 0.63 |
| **Pig** | 5 % | 0.33 | 0.24 | 0.09 | 0.19 |
| **Poultry** | 50 % | 2.79 | 0.83 | 0.67 | 1.35 |
| **BSF** | 66 % | 2.24 | 0.66 | 1.91 | 2.31 |
| **Mealworm** | 93 % | 2.60 | 0.17 | 1.77 | 2.79 |
| | **Adjusted for dry weight** | | | | |
| **Dairy cow** | 100 % | 5.2 | 3.0 | 0.8 | 5.7 |
| **Sheep** | 100 % | 4.6 | 2.7 | 0.9 | 5.3 |
| **Pig** | 100 % | 6.3 | 4.6 | 1.7 | 3.7 |
| **Poultry** | 100 % | 5.6 | 1.7 | 1.3 | 2.7 |
| **BSF** | 100 % | 3.4 | 1.0 | 2.9 | 3.5 |
| **Mealworm** | 100 % | 2.8 | 0.2 | 1.9 | 3.0 |

### 1.4.3 Insect skin residue and chitin in frass

As mentioned, frass fertilizer products often contain exoskeleton fragments, which are rich in chitin. When growing in size, many insects need to shed their skin and synthesize a new one, a process called molting (Nijhout, 1981). As an example, the mealworm larva molts up to 20 times before it transforms into an adult beetle (Vigneron et al., 2019). The skin residue fragments into small particles and ends up together with the frass in most industrial production systems. So far, the proportion of insect skin residue (ISR) in frass is not known, and measurements of this should be conducted in future research.

Chitin can also be excreted together with the feces due to the chitin content of the peritrophic membrane, which is present in the gut of all insects. This membrane is important in insect digestion, as well as protection of chemical and mechanical damage in the insect gut, and consists of chitin microfibrils and proteins in a matrix (Terra, 2001). The peritrophic membrane is constantly renewed, and excess is therefore excreted together with the frass (Fescemyer et al., 2013). There is also evidence that frass pellets are covered in the peritrophic membrane when excreted (Brandt et al., 1978; P. Wang & Granados, 2001). This has been shown for the larder beetle (*Dermestes lardarius*) (Magni et al., 2010), which is related to the yellow mealworm through the order Coleoptera.

### 1.4.4 Current scientific evidence for the use of frass as fertilizer

**Frass' role in an ecosystem**

Despite their relatively small cumulative biomass compared to plants and microbes, insects are regarded as key nutrient cyclers in natural ecosystems (Yang & Gratton, 2014), and can provide nutrients for both plants and microorganisms through their frass. This has been shown for a wide variety of insects, such as grasshoppers, beetles and moths (Poveda, 2021). As an example, the beetle *Paropsis atomaria* and the lepidopteran *Doratifera quadriguttata* feeding on eucalyptus were able to produce up to 270 kg/ha of frass over the course of 1 year, which translates to 4 kg/ha of nitrogen (Gherlenda et al., 2016). Studies with $N^{15}$-labelled nitrogen have shown that nitrogen in frass from the herbivore insect gypsy moth (*Lymantria dispar*) is quickly incorporated in the soil, while 80% of nitrogen from non-eaten leaf litter remained undecomposed in the leaf for 2 years, showcasing the importance of insects in making nitrogen available in a forest (Christenson et al., 2002).

**Black soldier fly frass**

As one of the most common insects for industrial production in Western countries, the black soldier fly (BSF) will be produced in great volumes the coming years. Several studies document the beneficial fertilizing capabilities of its frass.

It has for example been reported to act as rapidly as $NH_4NO_3$ when it comes to growth of ryegrass (*Lolium perenne*) (Klammsteiner et al., 2020), suggesting that the nitrogen mineralizes quickly enough for the nitrogen requirements of this plant. In fertilization trials with chili peppers and shallots, Quilliam et al. showed that BSF frass from a brewery waste diet is better at increasing growth compared to chicken manure. However, frass combined with inorganic NPK fertilizer yielded the best results, also better than inorganic NPK alone (Quilliam et al., 2020). Beesigamukama et al. however showed that nitrogen release from BSF frass was too slow to provide sufficient N for maize at periods of peak N demand, but it still performed better than the commercial organic fertilizer control (Beesigamukama et al., 2020). BSF fed on organic household waste produced a frass that performed better than the same organic waste applied as a fertilizer, and also better than manure from cow, horse and poultry in growth trials with komatsuna (*Brassica rapa* var. *perviridis*) (Kawasaki et al., 2020). As a replacement of commercial peat, an inclusion of 10% BSF frass by volume was shown to increase the growth of baby leaf lettuce, tomato, and basil compared to the controls (Setti et al., 2019).

Alternatively, Gärttling et al. found that BSF frass showed low N-fertilization effects compared to organic and mineral fertilizer control groups. It was concluded that BSF frass is more suited as a basic fertilizer, supplemented by other fertilizers depending on the crop (Gärttling et al., 2020).

Due to these somewhat contradicting results, more research should be conducted on the fertilizing aspects of BSF frass. Also, high application rates of BSF frass have been shown to lead to growth inhibition and yield reduction, possibly due to ammonia toxicity according to Gärttling et al., 2020. This phenomenon should also be investigated, to establish an upper limit for frass application to soil.

**Mealworm frass**

Poveda et al. showed that the diet of the yellow mealworm greatly influenced both the nutritional and the microbial content of its frass, yielding fertilizers with significantly different growth promoting abilities. It was found that a diet containing 66% carbohydrates, 6% fat and 28% protein resulted in the best performing frass fertilizer, with NPK values of approximately 3-2-2. In addition to improving plant growth, treatment with frass from this particular diet also increased plant tolerance to abiotic stresses, such as drought and flooding. It was also shown that when sterilized, frass was somewhat less effective as a fertilizer and these plants displayed lower tolerance when challenged with abiotic stresses. The reason for this is likely a reduced contribution of beneficial microorganisms to the soil by sterilized frass (Poveda et al., 2019).

Houben et al. found frass to be as effective as mineral NPK fertilizer in growth trials with barley (*Hordeum vulgare*), and nutrient content of leaves were similar between the two fertilizer treatments. The authors propose that frass can substitute mineral NPK fertilizer partially or completely, without compromising biomass output. They also found that the presence of frass increases the diversity as well as metabolic activity of soil microbiota, supporting the research on microorganisms in frass by Poveda et al. (Houben et al., 2020; Poveda et al., 2019).

Earthworms (*Lumbricus terrestris*) were able to improve the fertilizing efficiency of mealworm frass leading to increased nitrogen, phosphorus, potassium and calcium uptake in leaves of barley (*Hordeum vulgare L.*) (Dulaurent et al., 2020). This suggests that frass can have a positive synergy with soil fauna, which in turn can be crucial for soil structure, soil organic matter and nutrient cycling.

Some insects in the family Tenebrionidae, most notably mealworms and superworms (*Zophobas morio*), are capable of biodegrading plastics (Wu & Criddle, 2021). This fascinating trait is receiving increased attention as plastic pollution and circular economy are getting more relevant. Interestingly, superworm frass from a polystyrene diet promoted growth and root development of dragon fruit cacti (*Hylocereus undatus*) compared to the control treatment (Koh et al., 2020).

**Microbiota**

The gut microbiota of insects can include bacteria, archaea, protists, and fungi, which have various functions inside the insect, such as the enzymatic breakdown of cellulose, or even certain plastics. The microbiota is also important in the synthesis of essential nutrients for the insect, as well as elimination of toxic compounds present in the insect feed (Poveda, 2021). This microbiota ends up in the frass and can have numerous ecological functions when incorporated in soil.

To further investigate this, Poveda et al. conducted several analyses on the microbial community in mealworm frass. Massive parallel sequencing detected a total of 4772 bacterial and 1225 fungal species, many of which are considered PGPMs. Plant growth promoting (PGP) traits were analyzed in 188 microbial isolates cultivated from the frass and most of these showed at least one PGP trait, such as siderophore production, auxin production or nitrogen fixation (Poveda et al., 2019).

Current regulations in most western countries demand sanitation of animal manure for use in agriculture, in order to remove any microbial threat (Lovdata.no, 2021; Poveda, 2021). Such treatments, that can include pressure sterilizing and high temperatures, may weaken or totally eliminate the beneficial microflora in frass. Effort should be put in investigating the safety of unsterilized frass. Interestingly, it has been shown that BSF can neutralize the pathogens *Escherichia coli* and *Salmonella enterica* that are present in its feed (Erickson et al., 2004; Varelas, 2019).

## 1.5 Plant defense mechanisms

### 1.5.1 How plants defend themselves against their enemies

Over the course of millions of years, plants have evolved numerous ways to protect themselves against their enemies. Being rooted to the ground, they have no means of escaping a threat, and must use their arsenal of sophisticated defense mechanisms to survive in a world full of plant-eating organisms. Conversely, bacteria, fungi, and herbivorous insects have developed devious ways to thwart these defense mechanisms, leading to an ever-evolving arms race between plants and pathogens/pests. Nevertheless, most plants in nature remain unharmed and uninfected, demonstrating how effective their intricate defense systems are when plants are exposed to their natural enemies (Balmer et al., 2013; Taiz et al., 2015a).

The first layer of plant protection is the constitutive defense mechanisms, such as waxy epidermal cuticles, thick cell walls, and stored antimicrobial compounds that can be released upon attack. If these defense layers are penetrated by a pathogen or herbivore, numerous inducible defenses can be activated, resulting in a wide range of defense mechanisms that vary depending on the type of intruder (Balmer et al., 2013; Saijo et al., 2018).

Pattern-recognition receptors (PRRs) imbedded in plant cell membranes can recognize so-called molecular patterns, which are molecules originating from the attacker. This leads to signaling cascades ultimately resulting in defense responses tailor-made for the situation, called pattern-triggered immunity (PTI) (Jones & Dangl, 2006; Saijo et al., 2018). Microbe-associated molecular patterns (MAMPs) are pathogen-related molecules such as the fungal cell wall polysaccharide chitin or the bacterial protein flagellin, that upon recognition lead to defense responses against the respective microbial diseases (Balmer et al., 2013). Herbivore-associated molecular patterns (HAMPs) are compounds typically found in insect saliva, regurgitant or frass, leading to recognition of the herbivore by the plant (Ray et al., 2016). Damage-associated molecular patterns (DAMPs) are signs of damage from the plant itself. Such compounds can typically be polysaccharides released from plant cell walls after damage by a herbivore or necrotrophic pathogen (Taiz et al., 2015a).

After a molecular pattern is recognized by a PRR, a complex immune response is initiated. Within a few minutes after elicitation, an influx of $Ca^{2+}$ is activated, which in turn leads to a burst of reactive oxygen species (ROS). ROS can be directly toxic to the pathogen and work as signaling molecules for the plant inducing other defense responses. Further, a

cascade of signaling events, often including the mitogen-activated protein kinase (MAPK) pathway will lead to transcription of a wide range of defense genes. These defense responses can include reinforcement of cell walls with callose or lignin, production of secondary metabolites with antimicrobial properties, or production of pathogenesis-related (PR) proteins that help against secondary infections (Saijo et al., 2018; Taiz et al., 2015a).

Not surprisingly, such an immune response can be very costly for the plant. Many nutrients and metabolites must be re-allocated from processes such as growth and seed production in order to produce an effective counterattack against pathogens or herbivores. Wasting resources on ineffective or unnecessary responses can be disadvantageous for a plant's survival and competitiveness. That is why plant immune responses have evolved into inducible fine-tuned processes under strict regulation by phytohormones (Denancé et al., 2013).

Phytohormones are molecules that can regulate biological processes in the plant, just as hormones do in animals. Several phytohormones are involved in the regulation of plant immune responses, the two key defense hormones being arguably jasmonic acid (JA) and salicylic acid (SA), while ethylene (ET) and abscisic acid (ABA) also play important roles (Aranega-Bou et al., 2014; Denancé et al., 2013). Although hormonal crosstalk in plant defense is complex, JA is generally important for resistance against necrotrophic pathogens and herbivores, i.e., organisms that degrade plant tissue during infection/feeding, while SA is involved against biotrophic pathogens that infect plants without causing much damage, keeping the tissue alive during infection (Denancé et al., 2013; Taiz et al., 2015a). These two phytohormones are known to be mostly antagonistic, with one downregulating the expression of the other (Ray et al., 2015; Robert-Seilaniantz et al., 2011). There are however exceptions to this rule and JA has been found to be essential for resistance against particular biotrophic pathogens, while SA can be required for immune responses against necrotrophs (Ray et al., 2015; Robert-Seilaniantz et al., 2011). An interesting example of JA/SA regulation occurs during attack by aphids, herbivore insects that penetrate plant tissue with their stylet and suck out sap from the phloem. An aphid doesn't break down tissue while feeding, and thus activates the SA pathway of resistance (Taiz et al., 2015a).

In order to overcome PTI, many pathogens can produce different molecules called effectors, designed to inhibit PRRs or in other ways mask the presence of MAMPs. In response, plants have evolved the so-called R (resistance) genes encoding for NB-LRR proteins (nucleotide-binding leucine rich repeats). These receptor proteins are located in the cytoplasm and are tasked with detection of effectors, which leads to effector-triggered immunity (ETI) (Balmer et al., 2013; Saijo et al., 2018). ETI often results in the hypersensitive response (HR), which is a mechanism based on deliberate plant cell suicide at the infection site. This limits the pathogens supply to water and nutrients, thereby hindering its further growth (Saijo et al., 2018).

### 1.5.2 The role of priming in plant defense

Induced resistance allows plants to react at the moment of an attack with different responses against different threats, thereby avoiding waste of resources on constitutive defenses or responses that might not work against the pathogen in question. However, the complex

regulatory network involved in this immune system takes time, meaning attackers can inflict serious damage upon the plant even before the defense response starts. In order to compensate for this vulnerability, many plants have evolved a mechanism called priming (Aranega-Bou et al., 2014).

A primed plant is in a physiological state where it can respond faster and more robustly towards biotic stresses (Aranega-Bou et al., 2014). The improved response comes from an increased alertness towards defense activating signals such as MAMPs, and improved amplification of the signals downstream of such a recognition, as well as accumulation of defense proteins. In this way, a primed plant is more resistant against certain pathogens/pests, while it can continue to invest its resources in processes important for growth and reproduction when a defense response is not needed (Aranega-Bou et al., 2014; Ton et al., 2009).

A well-documented priming mechanism is systemic acquired resistance (SAR), where signaling molecules are produced after a pathogen attack, leading to systemic expression of defense genes throughout the whole plant, often leading to accumulation of antimicrobial PR proteins. This is regulated by SA and is mainly activated by biotrophic pathogens or phloem feeders such as aphids (Taiz et al., 2015a).

Symbiotic soil microorganisms colonizing plant roots can induce resistance in plants, even without being pathogens themselves. This type of priming is called induced systemic resistance and will primarily lead to an increased resistance against necrotrophs, regulated by JA (Balmer et al., 2013; Taiz et al., 2015a; Ton et al., 2009).

It is also possible to exogenously apply elicitor molecules in order to prime a plant. These molecules can be e.g. MAMPs, DAMPs, or synthetic compounds called plant defense stimulators (Gong et al., 2019).

There is also evidence of transgenerational priming, where the primed state is inherited by the plant's offspring. This suggests epigenetic regulation of priming, which can explain the lack of transcriptional changes found in primed plants until an actual attack occurs (Denancé et al., 2013; Luna et al., 2012).

Because priming prepares the plant against one type of attack, it can become more vulnerable against a different type of attack. Research on *A. thaliana* found that while more resistant against the biotrophic pathogen the plant was primed for, it showed more susceptibility against a necrotrophic pathogen. This phenomenon was suggested to come from a shift in gene responsiveness towards SA, at the expense of JA. This shift was not related to an actual change in hormone levels, but to epigenetical changes (Luna et al., 2012).

Priming against abiotic stress is also a well-known mechanism, where a primed plant responds faster and stronger towards stresses such as drought and flooding, thereby increasing its tolerance against these stresses (Xiao Wang et al., 2017).

## 1.6 Chitin

Chitin is a biopolymer that can be found in the insect exoskeleton and gut, crustacean shells, and in the cell walls of fungi, to name a few examples. It is in fact the second most abundant polysaccharide on the planet, after cellulose (Sun et al., 2018).

As already mentioned, the presence of chitin in frass is interesting due to its potential as a plant immune stimulant. Due to the complex nature of frass, it might contain compounds other than chitin, as well as different microorganisms, that can trigger immune responses in plants, but as chitin is the most well-documented in this regard, it will be the focus in this thesis.

### 1.6.1 Chemical composition and structure of chitin

Like cellulose, chitin is a long-chained linear polysaccharide with no charges and $\beta(1-4)$-linkages. It is robust and stable, suitable for its purpose as a mechanical barrier and structural stability (Sharp, 2013). However, unlike cellulose, chitin consists of repeating monomers of N-acetyl glucosamine (Figure 1.5) (Ramírez et al., 2010).

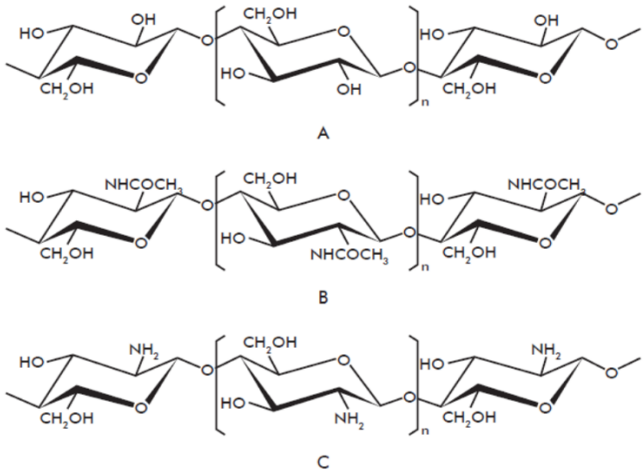



**Figure 1.4:** Structure of A) cellulose, B) chitin and C) chitosan (fully deacetylated). Protonation of the amino groups of chitosan leads to positive charges, which is how it usually appears in nature. From (Ramírez et al., 2010)

Chitosan, a derivative of chitin, is also of interest in agriculture. It is created when chitin is deacetylated, yielding positively charged amino groups on each monomer. Chitosan is one of few cationic polymers in nature, leading to its many functional properties (El Hadrami et al., 2010; Sharp, 2013).

Chitin polymers can form cross linkages with other chitin molecules, or with other compounds such as proteins, glucans and minerals, yielding even more robust and specialized structures (Tsurkan et al., 2021). For example, the insect exoskeleton consists of chitin nanofibers in complex with proteins and calcium minerals in various ratios, resulting in a highly specialized exoskeleton that is both light and robust (Vincent & Wegst, 2004).

### 1.6.2 Use of chitin and chitosan in agriculture

According to Sharp (2013), "positive responses to chitin and its derivatives have been reported in numerous economically important crop species …, including monocotyledons, eudicotyledons, magnoliids and gymnosperms" (Sharp, 2013). Indeed, chitin has been used in agriculture for decades, both as fertilizer, soil conditioner, disease control agent, plant growth regulator, and plant defense enhancer (Shamshina et al., 2020; Sharp, 2013).

However, there's a very limited amount of literature that focuses on the use of insect chitin in agriculture, as most research uses chitin sourced from crustaceans. Chitin is an abundant waste product from the seafood industry, and chitin-based products have also been on the commercial market for decades in many different areas of applications, primarily in medicine and human diet supplementation (Khoushab & Yamabhai, 2010). It must be noted that the exoskeleton of insects and crustaceans have significant structural differences (Bentov et al., 2016). However, after appropriate demineralization and deproteinization, and further degradation of chitin fibers to single strand oligomers, there should be no difference between the two. We can therefore assume that research on crustacean chitin also will apply for insect chitin, as long as it exists as oligomers and is not bound to other compounds.

### 1.6.3 Chitin as a plant elicitor

Plants do not synthesize chitin, while many of its natural enemies do. It is therefore not a surprise that chitin have been shown to work as elicitors recognized by plants (El Hadrami et al., 2010; Sharp, 2013). However, many symbiotic microorganisms also contain chitin, suggesting complex signaling mechanisms that enables the plant to distinguish between beneficial and pathogenic microorganisms. As an example, the plant symbionts *Rhizobium* bacteria and mycorrhiza fungi use chitin-containing compounds as signals for symbiosis initiation (Sharp, 2013).

Research has shown that chitin oligomers with a degree of polymerization (DP) of 6-8 triggers immune responses in plants most effectively (Li et al., 2020; B. Zhang et al., 2002), but larger chitin fragments and nanofibers can also stimulate this response (Egusa et al., 2015).

In addition to immediate defense responses, chitin recognition can also prime plants against future attacks, as shown with *Botrytis cinerea* (De Tender et al., 2021) and *Colletotrichum fructicola* infections in strawberry (*Fragaria ananassa*), and *Alternaria brassicicola* infection in cabbage (*Brassica oleracea*) (Parada et al., 2018).

### 1.6.4 Recognition of chitin and signaling pathway

Plants and many soil microbes can secrete chitinases that hydrolyze fungal cell walls, or other chitin containing structures. This will lead to the release of chitin oligomers that can be detected by plants through membrane-bound receptors (Gong et al., 2020). These receptors are LysM-RLKs (Lysin motif-containing receptor like kinases) or LysM-RLP (Lysin motif-containing receptor like proteins), that recognize chitin oligomers via one or more LysM motifs (a lysin

rich part of the protein) (Gong et al., 2020; Taiz et al., 2015a). The signaling responses after recognition of chitin by *Arabidopsis* are summarized in Figure 1.6.

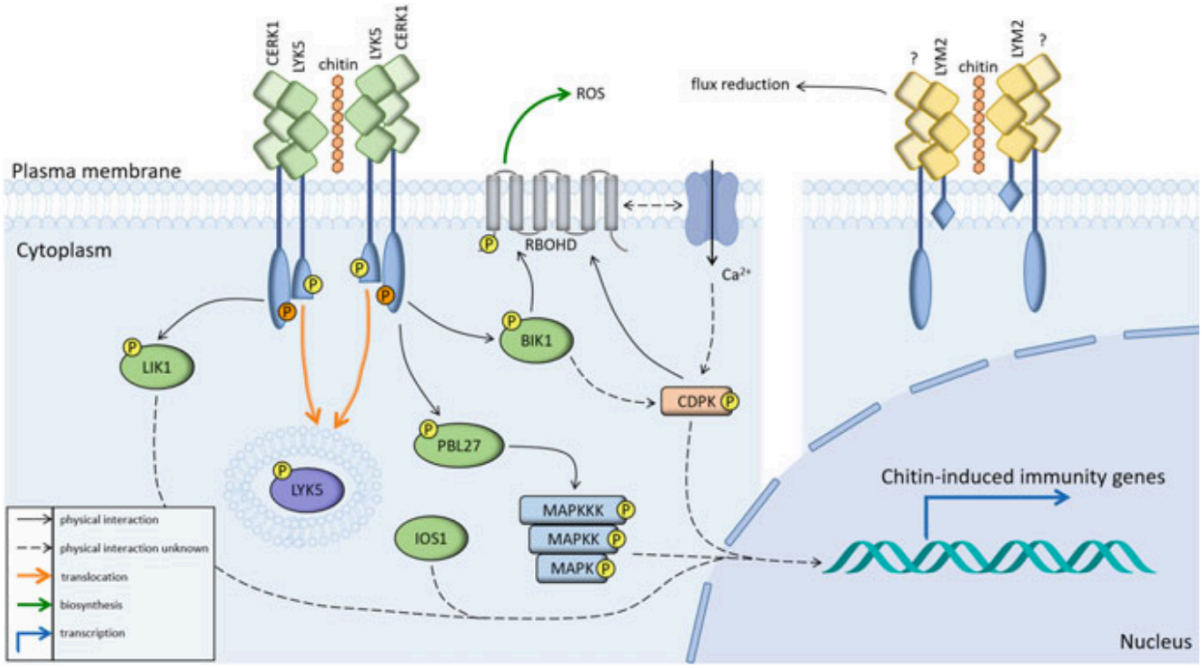



**Figure 1.6: Signaling response after recognition of chitin oligomer by CERK1 and LYK5 in *Arabidopsis*.** Chitin oligomer recognition leads to phosphorylation of kinase domains of CERK1 (chitin elicitor receptor kinase 1) and LYK5 (LysM-containing receptor-like kinase 5), and the latter is internalized by endocytosis. CERK1 activates BIK1 (*Botrytis*-induced kinase 1) leading to ROS bursts by the membrane bound protein RBOHD (Respiratory burst oxidase homolog protein D) and increased $Ca^{2+}$ influx. CERK1 also activates the receptor-like cytoplasmic kinase PBL27 which in turn triggers the MAPK (mitogen activated protein kinase) cascade, resulting in defense responses. LIK1 (LysM RLK1-interacting kinase 1) and IOS1 (impaired oomycete susceptibility 1) regulates chitin signaling and gene expression, while CDPK ($Ca^{2+}$-dependent protein kinases) affects ROS production and gene expression upon elevated $Ca^{2+}$ levels in the cytosol. LYM2 leads to plasmodesmatal closure and cell isolation upon chitin recognition (reduction of molecular flux between cells). From (Jiang et al., 2019).

In *Arabidopsis,* at least four LysM-RLK/RLPs seems to be involved in chitin perception. The one receiving most attention is CERK1 (chitin elicitor receptor kinase 1, also called LYK1 for LysM-containing receptor-like kinase 1) (Miya et al., 2007). Together with the receptor LYK5, CERK1 forms a receptor complex that binds to chitin oligomers. This leads to phosphorylation of the kinase domains of CERK1 and LYK5 (Gong et al., 2020). The latter is then internalized by endocytosis, allowing regulation of chitin signaling sensitivity, amplitude and duration (Erwig et al., 2017). CERK1 phosphorylates the receptor-like cytoplasmic kinases PBL27 and BIK1. Activation of BIK1 leads to increased $Ca^{2+}$ influx, as well as ROS bursts through phosphorylation of RBOHD (respiratory burst oxidase homolog protein D), which is also stimulated by increased $Ca^{2+}$ levels (Gong et al., 2020). ROS is toxic to pathogens and can stall

the attack, as well as work as a signaling molecule, leading to increased activation of defense responses (Taiz et al., 2015a), although this is not shown in Figure 1.6.

PBL27 activates a MAPK (mitogen activated protein kinase) cascade, leading to induction of defense related genes (Gong et al., 2020), which results in various defense responses such as production of the defense compounds phytoalexins, phenolics and terpenes. In addition, chitin perception can induce formation of physical barriers, such as callose and lignin. This will help in wound healing and compartmentalization of the pathogen, reducing further spread of the infection (Jiang et al., 2019; Sharp, 2013). Several PR proteins, including chitinases, have been found to be activated following recognition of chitin, and can help reduce the infection (Sharp, 2013).

As stressed by Gong et al., CERK1 is the active kinase in the receptor complex, responsible for intracellular signaling. LYK5 seems to mainly contribute to chitin binding, as it has a higher affinity for chitin oligomers than CERK1. Another LysM-RLK, LYK4, is also shown to bind chitin oligomers, and may be a part of the chitin receptor complex, contributing to chitin affinity (Gong et al., 2020).

The LysM-RLK chitin receptor LYM2 has also been found in *Arabidopsis*, and it has high affinity towards chitin oligomers. It is not associated with CERK1 and mediates chitin-triggered plasmodesmatal closure, which leads to isolation of the infected cell (Cheval et al., 2020).

Numerous studies have shown that activation of the phytohormone jasmonate (JA) often follow detection of chitin, and that exogenous application of methyl-jasmonate will activate the same systemic defense responses as chitin (Sharp, 2013). This suggests that chitin induces an immune response against necrotrophic pathogens and herbivores, i.e. the JA response pathway.

Interestingly, it has been shown that *Arabidopsis* CERK1 can be primed by bacterial pathogens as well as the bacterial elicitors flg22 (flagellin) and elf18 (elongation factor Tu). This happens through phosphorylation of CERK1 (not in the kinase domain of the protein which results in a defense response), that in turn leads to improved chitin signaling and increased resistance against fungal pathogens, such as *Botrytis cinerea* (Gong et al., 2019).

### 1.6.5 Other benefits of chitin in agriculture

It is well documented that chitin can increase plant health and resistance towards pests and pathogens (Shamshina et al., 2020). This includes the abovementioned stimuli of plant defenses, but there are several other modes of action.

When present in soil, chitin will recruit chitinolytic microorganisms, that feed on chitin (Sharp, 2013). The increased growth of these microbes (that are often PGPMs) will in turn retard the growth of chitin containing pathogens, as well as aid in suppression of other bacterial pathogens due to competition. This mechanism is often found to be the course of action when chitin is used as a biological control agent today (Sharp, 2013). An example is the use of the bacterium *Bacillus subtilis*, which is used as a biological agent against fungal infections. It secretes chitinases, which can break down the fungal cell walls. The addition of chitin together with the bacterium enhanced the bacterial growth and improved its fungicidal action (Manjula

& Podile, 2001). This effect is demonstrated with a wide range of other chitinolytic microorganisms as well, in the control of many different plant diseases, as well as animal pests like nematodes, insects and mites. In fact, it is now common for commercial biopesticides to supplement or encapsulate the active microbe strain with chitin to enhance its abilities (Sharp, 2013).

The stimulation of PGPMs by chitin can also lead to inhibition of biofilm formation by pathogens. This can be an important action in the control of certain pathogenic microbes, particularly in disruption of quorum sensing, a strategy many bacteria use to detect population density, in order to have a coordinated release of e.g. toxins (Bhattacharyya & Jha, 2012; Sharp, 2013).

It is well known that *Rhizobium* bacteria colonizing roots of legumes can provide the plant with nitrogen fixated from atmospheric $N_2$ (Sessitsch et al., 2002). These bacteria live mutualistically in nodules in the plant roots. The mechanism for the initiation of this symbiosis is well studied. Flavonoids from the plant stimulate the production of so-called 'Nod' factors from the bacterium, which is recognized by the plant, and nodule formation is initiated. These Nod factors are in fact chitin-based as they are lipochitooligosaccharides (LCOs) (Taiz et al., 2015a). It has been studied if the addition of chitin oligomers can enhance this beneficial symbiosis in legumes (Sharp, 2013). Indeed, chitin with structural similarities to LCOs can induce nodulation in alfalfa (*Medicago sativa*) (Staehelin et al., 2000). This can be a potential positive application in the cultivation of legumes but requires more research.

Related to this, arbuscular mycorrhizal fungi that live in symbiosis with 90% of all plant species, also secrete LCOs to signal for symbiosis, the so-called 'Myc' factors (Parniske, 2008). Addition of chitin or chitosan to soil is well known to enhance root colonization and benefits of mycorrhiza fungi, in turn leading to increased plant growth and health. This could indicate that chitin enhances symbiosis signaling by LCOs in the arbuscular mycorrhiza-plant relationship (Gryndler et al., 2003; Sharp, 2013).

The chitin receptors used in plant defense and the receptors used during *Rhizobium* and mycorrhiza symbiosis signaling are structurally very similar and are thought to be closely related to each other (Taiz et al., 2015a). In fact, the CERK1 ortholog in rice (OsCERK1) is known to be required for arbuscular mycorrhizal symbiosis, as well as activation of defense responses to chitin (Gong et al., 2020). However, it is important that plants are able to distinguish between these very different signals. Studies have shown that upon recognition of LCOs, immune responses are suppressed, while a combination of LCOs and chitin oligomers enhance the symbiosis signaling (Li et al., 2020).

As mentioned, chitin oligomers with DP 6-8 are most recognizable by plant chitin receptors. Interestingly, it has been found that short-chain chitin oligomers (DP 4) stimulated genes related to plant development, biogenesis and amino acid metabolism (Winkler et al., 2017). This indicates that chitin also might play a role in the regulation and stimulation of plant growth and development.

## 1.7 Activation of plant defense responses by insect frass

There is a growing, albeit limited, amount of literature suggesting that insect frass applied to soil can activate plant defense responses, and potentially enhance plant resistance against pests and pathogens through priming. In a 2021 review on frass fertilizers, Poveda states that "the recognition by the roots of microorganisms and biomolecules present in insect frass may be involved in the activation of plant systemic resistance through the SA and/or JA pathways" (Poveda, 2021). The strongest argument for this defense stimuli is the presence of chitin in frass, originating from molted larval skins and excreted peritrophic membrane residue. Other compounds or microorganisms existing in frass can also be contributing factors to plant defense activation (Poveda, 2021; Schmitt & de Vries, 2020). All available literature on the topic is described below, largely based on the review article by Poveda (2021).

The fall armyworm (*Sporoptera frugiperda*) is a notorious insect pest causing devastating losses to maize crops worldwide (Goergen et al., 2016). Ray et al. have shown that its frass, when applied to wounds in maize leaves actually increases herbivore performance of fall armyworm, while it decreases the effect of a necrotrophic fungi (Ray et al., 2020; Ray, Alves, et al., 2016). This is likely due to the suppression of the JA pathway after frass application, while SA levels and pathogen defense gene transcripts increased (Ray et al., 2015). Subsequently, it was found that two chitinases (Pr4 and Endochitinase A) in fall armyworm frass, originating from the maize plant which the insect was feeding on, was responsible for the suppressed defense against the herbivore, while increasing pathogen defenses in the plant (Ray, Alves, et al., 2016).

Ray et al. also found that frass from different caterpillars can induce plant defenses that are specific to each host-herbivore system (Ray, Basu, et al., 2016). Frass protein extract from different insect species applied to wounds in maize, rice, cabbage, or tomato, resulted in very distinctive defense responses. Some responses suppressed herbivore defenses while inducing pathogen defenses in the plant, while other responses did the exact opposite. This shows the complexity of frass' role in plant-insect interactions, and more research should be conducted in the field of frass-induced defenses (Ray, Basu, et al., 2016).

Some studies have also shown that frass can contain volatile organic compounds (VOCs) capable of deterring certain pests and reducing oviposition rate of moths when deposited on plants (Ahmed et al., 2013; X. G. Zhang et al., 2019). VOCs in frass have also been shown to attract ectoparasitic insects such as wasps, preying on plant pests, thus helping the plant through tritrophic interactions (Poveda, 2021; Schmitt & de Vries, 2020).

While the above-mentioned studies mainly focus on frass deposited in wounds or injected into leaves, it might not be comparable to the use of frass as a fertilizer. There is only limited literature looking at frass' effect on plant defense when mixed in soil, and some of the results seem to have emerged more by chance rather than by intention.

During a field experiment testing BSF frass on growth of cowpea (*Vigna unguiculata*), all crops where accidentally infected with Fusarium wilt (*Fusarium oxysporum*). Plants receiving frass had significantly fewer dead plants, indicating an effect by frass. This effect was not seen when inorganic NPK was added in addition to frass. The authors hypothesize that the

fragments of chitin activated defense responses leading to an increased resistance (Quilliam et al., 2020).

A study testing disease suppression in sugar beet (*Beta vulgaris*) and cress (*Lepidium sativum*) by BSF frass, showed no significant effect of any of the BSF treatments against the pathogenic fungi *Rhizoctonia solani* or *Pythium ultimum.* However, in the cress control group (no pathogen applied) where a most likely naturally occurring infection with *Pythium* was observed, frass treatment resulted in a significantly lower infection rate and an increased growth of infected plants (Elissen et al., 2019).

17-year-old Sarah Choi and Neelah Hassanzadeh won the silver medal in a Canadian science fair in 2019 with their research on BSF frass as a fertilizer and plant health improver. Their experiments showed a significantly higher growth of *Pythium*-infected green bush beans (*Phaseolus vulgaris*) when fertilized with BSF frass compared to the control. The addition of humic acid and *Trichoderma*, a plant symbiotic soil fungus, increased disease resistance even further. They also demonstrated that plant pathogens *Fusarium oxysporum* and *Rhizoctonia solani* were not able to grow in 100% BSF frass (Choi & Hassanzadeh, 2019).

In a report from Canadian insect producers Enterra, BSF frass showed positive results on plant health when used for bok choi, lettuce and potatoes. Close to 100% of lettuces not receiving frass died to the herbivory of wireworms (larval stage of the click beetle, order: Coleoptera) in the soil, while frass-fertilized plants stayed healthy. Similar results were found with bok choi. Interestingly, the number of wireworms per plant was not significantly different between treatments, suggesting that frass did not deter the pests, but increased the plant's resistance against them. The improved resistance might also be attributed to an increased growth of frass fertilized plants, thereby making them more tolerant to biotic stresses, suggests to the authors (Temple et al., 2013).

Needless to say, there is a need for more legitimate research in order to conclude if frass used as a fertilizer will indeed stimulate plant defenses and increase resistance against pests and pathogens.

# 2 Aims of the study

Insect frass has the potential to become a significant organic fertilizer in agriculture when produced at levels expected for future insect production. It is therefore important to assess the fertilizing qualities of this product compared to the organic and inorganic fertilizers used today, so that farmers need not be concerned with its effect on crop plants. Hence, fertilization experiments on important crop plants or model plant species should be conducted with frass from the insect species most commonly used in industrial production.

A biostimulating effect of frass leading to increased plant resistance against pests and pathogens can be of great importance in agriculture. This effect should be thoroughly investigated as it might help reduce the use of harmful pesticides, contributing to a more sustainable agriculture.

The aims of the study can be summarized as:

1. Assessing the fertilizing capabilities of mealworm frass through growth experiments and evaluation of nutrient content.
2. Study potential immune responses in plants triggered by mealworm frass or by pure insect skin residue.
3. Evaluate any priming effect by frass using pathogen assays and gene expression analyses.

# 3 Materials and Methods

## 3.1 Mealworm frass and skin residue

Mealworm frass and skin residue were provided by the mealworm producers Invertapro. The larvae of the yellow mealworm (*Tenebrio molitor*) were reared on wheat bran and food waste slurry in 70% air humidity and 25-27 °C in an indoor production facility. 8 weeks after hatching, accumulated frass was collected mechanically by sieving, as well as large particle larval skin residue separated from the frass.

Normally, the frass is then heat-treated at 70 °C for 1 hour according to local legislation (Lovdata.no, 2021). However, untreated frass was used in most experiments in order to maintain the microbiota. Heat-treated frass and autoclaved frass (at 121 °C for 20 minutes) were also used in the fertilization experiment with tomato.

Invertapro inform that their frass fertilizer has an NPK of 2.6-1.8-2.8. The full nutrient profile is provided in Appendix 1.1.

## 3.2 Plant material

The *Arabidopsis thaliana* accession Columbia-0 (Col-0) seeds had been purchased from Lehle seeds (CatNb WT-2-8) and propagated in the lab before the start of this project. *A. thaliana* seeds were stratified for 3 days at 4 °C in a 0.1% agarose (Sigma-Aldrich) solution before being sown when used in soil.

Tomato (*Solanum lycopersicum*) seeds of the varieties Moneymaker and Roma VF were purchased from Plantasjen.

Sunflower (*Helianthus annuus*) seeds were purchased from Plantasjen.

## 3.3 Pathogens

Before the start of this project the *Botrytis cinerea* isolate CECT2100 had been obtained from Imre E. Somssich (Max Planck institute for Plant Breeding Research, Cologne, Germany) and spores had been produced as described by Birkenbihl et al (2012). Spores were stored at -80 °C.

The *Pseudomonas syringae* DC3000 bacterial culture was obtained from Javad Najafi at Institute for Biologi at NTNU, and was stored at -80 °C.

## 3.4 Fertilization experiments

### 3.4.1 Fertilizing effect of different frass forms and inclusion rates with tomato

**Sowing, repotting, and harvesting of tomato plants**

Seeds of tomato (*Solanum lycopersicum* var. Moneymaker) were sown into 15 pots containing P-soil (peat-based soil, Tjerbo) and germinated under a plastic sheet in a greenhouse at Ringve Botanical Garden (Trondheim, Norway). After 34 days, 144 tomato plants were transplanted into individual 2-liter pots in P-soil containing perlite in a 1:4 ratio, and a fertilizer treatment (Table 3.1). In this way each treatment was applied to 12 plants. The pots were placed under artificial growth lights in a greenhouse from January to April (12-hour photoperiod, 145 µmol $s^{-1}m^{-2}$, 40-65% humidity, 20 °C), and randomized to avoid trends caused by placement. Plants were watered regularly and bound up with rope to a support stick when necessary.

After 1.5 months from fertilization treatment, 6 plants from each treatment were harvested. The shoot was cut off aboveground, the number of flowers were counted, the shoot length was measured, and the fresh shoot biomass was immediately weighed. Roots were extracted from soil and thoroughly rinsed with water. Some soil fibers could not be removed, and some thin lateral roots were occasionally lost. It is assumed that this error is evenly distributed among the groups. Roots were air dried for 1 hour to evaporate water after rinsing, and root fresh biomass was weighed. Shoot and root biomass was subsequently dried at 70 °C for 3 days to determine the dry weight.

After almost 2 months from fertilization, the remaining 6 plants per treatment were transplanted into 5-liter pots containing P-soil supplemented with the same fertilization treatment as at the beginning of the trial. To do this, the plants were extracted from the 2-liter pots without removal of any soil and placed in the 5-liter pots containing approximately 3 liters of new soil.

After 2.5 months from the first fertilization, these plants were harvested in the same way as done during the first harvest. Only 4 out of 6 roots were harvest due to time restrictions (as a result of limited access to the greenhouse when the corona pandemic arrived to Trondheim). In addition, the number of tomato fruits, tomato fresh weight, and tomato dry weight was measured. To determine the fruit dry weight, the tomatoes fruits were dried at 70 °C for 3 days.

**Fertilizer treatments**

The different fertilizer treatments are shown in table 3.1. As a positive control, the organic fertilizer NaturGjødsel (NG) (Hageland) was used. It consists of pelletized chicken manure and has a NPK value of 6-4.5-5, which is quite close to the NPK ratio of frass, however more than twice as high values. NG pellets were crushed to the size of frass particles, and half the amount by volume compared to frass was used as positive control to balance out differences in NPK.

There were two negative control treatments: CTRL (only P-soil), and CTRL+ (P-soil + 0.15% NG by volume). This was done to increase the NPK-content of the base soil to a level more comparable to what is used by professional tomato farmers and home growers in Norway (based on personal communication). CTRL+ was used as a base soil for the treatments with the organic fertilizer (0.25% and 1%) and frass (0.1%, 0.5% and 2%). However, for the 10% frass

treatment not CTRL+ but P-soil without NG fertilizer (CTRL) was used as base soil due to the high amounts of nutrients added with 10% frass by volume.

Frass was applied under three different forms to test whether the processing of frass affects its performance: Untreated frass (UF), frass heat-treated at 70 °C for 1 hour (HF) and sterilized (autoclaved) frass (SF).

**Table 3.1: Fertilizer treatments for growth trial with tomato and mealworm frass**. CTRL+ was used as a base soil for all treatments except HF 10%, where CTRL was used. NaturGjødsel (NG) = Commercial organic fertilizer based on chicken manure used as positive control. It was used as ½ the amount of frass by volume, to balance out differences in NPK. CTRL+ received NG fertilizer at 0.15%.

| | CTRL | CTRL+ | NaturGjødsel (NG) | | Untreated frass (UF) | | Sterilized frass (SF) | | Heat-treated frass (HF) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Amount of fertilizer (V%)** | 0% | 0,15% | 0,25% | 1% | 0,5% | 2% | 0,5% | 2% | 0,1% | 0,5% | 2% | 10% |
| **Replicates** | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 |

## 3.4.2 Nutrient deficiency assay with sunflower

This trial was conducted with the plant physiology course at NTNU (BI1007), and Professor Richard Strimbeck was the chief designer of the experimental setup. Students helped out with planting, watering, and harvesting, and used the data in their own lab reports on plant mineral nutrients.

Sand was acid-washed to remove any nutrients and combined with perlite in a 2:3 sand/perlite mixture. Another mixture was made where frass was included at 5% by volume into the 2:3 sand/perlite mixture. Forty-eight 2-week-old sunflower seedlings (*Helianthus annuus*) were transplanted into each of the two mixtures in 0.2 liter pots. The plants were grown under artificial lights in a growth room (18-hour photoperiod, 200 µmol $m^{-2}$ $s^{-1}$, 21 °C), with additional natural light coming from large windows on one side. They were randomized in order to avoid growth variations due to uneven light availability.

The experiment used 6 different nutrient solutions and 8 replicate plants for each treatment. Nutrient solutions were prepared using modified Hoagland solutions (Hoagland, 1920), see Appendix 1.2 for details. One of the solutions contained the complete Hoagland solution, the other five lacked either N, P, K, S or Ca. The plants were watered with approximately 15 ml nutrient solution once a day for 5 weeks, then with approximately 40 ml once a day for 3 weeks. Table 3.2 presents total nutrient content (N, P, K, Ca, and S) provided over the 8-week period by the complete Hoagland solution, assuming no leakage of water out of the pots, and mealworm frass at 5% by volume, assuming all nutrients are available to plants (although the latter assumption probably isn't correct, particularly for nitrogen).

**Table 3.2:** Nutrients provided to sunflowers by Hoagland solution and frass. Total amount of N, P, K, Ca, and S provided by the complete Hoagland solution and mealworm frass to sunflowers over the 8-week period. For more details on the Hoagland solution and the nutrient content of mealworm frass, see Appendix 1.1 and 1.2.

| | Nitrogen | Phosphorus | Potassium | Calcium | Sulfur |
|---|---|---|---|---|---|
| **Hoagland solution** | 230 mg | 20 mg | 290 mg | 140 mg | 90 mg |
| **Mealworm frass** | 95 mg | 65 mg | 100 mg | 25 mg | 15 mg |

At the end of the growth period (i.e. day 244), deficiency symptoms were registered for each plant, before the plants were harvested. Shoot height, shoot dry weight, root dry weight, leaf lengths, and number of leaves were measured for each plant, and root/shoot biomass ratio was calculated. For dry weight measurements, plant material was dried at 70 °C for 3 days.

## 3.5 Plant defense activation by frass

Several different materials and methods were utilized in the investigation of defense responses in plants activated by frass. Figure 3.1 shows a flow chart for a better overview. The fertilization trials with tomato and sunflower are not included here.

In most experiments, frass and insect skin residue (abbreviated ISR) were used as treatments, applied to plants in various forms.

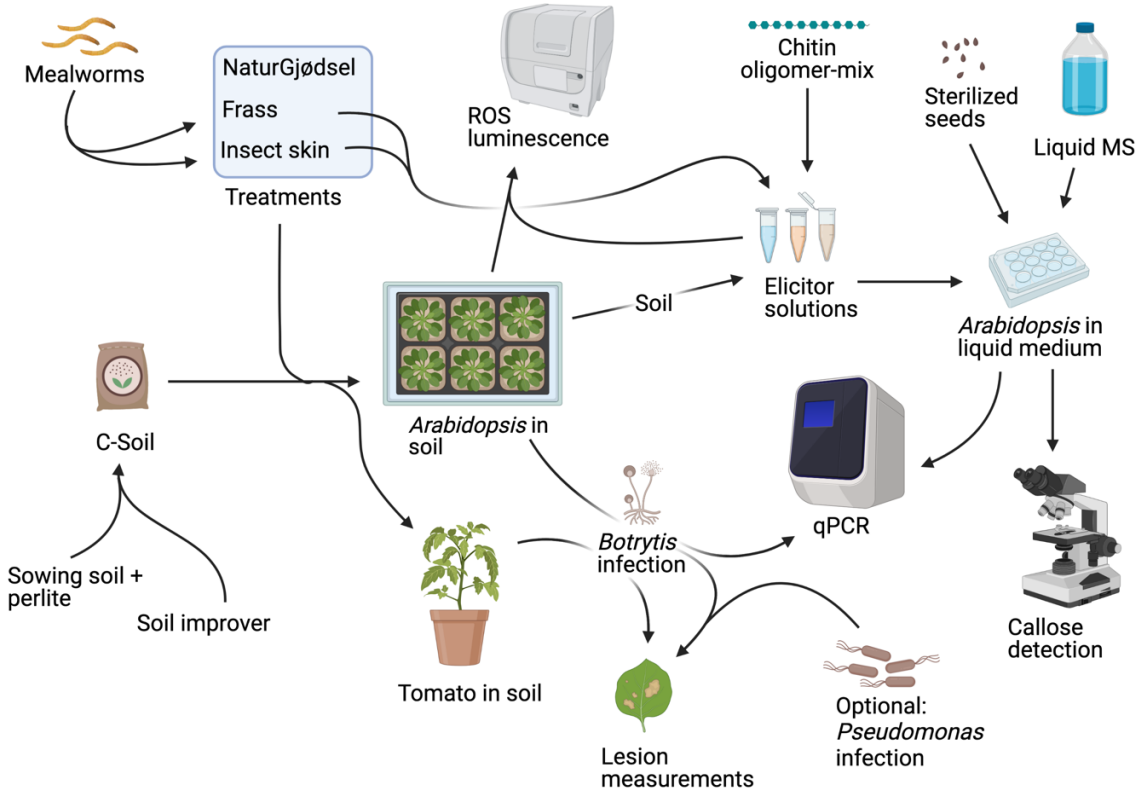



**Figure 3.1:** Flow chart giving a simplified overview of materials and experiments used in investigation of plant defense responses to frass and ISR (insect skin residue). Fertilization experiments are not included. Created with BioRender.com

### 3.5.1 Soil used in experiments

Sowing soil (Hasselfors S-jord, Norgro, CatNb 191-484120) was first mixed with perlite (Agra-Perlite, Norgro, CatNb 199-440115) at a 4:1 ratio by volume and further supplemented with soil improver consisting of 60% compost and 40% sand (NorgesJord Jordforbedring 60, LOG, CatNb 6741026) at 4:1 ratio by volume. This mixture was used for all lab experiments and is referred to as 'C-soil' (Compost-soil) throughout this thesis.

### 3.5.2 Preparation of elicitor solutions

Different elicitor solutions containing pure chitin, frass, or insect skin residue (ISR), were used in several experiments. These were prepared as described below.

**Elicitor solutions from frass or ISR in soil**
To prepare elicitor solutions, *Arabidopsis thaliana* Col-0 seeds were sown into C-soil to which either frass (2% or 5% by volume), ISR (0.5 g/l) + NG, or just NG (control treatment) had been added. NG was applied at equivalent NPK to that of frass. Plants were grown under a 12-hour photoperiod, 75 to 90 µmol $m^{-2}s^{-1}$), relative humidity of 40% and temperature of 22°C in a Vötsch VB 1514 growth cabinet. Soil (approx. 15 g) was sampled from the pots at different time points, mixed with $ddH_2O$ or liquid growth medium (10 ml), and filtered through 0.45 µm and 0.2 µm pore size filters using a needle-less syringe in order to produce a clear elicitor solution.

**Solution of insect skin residue (ISR)**
Insect skin residue (20 ml) and frass (5 ml) was mixed with 45 ml $ddH_2O$ and left for 24 hours at room temperature. 10 ml of this solution was filtered through syringe filters, first 0.45 µm then 0.2 µm pore size, in order to remove particles and obtain a clear elicitor solution.

**Pure chitin solution**
A shrimp-based chitin oligomer-mix was kindly provided by NOBIPOL at NTNU, originally acquired from Seikagaku. Size exclusion chromatography (SEC) was conducted by Olav Aarstad from NOBIPOL on the chitin sample to examine its oligomer ratios. In short, the samples were dissolved in 4 ml 0.15 ammonium acetate buffer (pH 4.5) and passed through a syringe filter (0.45 µm pore size). The sample were analyzed on a SEC system consisting of a manual Rheodyne 7125 injector, a Shimadzu LC10 pump, a Shodex RI-101 refractive index detector, and three serially connected (2.6 x 60 cm) Superdex30 columns (GE Healthcare). Flow was 0.8 ml/min and 0.15 M ammonium acetate buffer as mobile phase.

NMR spectroscopy was also conducted by Olav Aarstad from NOBIPOL on the chitin sample by NOBIPOL, in order to confirm its purity (Appendix 1.7).

To be used as elicitor solution, the chitin oligomer-mix was dissolved in $ddH_2O$ at 25 mg/ml to prepare a stock solution that was stored at -20 °C. Based on SEC results, 25 mg/ml corresponds to 2 mM oligomers of DP 6-8, which are the eliciting oligomers (calculations are

shown in Appendix 2.1). From this stock solution chitin elicitor working solutions were prepared in $ddH_2O$.

### 3.5.3 Measurement of reactive oxygen species

The production of reactive oxygen species (ROS) was measured through a chemiluminescence assay based on protocols described by (M. Albert et al., 2015) and (Bisceglia et al., 2015), as follows.

**Plant growth conditions**

*Arabidopsis thaliana* Col-0 seeds were sown into C-soil in 80 ml pots (one seed per pot), watered thoroughly and put on a covered tray (Minidrivhus 52x42cm, Willab Garden, Sweden). Plants were grown in a Vötsch VB 1514 growth cabinet under a light intensity of 75 to 90 µmol $m^{-2}s^{-1}$, 16-hour photoperiod, temperature of 22°C, and a relative humidity of 40%.

After 3 weeks, leaf disks were cut from the youngest fully developed leaves using a cork borer. Leaf disks were floated on $ddH_2O$ water overnight in room temperature and in the dark, in order to remove any ROS generation resulting from wounding. Water was replaced twice before leaf disks were used in the ROS detection assay.

**ROS detection by chemiluminescence assay**

For ROS detection, horse radish peroxidase (Sigma-Aldrich, CatNb P8125) and the luminol analog L-012 (Sigma-Aldrich, CatNb SML2236) were added as a 200 µl mix per well to a 96-well plate (ThermoFisher Scientific, CatNb 236105) to reach a final concentration of 10 µg $ml^{-1}$ and 50 µM, respectively. One leaf disk was carefully placed in each well.

In the first experiment (presented in Figure 4.5), elicitor solutions were prepared as follows: chitin (1250 µg/ml) was dissolved in $ddH_2O$, filtered frass solution (0.45 µm or 0.2 µm pore size filter), or filtered soil solution (see 3.5.2) containing frass (0.45 µm pore size filter), as well as each solution without chitin. Elicitor solutions were added at 50 µl per well, yielding a final concentration of 250 µg/ml chitin, and 5x dilution of soil and frass solutions. For each treatment 4 replicate wells (i.e. 4 leaf disks) were prepared.

In the second experiment (presented in Figure 4.6), either the chitin oligomer-mix (625 µg/ml and 62.5 µg/ml) or ISR elicitor solution (see 3.5.2) were added at 50 µl per well, yielding a final concentration of 125 µg/ml and 12.5 µg/ml chitin, and 5x dilution of ISR solution. 50 µl $ddH_2O$ was added to the mock treatment. For each treatment 12 replicate wells (i.e. 12 leaf disks) were prepared.

Luminescence was measured ever 2 mins for 1 hour and 12 minutes (first experiment) or 3 hours (second experiment) using a Bio-Tek Cytation 5 Cell Imaging Multi-Mode Reader with a signal integration time of 2 seconds and a gain of 200.

## 3.5.4 Callose staining

Callose was stained with aniline blue using a combination of the methods described by (Schenk & Schikora, 2015) and (Millet et al., 2010), as described below.

**Plant growth conditions**
*Arabidopsis thaliana* Col-0 seeds were sterilized with chlorine gas (3 ml HCl (37%) for 100 ml of commercial bleach (Klorin, Orkla)) for 3 hours in a sealed 29x24x9 cm box (Clough & Bent, 2008). Liquid growth medium was prepared according to Millet et al. (2010): 4.3 g/L Murashige and Skoog basal salt mixture (Sigma-Aldrich, CatNb M5524), 5 g/l sucrose and 0.5 g/l 2-morpholinoethanesulfonic acid monohydrate (Duchefa, M1503), adjusted to pH 5.7 and autoclaved. The medium was cooled to room temperature before filter-sterile vitamins (Sigma-Aldrich, CatNb M3900) were added. Seeds were germinated in a 24-well plate (Sigma-Aldrich, CatNb CLS3526) containing 1 ml liquid medium per well, each well containing 2 seeds. Seedlings were grown for 10 days in a growth room at 16-hour photoperiod at a light intensity of 125-140 µmol $m^{-2}s^{-1}$, a relative humidity of 40%, and a temperature of 22°C. The medium was changed after 8 days.

**Callose staining**
The liquid medium in wells were replaced by 1 ml elicitor solutions obtained either from soil with frass (2% by volume), soil with ISR (0.5 g/l) + NG, or soil + NG (control treatment) after 3 weeks, prepared as described in 3.5.2. In addition, the solution from soil with just NG was used to obtain a 250 µg/ml chitin solution. This yields four treatments all containing soil in solution (frass, ISR, control and chitin 250 µg/ml) that were applied to six replicate wells (i.e. 12 seedlings) each. The plate was placed back into the growth room for approximately 20 hours. Elicitor solutions were then replaced with 1 ml 3:1 ethanol/acetic acid fixation solution and put on shaker at 90 rpm for 24 hours. The fixative was changed 3 times during this time to ensure thorough clearing of tissue. Seedlings were then rehydrated in 70% ethanol for 4 hours, 50% ethanol for 2 hours, and finally in $ddH_2O$ overnight. The water was then replaced with 1 ml 10% NaOH per well for 90 mins at 37 °C. After 2 washes with $ddH_2O$, seedlings were incubated in 1 ml 0.01% aniline blue (Sigma-Aldrich, CatNb 415049) in 150 mM $K_2HPO_4$, covered with aluminum foil, and put on shaker at 90 rpm for 2 hours.

Whole seedlings were mounted on slides in 50% glycerol and leaves and roots were observed with a Nikon Eclipse E800 epi-fluorescence microscope under UV light.

## 3.5.5 Gene expression analysis

Two different experiments were designed to analyze the expression of defense related genes following stimuli by frass and either chitin or ISR.

*3.5.5.1 Gene expression after elicitor treatments (GE-ET)*

**Plant material**

To monitor the gene expression response after treatment with pure chitin oligomers or frass solution from soil, called hereafter GE-ET, *Arabidopsis thaliana* Col-0 seeds were sterilized with chlorine gas for 3 hours and sown on liquid growth medium that was prepared as described in 3.5.4. Seeds were germinated in two 24-well plates (Sigma-Aldrich, CatNb CLS3526) containing 1 ml liquid medium per well, each well containing 3 seeds. Seedlings were grown in a growth room for 10 days at 22°C, a relative humidity of 40%, and in 16-hour photoperiod at a light intensity of 125-140 µmol $m^{-2}s^{-1}$. The medium was changed after 8 days.

**Elicitation and harvesting of plant material**

In GE-ET, soil prepared as described in 3.5.2 for 2 months, with or without 5% frass by volume, was mixed with dd$H_2O$, and filtered through Miracloth (Merck, CatNb 475855). A 62.5 µg/ml chitin solution was made with the control soil solution and chitin oligomer-mix, resulting in 3 different treatments: 62.5 µg/ml chitin, frass, and mock. The liquid medium in wells were replaced by 1 ml elicitor solutions, and the plates were kept at the lab bench. After 30 mins, 1 hour, and 3 hours plant tissue from 4 wells per treatment were washed in dd$H_2O$, quickly dried with a paper tissue and frozen in liquid nitrogen. Plant tissue from each well was considered as a biological replicate. Frozen plant tissue was stored at -80 °C.

*3.5.5.2 Gene expression after Botrytis cinerea infection of plants primed with frass or ISR (GE-BC)*

**Plant material**

To monitor the gene expression response after *Botrytis cinerea* infection of plants primed by frass or ISR in soil, called hereafter GE-BC, *Arabidopsis thaliana* Col-0 seeds were germinated in C-soil (3.5.1) under a 12-hour photoperiod (75 to 90 µmol $m^{-2}s^{-1}$, relative humidity of 40%, temperature of 22°C) in a Vötsch VB 1514 growth cabinet. 3-week-old seedlings were transplanted into individual 80 ml pots containing C-soil supplemented with either frass (2% by volume), ISR (0.5 g/l) + NG, or NG only (control), where the amount of NG matched the amount of NPK provided by frass. Twenty-four plants were used for each soil type, and these were grown under said conditions in a growth chamber for 2 weeks after transplanting.

**Elicitation and harvesting of plant material**

For GE-BC, the *B. cinerea* spore stock solution ($10^7$ spores/ml) was diluted to $10^6$ spores/ml with filter-sterile Vogel solution (Appendix 1.3, Birkenbihl et al., 2012). Two 2 µl droplets of the spore solution were applied on each side of the midrib on the adaxial side of two young but fully developed leaves per plant, on 36 plants (12 per soil treatment). Vogel solution was used as mock treatment and 2 µl droplets were placed similarly on 36 plants. Due to space restrictions plants were put in two separate covered trays with *B. cinerea* treated plants in one and mock treated plants in the other, randomized within each tray. The trays were then sealed with cling film to guarantee high humidity and returned to growth chambers. After 8, 24 and 48 hours, the *B. cinerea* and mock inoculated leaves from 4 plants per soil treatment were harvested and

frozen in liquid nitrogen. The tissue pooled from one plant was considered as a biological replicate. Frozen plant tissue was stored at -80 °C.

### *3.5.5.3 RNA isolation*

Total RNA was isolated with the Spectrum Plant Total RNA Kit (Sigma-Aldrich, CatNb STRN250), following the supplier's instructions.

In short, frozen plant tissue (50-100 mg) was crushed with a TissueLyserII (Qiagen) for 2 minutes at 25 Hz, and 500 µl lysis-buffer containing 10 µl/ml β-mercaptoethanol was added to each tube. Samples were then run in the TissueLyser once more for 2 minutes at 25 Hz, and incubated for 5 min at 56 °C. Samples were centrifuged in a tabletop centrifuge at 13000 RPM for 3 min and the supernatant was transferred to a filtration column. This was centrifuged for 1 min at 13000 RPM, 500 µl binding solution was added to the flow through, and the mixture was transferred to a binding column. This was centrifuged for 1 min at 13000 RPM and the flow through was discarded. Wash solution 1 (300 µl) was added to the column, which was centrifuged for 1 min at 13000 RPM. Potential genomic DNA in the samples were removed by the addition of DNase (RNase-Free DNase Set, Qiagen, CatNb 79254) to the column and an incubation for 15 mins. Wash solution 1 (500 µl) was then added to the column, and the column was centrifuged for 1 min at 13000 RPM. Wash solution 2 (500 µl) was then added, followed by a centrifugation for 1 min at 13000 RPM. This last step was repeated, before the tube was centrifuged for 1 min at 13000 RPM for drying of the binding column. The column was transferred to a clean tube, and 50 µl elution solution was added for 1 min, before it was centrifuged for 1 min at 13000 RPM to elute the RNA. RNA concentration and quality were assessed with the NanoDrop One spectrophotometer (ThermoFisher Scientific), before it was stored at -80 °C.

### *3.5.5.4 cDNA*

For cDNA synthesis, the QuantiTect Reverse Transcription Kit from Qiagen was used. For GE-ET, a -RT control (lacking reverse transcriptase) was included as a half volume reaction to test for contaminating DNA. This was not done in GE-BC, as it was deemed sufficient to do this in one experiment only. All steps relating to the -RT control are therefore only applicable for GE-ET.

In a 96-well plate, RNAse-free water, the isolated RNA and 2µl gDNA wipeout buffer were mixed to a final volume of 21 µl for the GE-ET experiment and 14 µl for the GE-BC experiment. The amount of RNA was 1 µg RNA per sample for the GE-ET, and 0.5 µg RNA per sample for the GE-BC experiment. The plate was then incubated at 42 °C for 2 mins in a Bio-Rad T100 Thermal Cycler. For the -RT control, 7 µl of each 21 µl reaction was transferred to another well.

A reverse transcriptase (RT) master mix was prepared containing Quantiscript RT enzyme, RT primer-mix and RT-buffer, in a 1:1:4 ratio. For the -RT control, a master mix was prepared with RNAse free $H_2O$ replacing the RT enzyme. 6 µl of RT master mix were added to each well and mixed with the pipette. -RT master mix (3 µl) were added to each well for the

-RT control. The plate was then incubated in a Bio-Rad T100 Thermal Cycler for 15 mins at 42 °C, 3 mins at 95 °C, and then kept at 4 °C. Each cDNA reaction was then diluted 5x with RNAse free $H_2O$ and stored at -80 °C.

*3.5.5.5 Quantitative real time polymerase chain reaction (qPCR)*

For qPCR, the LightCycler 480 SYBR Green I Master Kit (Roche Life Science, CatNb 04887352001) was used according to the supplier's instructions.

In brief, a master mix was created by mixing PCR grade $H_2O$, primer working solution (each primer at 5 µM), and LightCycler 480 SYBR Green I Master (2x concentrated), in a 3:2:10 ratio. Master mix (15 µl) was pipetted into each well in a 96-well qPCR plate (Roche Life Science, CatNb 4729692001) and 5 µl of cDNA was added. Non template controls where the cDNA was replaced with PCR grade $H_2O$ were included on the plate as negative controls. The plate was covered with a LightCycler 480 Sealing Foil (Roche Life Science, CatNb 04729757001) and centrifuged at 1500xg for 2 mins. The plate was then run in a LightCycler96 (Roche Life Science) machine, starting with a preincubation at 95 °C for 10 mins, then 45 cycles of 95 °C for 10 sec, 55 or 59 °C (see Appendix 1.5) for 10 sec and 72 °C for 15 sec, finishing with a melting point analysis.

The setup of qPCR plates was according to the sample maximization strategy (Hellemans et al., 2007): GE-ET had room for two genes per plate per qPCR run, or one gene including -RT control; GE-BC had room for one gene per plate. In GE-ET, *ZAT10* were tested with -RT control.

Table A1.5 in Appendix gives an overview of the target genes used in each experiment, including forward and reverse primer sequence.

## 3.5.6 Pathogen resistance assays on *Arabidopsis thaliana* and *Solanum lycopersicum*

**Plant material**

*Arabidopsis*

For the first experiment, seeds of *Arabidopsis thaliana* Col-0 were sown in unfertilized soil and grown for 3 weeks in a Vötsch VB 1514 growth cabinet under a 12-hour photoperiod (75 to 90 µmol $m^{-2}s^{-1}$, relative humidity of 40% and temperature of 22°C). Seedlings were then transplanted to 80 ml pots with C-soil containing the following treatments: 2% frass, ISR (5 g/l) + NG, or NG (control), where NG was applied equivalent to the NPK of the frass treatment. Plants were then grown for another 2.5 weeks under the same conditions.

For the second experiment, *A. thaliana* Col-0 seeds were sown directly into identical soil treatments as described above and grown for 4.5 weeks in a Vötsch VB 1514 growth cabinet, with the same conditions as described above.

Tomato

Tomato seeds (*Solanum lycopersicum* var. Roma VF) were sown into C-soil and germinated for 12 days in a growth chamber under a 12-hour photoperiod (125 to 140 µmol $m^{-2}s^{-1}$, relative humidity of 40% and temperature of 22°C). Seedlings were then transplanted into 400 ml pots containing C-soil and the following treatments: 2% frass, ISR (5 g/l) + NG, or NG (control), where NG was applied equivalent to the NPK of the frass treatment. Plants were then grown for another 2 weeks under the same conditions.

### ***Botrytis cinerea*** **and** ***Pseudomonas syringae*** **inoculation**

*Arabidopsis*

In the first experiment, *B. cinerea* stock solution ($10^7$ spores/ml) was diluted to $2.5x10^5$ spores/ml with filter-sterile Vogel solution. 2 µl droplets of this solutions were placed on each side of the midrib on two fully grown leaves per plant. 2 µl droplets of Vogel solution was placed similarly on one plant per elicitor treatment as control (mock) inoculation. Plants were randomly distributed in two separate covered trays, sealed with cling film (resulting in 100% relative air humidity), and placed in the dark for 24 hours, before they were returned to the growth chamber (12-hour photoperiod) for 6 days.

In the second experiment, a $10^6$ spores/ml *B. cinerea* solution was prepared with filter-sterile Vogel solution. 2 µl droplets were placed on leaves as described above, and plants were inoculated in the dark for 24 hours and in growth chamber for 8 days, also as described above.

Since no infection was detected, inoculation of leaves with *Pseudomonas syringae* was conducted, as described by (Lee et al., 2011). Frozen bacteria stock solution (stored at -80 °C) was dissolved in KingsB liquid medium (Appendix 1.4) with 50 µg/ml rifampicin and left on shaking (220 rpm, 28 °C) for 8 hours. This solution was then resuspended in fresh KingsB medium (with 50 µg/ml rifampicin) and inoculated for another 12 hours in the same conditions. The resulting *P. syringae* culture were then washed three times with $MgCl_2$ (10 mM) by centrifugation (13000 RPM for 10 minutes each time) and diluted with $MgCl_2$ (10 mM) to an optical density (at 600 nm) of 0.02. *Arabidopsis* plants were then infected with this solution, choosing 2-4 fully grown leaves per plant, avoiding leaves who were already infected with *B. cinerea*. The pathogen solution was injected into stomata on abaxial side of leaves by needle-less syringe injection. $MgCl_2$ (10 mM) was injected in the same way into leaves on one plant per soil treatment as mock inoculation. Plants were replaced in growth cabinets in covered trays similar to the *B. cinerea* infection for 4 days.

Tomato

For inoculation of tomato plants *B. cinerea* stock solution ($10^7$ spores/ml) was diluted to $10^6$ spores/ml with filter-sterile Vogel solution. 5 µl droplets of *B. cinerea* solution were placed on each of 5 leaflets on the two first true leaves per plant, yielding up to 10 droplets per plant, on 7 plants per elicitor treatment. Leaves with less than 5 leaflets got fewer droplets. Droplets of Vogel solution (mock) were placed similarly on one plant per elicitor treatment to serve as control (mock) inoculation. Plants were randomly distributed in two separate covered trays, sealed with cling film, and placed in the dark for 24 hours, before they were returned to the growth chamber (12-hour photoperiod) for 48 hours.

**Lesion measurements**

*Botrytis* infection in *Arabidopsis* was visually assessed, rating the severity of each infection from 0 (no lesion) to 5 (whole leaf infected).

The *Pseudomonas* infection was also visually assessed. Two infected leaves from each treatment were cut off and pictures were taken with an iPhone 6s.

Infected tomato leaves were cut off the plant and pictures were taken with an iPhone 6s. The diameters of necrotic lesions were measured with the ImageJ software.

## 3.6 Data presentation and statistical analyses

GraphPad Prism (GraphPad Software, CA, USA) was used for data representation and statistical analyses in tomato and sunflower fertilization experiments, ROS assays, and pathogen assays. Using this software, a one-way ANOVA analysis and Tukey-Kramer multiple comparison test was conducted on all results (unless otherwise specified), except the pairwise comparisons between frass and no frass in the sunflower experiment, where a Sidák test was used. A p-value below 0.05 was considered statistically significant.

Microsoft Excel (Microsoft Corporation, WA, USA) was used for data representation of the chitin oligomer-mix SEC analysis as well as both gene expression analyses (GE-EC and GE-BC). LinRegPCR (Ramakers et al., 2003; Ruijter et al., 2009) was used to determine PCR efficiencies and Cq values based on qPCR raw data. Statistical analysis of qPCR results was done with a one-way ANOVA analysis in qBase+ (Hellemans et al., 2007), with integrated Tukey-Kramer multiple comparison analysis. A p-value below 0.05 was considered statistically significant.

# 4. Results

## 4.1 Growth trials

Two fertilizer trials were conducted with frass, in order to establish its effect as a fertilizer. One looked at the ability of frass to increase growth of tomato compared to a commercial organic fertilizer, the other looked at its ability to complement deficient nutrient solutions in a growth experiment on sunflower.

### 4.1.1 Effect of frass on the growth of tomato

To assay the fertilizing effect of mealworm frass, tomato plants (*Solanum lycopersicum* var. Moneymaker) were grown in commercial plant soil and fertilized with different treatments of frass (Table 3.1). Frass processed in three different ways (heat-treated frass at 70 °C for 1 hour (HF), sterilized frass (SF), and untreated frass (UF)) was added in different proportions to a base soil (CTRL+). NaturGjødsel (NG), a commercial organic fertilizer based on chicken manure, was used to increase the nutrient values of the base soil as well as included as a positive control. Growth parameters of tomato plants were measured at 76 days and 111 days after sowing.

When included to the soil at 2%, frass seems to have led to a general increase in the growth of tomato plants (Figure 4.1; Appendix 1.6). However, there are little statistical significance between treatments.

After 76 days, all frass treatments led to a higher shoot biomass than the base soil, similar to the positive control treatment (NG), although the differences were non-significant. For root growth, the results were the exact opposite, with negative control having the highest root weight.

After 111 days, the positive control performed better than the other treatments on promoting shoot biomass, however only significantly different from the base soil. Frass-treated plants showed higher growth than plants grown in base soil (CTRL+), but this was not significant. It must be noted that the positive control received twice the amount of NPK compared to frass treatments, which is explained in Discussion section 5.1.1.

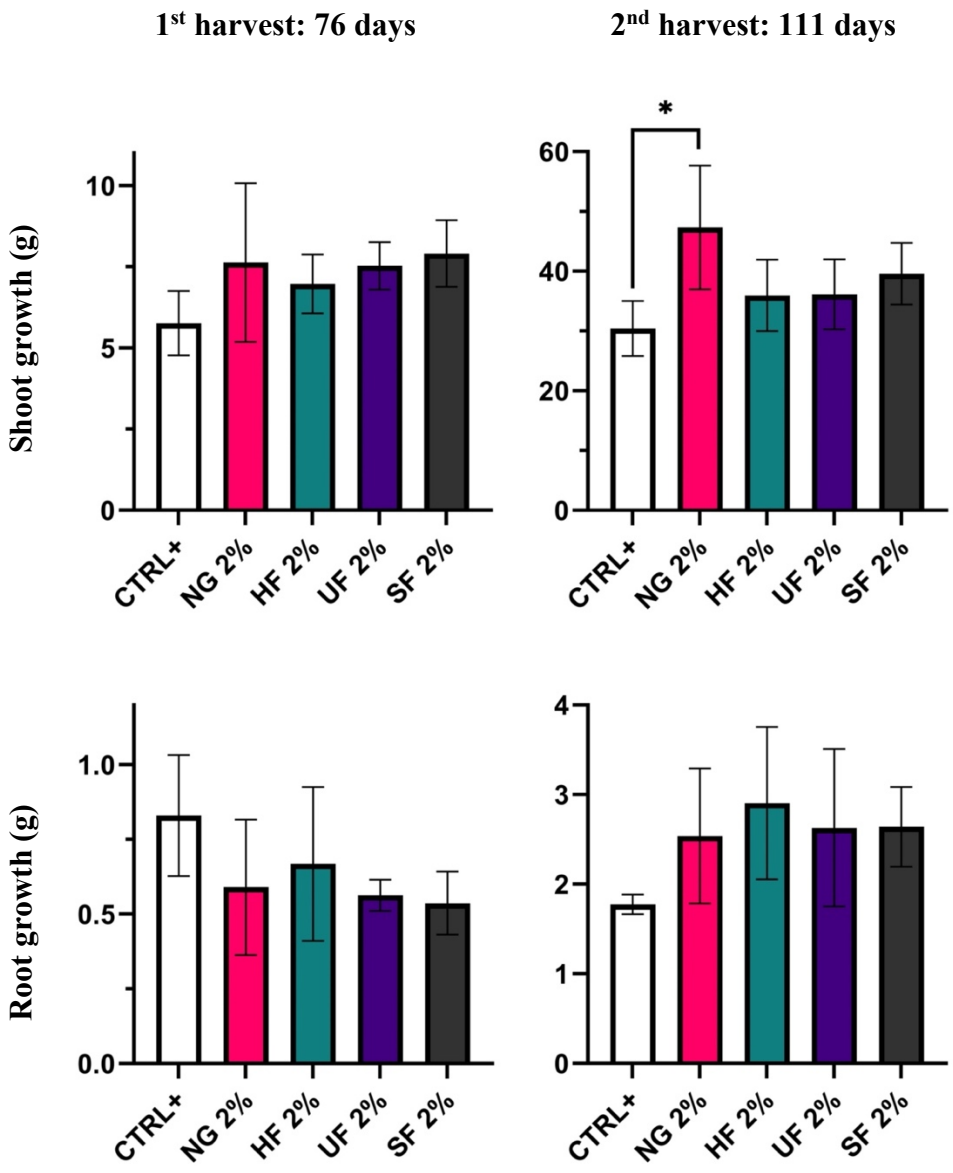



**Figure 4.1: Fertilizing effect of different frass treatments at 2% volume inclusion.** Shoot and root dry weights for tomato (*Solanum lycopersicum* var. Moneymaker) grown in CTRL+ soil fertilized with frass or NG treatments (2% and 1% by volume, respectively), harvested after 76 days or 111 days. Each bar represent the mean of 6 biological replicates ± SD. Asterisks denote statistically significant differences between treatments, Tukey-Kramer multiple comparison test ($p < 0.05$). CTRL+ = control treatment with soil added extra nutrients, NG = NaturGjødsel (pelletized chicken manure, Hageland), HF = heat treated frass (70 °C for 1 hour), UF = untreated frass, SF = sterilized frass (autoclaved at 121 °C for 20 minutes).

As a heat treatment of insect frass is currently required by local legislation before being used as fertilizer, four inclusion rates were tested for the heat-treated frass (HF) (Figure 4.2; Appendix 1.6). After 76 days, the 2% inclusion performed best in promoting shoot growth (Figure 4.2). It was however not significantly different from other treatments, except from the 10% treatment, which led to reduced shoot biomass. After 111 days, the HF 10% treatment had caught up with the remaining treatments. It caused the highest average shoot weight, but the difference was only significant when compared to HF 0.5%. CTRL+ yielded the highest root weight after 76 days, while the 2% treatment had the highest after 111 days. 10% inclusion had

significantly lower root weight than 2% after 76 days, while this had improved somewhat after 111 days.

CTRL+ plants had some signs of chlorosis in both harvests, suggesting some nutrient deficiency (data not shown). All other treatments looked healthy, except HF 10%, which yielded tall but skinny plants, with curled leaves that looked burnt on the edges. This treatment also resulted in plants with almost no flowers, and no tomatoes (Appendix 1.6). All other treatments got similar number of flowers and tomatoes, except the CTRL+ treatment which led to a reduced number of flowers and fruit yield (Appendix 1.6).

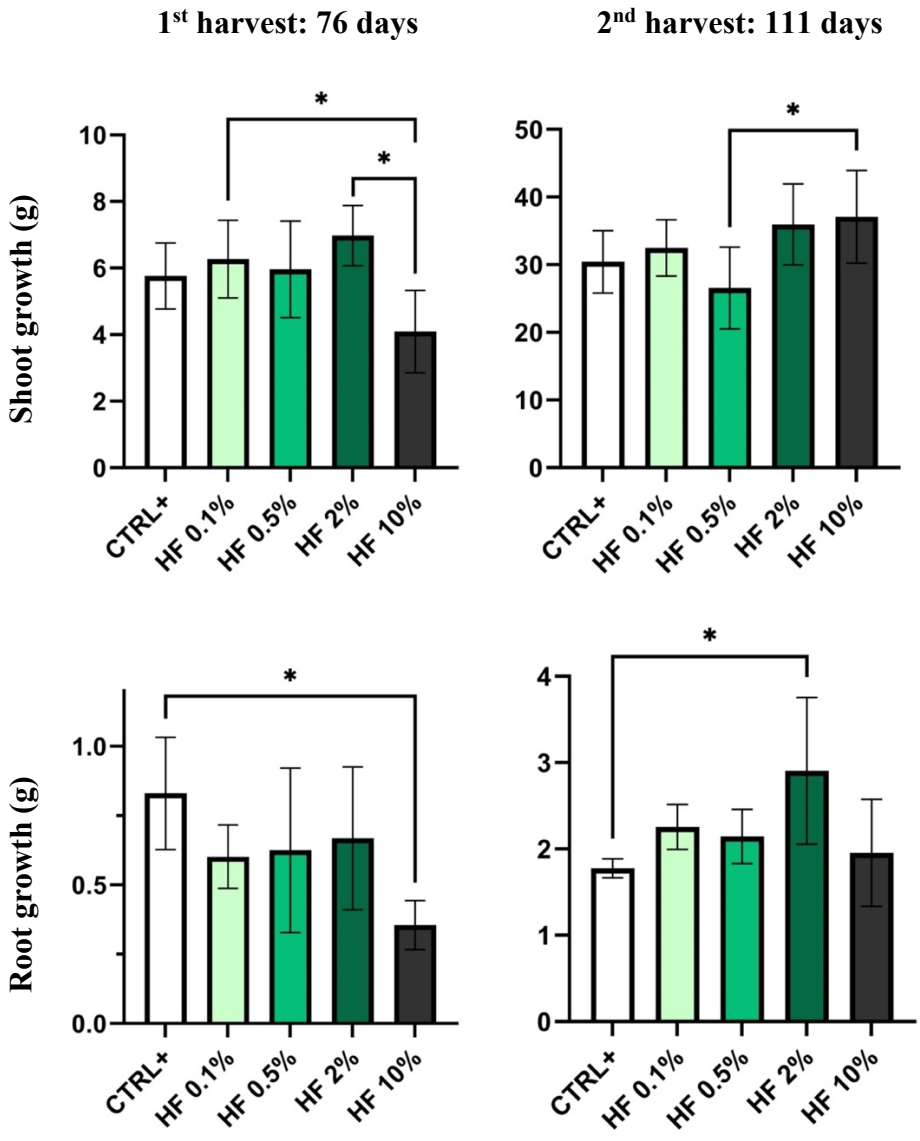



**Figure 4.2: Fertilizing effect of different inclusion rates of heat-treated frass (HF)**. Shoot and root dry weights for tomato (Solanum lycopersicum var. Moneymaker) grown for 76 or 111 days in Ctrl+ soil fertilized with 0.1-10 % by volume HF (frass heat-treated at 70 °C for 1 hour). HF 10% used Ctrl soil as base (less nutrients than Ctrl+). Data represent the mean of 6 biological replicates ± SD. Asterisks denote statistically significant differences between treatments, Tukey-Kramer multiple comparison test ($p < 0.05$).

### 4.1.2 Sunflower nutrient deficiencies

Frass' ability to contribute specific macronutrients for plant growth where assayed. Sunflowers were grown in a nutrient free sand/perlite mixture (2:3) with or without inclusion of 5% by volume frass. Plants were watered each day for 8 weeks with either a complete nutrient solution or five solutions lacking one vital macronutrient each (Figure 4.3).

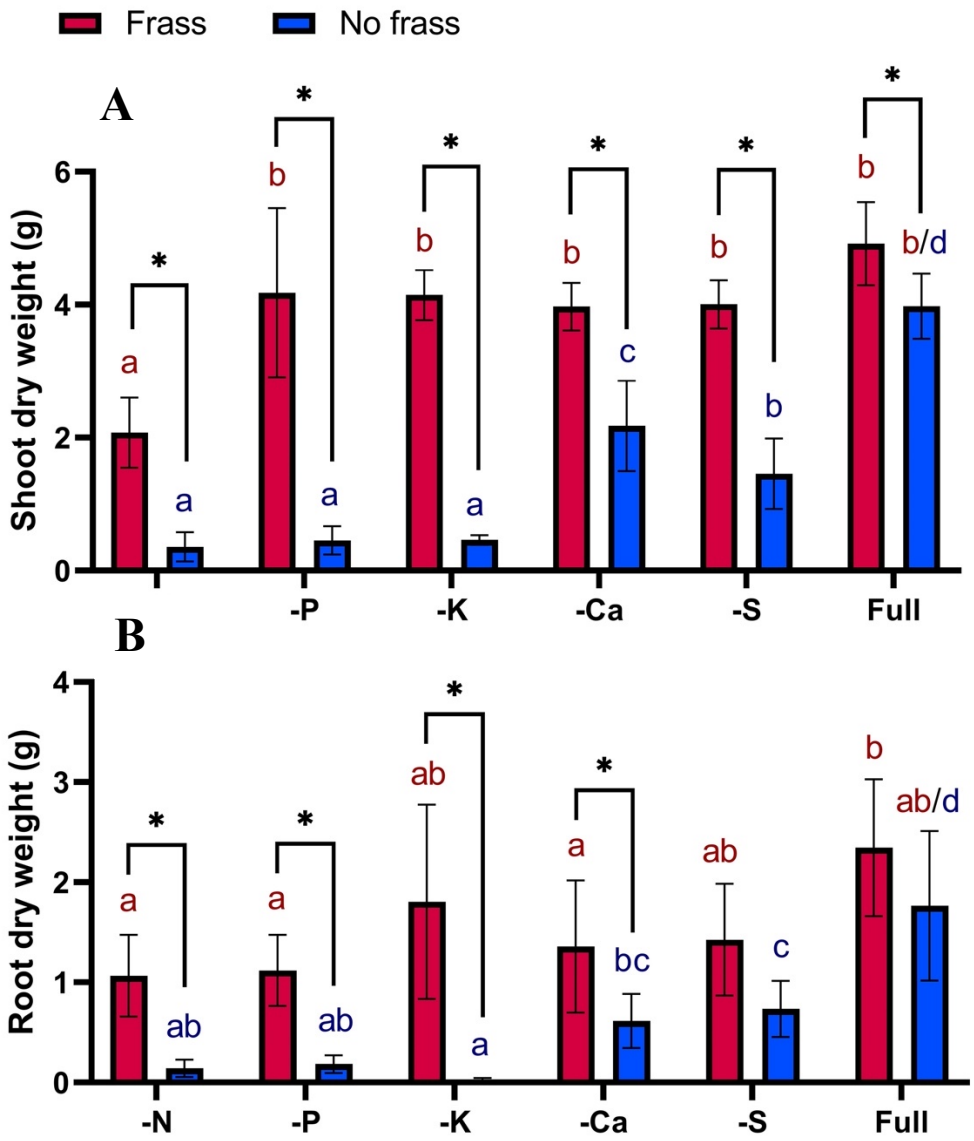



**Figure 4.3: Growth of nutrient deficient sunflowers with or without frass.** Shoot (A) and root (B) dry weights for sunflowers (*Helianthus annuus*) fertilized with or without frass and watered with nutrient solutions lacking nutrient -X (indicated below bars on the x-axis). Full = complete nutrient solution. Each bar represents the mean of 8 biological replicates ± SD. The letters denote statistically significant differences between nutrient treatments within each frass treatment (red letters = frass, blue letters = no frass), Tukey-Kramer multiple comparison test ($p < 0.05$). Asterisks represent statistically significant differences between frass and no frass within each nutrient treatment, Sidák test ($p < 0.05$).

Nutrient deficiency in N, P and K proved most detrimental to plant growth, while Ca and S deficiencies also reduced growth significantly. Frass restored normal shoot growth of plants watered with treatments deficient in P, K, Ca, or S, suggesting that frass contributed these missing nutrients (Figure 4.3A). Shoot weight was also significantly increased by frass in the -N treatment, but it was still lower than for plants receiving the complete nutrient solution.

The addition of frass greatly improved root growth for all nutrient deficient treatments (Figure 4.3B). However, these plants had somewhat smaller roots compared to the Full treatment without frass, even though this difference was not significant. It seems as if the shortage of nitrogen was less detrimental for root growth than for shoot growth, as -N treatment with frass displayed similar root growth to the other nutrient deficient frass treatments.

The complete nutrient solution with frass yielded the highest shoot and root weight. Plants receiving this treatment also showed some curling of young leaves similar to the 10% frass treatments from the tomato growth trial (data not shown).

## 4.2 Plant defense activation by frass or insect skin residue (ISR)

Several molecular methods were utilized in order to investigate the potential immune stimulating effects of frass and its chitin content. Some of the methods were unsuccessful in proving this, but are included in this section nevertheless, as much time were spent on these and some showed positive results for the chitin standard.

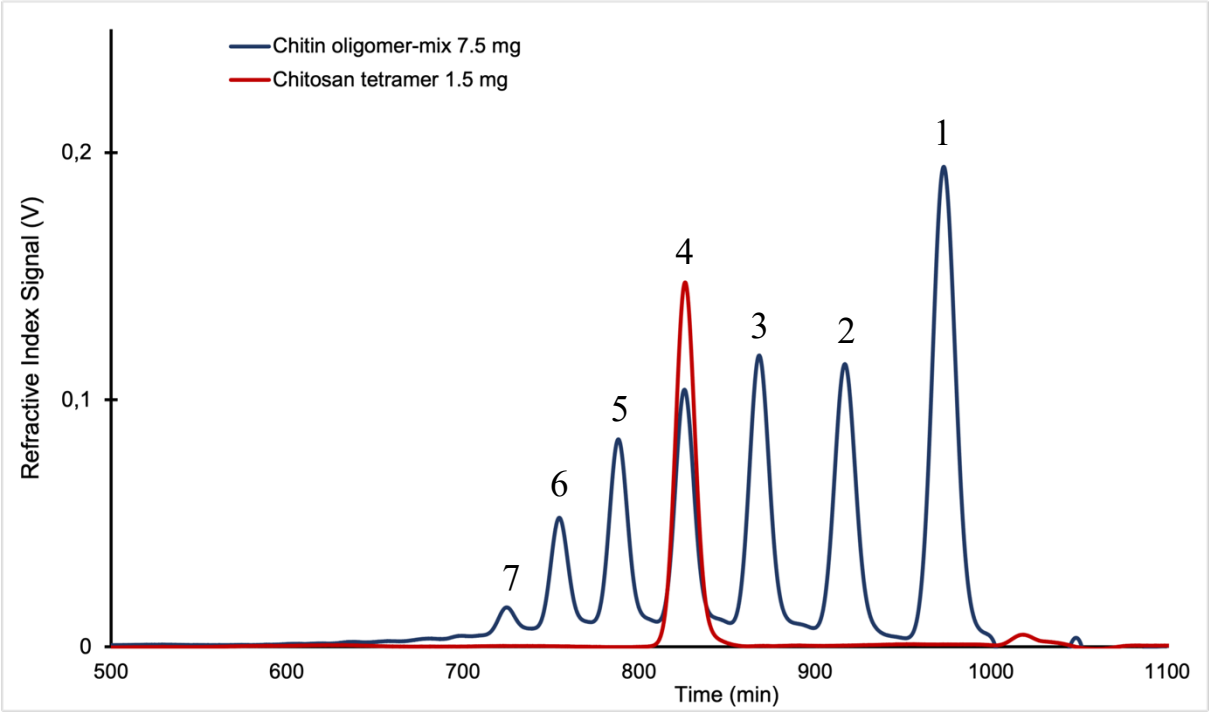
Due to the complex structure of the insect exoskeleton, it was eventually assumed that ISR would not be able to trigger immune responses in plants without any degradation by soil microbes. To overcome this, C-soil was mixed with frass or ISR, and left to decompose for several weeks, as described in Methods section 3.5.2. Soil solutions were prepared with dd$H_2O$ or liquid medium and used in several experiments. These are referred to as “soil solutions”. Frass and ISR solutions without soil were also prepared and are referred to as “frass/ISR solutions”.

### 4.2.1 Chitin standard used as positive control

A pure chitin oligomer-mix derived from shrimp was used as a positive control in several experiments throughout this project. It was kindly provided by the biopolymer research group NOBIPOL at NTNU. Size exclusion chromatography (SEC) was conducted on the sample by Olav Aarstad from NOBIPOL, in order to evaluate its oligomer content.

The SEC showed that most chitin molecules in the sample were short-chained oligomers with a degree of polymerization (DP) <6 or the monomer N-acetyl glucosamine. Only 10.8% by weight of the oligomers in the sample are of DP 6 or higher, which are the main plant immune defense eliciting chitin molecules (Li et al., 2020).

NMR spectroscopy was also conducted on the sample by NOBIPOL, showing pure chitin (Appendix 1.6).




| **Oligomer DP** | **>7** | **7** | **6** | **5** | **4** | **3** | **2** | **1** |
|---|---|---|---|---|---|---|---|---|
| **Weight %** | 1.4 | 2.4 | 7.0 | 11.6 | 14.3 | 17.0 | 17.0 | 29.3 |

**Figure 4.4: Oligomer weight fractions in chitin oligomer-mix from SEC.** Size Exclusion Chromatography of the chitin oligomer-mix (blue), run through 3 serially connected Superdex30 columns with ammonium acetate (0.15 M) mobile phase (flow: 0.8 ml/min), conducted by NOBIPOL. Chitosan tetramer (red) is used as a positive control. Numbers above peaks represent degree of polymerization (DP). The table displays weight percentage of each oligomer in the solution calculated from the relative peak area.

## 4.2.2 Detection of Reactive Oxygen Species (ROS)

In order to assess whether frass components trigger the production of reactive oxygen species (ROS), an early signal of plant defense responses, several experiments were conducted with leaf disks from *Arabidopsis* Col-0 treated with different solutions of the chitin standard, frass, ISR, or control ($ddH_2O$) solutions, before chemiluminescence was measured. Solutions from soil premixed with frass or ISR, as explained in methods section 3.5.2, was also used in order to test for degradation of frass or insect skin in soil, potentially leading to immune stimulating chitin oligomers. Early attempts showed ROS production from chitin solutions, but not from frass, ISR, or soil solutions (data not shown).

To assess if filtered frass or soil solutions interfere with luminescence production or detection (e.g. by presence of particles), chitin (250 µg/ml) was dissolved in $ddH_2O$, filtered frass solution (0.45 µm or 0.2 µm pore size filter), or filtered soil solution containing frass (0.45 µm pore size filter), as well as each solution without chitin. Leaf disks from 3-week-old *Arabidopsis* Col-0 grown in unfertilized C-soil were treated with these solutions, before chemiluminescence was measured.

Chitin in $ddH_2O$ showed a strong response with a peak after approximately 20 minutes (Figure 4.5). Chitin in soil solution yielded some ROS production, but a lot less than chitin in $ddH_2O$. All other treatments, including chitin in frass solution, led to no detectable increase of ROS production (from background level).

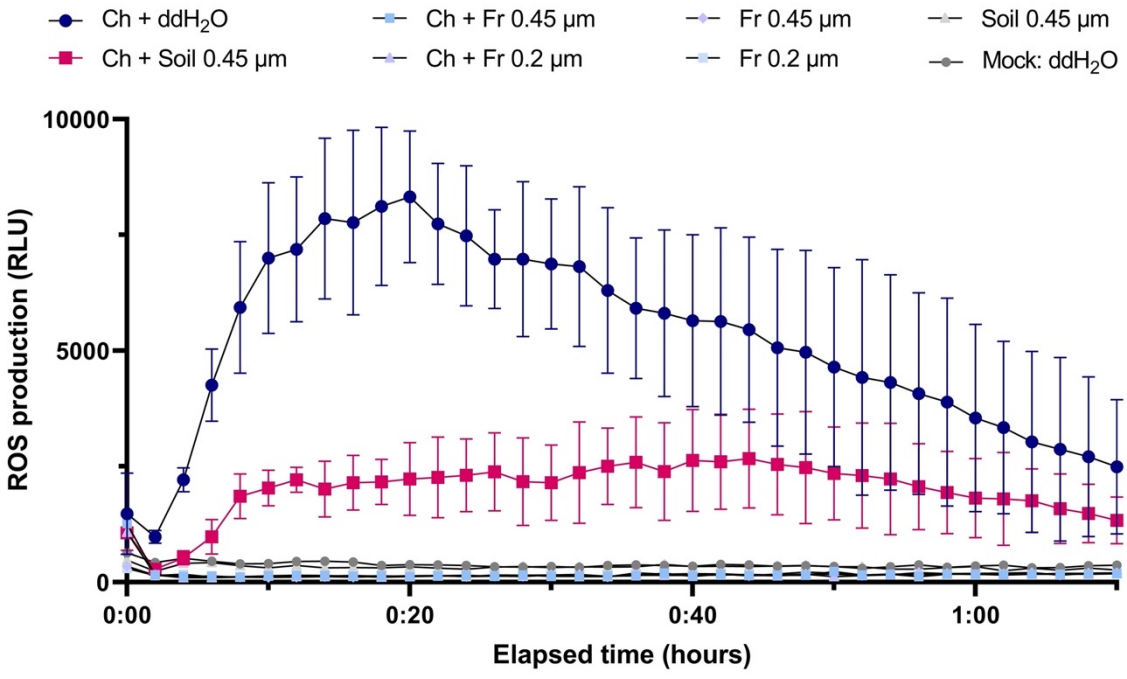



**Figure 4.5: Production of ROS by plants elicited with chitin dissolved in filtered frass or soil solutions.** ROS production of 3-week-old Col-0 leaf disks treated with $ddH_2O$ (Mock), chitin (Ch, 250 µg/ml) either in $ddH_2O$, filtered frass solution (Fr, filtered through 0.45 or 0.2 µm pore size filter) or filtered soil solution containing frass (0.45 µm pore size filter), as well as each solution without chitin. Production of ROS where measured every 2 minutes for 1 hour and 12 mins with a Bio-Tek Cytation 5 Cell Imaging Multi-Mode Reader. Each data point represents the mean of 4 biological replicates ± SD. Some data points are not seen in the graph due to layering of symbols. These values can be assumed to follow the mock treatment trend. RLU: Relative luminescence units.

Two additional concentrations of the chitin standard (in $ddH_2O$) were tested as elicitor solutions in *Arabidopsis*, together with an ISR solution prepared in $ddH_2O$ as explained in methods section 3.5.2. Chemiluminescence was measured over 3 hours in order to detect a potential second wave of ROS production. Leaf disks from 3-week-old *Arabidopsis* Col-0 grown in unfertilized C-soil were treated with the chitin, ISR or control ($ddH_2O$) solutions, before chemiluminescence was measured.

The chitin treatments led to rapid and strong production of ROS in the leaf disks, with approximately twice as strong response for 125 µg/ml chitin compared to 12.5 µg/ml chitin (Figure 4.6). The response peaked after 15-20 minutes, then declined for about 40 minutes, before it increased again for a second wave. No ROS production were detected in mock and ISR treatments. In fact, the ISR treatment displayed even lower luminescence than the mock.

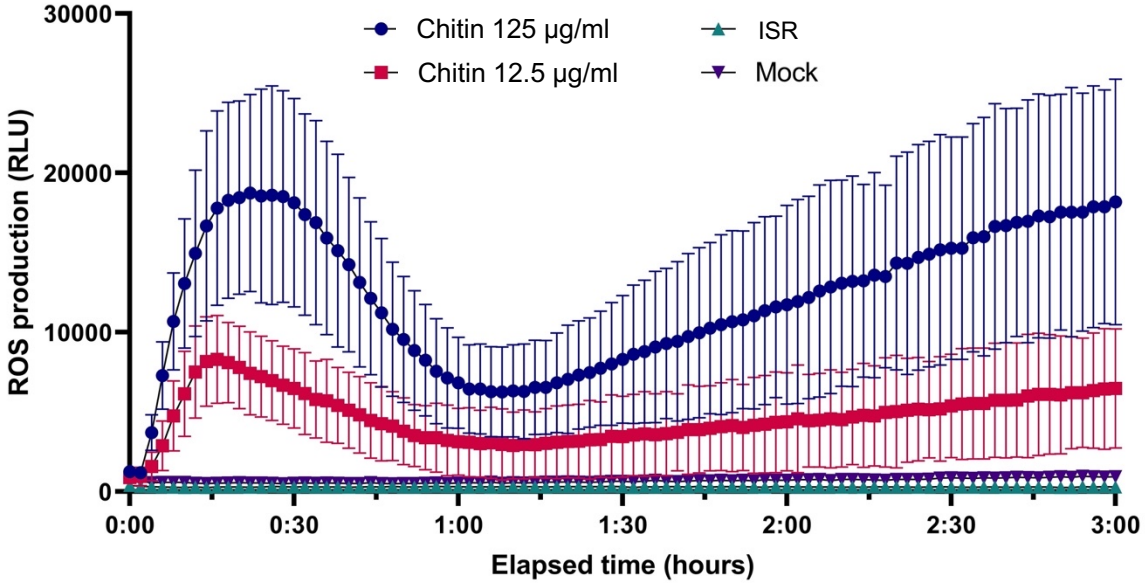



**Figure 4.6: Production of ROS by plants elicited with chitin and ISR.** ROS production of 3-week-old Col-0 leaf disks treated with chitin (125 µg/ml or 12.5 µg/ml), ISR solution or water (Mock). Production of ROS were measured by chemiluminescence every 2 minutes for 3 hours with Bio-Tek Cytation 5 Cell Imaging Multi-Mode Reader. Each data point represents the mean of 12 biological replicates ± SD. RLU: Relative luminescence units.

### 4.2.3 Callose

Another event in the plant immune response is the deposition of the polysaccharide callose. To detect callose, *Arabidopsis* Col-0 were grown in liquid medium for 10 days before seedlings were treated with different elicitor solutions for 20 hours. In Figure 4.7, soil solutions containing frass, ISR, or chitin (250 µg/ml, added to control soil solution) was used. Callose was stained with aniline blue and observed under UV light in a microscope.

Callose was clearly observed in the roots of chitin treated plants (Figure 4.7D). The treatments with frass in soil or ISR in soil displayed very low levels of callose in roots. This experiment was also conducted with pure frass or ISR solutions (in $ddH_2O$), which also resulted in very little callose deposition in roots compared to the chitin positive control (data not shown).

Callose was also observed in cotyledons of the seedlings treated with chitin, frass, or ISR in soil solutions (data not shown). Chitin generally led to more callose deposition than the two other treatments, but there were large variations in callose deposition within each treatment, and callose was also observed in the control treatment.

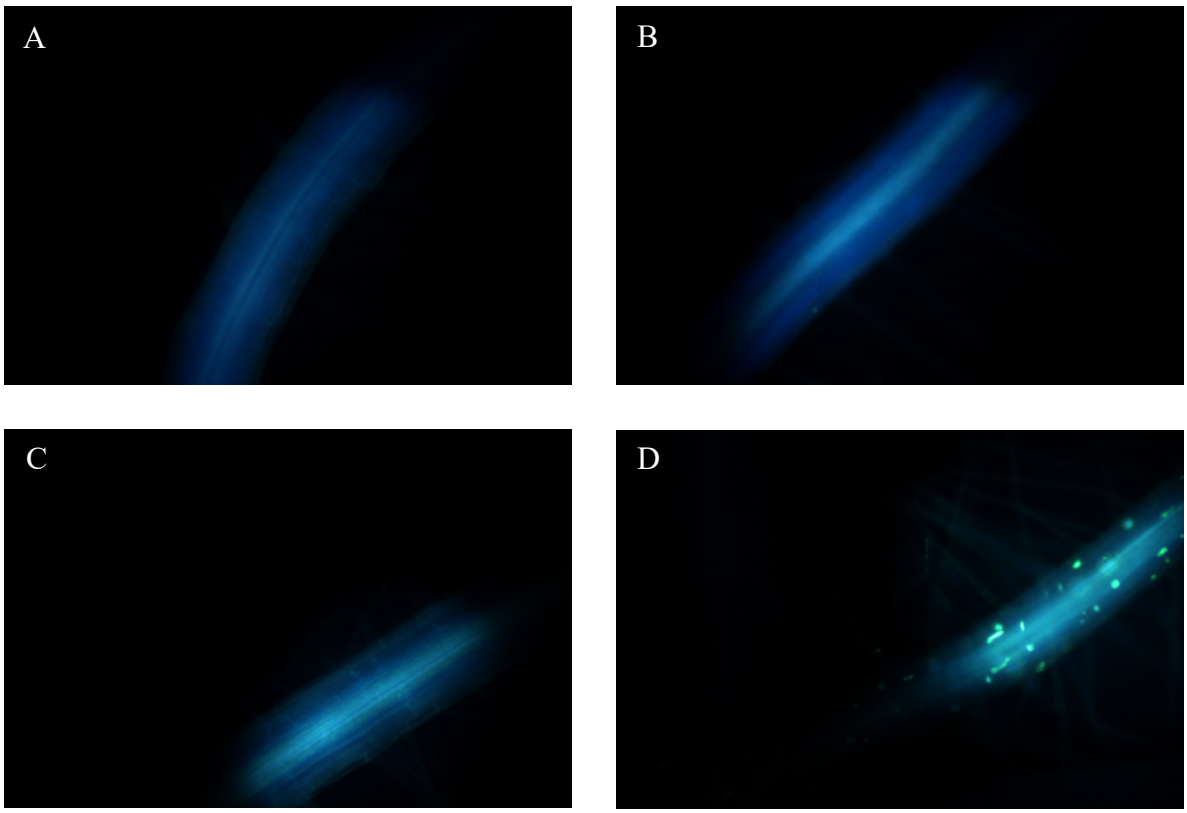



**Figure 4.7: Callose deposition in *A. thaliana* roots.** Representative pictures of callose deposition in roots of 10-day-old *A. thaliana* seedlings grown in liquid medium, treated with A: a filtered soil solution (Ctrl) containing B: frass, C: ISR or D: chitin (250 µg/ml) for 20 hours. Callose was stained with aniline blue and observed under UV light in a Nikon Eclipse E800 epi-fluorescence microscope (10x magnification).

### 4.2.4 Early changes in defense gene expression after elicitor treatment with chitin or frass

Two different gene expression analyses were designed. One investigates the early immune response when pure chitin oligomers or soil-degraded frass is applied to *Arabidopsis* seedlings, while the other looks at more long-term responses activated by the pathogenic fungus *Botrytis cinerea* isolate 2100 (BC) in *Arabidopsis* primed by frass or ISR in soil. For simplicity, throughout this thesis the short-term gene expression experiment will be referred to as GE-ET (gene expression elicitor treatment), while the *Botrytis* priming experiment will be called GE-BC (gene expression *Botrytis cinerea)*.

Transcription of defense related genes is a common immune response in plants, and the expression of these genes can be used to monitor an immune stimulating effect in plants treated with elicitors. The early changes in expression of the defense response genes *ZAT10* (*zinc finger of Arabidopsis thaliana 10*), *ERF5* (*ethylene-responsive transcription factor 5*) and *PER4* (*peroxidase 4*) were investigated after 30 min, 1 hour or 3 hours in 10-day-old *Arabidopsis* Col-0 seedlings treated with chitin or frass solutions.

Both *ZAT10* and *ERF5* are transcription factors involved in biotic and abiotic stress responses and are linked to jasmonate signaling. They are both documented to be responsive to chitin oligomers (Egusa et al., 2015; Kazan & Manners, 2011; Son et al., 2011). *PER4* encodes a member of peroxidases that are linked to lignin formation as a response to biotic stress (Rasul et al., 2012).

Chitin clearly induced expression of all three genes, particularly after 30 min and 1 hour. *ZAT10* and *ERF5* were significantly higher expressed after 30 min in the chitin treatment compared to frass and mock treatments. This effect decreased over time. *PER4* was significantly higher expressed in chitin treatments than frass and control after 1 hour.

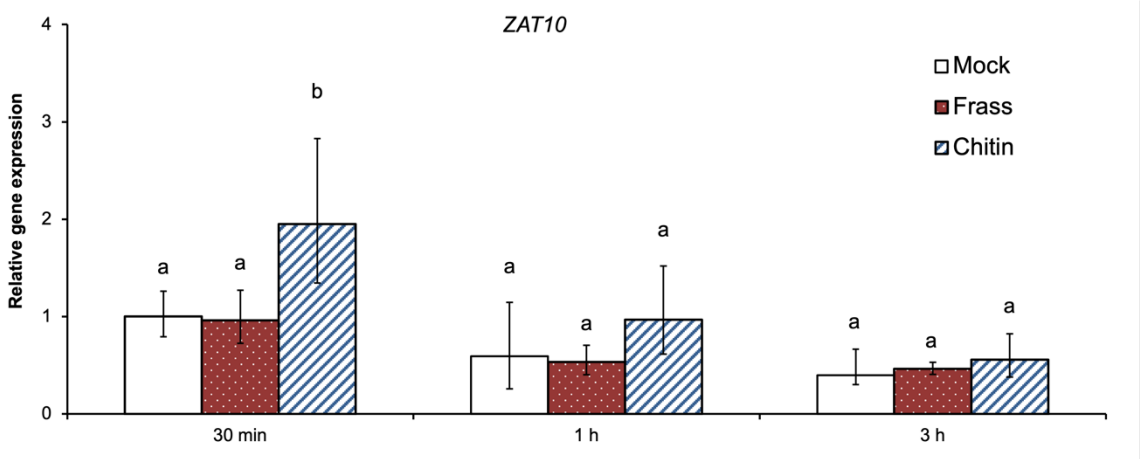



**Figure 4.8:** Gene expression analysis of *ZAT10* in 10-day-old Arabidopsis Col-0 seedlings grown in liquid medium and treated with chitin (62.5 µg/ml) in soil solution, frass soil solution, or mock soil solution. Seedlings were harvested 30 min, 1 hour or 3 hours post elicitation. All expressions are relative to *ZAT10* expression for the Mock treatment after 30 min (set to 1). Bars represents the mean of 4 biological replicates, and error bars show 95% confidence intervals. Different letters denote significant differences within the same time point (no comparisons between time points), Tukey-Kramer multiple comparison test, $p < 0.05$

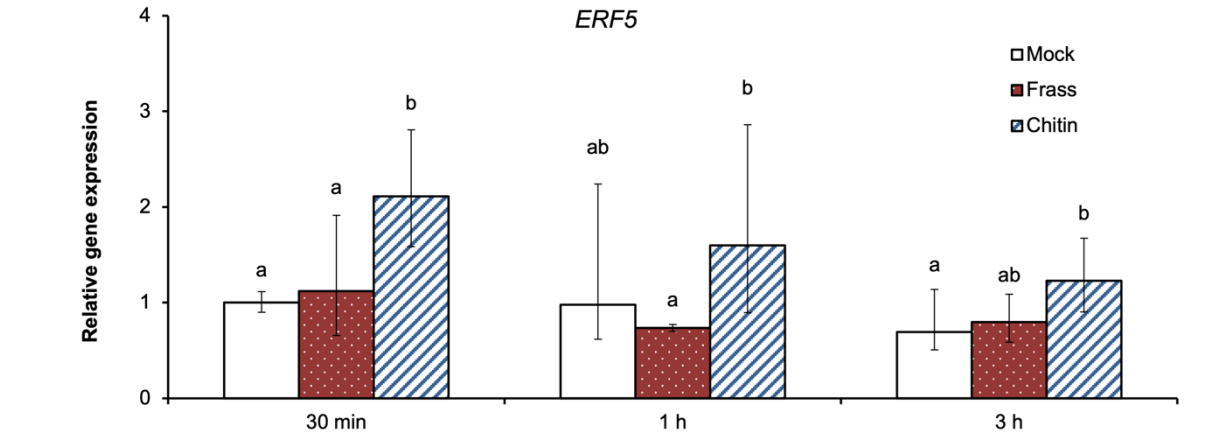



**Figure 4.9:** Gene expression analysis of *ERF5* in 10-day-old Arabidopsis Col-0 seedlings grown in liquid medium and treated with chitin (62.5 µg/ml) in soil solution, frass soil solution, or mock soil solution. Seedlings were harvested 30 min, 1 hour or 3 hours post elicitation. All expressions are relative to *ERF5* expression for the Mock treatment after 30 min (set to 1). Bars represents the mean of 4 biological replicates, and error bars show 95% confidence intervals. Different letters denote significant differences within the same time point (no comparisons between time points), Tukey-Kramer multiple comparison test, $p < 0.05$.

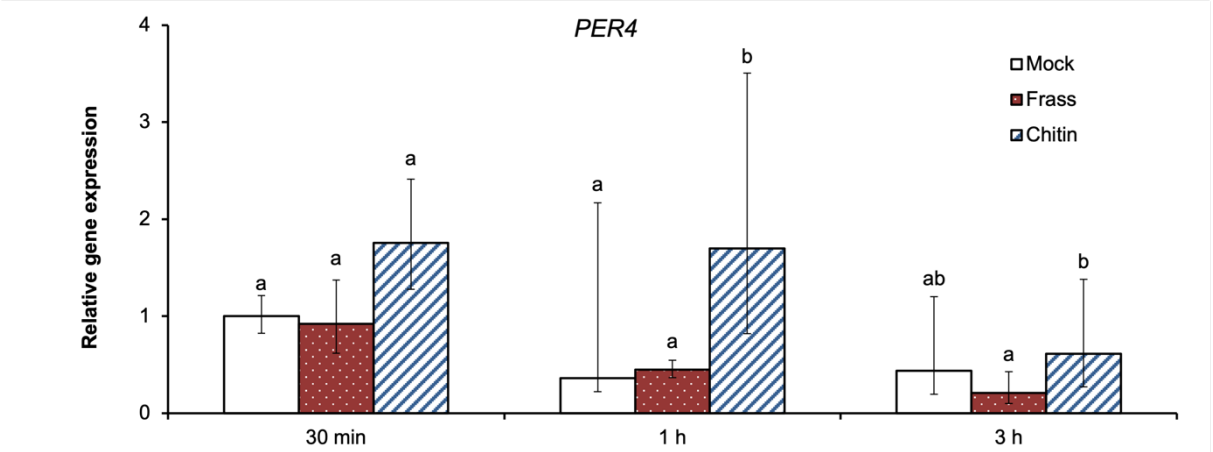



**Figure 4.10:** Gene expression analysis of *PER4* in 10-day-old Arabidopsis Col-0 seedlings grown in liquid medium and treated with chitin (62.5 µg/ml) in soil solution, frass soil solution, or mock soil solution. Seedlings were harvested 30 min, 1 hour or 3 hours post elicitation. All expressions are relative to *PER4* expression for the Mock treatment after 30 min (set to 1). Bars represents the mean of 4 biological replicates, and error bars show 95% confidence intervals. Different letters denote significant differences within the same time point (no comparisons between time points), Tukey-Kramer multiple comparison test, $p < 0.05$.

### 4.2.4 Effect of *B. cinerea* infection after priming with frass or insect skin residue on defense gene expression

Expression of several defense related genes were also investigated in plants infected with *Botrytis cinerea* (BC) after they had been exposed to frass and insect skin residue (ISR) for a longer time period, potentially showcasing a priming effect triggered by the frass and ISR treatments.

*Arabidopsis* Col-0 were grown for 3 weeks in C-soil before transplanted to a frass, ISR or control fertilizer treatment for 2 weeks.

Leaves were inoculated with *B. cinerea* spores and gene expression was analyzed 8, 24 and 48 hours post inoculation (hpi). In addition to *ZAT10*, *ERF5*, and *PER4* used for the elicitor treatment described above, the expression of three WRKY transcription factors (*WRKY33*, *WRKY53* and *WRKY75*) and a myeloblastosis transcription factor (*MYB51*), two cytochrome P450 monooxygenases (*CYP71A13* and *CYP71B15*), *CML37* (*calmodulin-like protein 37*), and an uncharacterized Chitinase were monitored (Figures 4.11 to 4.16; Appendix 1.8).

The WRKY gene family are transcription factors that are important in the modulation of defense responses as well as many other functions in the plant. Both *WRKY33*, *WRKY53* and *WRKY75* are involved in the *Arabidopsis* defense against fungal pathogens, especially *Botrytis cinerea* (Aranega-Bou et al., 2014). *MYB51* is a transcription factor regulating the production of camalexin and glucosinolate, compounds involved in plant immunity (Frerigmann et al., 2015), while *CYP71A13* and *CYP71B15* encode proteins important in camalexin biosynthesis (Birkenbihl et al., 2012). *CML37* is a $Ca^{2+}$ sensitive defense regulator in plants, connecting $Ca^{2+}$ signaling with the jasmonate response pathway (Scholz et al., 2014). Chitinases are enzymes that hydrolyses chitin and can be synthesized by plants as a defense response against fungal pathogens (Kumar et al., 2018).

Expression of *ZAT10* was elevated in frass treatments compared to the ISR and control soil treatments, particularly in the BC infected plants (Figure 4.11). ISR treatments with BC infection also led to a significantly induced *ZAT10* expression compared to the control after 24 and 48 hours. After 48 hpi, BC infected plants treated with frass or ISR displayed considerably higher expression values than mock treated plants.

Both *ERF5* (Figure 4.12) and *WRKY33* (Figure 4.13) were overall higher expressed in frass and ISR treatments than in the control treatment. However, BC inoculation seemed to have little effect on the expression levels of these genes, except after 48 hours, where the BC infestation led to higher expression levels in both genes compared to mock (only significant for ISR). *PER4* (presented in Appendix 1.8) showed quite similar results as *ERF5* and *WRKY33*, with an inducing effect by frass and ISR, but the treatment that led to the highest expression varied considerably depending on the time point.

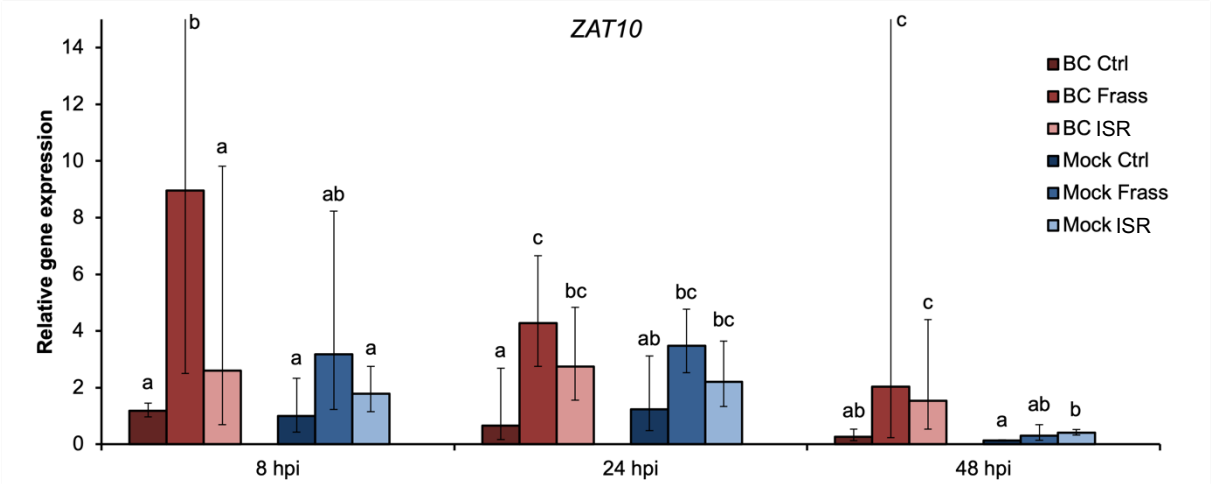



**Figure 4.11**: **Gene expression analysis of *ZAT10* target gene after *B. cinerea* infection.** 5-week-old *Arabidopsis* Col-0 leaves grown for 2 weeks in control (Ctrl), frass or ISR soil treatments. Leaves were inoculated with *Botrytis cinerea* spores (BC, red bars) or Vogel solution (Mock, blue bars), and harvested at 8 hours, 24 hours or 48 hours post inoculation (hpi). All values are relative to *ZAT10* expression in plants grown on Ctrl soil, mock-inoculated and subsequently incubated for 8 h (set to 1). Error bars show 95% confidence intervals (CI), but the upper CI for BC Frass 8 h (32.0) and for BC Frass 48 h (17.9) are capped in the figure for presentation purposes. Different letters denote significant differences within the same time point (no comparisons between time points), Tukey-Kramer multiple comparison test, $p < 0.05$.

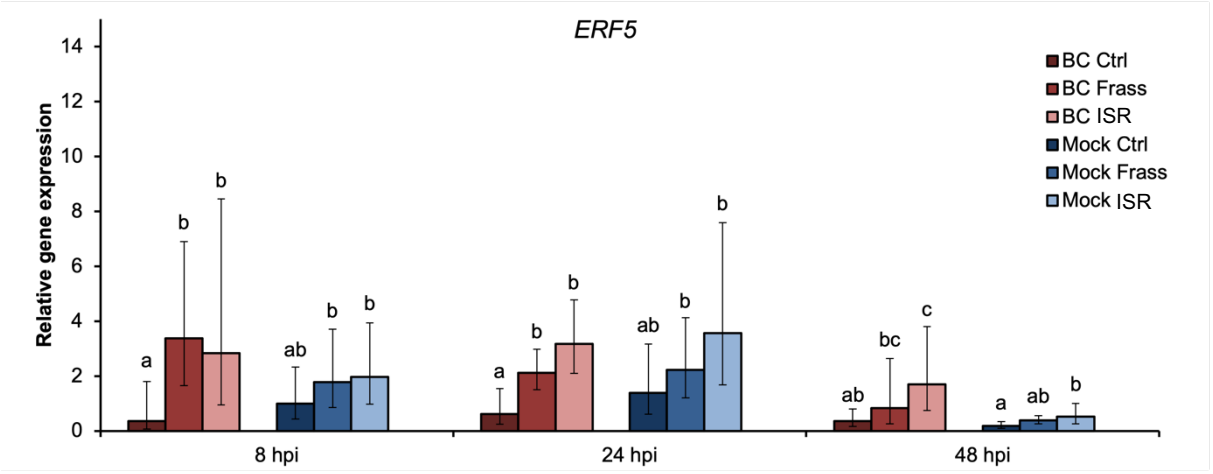



**Figure 4.12**: **Gene expression analysis of *ERF5* target gene after *B. cinerea* infection.** 5-week-old *Arabidopsis* Col-0 leaves grown for 2 weeks in control, frass or ISR soil treatments. Leaves were inoculated with *Botrytis cinerea* spores (BC, red bars) or Vogel solution (Mock, blue bars), and harvested at 8 hours, 24 hours or 48 hours post inoculation (hpi). All values are relative to *ERF5* expression in plants grown on Ctrl soil, mock-inoculated and subsequently incubated for 8 h (set to 1). Error bars show 95% confidence intervals. Letters denote significance within the same time group (no comparisons between time groups), Tukey-Kramer multiple comparison test, $p < 0.05$.

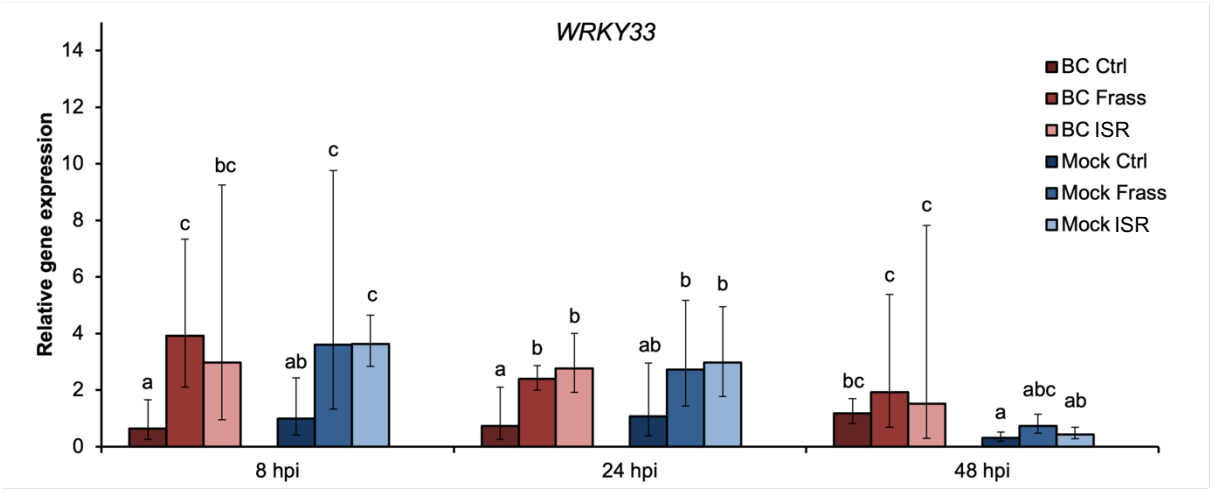
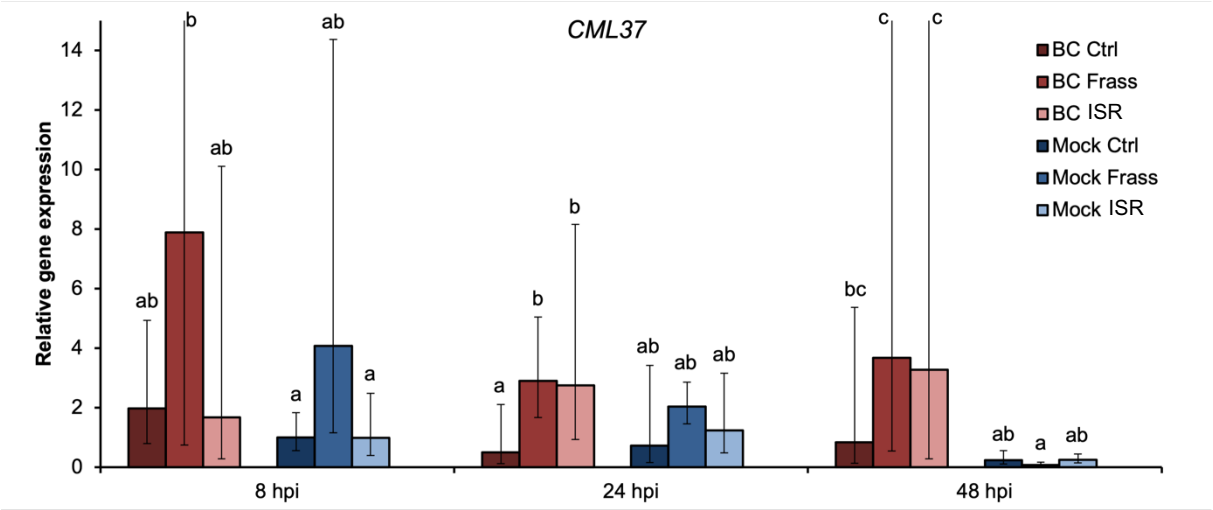



**Figure 4.13**: **Gene expression analysis of *WRKY33* target gene after *B. cinerea* infection.** 5-week-old *Arabidopsis* Col-0 leaves grown for 2 weeks in control, frass or ISR soil treatments. Leaves were inoculated with *Botrytis cinerea* spores (BC, red bars) or Vogel solution (Mock, blue bars), and harvested at 8 hours, 24 hours or 48 hours post inoculation (hpi). All values are relative to *WRKY33* expression in plants grown on Ctrl soil, mock-inoculated and subsequently incubated for 8 h (set to 1). Error bars show 95% confidence intervals. Letters denote significance within the same time group (no comparisons between time groups), Tukey-Kramer multiple comparison test, $p < 0.05$.




**Figur 4.14: Gene expression analysis of *CML37* target gene after *B. cinerea* infection.** 5-week-old *Arabidopsis* Col-0 leaves grown for 2 weeks in control, frass or ISR soil treatments. Leaves were inoculated with *Botrytis cinerea* spores (BC, red bars) or Vogel solution (Mock, blue bars), and harvested at 8 hours, 24 hours or 48 hours post inoculation (hpi). All values are relative to *CML37* expression in plants grown on Ctrl soil, mock-inoculated and subsequently incubated for 8 h (set to 1). Error bars show 95% confidence intervals (CI), but the upper CI for BC Frass 8 h (76.6), BC Frass 48 h (21.4), and BC IS 48 h (35.4) are capped in the figure for presentation purposes. Letters denote significance within the same time group (no comparisons between time groups), Tukey-Kramer multiple comparison test, $p < 0.05$.

As to the expression of *CML37*, a positive effect from frass was seen after 8 hours in both BC and mock inoculation, although non-significant to the control (Figure 4.14). After 24 hours, frass and ISR have a clear effect on *CML37* expression levels when infested with BC, while this effect is less clear in mock treatments. After 48 hours, plants exposed to BC show a lot higher expression of *CML37* compared to mock-inoculated ones, and frass and ISR treated plants are higher expressed but not significantly different compared to BC control. Considerable variations within treatments led to large confidence intervals and consequently less significant results.

*WRKY53* displayed similar expression levels in mock and BC treated plants in all time points (Figure 4.15). Frass and ISR seemed to lead to increased induction also for this gene, especially at 8 hpi. However, after 48 hpi, all treatments exhibited the same low expression values.

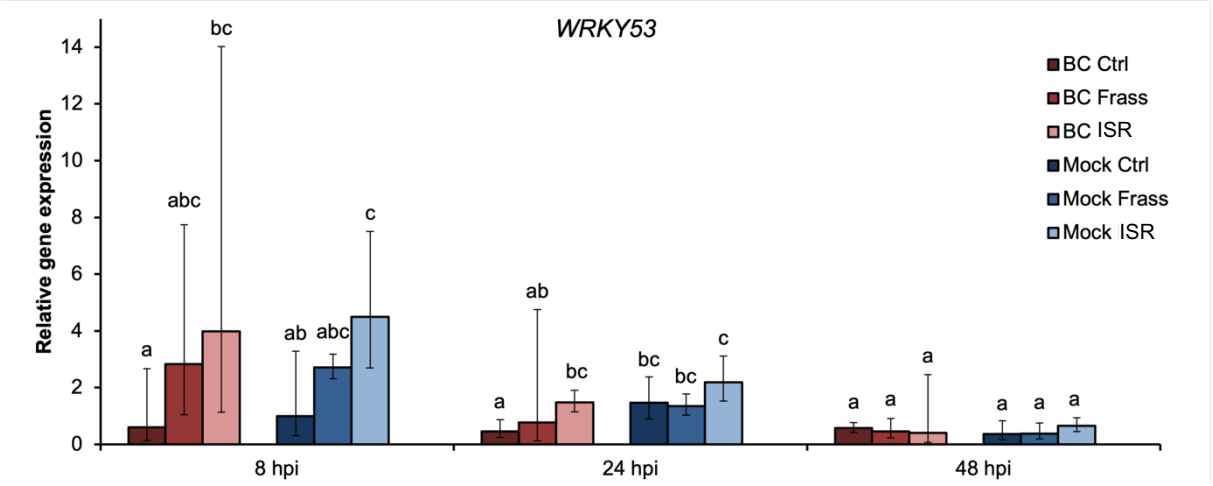



**Figure 4.15**: **Gene expression analysis of *WRKY53* target gene after *B. cinerea* infection.** 5-week-old *Arabidopsis* Col-0 leaves grown for 2 weeks in control, frass or ISR soil treatments. Leaves were inoculated with *Botrytis cinerea* spores (BC, red bars) or Vogel solution (Mock, blue bars), and harvested at 8 hours, 24 hours or 48 hours post inoculation (hpi). All values are relative to *WRKY53* expression in plants grown on Ctrl soil, mock-inoculated and subsequently incubated for 8 h (set to 1). Error bars show 95% confidence intervals. Letters denote significance within the same time group (no comparisons between time groups), Tukey-Kramer multiple comparison test, $p < 0.05$.

In contrast to *WRKY33* and *WRKY53*, *WRKY75* (Figure 4.16) showed very low expression values and no significant differences between treatments until 48 hours post inoculation, where the BC infestation led to a substantial increase in expression compared to the mock treatment. Although this was especially prominent in plants grown on soil supplemented with frass and IS, the levels were not significantly different from plants grown on control soil. The same trend was seen for the expression of *CYP71A13* (Figure 4.17) and *CYP71B15* (Appendix 1.8). Chitinase also showed increased expression after 48 hours in BC-infested plants, but only 3-5 times more than in mock-inoculated ones, and without any significant differences between control, frass and IS treatments (results in Appendix 1.8).

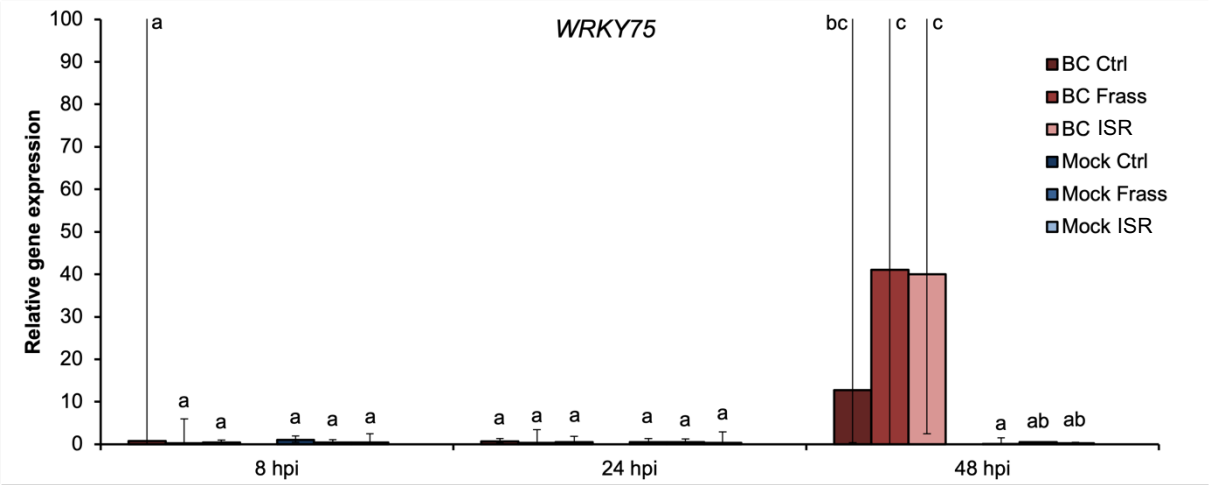



**Figure 4.16**: **Gene expression analysis of *WRKY75* target gene after *B. cinerea* infection.** 5-week-old *Arabidopsis* Col-0 leaves grown for 2 weeks in control, frass or ISR soil treatments. Leaves were inoculated with *Botrytis cinerea* spores (BC, red bars) or Vogel solution (Mock, blue bars), and harvested at 8 hours, 24 hours or 48 hours post inoculation (hpi). All values are relative to *WRKY75* expression in plants grown on Ctrl soil, mock-inoculated and subsequently incubated for 8 h (set to 1). Error bars show 95% confidence intervals (CI), but the upper CI for BC Ctrl 8 h (133.8), BC Ctrl 48 h (481.2), BC Frass 48 h (42940.0), and BC IS 48 h (635.6) are capped in the figure for presentation purposes. Letters denote significance within the same time group (no comparisons between time groups), Tukey-Kramer multiple comparison test, $p < 0.05$.

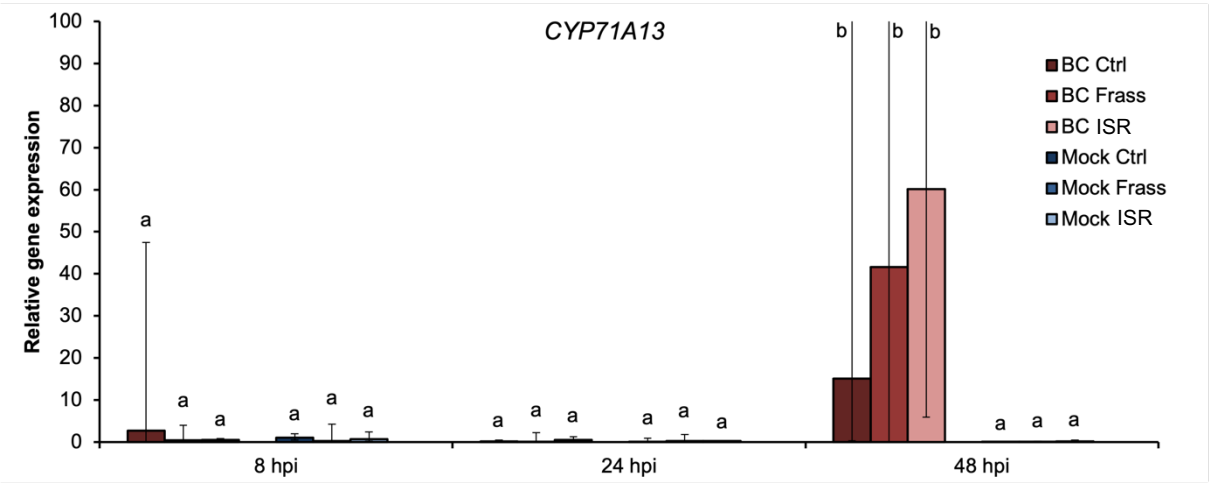



**Figure 4.17: Gene expression analysis of *CYP71A13* target gene after *B. cinerea* infection.** 5-week-old *Arabidopsis* Col-0 leaves grown for 2 weeks in control, frass or ISR soil treatments. Leaves were inoculated with *Botrytis cinerea* spores (BC, red bars) or Vogel solution (Mock, blue bars), and harvested at 8 hours, 24 hours or 48 hours post inoculation (hpi). All values are relative to *CYP71A13* expression in plants grown on Ctrl soil, mock-inoculated and subsequently incubated for 8 h (set to 1). Error bars show 95% confidence intervals (CI), but the upper CI for BC Ctrl 48 h (734.), BC Frass 48 h (28682.6), and BC IS 48 h (548.5) are capped in the figure for presentation purposes. Letters denote significance within the same time group (no comparisons between time groups), Tukey-Kramer multiple comparison test, $p < 0.05$.

### 4.2.5 Pathogen assays with *B. cinerea* and *P. syringae* on *Arabidopsis thaliana*

To monitor the effect of frass and ISR on pathogen resistance of *Arabidopsis thaliana* two separate experiments were conducted.

In the first pathogen assay, *A. thaliana* Col-0 was germinated and grown for 3 weeks in soil, before being transplanted into new pots with control, frass, or ISR treatments in C-soil and grown for another 2.5 weeks. Two leaves per plant were inoculated with spores of the necrotrophic fungus *B. cinerea* and incubated for 7 days before disease symptoms were registered. Severity of infection was rated from 0 (no lesion) to 5 (whole leaf infected) and the distribution of infection severity among the different treatments was assessed (Figure 4.17). Many *B. cinerea* inoculations led to no infection at all, while others resulted in small to large lesions. Due to the large variations within treatments, the mean lesion size and standard error are not presented, and statistical analyses were not conducted on these results. Figure 4.17 merely shows a potential trend rather than conclusive results.

Plants treated with ISR seemed to suffer less severe *B. cinerea* infections compared to control and frass treatments, which had somewhat more cases of severe infection. However, most plants resisted the pathogen quite well, as indicated by the left-shifted graph.

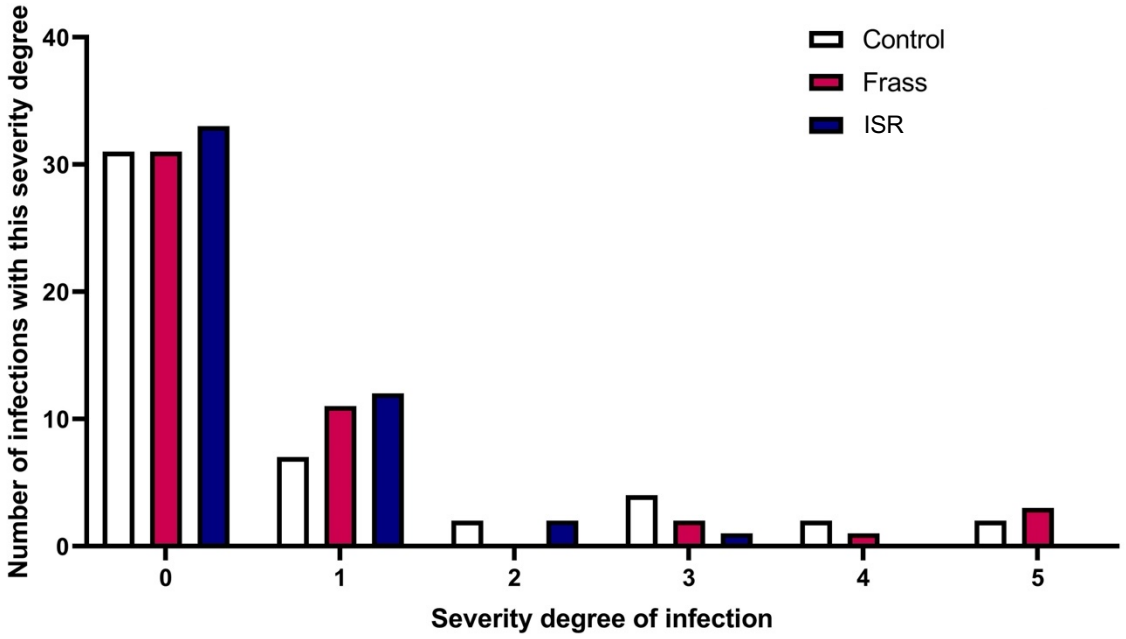



**Figure 4.17: Severity degree of *B. cinerea* infections in *A. thaliana*.** *Arabidopsis thaliana* Col-0 were grown for 3 weeks on soil before being transplanted to new soil (control) or soil supplemented either with frass or ISR (insect skin residue) and grown for 2.5 additional weeks. Leaves where inoculated with a *B. cinerea* spore solution (2 µl droplet, $2.5x10^5$ spores/ml, 1-2 droplets per leaf) and incubated for 7 days. Severity degree of infection from each droplet were rated from 0 (no lesion) to 5 (whole leaf infected). The graph displays the number of droplets within each treatment that led to the different severity degrees.

In the second pathogen assay, we attempted to repeat the previous procedure with sowing plants directly on soil supplemented with frass and ISR (i.e. resulting in a longer exposure to the treatments) and inoculating them with a 4 times higher *B. cinerea* spore concentration. *A. thaliana* was thus grown for 4.5 weeks before two leaves per plant were inoculated with *B. cinerea* spores. No visible disease symptoms were detected after 9 days. Instead of discarding the plants, other leaves of these plants were inoculated with the hemibiotrophic bacterium *Pseudomonas syringae* DC3000 by needle-less syringe injection and incubated for 4 days.

Disease symptoms were visually assayed. All inoculated leaves showed symptoms of infection, but no clear difference in lesion size was detected between treatments. However, it was observed that in the ISR treatment infections primarily led to chlorosis of leaf tissue, while control and frass treatments frequently showed symptoms of necrosis (Figure 4.18).

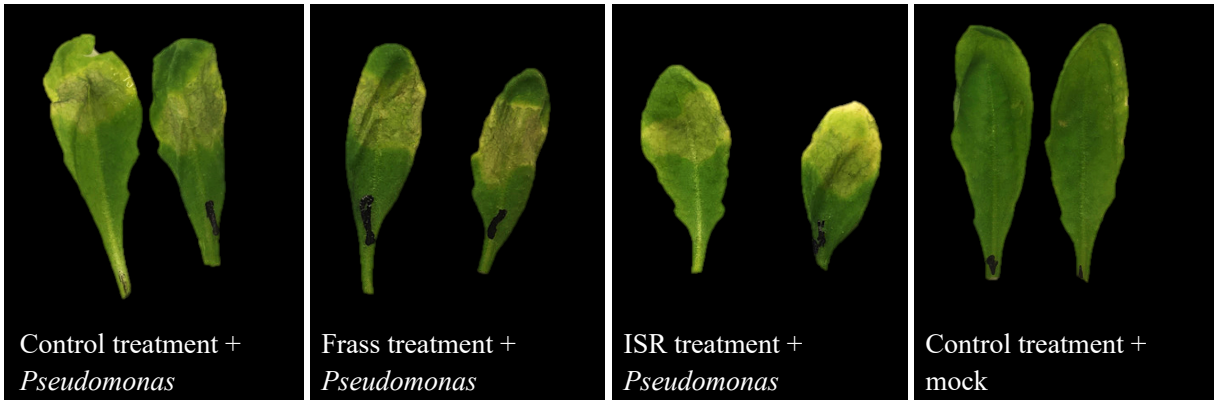



**Figure 4.18: *P. syringae* infections in *A. thaliana*.** Representative *Arabidopsis thaliana* leaves (grown for 4.5 weeks in control, frass, or ISR (insect skin residue) soil treatments) were inoculated with *B. cinerea* droplets ($10^6$ spores/ml) and incubated for 9 days, before other leaves on the same plants were infected with *Pseudomonas syringae* ($OD_{600}$ = 0.02, in 10 mM $MgCl_2$) for 4 days, with two injections per leaf, on each side of the midrib on abaxial side of leaf. Mock treated leaves were inoculated with 10 mM $MgCl_2$.

### 4.2.6 Pathogen assay with *B. cinerea* on tomato

Tomato (*Solanum lycopersicum* var. Roma VF) plants were grown for 12 days in C-soil before transplanted into new C-soil (control) or C-soil supplemented with either frass or ISR and grown for another 2 weeks. Two leaves per plant were inoculated with *B. cinerea* spores and incubated for 72 hours before lesions diameters were measured.

As seen in Figure 4.19, there was little difference in lesion size between treatments. ISR treatment led to slightly smaller lesions, but this was not significantly different compared to control or frass treatments.

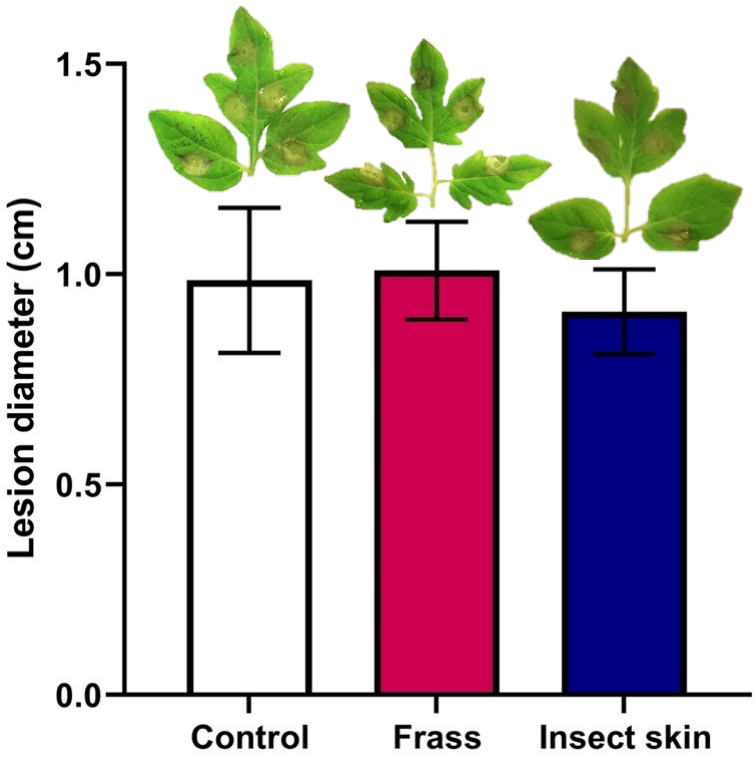



**Figure 4.19**: ***B. cinerea*** **lesions on leaves of tomato plants.** Tomato plants (*Solanum lycopersicum* var. Roma VF) were grown for 2 weeks in control, frass, or ISR (insect skin residue) treatments before infected with *B. cinerea* for 72 hours. Representative pictures of a leaf with lesions are shown for each treatment (contrasts are increased for better visibility of lesions). Each bar represents average lesion diameter (cm) of 14 replicates ± SD. Each replicate is the average of up to 5 lesions on a single leaf. No statistical significance was observed between treatments by a Tukey-Kramer multiple comparison test, $p < 0.05$.

# 5. Discussion

Insect frass fertilizer is a novel product unknown to many and is often met with skepticism. There has been a growing interest in this product the last few years, as the insect production industry has received an increasing amount of attention. However, there is still a very limited amount of literature on this topic. This thesis is therefore not only reporting and discussing the positive results achieved but is also structured as an exploration of what methods one can use in order to investigate the fertilizing and potential immune stimulating properties of frass. Unsuccessful as well as successful experiments are explained in detail, as are the reasoning behind the method selections, and plausible reasons for why it sometimes didn't work out as planned. This way, future research on the topic can avoid inappropriate methods and save time.

## 5.1 Fertilizer qualities of mealworm frass

Although the potential plant stimulant properties of frass are intriguing, it was deemed necessary to prove its effect as a fertilizer before looking at these characteristics, as summarized by the first study aim. After all, plant fertilization is its intended use as of today.

Firstly, the theoretical fertilizer capabilities of frass are assessed. The nutrient profile of the mealworm frass obtained from Invertapro (Appendix 1.1) shows good levels of both macronutrients as well as several micronutrients, which suggests promising fertilizing qualities. Poveda et al. reported that mealworm frass can have a nitrogen content ranging from 2.7% to 7.8%, indicating that the frass from Invertapro (2.6% N) is somewhat low in nitrogen. Yet, during growth trials and abiotic stress experiments, the authors found that frass with 2.9% nitrogen performed much better than the one containing 7.8% nitrogen at a 2% volume inclusion, a result attributed to the low carbon to nitrogen ratio in the latter frass version (Poveda et al., 2019).

The nitrogen to phosphorus ratio in the mealworm frass is quite low compared to other manures (Table 1.1), and especially low compared to most inorganic fertilizers. P toxicity might therefore be of concern when using frass as the sole fertilizer for nitrogen-demanding plants, so a combination of frass and a N rich fertilizer can be sensible in some cases.

Lab analyses alone are not sufficient proof, so growth trials were designed to demonstrate the fertilizing qualities of mealworm frass. Fortunately, since 2019, when this thesis was first designed, much more research has emerged on the topic, supporting the claims of frass being an able fertilizer. Most research has focused on frass from the black soldier fly larvae, but those that look at mealworm frass conclude that it is a very promising fertilizer (Houben et al., 2020; Poveda et al., 2019).

### 5.1.1 Frass has a fertilizer effect when applied to tomato (*Solanum lycopersicum*)

The growth trial with tomato tested different inclusion rates of frass, among them the 2% by volume used by Poveda et al. 2019. At this dose frass had a fertilizing effect, increasing shoot growth compared to the negative control, although not significant (Figure 4.1). This effect of frass was comparable to that of the positive control treatment (i.e. organic fertilizer NG) after 76 days. However, after 111 days the positive control showed significantly higher shoot growth compared to the negative control, while the frass treatments did not.

Initially, the reason for the increased effect of the organic fertilizer was thought to be higher amounts of nitrogen available to plants in chicken manure compared to mealworm frass (Table 1.1). This can indeed be part of the explanation, but the main reason was likely that the NaturGjødsel (NG) treatments contained more than twice the amount of NPK than the frass treatments. Frass and crushed NG pellets were mistakenly assumed to have approximately the same volume weight, while later measurements revealed that crushed NG in fact has about the double volume weight of frass. This, combined with the fact that NG has a NPK of 6-4.5-5 while the NPK values for the mealworm frass is 2.6-1.8-2.8, the positive treatments were given a lot more nutrients than what was intended, explaining the high biomass of these plants.

This difference didn't become apparent before the second harvest, where the positive treatment yielded clearly larger plants than the frass treatment, although non-significant. An explanation to this delayed effect is that the young tomato plants received sufficient nutrients in their initial growth phase, helped by the fact that about 40% of mealworm frass nitrogen mineralizes during the first 20 days after fertilization, as seen in Figure 1.4 (Houben et al., 2020). The nutrient demanding older plants on the other hand benefited greatly from the increased NPK in the positive treatment.

The main base soil, Ctrl+, was potting soil with extra NPK added from NG. The reasoning was to increase the NPK values of the base soil, as many tomato farmers typically use a nutrient rich soil, supplied with extra fertilizer (personal communication). However, due to the miscalculations of NG amounts, the Ctrl+ treatment also ended up receiving quite high levels of NPK, probably leading to the non-significant differences between this and most frass treatments (Figure 4.1 and 4.2). The small number of replicates per treatment also contributed to the statistically weak results.

Nevertheless, it seems like a higher dose of frass increases tomato plant growth as expected (Figure 4.2), but that 10% leads to some kind of toxicity (reduced growth in first harvest). After 111 days, the 10% treatments yielded in fact most shoot biomass, but with malformed leaves and no tomato fruits. The reason for this toxicity is unknown, but it may be attributed to high amounts of phosphorus. Jones (1998) mentioned in his paper on P toxicity in tomato plants that "firing" of leaf margins can be a symptom of P toxicity (J. B. Jones, 1998), which might be the same leaf malformation that was observed in this experiment. Indeed, due to low N/P ratio in frass, the 10% treatments contained very high levels of P. Other possible explanations can be toxic levels of heavy metals such as zinc (Rout & Das, 2009), that are quite abundant in mealworm frass (Appendix 1.1), or some other compound in frass that might lead to stress responses in plants, such as chitin (Sharp, 2013).

Root growth seemed to be increased by lower nutrient values at early stages (Figure 4.1 and 4.2), while the second harvest yielded the biggest roots in the 2% fertilized treatments. This might be attributed to low nutrient content in soil leading to higher initial root growth because the plants are searching for nutrients, while later, the bigger plant can invest more recourses into its roots, while it also needs more water to sustain its increased biomass. As with shoot growth, 10% frass seemed inhibitory to root growth, particularly in young plants. The small roots in 10% treatments may actually be the reason for the stunted shoot growth in this treatment early on.

We expected that untreated frass would perform better than sterilized frass in this growth trial due to frass containing growth promoting microorganisms, as shown by Poveda et al. (Poveda et al., 2019). In our experiment the sterilized frass actually resulted in a slightly higher shoot biomass than untreated frass, however non-significant (figure 4.1). It might be that the process of sterilization by autoclaving changed the properties of the frass, such as increased mineralization of nutrients, leading to increased growth.

The different treatments did not lead to significant differences in tomato fruit production (based on dry weight), except for CTRL and HF 10% (Appendix 1.6). However, only green tomatoes were harvested, and many were small and still growing. The 10% treatment led to no flower production and these plants could therefore not develop fruits, which is attributed to the fertilizer toxicity mentioned above.

Even though this experiment indicated a fertilizing effect of frass, this was not statistically significant compared to control soil, and some questions remain unanswered. It should be repeated with the following modifications:

1. Correct amounts of positive control fertilizer calculated on weight not volume basis, and a lower amount of nutrients in base soil.
2. More biological replicates per treatment in order to achieve statistically significant results.
3. One or two more treatments with soil containing frass between 2% and 10% by volume could be included to disclose the threshold where frass leads to toxicity in tomato.
4. A longer experiment, until ripe tomato fruits are obtained.

### 5.1.2 Frass is able to restore growth of nutrient deficient sunflowers (*Helianthus annuus*)

Also supporting frass' capability to provide nutrients to plants, this experiment revealed that mealworm frass contains P, K, Ca, and S at high enough levels to be the sole nutrient provider for one sunflower at 5% volume inclusion (Figure 4.3). The reason why 5% volume inclusion was chosen instead of 2% as in the tomato growth experiment, was that we wanted to apply as much frass as possible per pot in order to get closer to the nutrient content provided by the Hoagland solution. 5% seemed reasonable as 10% led to toxicity in tomato.

Nutrient deficiency led to strong growth reductions, while application of frass restored plants to normal growth in these treatments. To use the Liebig analogy (Figure 1.3); the short plank in the barrel is replaced with a longer one, and the barrel can be filled to the brim with water, without leaking. This result is somewhat surprising, as frass provided a lot less nutrients to the soil compared to the Hoagland solution, except for phosphorus (Table 3.2). This suggests that the Hoagland solution contained many nutrients in excess compared to the requirements of the sunflower.

The only case where frass was not sufficient as the sole nutrient provider was in N deficient treatments. These plants did not exhibit normal growth when frass was mixed in the soil. The plants received approximately 2.4 times more nitrogen through the nutrient solution than through frass, which probably explains this difference in growth. The fact that nitrogen in frass is mainly bound to organic compounds instead of existing as plant-available ammonium or nitrate should also be contributing to the reduced growth. As mentioned, the nitrogen content in mealworm frass breaks down quickly the first month after fertilization, with approximately 40% mineralized N after 20 days, Figure 1.4 (Houben et al., 2020). However, after 60 days, only 10% additional nitrogen is reported to become available to the plant, with a subsequent slow release of the remaining nitrogen. So, after 8 weeks, it is likely that not more than 50% of frass N had been absorbed by the plant. This is not counting the fact that the potting substrate used was acid washed sand with perlite, which most certainly has a poor microbiota. Without microorganisms decomposing organic compounds, nitrogen mineralization goes even slower (Taiz et al., 2015b), possibly leading to even less than 50% of frass N being mineralized in this trial. Poveda et al. showed that a myriad of plant growth promoting microorganisms exists in mealworm frass (Poveda et al., 2019), which might have improved the situation.

Larger amounts of frass could have been used to compensate for this lack of nitrogen, but as shown in the tomato trial, 10% volume inclusion led to some kind of toxicity. The plants receiving the complete nutrient solution in addition to 5% frass showed already some signs of toxicity similar to that observed in the tomato trial, namely curling of young leaves.

This treatment (Full + frass) also had increased growth compared to all other treatments, suggesting that frass is able to increase plant growth even when there are ample amounts of nutrients in the soil.

These experiments on sunflower and tomato supports the claims that frass works as a fertilizer on different plant species, as summarized by Poveda (2021). It also showed the possibility of combining frass with other nitrogen rich fertilizers for nitrogen demanding plants.

## 5.2 Plant defense responses to frass

After confirming fertilizing properties of the mealworm frass, its effect on plant immune responses was assessed. Research on crustacean chitin has shown that chitin induces plant defense (Sharp, 2013). This is a well-known phenomenon, and a large bulk of research has been published describing everything from field studies investigating plant resistance against specific pathogens, to detailed studies on signal cascades following chitin recognition (Shamshina et al., 2020; Sharp, 2013). Hence, the presence of chitin in frass is often emphasized by frass fertilizer producers, claiming that their product can bolster plant's resistance to pests

and pathogens, but these claims are not yet backed by literature (Temple et al., 2013). Several frass researchers have also mentioned this aspect of frass and stressed the importance of more research (Elissen et al., 2019; Poveda, 2021; Quilliam et al., 2020), but no convincing papers have actually confirmed this hypothesis so far. It is evident that research on plant immune stimulating effects by frass is needed and that it will benefit both researchers, farmers, and producers of frass.

### 5.2.1 Documentation of immune responses triggered by mealworm frass: the initial attempts

The most natural approach to this issue is to look at how pure chitin leads to defense responses in plants, and how this is commonly measured. Two methods widely used for detection of plant immune responses by chitin are production of reactive oxygen species (ROS) and callose deposition (Bisceglia et al., 2015; Schenk & Schikora, 2015). These are based on stimulation of the immune response with elicitors dissolved in solution, and thereafter measuring the triggered defense response. These methods were chosen as appropriate starting points, addressing study aim 2.

After much testing and optimization of the ROS assay, positive results were achieved with the chitin oligomer-mix (as also shown in Figure 4.5 and 4.6). However, no ROS formation was observed with ISR (Figure 4.6) or frass solutions (Figure 4.5). Similarly, in the pilot callose assays, plants treated with chitin showed clear callose production in roots, while both frass and ISR solution showed very little callose deposition (Figure 4.7).

The results achieved with the chitin oligomer-mix are consistent with what has been previously reported for ROS production (Albert et al., 2006) and callose deposition in roots (Millet et al., 2010) as a response to chitin oligomers, showing that the protocols worked.

However, most studies use chitin processed to contain only a desired oligomer size of 6-8 (usually the octamer), as these are documented to possess the strongest immune stimulating effect in plants (Li et al., 2020). Contrarily, the chitin in frass originating from insect skin residue (ISR) probably exists in the form of nanofibers bound to minerals and proteins (Vincent & Wegst, 2004) and might therefore not be able to activate plant immune responses without degradation. Separation of chitin nanofibers from proteins and minerals will likely lead to some plant recognition, as chitin nanofibers also have been documented to activate plant defense responses (Egusa et al., 2015), whereas fragmentation of these fibers will result in more chitin oligomers that can be detected by the chitin receptor complexes.
The chitin contribution to frass by the insect gut peritrophic membrane (PM) might be significant, as some studies show that fecal pellets of some insect species are covered in PM when excreted (Brandt et al., 1978; P. Wang & Granados, 2001). As the PM is less structurally robust than the insect cuticle, it might be more easily degradable, potentially resulting in faster generation of the eliciting oligomers in frass.

In order to break down insect skin to chitin fragments, a wide range of enzymes are needed, including proteases and chitinases (Ali et al., 2010). It is known that the insect exoskeleton can be degraded by soil microorganisms such as entomopathogenic fungi (Ali et al., 2010) or

chitinolytic bacteria that feed on chitin (Sharp, 2013). Plants also secrete chitinases, contributing further to chitin degradation (Ramonell et al., 2005). It is likely that degradation of the PM also requires various enzymes such as chitinases. One cannot assume that these are already present in frass and must therefore be contributed by external sources. According to this hypothesis, the frass and ISR solutions were unable to stimulate immune responses in plants because they were not sufficiently degraded.

In contrast, when frass is incorporated in soil for a longer time period, it is thought that chitin fibrils and oligomers are generated by the enzymatic activity of microorganisms, and that these compounds will stimulate immune responses in nearby plants. However, research on this has to the best of our knowledge not yet been published, so the time frame in which this happens is unknown.

Consequently, an alternative experimental setup was designed to facilitate degradation of frass and ISR, as described in Methods section 3.5.2. Frass and ISR were premixed in C-soil and one *Arabidopsis* plant was planted into the soil mix. The compost in the C-soil aided the development of a rich microbiota, while the plant was included to stimulate growth of microorganisms and for potential secretion of chitinases. Soil samples were extracted at different time points due to the unknown time frame of the degradation and tested for potential immune stimulating effects. Both ROS and callose assays was repeated with these new solutions. Neither methods showed any convincing results for the frass or ISR soil treatments (Figure 4.5 and 4.7), nor did the short-term gene expression assay (Figure 4.8 to 4.10).

There could be many reasons for the lack of results, such as insufficient degradation, or that the solutions were too dilute. Alternatively, the organic matter in soil (or frass) could potentially be interfering with the methods used.

To test the latter suggestion, a ROS experiment was designed where the chitin positive control (250 µg/ml) was prepared with filtered soil or frass solutions (Figure 4.5). In contrast to chitin prepared with $ddH_2O$ (250 µg/ml), chitin prepared with soil solutions showed much lower luminescence signals. The reason for this can probably be attributed to the fact that the soil solutions had a slight brown color, i.e. contained substances that might have interfered with the reaction or absorbed the light produced, thereby reducing luminescence detected by the plate reader. Similarly, chitin added to pure frass solutions (i.e. frass prepared in $ddH_2O$), which had a light brown color after filtration, yielded no luminescence response at all (Figure 4.5).

Similar solutions were tested in the callose assay, showing a clear defense response triggered by the chitin treatment (dissolved in control soil solution), suggesting no interference by soil with that specific method (Figure 4.7).

These experiments revealed that the widely used ROS assay by chemiluminescence is not an appropriate method to detect immune responses in plants triggered by frass unless extensive modifications are made to the procedure.

As mentioned, it can also be argued that the soil solutions with frass were too dilute, containing insufficient concentrations of the active chitin elicitors. A very rough calculation (shown in Appendix 2.2) estimates that about 1-14 µM immune stimulating chitin fragments (DP 6-8) exist at any given time point in soil solutions with 2% frass (2.5-35 µM in 5% treatment). For ROS assays that will be further reduced to 0.2-2.8 µM, as elicitor solutions are diluted 5x in

wells. This concentration range should be sufficient for the ROS assay as we were able to detect consistent ROS production from leaf disks elicited with a 12.5 µg/ml chitin oligomer mix (Figure 4.6), which should correspond to approximately 1 µM of chitin oligomers with DP 6-8 (based on the calculations presented in Appendix 2.1). However, concentrations might have been even lower, perhaps explaining the lack of a defense response (ROS, callose, gene expression) when treating with frass soil solutions. Other factors might however have contributed as research has demonstrated that chitin octamer concentrations down to 1 nM can trigger immune responses in plant (e.g. gene expression changes reported by Zhang et al., 2002).

### 5.2.2 Plants exposed to frass and insect skin in soil exhibits possible priming effects

The abovementioned difficulties indicated that trying to elicit plants with frass or insect skin residue (ISR) and shortly thereafter measure the defense response was the wrong methodology, mainly because the appearance of immune stimulating chitin molecules could not be confirmed in the elicitor solutions.

A new approach was therefore investigated, with study aim 3 in focus. Plants grown for several weeks in soil containing frass or ISR should in theory be stimulated at some point. Detecting the initial immune response could prove difficult, as it could happen at any time point during this period and would likely be reduced over time. To overcome this issue, plants grown in soil containing frass or ISR were infected with the necrotrophic fungus *Botrytis cinerea* and monitored for increased pathogen resistance due to a priming effect of frass and ISR. A pathogen assay measuring lesions in infected leaves was thus conducted with *Arabidopsis thaliana* and tomato (*Solanum lycopersicum*), and a gene expression analysis of defense related genes was done with *Arabidopsis thaliana* (GE-BC).

The latter experiment showed some interesting results. Although not always statistically significant, a general trend was seen in all genes tested: fertilization with frass or ISR led to higher expression of target gene, often in both mock and *B. cinerea* treated plants. Two very distinct response patterns were observed:

- **Group 1** (Figures 4.11 to 4.15; Appendix Figure A1.1): 2 to 10-fold induction of genes treated with frass or ISR at 8 hpi (compared to control plants at 8 hpi), with a slight decrease in induction over time. Quite similar for BC and mock treatments at 8 and 24 hpi, while BC infection resulted in higher gene expression than mock at 48 hpi, except for *WRKY53*.
- **Group 2** (Figures 4.16 and 4.17; Appendix Figure A1.3): Very low levels of expression in all treatments until 48 hpi, where the BC infection led to a 10 to 60-fold induction (compared to control plants at 8 hpi), with the highest expression values for frass and ISR treatments.

Some rather large biological variation, possibly due to an uneven infection rate, was observed. The assay should therefore be repeated with an *Arabidopsis thaliana* accession or mutant

showing a higher susceptibility for the *Botrytis cinerea* isolate CECT2100 used (Birkenbihl et al., 2012). Alternatively, *Botrytis cinerea* isolates with a higher virulence for the *Arabidopsis thaliana* accession Col-0 could be tested (Denby et al., 2004).

Nevertheless, our results strongly indicate that frass and ISR was indeed recognized by the plant and induced the expression of defense genes, particularly if infected by *B. cinerea*, which implies a priming effect. The fact that pure ISR in most cases led to as high (or higher) expression as frass, suggests that this is the stimulating factor, and not some other compound in frass.

In the gene expression analysis with elicitor treatments (GE-ET), chitin led to increased expression *of ZAT10, ERF5* and *PER4* as early as 30 mins post elicitation, before decreasing after 3 hours (Figure 4.8 to 4.10). This is consistent with earlier reports on the chitin-triggered changes in expression for these genes (Ramonell et al., 2002; Zhang et al., 2002, Wan et al., 2008). Chitin in frass should be able to trigger this response as well, which might be an indication of the initial stimuli leading to increased responsiveness towards frass (priming).

Interestingly, the frass and ISR treatments led to an increased expression of genes in Group 1 even without infection of *B. cinerea* (Figures 4.11 to 4.15). Expression of defense genes when there is no actual attack can be very costly for plants, as resources must be re-allocated from important functions such as growth and seed production (Denancé et al., 2013). This is why the priming mechanism exists, and a long-lasting defense response after frass application is not necessarily a good thing.

However, these gene expression levels in mock treatments had decreased substantially after 48 hours, in contrast to the BC treatments. This may reveal that plants treated with the mock solution became stressed by the procedure, perhaps due to handling of plants and the sudden change of environment during the inoculation (removed from growth room to lab bench, with the following change in humidity, temperature, and light conditions). Indeed, several genes tested including *ZAT10*, *ERF5* and *WRKY33*, are linked to responses to abiotic stress as well as biotic stress, supporting this theory (Mittler et al., 2006; Pan et al., 2012; Xinjing Wang et al., 2013). This can be the reason why plants inoculated with *B. cinerea* also displayed increased expression of several target genes (Group 1) in frass and ISR treatments at 8 and 24 hpi and might suggest that the pathogen wasn't actually recognized by the plant this early.

Since plants reacted more strongly to this potential abiotic stress when treated with frass and ISR compared to control treatment, it might suggest that these treatments also affect plant responses to abiotic stresses, as described by Poveda et al. (2019). In fact, the CERK1 receptor has been linked to responses against abiotic stress, supporting this (Espinoza et al., 2017; Gong et al., 2020).

As seen with *WRKY75*, *CYP71B15* and *CYP71A13* (Group 2), it is first after 48 hpi that these genes get significantly expressed, also suggesting that the plant recognized the infection somewhere between 24 and 48 hpi (Figures 4.16 and 4.17; Appendix Figure A1.3).

This delayed infection corresponds quite well with previous research looking at gene responses in *Arabidopsis* after *Botrytis cinerea* infection. AbuQamar et al. used 24 hpi as the first time point after infection where gene expression was analyzed. At this time point, *ZAT10* and *MYB51* had a fold-increase of approximately 10 (compared to mock treatments), while

*WRKY53* and *ERF5* had a fold-increase of about 4 (AbuQamar et al., 2006). Sham et al. used 18 hpi as the first time point analyzed, where 2-3-fold increase was observed in gene expressions of *WRKY53*, *MYB51* and *ERF5*, 15-fold in *WRKY33* and 83-fold in *CYP71A13* (Sham et al., 2019).

The results obtained in this thesis indicate as mentioned a successful infection between 24 and 48 hpi. This is even more delayed than the abovementioned studies. The reason for this might be attributed to the low virulence of the *B. cinerea* strain on the *Arabidopsis* ecotype used. More similarly to our experiment, Ferrari et al. showed that *CYP71A13* was induced only 8-fold after 18 hpi, while it was induced 45-fold after 48 hpi (Ferrari et al., 2007).

For *WRKY75*, *CYP71B15* and *CYP71A13*, frass and ISR also led to an increased expression compared to control. This indicates a priming effect, where expression only happens after the pathogen is detected, and more quickly in plants treated with frass and ISR.

It has been shown that *WRKY75* is not triggered by the abiotic stresses drought and heat (López-Galiano et al., 2018), which supports the theory that abiotic stress led to the early responses in Group 1, while Group 2 only reacted to the biotic stress of the pathogen. However, *CYP71A13* and *CYP71B15* might have a role in abiotic as well as biotic stress response through their involvement in the synthesis of the phytoalexin camalexin (Xu et al., 2008).

Both *B. cinerea* and chitin is known to trigger the jasmonic acid (JA) pathway in *Arabidopsis*, leading to increased resistance against necrotrophic pathogens and herbivores (Sharp, 2013; Windram et al., 2012). Most genes investigated in this experiment are known to be JA dependent, suggesting that JA production in the plant was triggered by frass or ISR in soil.

As an exception, *WRKY53* is mainly thought to be connected to the SA pathway (Hu et al., 2012). In our experiment, *WRKY53* expression was induced after 8 and 24 hpi, but not after 48 hpi (Figure 4.15), possibly the time point where the pathogen is perceived by the plant. This indicates that increased expression of *WRKY53* is a response to the abiotic stresses but not to the biotic stressed inflicted upon the plant in this experiment. Previous research has documented that *WRKY53* is indeed expressed during abiotic stresses (Van Eck et al., 2014). Even though *WRKY53* is linked to the SA pathway, it seemed to be stimulated by frass and ISR treatments, similar to the JA associated target genes. The increased expression of *WRKY53* in these treatments at 8 hpi might indicate that frass/ISR also influences SA levels related to abiotic stress (Ahmad et al., 2019).

These results suggest that frass can prime plants against pathogens and pests, probably through the JA pathway. As JA and SA are thought to be antagonistic, stimulation of JA synthesis might lead to suppression of SA synthesis. This means that if frass is increasing the plant's resistance against necrotrophic pathogens due to induction of the JA pathway, it can potentially lead to susceptibility against biotrophic pathogens and phloem feeding insects as SA is important in the defense against these. Analyzing the expression of additional genes linked to SA biosynthesis and signaling could strengthen these claims. Also, the measurements of JA and SA levels in plant tissue exposed to frass should be considered in future experiments, as it can give a clear indication of which type of defense response that are induced.

### 5.2.3 Pathogen assays

It is easy to assume that a defense stimulus activated by frass triggers a response against insect pests. However, many insect pests operate aboveground, feeding on plant leaves. Obviously, a defense response against such an insect attack that only occurs after its frass has been incorporated into soil, would be far too delayed and probably not evolutionary viable. There are also insect pests that live in soil and feed on plant roots, but recognition of chitin or frass is likely not the fastest way for a plant to recognize these either. Damage associated molecular patterns (DAMPs) that occur during wounding of plant tissue, or herbivore associated molecular patterns (HAMPs) from insect saliva or regurgitant are more likely cues of an insect attack (Ray, Alves, et al., 2016). On the other hand, plant responses against fungal pathogens are often a result of chitin recognition (Gong et al., 2020). It is therefore reasonable to think that recognition of chitin in soil signals the appearance of a fungal pathogen, not an insect pest, regardless of the chitin's origin.

This means that if chitin is the immune stimulating component of insect frass, it might not activate a defense response against insects, but rather against fungal pathogens. There might however be other compounds in frass that plants could recognize (HAMPs), actually leading to an insect directed defense response (Ray, Alves, et al., 2016). Interestingly, Ray et al. showed how maize can detect proteins in fall armyworm (*Sporoptera frugiperda*) frass deposited in leaf wounds, leading to increased performance by the insect instead of suppressing it, while improving plant resistance against a fungal pathogen, showcasing how complex these plant-herbivore relationships can be (Ray et al., 2015).

To assess whether the defense gene expression triggered by frass or ISR resulted in an increased plant resistance against fungal diseases, pathogen assays were performed.

Disease assay with *Botrytis cinerea* in *Arabidopsis* only led to symptoms in less than half the infected leaves in each treatment (Figure 4.17). The reason for this was likely that the wild-type *Arabidopsis* Col-0 is highly resistant against the selected *B. cinerea* isolate CECT2100, as already mentioned for the GE-BC experiment. A different *B. cinerea* isolate, such as the B05.10 isolate (Liu et al., 2017), should therefore be used in further experiments on *Arabidopsis* Col-0 in order to get consistent infection and assess a positive effect of frass and ISR. Unfortunately, no other *B. cinerea* isolate was available to us, and legal procedures and shipping time for a new *B. cinerea* isolate was too long due to the corona pandemic in order to be included in this project.

Nevertheless, as seen in Figure 4.17, a trend can be observed where ISR treatment resulted in less severe infections compared to frass and control treatments.

When this experiment was repeated with plants sown and grown for 4.5 weeks in treatments (in order to increase exposure time to frass and ISR), and higher *B. cinerea* spore concentration, lesions were not observed in any leaves. The reason for this might be that the fungal spores were inactive, changes in the procedure, or that plants were strong enough to resist the infection completely. Indeed, plants looked a lot healthier compared to the preceding experiment, where many plants were noticeably stressed. This might be attributed to the transplanting of seedlings in the first experiment.

It was therefore decided to inoculate these plants with *Pseudomonas syringae*, a hemibiotrophic bacterium, in order to test the hypothesis that frass primes plants against necrotrophic pathogens but might render them more vulnerable against biotrophic ones. This was particularly interesting since plants already responding to *B. cinerea* might have a defense hormone shift largely towards JA. Interestingly, it has been shown that *P. syringae* infection in *Arabidopsis* can lead to a priming effect of CERK1, resulting in a faster response towards a simultaneous *B. cinerea* infection (Gong et al., 2019). Whether this works the other way around, with *B. cinerea* infection leading to improved resistance against *P. syringae*, is not known.

It was observed that the *P. syringae* symptoms on plants treated with ISR were mostly chlorotic, while frass and control treated plants displayed necrosis in many leaves (Figure 4.18). This might indicate that the transition of *P. syringae* from the biotrophic (chlorosis) to the necrotrophic (necrosis) lifestyle was retarded in ISR treated plants. Frass treatment did not seem to lead to increased susceptibility towards the pathogen as postulated. Optimally, the development of *P. syringa*e in plant tissue by bacterial count or DNA quantification should have been conducted in order to get a more reliable result, but due to time restrictions only a visual assessment was possible.

In contrast to *Arabidopsis thaliana* Col-0 wild-type, the *B. cinerea* isolate CECT2100 is virulent against tomato (*Solanum lycopersicum*) (Finiti et al., 2014). In this experiment, all infected leaves displayed clear necrotic lesions, but with little difference between treatments (Figure 4.19). ISR treatment resulted in slightly smaller lesions compared to frass and control, but this difference was non-significant. Nevertheless, as indicated in our previous pathogen assays, ISR treatment might have an effect on necrotrophic pathogens, and this experiment showed a similar trend.

The results mentioned above indicate that ISR increases resistance against necrotrophs. This partly contributes to the hypothesis that frass (which contains ISR) induces resistance, even though frass treatments showed no tendency to do so in our assays. Hence, more research must be conducted in this field in order to reach a conclusion.

According to Quilliam et al., BSF frass application led to a potential increase in plant resistance against the hemibiotroph *Fusarium oxysporum* (Quilliam et al., 2020). Apparently, both JA and SA are involved in defense responses against *F. oxysporum* in *A. thaliana* (Lyons et al., 2015).

Choi & Hassanzadeh demonstrated resistance against the necrotroph *Pythium myriotylum* by plants fertilized with BSF frass (Choi & Hassanzadeh, 2019). Similarly, Elissen et al. showed increased defense by BSF frass fertilized plants against a natural *Pythium* infection (Elissen et al., 2019). The *Pythium* pathogen is usually a necrotroph, but can also have hemibiotrophic infection patterns, depending on the *Pythium* species (Lévesque et al., 2010).

These recent studies strengthen the hypothesis that frass can increase plant resistance against necrotrophs, and also indicate a possible effect on resistance against hemibiotrophs. The hypothesis that frass might induce resistance against some pathogens and lead to susceptibility against other pathogens, is very important to clarify, especially for farmers struggling with a specific type of disease or pest.

## 5.3 Potential impacts of insect frass in future agriculture

The fertilizing capabilities of frass should be sufficient to make it a utilizable product as there is an increasing global demand for organic fertilizers due the negative environmental impacts of inorganic fertilizers (Farooq et al., 2019). Its slow-releasing nitrogen might be of benefit for some farming practices, while others can combine it with more nitrogen rich fertilizers such as chicken manure, urea, or synthetic nitrogen in order to reach a desired nitrogen level. Compared to other common manure fertilizers such as poultry and dairy cow manure, frass is somewhat low in NPK (Table 1.1) on a dry weight basis. However, per wet weight, which is the most common condition of manure used by farmers, insect frass with its low moisture content has high levels of plant nutrients per kilo. Therefore, insect frass also has potential as an alternative to conventional manure fertilizers.

Results obtained in this thesis suggest that frass are able to trigger an immune stimulating and priming effect in plants, probably due to the content of chitin from insect skin residue in frass. These results alone are not enough to prove that frass is able to improve plant resistance against pathogens but can be an important step towards a possible confirmation of this theory. If future research is able to confirm this, frass can become a valuable tool for farmers in combating plant pests and diseases, as a sustainable alternative to synthetic pesticides. A possible priming effect by frass against abiotic stress was also observed during this thesis, which can become relevant in agriculture in order to cope with future challenges related to climate change.

Demonstrating the fertilizing effect of frass will also help the insect industry, as their by-product probably can be sold with profit. Further, a documentation of immune stimulating properties can significantly increase the value of frass, perhaps making it a noteworthy source of income for insect producers worldwide. This can in turn boost the viability and growth of this novel industry. As it addresses many sustainability issues our world faces today, this should be a very positive side-effect.

# 6 Conclusion and future perspectives

The aims of this thesis were to document the fertilizing properties of mealworm frass and to investigate its potential plant immune stimulating characteristics, with a focus on chitin.

The tomato growth trial showed that there is a fertilizing effect of mealworm frass, but due to a suboptimal experiment design, these results were not statistically robust, and the experiment should preferably be repeated with modifications. On the other hand, the nutrient deficiency trial with sunflowers revealed that the mealworm frass can contribute sufficient amounts of several macronutrients to support plant growth, with the exception of nitrogen, which was somewhat in shortage when frass was the only nitrogen source. Recent literature that has been published on the topic with mostly positive results give a strong indication towards frass being a valuable fertilizer, and that it can work as a substitute for less sustainable nutrient sources used in agriculture today. Still, more research should be conducted in this field, especially on mealworm frass, as most literature focuses on the frass from the black soldier fly larvae.

Different methods were tested in the attempt to document immune stimulating effects of frass in the model plant *Arabidopsis thaliana*. The initial experiments using ROS chemiluminescence and callose staining with frass or ISR (insect skin residue) suspended in water as elicitor solutions were not successful, possibly due to a lack of degradation of the material. To facilitate degradation, frass and ISR were mixed with a compost-containing soil for several weeks, and elicitation solutions were made with these mixtures. However, these solutions were also not able to produce any convincing responses from the ROS and callose assays, perhaps due to interference with the methods by soil particles. A gene expression analysis investigating short term changes in expression of defense related genes did also not reveal an effect of the frass soil solution.

A new approach was tested where *Arabidopsis thaliana* plants were grown in soil already treated with frass or ISR to expose the plant to the treatments over a longer time period. An infection with the fungal pathogen *Botrytis cinerea* in these plants led to a substantially increased expression of defense related genes in frass and ISR treatments, suggesting a priming effect possibly through activation of the jasmonic acid pathway. There were also indications of an increased response towards abiotic stresses in these treatments. Pathogen assays with *Botrytis cinerea* and *Pseudomonas syringae* yielded no convincing results but may have shown a positive effect against necrotrophic pathogens by the ISR treatment, supporting the theory that jasmonic acid is involved. These results indicate a potential immune stimulating effect by mealworm frass and can contribute to future research on this topic. However, more documentation is needed to confirm this theory. Improved pathogen assays, particularly in real farming systems, seems like a sensible future step.

It is concluded that frass can become a useful fertilizer in future agriculture, as its production is expected to significantly increase in the coming decades. If an immune stimulating and priming effect is documented for frass, it can become a very valuable resource for a transition towards a more sustainable agriculture, as well as help the insect industry in its future growth.

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# Appendix 1 – Supplementary figures and tables

## A1.1 Nutrient profile of the mealworm frass from Invertapro used in this thesis

**Table A1.1: Full nutrient profile of mealworm frass from Invertapro.** Ammonium, nitrate, and available P and K are included as these numbers represent the amounts of these nutrients available for absorption by plants without degradation of organic content. Data on the plant availability of other nutrients are not known. Analysis conducted by Eurofins Norway. n/a = data not available.

| Nutrient | mg/kg dry weight | % of dry weight |
|---|---|---|
| **NPK** | | |
| Total Nitrogen (N) | 28 000 | 2.8 |
| Ammonium ($NH_4^+$) | 1 800 | 0.18 |
| Nitrate ($NO_3^-$) | 100 | 0.01 |
| Total Phosphorus (P) | 19 000 | 1.9 |
| Available P | 13 000 | 1.3 |
| Total Potassium (K) | 30 000 | 3 |
| Available K | 23 000 | 2.3 |
| **Other macronutrients** | | |
| Calcium (Ca) | 6 400 | 0.64 |
| Magnesium (Mg) | 8 700 | 0.87 |
| Sulphur (S) | 3 900 | 0.39 |
| **Micronutrients** | | |
| Sodium (Na) | 500 | 0.05 |
| Boron (B) | 11 | 0.0011 |
| Cobalt (Co) | n/a | n/a |
| Copper (Cu) | 18 | 0.0018 |
| Iron (Fe) | 380 | 0.038 |
| Manganese (Mn) | 230 | 0.023 |
| Molybdenum (Mo) | 1.9 | 0.00019 |
| Zinc (Zn) | 150 | 0.015 |
| **Heavy metals** | | |
| Chromium (Cr) | < 0.5 | ≈ 0 |
| Nickel (Ni) | 1.3 | ≈ 0 |
| Cadmium (Cd) | 0.26 | ≈ 0 |
| Mercury (Hg) | < 0.05 | ≈ 0 |
| Lead (Pb) | < 0.5 | ≈ 0 |
| **Other properties** | | |
| Organic content | | 43.6 |
| Dry matter | | 93.2 |
| Water content | | 6.8 |

# A1.2 – Modified Hoagland solution

**Table A1.2: Recipe for the modified Hoagland solution used in the sunflower growth experiment.** The table displays the chemicals used to reach the desired amount of nutrients in each solution. All solutions ended up containing the same amount of N, P, K, Ca, and S, except for one nutrient which was removed completely from each solution. Full = the complete Hoagland solution without any missing nutrients. In addition, Mg, Na, Fe, and Cl was added to all solutions in appropriate amounts, as well as a concentrated micronutrient stock solution which reached a desired concentration when included at 1 ml/liter.

| **Stock solution** | **Stock conc. (M)** | ml of stock solution per liter of nutrient solution | | | | | |
|---|---|---|---|---|---|---|---|
| | | **-N** | **-P** | **-K** | **-Ca** | **-S** | **Full** |
| $Ca(NO_3)_2$ | 1 | 0 | 2.5 | 2.5 | 0 | 2.5 | 2.5 |
| $KNO_3$ | 1 | 0 | 5 | 0 | 5 | 5 | 5 |
| $MgSO_4$ | 1 | 2 | 2 | 2 | 2 | 0 | 2 |
| $KH_2PO_4$ | 1 | 0.5 | 0 | 0 | 0.5 | 0.5 | 0.5 |
| $NH_4NO_3$ | 1 | 0 | 1 | 3.5 | 3.5 | 1 | 1 |
| $MgCl_2$ | 1 | 0 | 0 | 0 | 0 | 2 | 0 |
| $NaH_2PO_4$ | 1 | 0 | 0 | 0.5 | 0 | 0 | 0 |
| $CaCl_2$ | 1 | 2.5 | 0 | 0 | 0 | 0 | 0 |
| KCl | 1 | 5 | 0.5 | 0 | 0 | 0 | 0 |
| FeNa-EDTA | 0.076 | 1 | 1 | 1 | 1 | 1 | 1 |
| Micronutrients | 1000x | 1 | 1 | 1 | 1 | 1 | 1 |

# A1.3 – Vogel buffer

**Table A1.3: Recipe for the Vogel buffer used in *Botrytis cinerea* assays.** From Birkenbihl et al. 2012.

| **Ingredient** | **Amount (g/liter)** |
|---|---|
| Sucrose | 15 |
| Na-citrate | 3 |
| $K_2HPO_4$ | 5 |
| $MgSO_4 \cdot 7H_2O$ | 0.2 |
| $CaCl_2 \cdot 2H_2O$ | 0.1 |
| $NH_4NO_3$ | 2 |

# A1.4 – KingsB liquid medium

**Table A1.4: KingsB liquid medium used in *Pseudomonas syringae* assay.** From (Cold Spring Harbor Protocols, 2009).

| Ingredient | Amount |
|---|---|
| Proteose peptone #2 (DIFCO) | 10 g |
| Anhydrous $K_2HPO_4$ | 1.5 g |
| Glycerol | 15 g |
| $MgSO_4$ (1 M; sterile) | 5 ml |

# A1.5 – List of genes used for gene expression analyses

**Table A1.5: Genes of interest used in GE-ET and GE-BC**. *TIP41* and *PP2AA2* were used as reference genes for both experiments. A -RT (minus Reverse Transcriptase control) was run with *ZAT10*.

| Gene | Gene ID | Experiment | Annealing temperature | Forward primer sequence | Reverse primer sequence |
|---|---|---|---|---|---|
| *PP2AA2* (ref. gene) | *At3g25800* | GE-ET/GE-BC | 55 °C | TGGCTCCAGTCT TGGGTAAG | ATCCGGGAACTC ATCTTTCA |
| *TIP41* (ref. gene) | *At4g34270* | GE-ET/GE-BC | 55 °C | GTGAAAACTGT TGGAGAGAAGC AA | TCAACTGGATAC CCTTTCGCA |
| *ZAT10* | *At1g27730* | GE-ET/GE-BC | 55 °C | TAGCTTCTCCGA TTCCTCC | GTGGAAATCGGA TCTTGATC |
| *ERF5* | *At5g47230* | GE-ET/GE-BC | 59 °C | TCTTCGGATCAT CGTCCTCTTC | GGTTTGCATACG GATTCAGAGAA |
| *PER4* | *At1g14540* | GE-ET/GE-BC | 59 °C | CTCACACATTA GGGCAAGCTC | GCTTACGGGTAC TGGAGAATCC |
| *WRKY33* | *At2g38470* | GE-BC | 55 °C | GACATTCTTGA CGACGGTTACA | CGATGGTTGTGC ACTTGTAGTA |
| *WRKY53* | *At4g23810* | GE-BC | 55 °C | GTTCTAGCGAG AGTCATCATCG | CATCATCTTGAG GTCCTTCTAAGC |
| *WRKY75* | *At5g13080* | GE-BC | 55 °C | TATGCGTTTCAA ACAAGGAG | CGATGGTTGTGC ACTTGTAGTA |
| *MYB51* | *At1g18570* | GE-BC | 55 °C | GCAACAAATGG TCTGCTATAGC | ATGCCCTTGTGT GTAACTGG |
| *CML37* | *At5g42380* | GE-BC | 55 °C | CGTTTGGGATG TATGTTATGG | CAAAGCTGAGA ACTCCATCG |
| *CYP71A13* | *At2g30770* | GE-BC | 55 °C | ATGGATAGATG GGATCCGT | GAAATCCGCTTT ATCGTTACTC |
| *CYP71B15* | *At3g26830* | GE-BC | 55 °C | CACCACTGATC ATCTCAAAGGA | CGGTCATTCCCC ATAGTGTT |
| *Chitinase* | *At4g01700* | GE-BC | 55 °C | TCTTACAAAGG AAGAGGTCCCA | TAAACCGTCGAA ACCCAATGCT |

# A1.6 – Tomato fertilizer experiment complete data set

**Table A1.6: Supplementary data from the tomato growth trials.** The table displays the measured growth parameter for harvest 1 (H1) and harvest 2 (H2). The data represents the mean of 6 biological replicates ± standard deviation (STD). Treatments are presented as the volume% inclusion in soil. CTRL = control base soil, CTRL = control base soil + extra nutrients, NG = NaturGjødsel (commercial organic fertilizer), UF = Untreated frass, SF = Sterilized frass, HF = heat-treated frass (at 70 °C for 1 hour).

| | **Height (cm) ± STD** | | **Fresh weight (g) ± STD** | | **Shoot DW (g) ± STD** | | **Root DW (g) ± STD** | | **# of flowers ± STD** | | **Tomato DW (g) ± STD** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | H1 | H2 | H1 | H2 | H1 | H2 | H1 | H2 | H1 | H2 | H2 |
| **CTRL** | 79 ± 8 | 161 ± 21 | 60 ± 3 | 247 ± 23 | 4,9 ± 0,9 | 18 ± 3 | 0,82 ± 0,16 | 1,3 ± 0,4 | 1,7 ± 1,5 | 8,5 ± 4,5 | 1,5 ± 1,1 |
| **CTRL+** | 78 ± 12 | 191 ± 16 | 78 ± 7 | 352 ± 41 | 5,8 ± 0,9 | 30 ± 5 | 0,83 ± 0,18 | 1,8 ± 0,1 | 1,5 ± 2,0 | 15,2 ± 3,6 | 4,4 ± 1,1 |
| **NG 0,25%** | 87 ± 12 | 201 ± 12 | 108 ± 11 | 416 ± 49 | 7,0 ± 1,2 | 33 ± 5 | 0,82 ± 0,27 | 2,6 ± 0,8 | 2,0 ± 1,4 | 22,3 ± 5,9 | 6,4 ± 2,5 |
| **NG 1%** | 85 ± 10 | 184 ± 13 | 125 ± 31 | 581 ± 65 | 7,6 ± 2,2 | 47 ± 10 | 0,59 ± 0,21 | 2,5 ± 0,8 | 1,8 ± 1,0 | 32,0 ± 13,8 | 5,5 ± 3,8 |
| **UF 0,5%** | 83 ± 10 | 179 ± 12 | 94 ± 10 | 384 ± 20 | 6,7 ± 0,9 | 34 ± 6 | 0,66 ± 0,18 | 2,2 ± 0,3 | 2,0 ± 1,3 | 21,2 ± 6,2 | 5,8 ± 1,7 |
| **UF 2%** | 93 ± 14 | 172 ± 10 | 126 ± 11 | 438 ± 31 | 7,5 ± 0,7 | 34 ± 6 | 0,56 ± 0,05 | 2,3 ± 0,7 | 1,5 ± 1,2 | 20,4 ± 10,2 | 6,4 ± 1,5 |
| **SF 0,5%** | 86 ± 14 | 180 ± 18 | 93 ± 11 | 361 ± 26 | 6,0 ± 0,7 | 33 ± 7 | 0,57 ± 0,10 | 2,3 ± 0,2 | 1,5 ± 1,2 | 17,8 ± 7,3 | 5,2 ± 2,0 |
| **SF 2%** | 88 ± 14 | 176 ± 18 | 126 ± 11 | 444 ± 34 | 7,9 ± 0,9 | 40 ± 5 | 0,54 ± 0,10 | 2,6 ± 0,4 | 1,0 ± 1,3 | 22,3 ± 10,3 | 6,5 ± 2,9 |
| **HF 0,1%** | 85 ± 8 | 172 ± 18 | 85 ± 10 | 344 ± 31 | 6,3 ± 1,1 | 32 ± 4 | 0,60 ± 0,10 | 2,3 ± 0,3 | 2,5 ± 1,9 | 19,3 ± 6,4 | 4,9 ± 2,0 |
| **HF 0,5%** | 87 ± 14 | 179 ± 15 | 86 ± 8 | 319 ± 45 | 6,0 ± 1,3 | 27 ± 6 | 0,63 ± 0,27 | 2,1 ± 0,3 | 1,2 ± 1,6 | 13,3 ± 6,1 | 5,9 ± 3,2 |
| **HF 2%** | 99 ± 6 | 183 ± 14 | 113 ± 7 | 421 ± 26 | 7,0 ± 0,8 | 36 ± 6 | 0,67 ± 0,24 | 2,9 ± 0,9 | 1,2 ± 1,2 | 27,5 ± 6,8 | 5,5 ± 1,6 |
| **HF 10%** | 90 ± 11 | 196 ± 29 | 79 ± 19 | 528 ± 75 | 4,1 ± 1,1 | 37 ± 7 | 0,36 ± 0,08 | 2,0 ± 0,6 | 0,0 ± 0,0 | 7,7 ± 2,3 | 0,0 ± 0,0 |

# A1.7 – NMR spectrum of chitin oligomer-mix

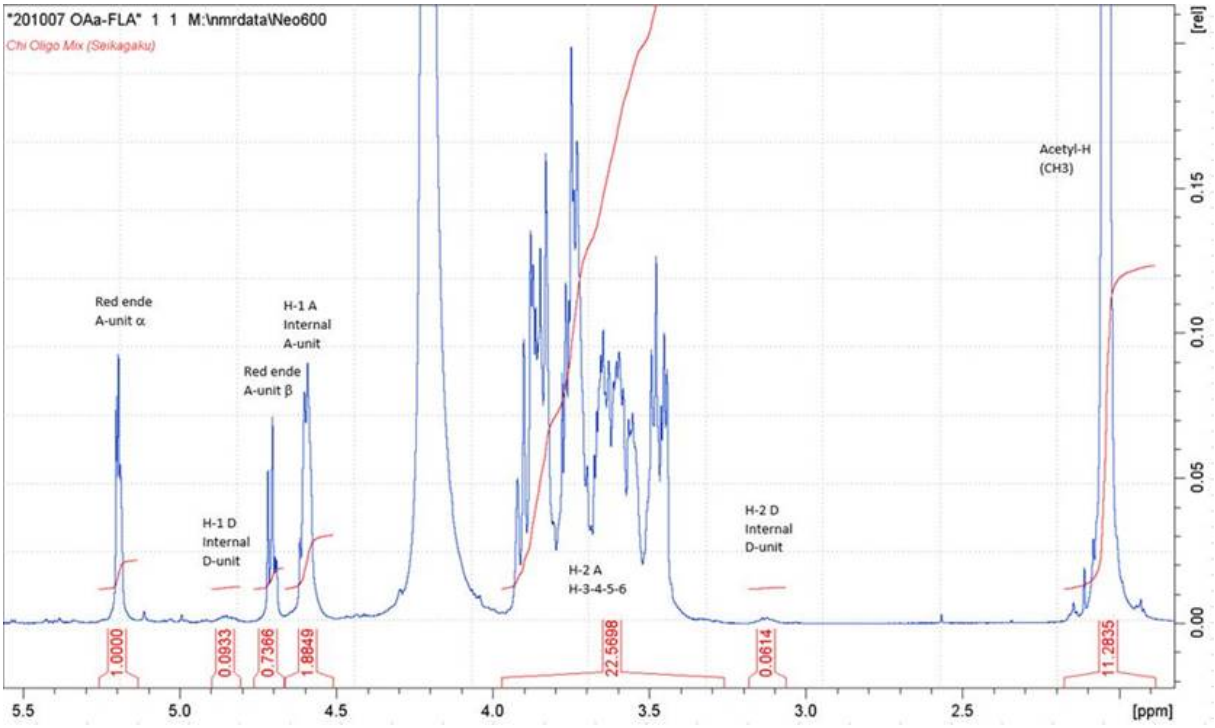


**Figure A1.6: NMR spectrum of chitin oligomer-mix used as positive control.** NMR spectroscopy and data analysis of results were conducted by Olav Aarstad at NOBIPOL (NTNU). A-unit = N-acetyl glucosamine, D-unit = D-glucosamine.

# A1.8 – Supplementary figures from the GE-BC experiment

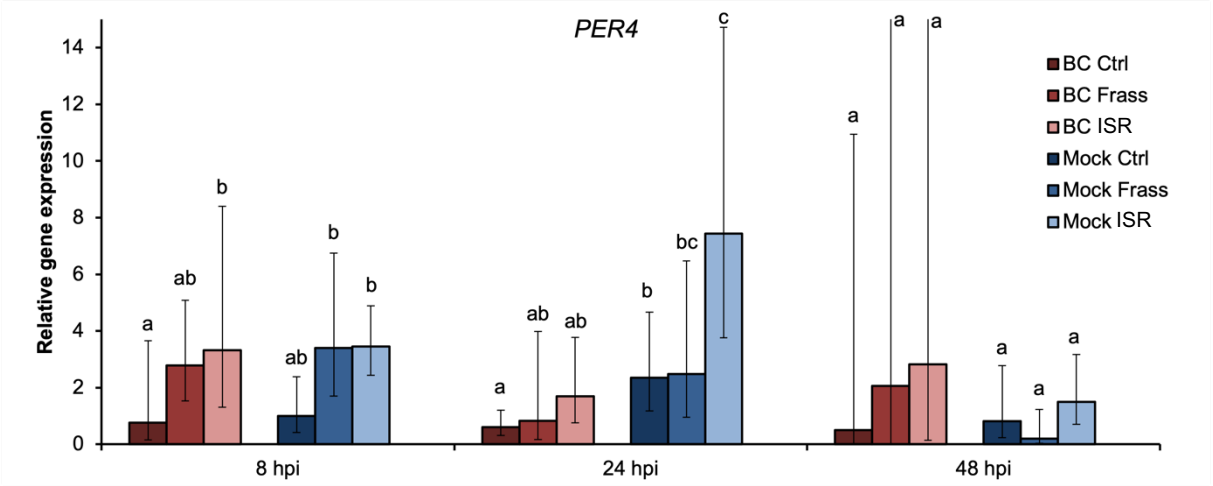


**Figure A1.1: Gene expression analysis of *PER4* target gene after *B. cinerea* infection.** 5-week-old *Arabidopsis* Col-0 leaves grown for 2 weeks in control, frass or ISR (IS) soil treatments. Leaves were inoculated with *Botrytis cinerea* spores (BC, red bars) or Vogel solution (Mock, blue bars), and harvested at 8 hours, 24 hours or 48 hours post inoculation (hpi). All values are relative to *PER4* expression in plants grown on Ctrl soil, mock-inoculated and subsequently incubated for 8 h (set to 1). Error bars show 95% confidence intervals (CI), but the upper CI for BC Frass 48 h (383.7) and for BC IS 48 h (55.7) are capped in the figure for presentation purposes. Letters denote significance within the same time group (no comparisons between time groups), Tukey-Kramer multiple comparison test, $p < 0.05$.

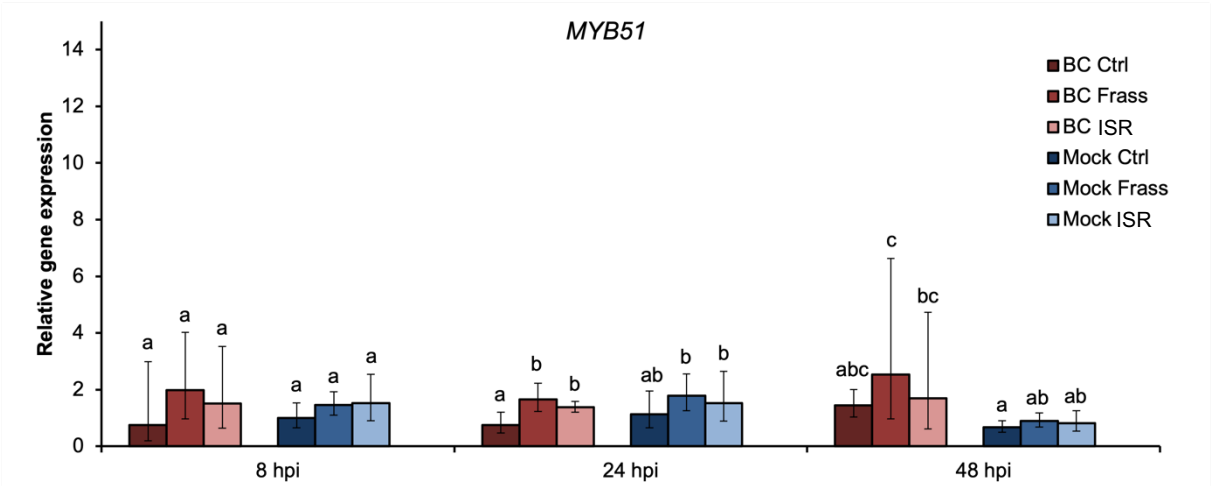


**Figure A1.2: Gene expression analysis of *MYB51* target gene after *B. cinerea* infection.** 5-week-old *Arabidopsis* Col-0 leaves grown for 2 weeks in control, frass or ISR (IS) soil treatments. Leaves were inoculated with *Botrytis cinerea* spores (BC, red bars) or Vogel solution (Mock, blue bars), and harvested at 8 hours, 24 hours or 48 hours post inoculation (hpi). All values are relative to *MYB51* expression in plants grown on Ctrl soil, mock-inoculated and subsequently incubated for 8 h (set to 1). Error bars show 95% confidence intervals. Letters denote significance within the same time group (no comparisons between time groups), Tukey-Kramer multiple comparison test, $p < 0.05$.

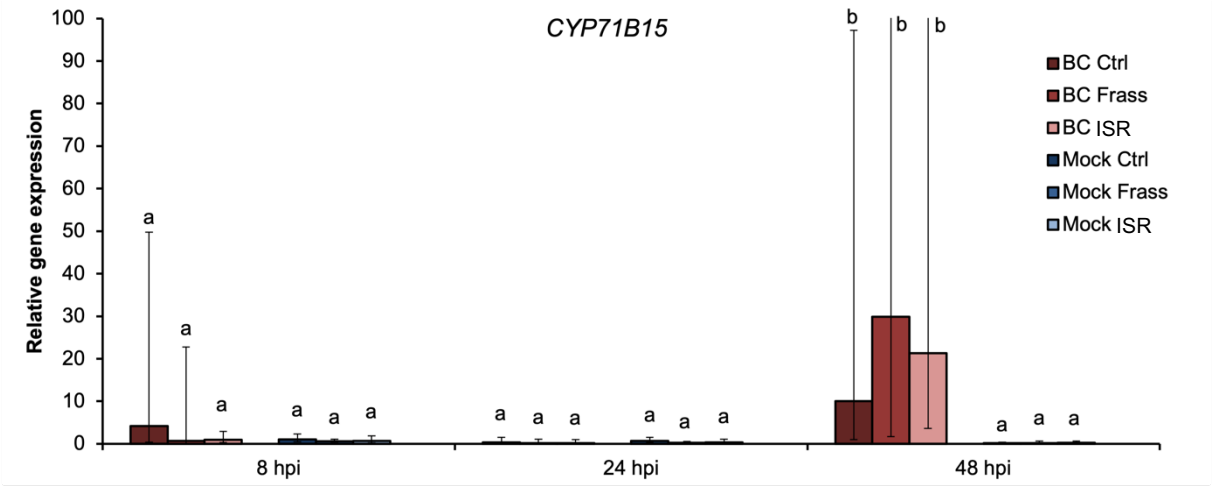


**Figure A1.3: Gene expression analysis of *CYP71B15* target gene after *B. cinerea* infection.** 5-week-old *Arabidopsis* Col-0 leaves grown for 2 weeks in control, frass or ISR (IS) soil treatments. Leaves were inoculated with *Botrytis cinerea* spores (BC, red bars) or Vogel solution (Mock, blue bars), and harvested at 8 hours, 24 hours or 48 hours post inoculation (hpi). All values are relative to *CYP71B15* expression in plants grown on Ctrl soil, mock-inoculated and subsequently incubated for 8 h (set to 1). Error bars show 95% confidence intervals (CI), but the upper CI for BC Frass 48 h (527.9) and for BC IS 48 h (125.4) are capped in the figure for presentation purposes. Letters denote significance within the same time group (no comparisons between time groups), Tukey-Kramer multiple comparison test, $p < 0.05$.

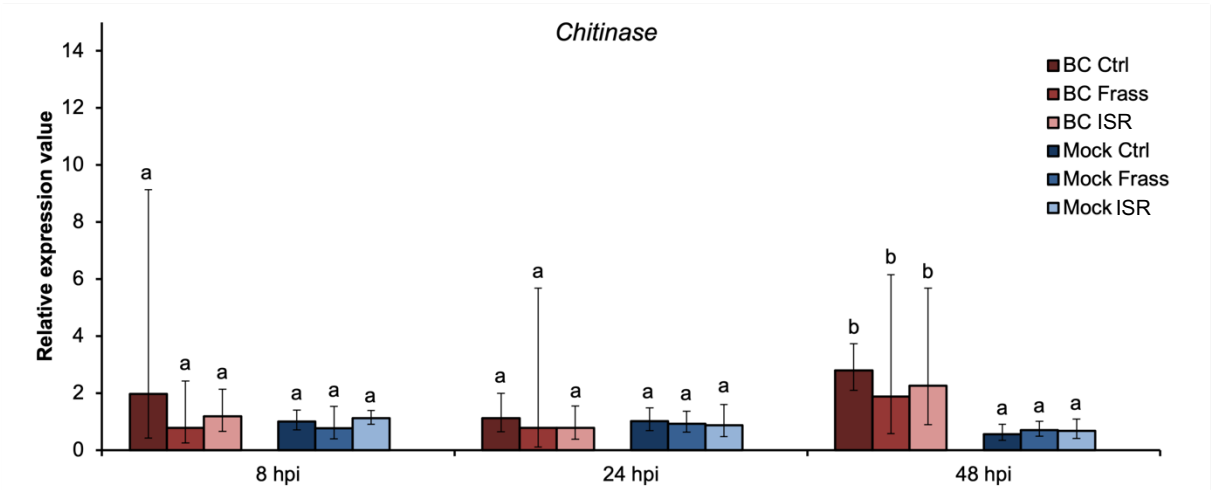


**Figure A1.4: Gene expression analysis of *Chitinase* target gene after *B. cinerea* infection.** 5-week-old *Arabidopsis* Col-0 leaves grown for 2 weeks in control, frass or ISR (IS) soil treatments. Leaves were inoculated with *Botrytis cinerea* spores (BC, red bars) or Vogel solution (Mock, blue bars), and harvested at 8 hours, 24 hours or 48 hours post inoculation (hpi). All values are relative to *Chitinase* expression in plants grown on Ctrl soil, mock-inoculated and subsequently incubated for 8 h (set to 1). Error bars show 95% confidence intervals. Letters denote significance within the same time group (no comparisons between time groups), Tukey-Kramer multiple comparison test, $p < 0.05$.

# Appendix 2 – Calculations

## A2.1 – Concentration of chitin oligomers with degree of polymerization (DP) of 6-8 in the 25 mg/ml chitin oligomer-mix stock solution

In order to calculate the concentration, one needs the molecular weight (MW) of the molecule. Here we have three different molecules, so an average of the molecular weights is used, based on the fraction of each oligomer compared to the total amount of eliciting oligomers (DP 6-8).

**Table A2.1:** Molecular weight of the three chitin oligomers with the strongest plant immune defense activation potential, including the fraction of each oligomer in the oligomer-mix used in this thesis.

| Oligomer name | DP | Molecular weight (g/mol) | Fraction in oligomer-mix |
|---|---|---|---|
| Chitin hexamer | 6 | 1237.2 | 7.0 % |
| Chitin heptamer | 7 | 1440.4 | 2.4 % |
| Chitin octamer | 8 | 1643.6 | 1.4 % |

Combined chitin oligomer DP 6-8 fraction: 10.8%

Average MW based on fractions:

$$\left(\frac{7\%}{10.8\%} \times 1237.2\frac{g}{mol}\right) + \left(\frac{2.4\%}{10.8\%} \times 1440.4\frac{g}{mol}\right) + \left(\frac{1.4\%}{10.8\%} \times 1643.6\frac{g}{mol}\right) = 1335.0\frac{g}{mol}$$

Weight of oligomers of DP 6-8 in 25 mg chitin oligomer-mix:

$$25\ mg \times 0.108 = 2.7\ mg = 0.0027g$$

Approx. moles of oligomers DP 6-8:

$$\frac{0.0027g}{1335\frac{g}{mol}} = 2.022 \times 10^{-6}\ moles = 2.022\ \mu moles$$

Dissolved in 1 ml:

$$\frac{2.022\ \mu moles}{0.001\ L} = 2.022\ mM \approx 2\ mM$$

# A2.2 Calculation of immune stimulating chitin fragments in soil solutions with frass

To get a picture of the amount of plant immune defense stimulating chitin fragments (DP 6-8) actually exist in a soil with frass solution, an example estimate was produced (Table A2.2 and A2.3). It is based on frass mixed in soil at 2% by volume. The fraction of ISR in frass is not known, so a range of 1-5% by weight is suggested based on a visual estimation. The fraction of chitin that can stimulate the plant immune system when insect skin is degraded in soil is also not known and is suggested as well. Also, the chitin heptamer (DP 7) molecular weight is used as an average molecular weight. However, longer chitin molecules than the octamer can also stimulate the plant defense response (Egusa et al., 2015), so this is also a potential error source. It must be noted that these suggestions are so-called educated guesses, so the calculation should not be regarded as more than a proposal to the question, not a finite answer.

The example calculation estimates that the 2% frass in soil solutions contains approximately 1-14 µM of plant eliciting chitin molecules.

**Table A2.2:** Suggested ratios of the relevant substances when mixing frass in soil at 2% by volume.

| Substance | Ratio | | Unit | Comment |
|---|---|---|---|---|
| | Min | Max | | |
| Frass in soil | 0,02 | | v/v | 2% volume inclusions used |
| Insect skin residue in frass | 0,01 | 0,05 | w/w | Suggested ratio |
| Chitin in mealworm exoskeleton | 0,18 | | w/w | From (Song et al., 2018) |
| Ratio of chitin that exists as elicitor at any given time point | 0,05 | 0,15 | w/w | Suggested ratio |

**Table A2.3:** Estimated range of eliciting chitin oligomers existing in a 10 ml soil solution.

| Substance | Amounts | | Comment |
|---|---|---|---|
| | Minimum | Maximum | |
| Soil | 15 g | | Equals 20 ml |
| Frass | 0,4 ml | | Equals 0,144 g |
| Insect skin residue | 0,00144 g | 0,0072 g | |
| Chitin | 0,0002592 g | 0,001296 g | |
| Elicitors | $1.3 \times 10^{-5}$ g | 0,0001944 g | |
| Moles of elicitor | $9.0 \times 10^{-9}$ mol | $1.4 \times 10^{-7}$ mol | The chitin heptamer MW is used (Table A2.1) |
| Dissolved in 10 ml | $9.0 \times 10^{-7}$ M | $1.4 \times 10^{-5}$ M | **= 0.9 – 14 µM** |

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